CENTRAL ROLE OF THE VACUOLE IN NUTRIENT MODULATION OF VOLATILE SULFUR COMPOUND FORMATION DURING FERMENTATION BY SACCHAROMYCES CEREVISIAE

A thesis presented for the degree of Doctor of Philosophy by

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Dedication

In memory of my dad, Nechemia Winter
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Statement of authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

[Signature]
Gal Winter
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<td>3MHA</td>
<td>3-mercaptophexyl acetate</td>
</tr>
<tr>
<td>4MMP</td>
<td>4-mercapto-4-methylpentan-2-one</td>
</tr>
<tr>
<td>ADY</td>
<td>Active dry yeast</td>
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<tr>
<td>ATP</td>
<td>Adenosine-5' triphosphate</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine β-lyase</td>
</tr>
<tr>
<td>Cd(OH)₂</td>
<td>Cadmium hydroxide</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>-CoA</td>
<td>-co enzyme A</td>
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<tr>
<td>COS</td>
<td>Carbonyl sulfide</td>
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<tr>
<td>CS₂</td>
<td>Carbon disulfide</td>
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<td>CSE</td>
<td>Cystathionine γ-lyase</td>
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<td>Cys-3MH</td>
<td>Cysteine-3MH</td>
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<td>Cys-4MMP</td>
<td>Cysteine-4MMP</td>
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<tr>
<td>DAP</td>
<td>Diammonium phosphate</td>
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<tr>
<td>DMDS</td>
<td>Dimethyldisulfide</td>
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<tr>
<td>DMS</td>
<td>Dimethyl sulfide</td>
</tr>
<tr>
<td>DMTS</td>
<td>Dimethyltrisulfide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EtSAc</td>
<td>Ethyl thioacetate</td>
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<td>FAN</td>
<td>Free amino nitrogen</td>
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<td>GC-SCD</td>
<td>Gas chromatography / Sulfur chemiluminescence detection</td>
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<td>Glu</td>
<td>Glutamate</td>
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<td>Gly</td>
<td>Glycine</td>
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<td>GO</td>
<td>Gene Ontology</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>H₂S</td>
<td>Hydrogen sulfide</td>
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<td>HCL</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>IDY</td>
<td>Inactivated dry yeast</td>
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<tr>
<td>IMP</td>
<td>Inosine monophosphate</td>
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<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>MBR</td>
<td>Methylene blue reduction</td>
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<td>MeSAc</td>
<td>Methyl thioacetate</td>
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<td>MTL</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NCR</td>
<td>Nitrogen catabolic repression</td>
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<td>NH₃</td>
<td>Ammonium</td>
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<tr>
<td>OAS</td>
<td>O-acetyl serine</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PLP</td>
<td>Pyridoxal 5' phosphate</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SO₂</td>
<td>Sulfur dioxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>V-ATPase</td>
<td>Vacuolar type ATPase</td>
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<td>VSC</td>
<td>Volatile sulfur compounds</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>YAN</td>
<td>Yeast assimilable nitrogen</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract, pepton, dextrose</td>
</tr>
<tr>
<td>YPDG</td>
<td>Yeast extract, pepton, dextrose, glycerol</td>
</tr>
<tr>
<td>ZnO</td>
<td>Zinc oxide</td>
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<tr>
<td>γ-GCS</td>
<td>γ-glutamylcysteine synthetase</td>
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Abstract

Volatile sulfur compounds (VSCs) are a key component of wine aroma, contributing both attractive (‘fruity’) and repulsive (‘rotten eggs’) odours. These compounds largely develop in wine by yeast metabolic activity, as yeast consume the sulfur nutrients available in the grape juice and release a range of VSCs. This thesis studies the formation of VSCs by the wine yeast Saccharomyces cerevisiae and its modulation through nutrient supplementation, focusing on organic sulfur nutrient sources- cysteine, glutathione (GSH) and their polyfunctional thiol conjugates.

The thesis first objective was to characterise the effect of an organic nutrient preparation on the resultant sulfur aroma composition. Using metabolite profiling we demonstrated two distinct effects; concentration of the ‘fruity’ polyfunctional thiols 3-mercaptohexan-1-ol (3MH) and 3- mercaptohexyl acetate (3MHA), was increased while the concentration of the ‘rotten egg’ aroma compound, hydrogen sulfide (H$_2$S), was decreased. Nutrient supplementation also changed the kinetics of H$_2$S production during fermentation by advancing its onset. Transcriptomic analysis revealed that this was not due to expression changes within the sulfate assimilation pathway, which is known to be a major contributor to H$_2$S production.

Further investigations suggested an organic sulfur component of the nutrient mix, GSH, as an activator of an alternative pathway for H$_2$S formation. GSH was previously shown to contribute to H$_2$S formation. Results here infer that this contribution is through GSH sulfur containing constituent- cysteine. Although cysteine catabolism to release H$_2$S was demonstrated in yeast, the cellular mechanisms regulating this pathway are not yet understood.

As S. cerevisiae is a model organism of the eukaryotic cell, the study of cysteine catabolism to release H$_2$S hold a broader relevance, rising from H$_2$S increasing prominence as an essential modulator of many physiological processes, particularly considering that cysteine is the main source for H$_2$S formation in mammalian cells. These research interests motivated the next objective of this thesis- a comprehensive genome-wide screen for cysteine catabolism to release H$_2$S, to shed light on the cellular mechanisms involved in this pathway.
A technical barrier in meeting this goal was the lack of available methodology for high throughput detection of cysteine-generated H$_2$S. To overcome this difficulty a novel, patent-pending method was developed, suitable for high throughput detection of cysteine-generated H$_2$S. The method utilizes a redox reaction in which sulfide ion reduces methylene blue, leading to its decolourisation. Incorporation of methylene blue into the fermentation medium allows real-time, in situ, detection of H$_2$S and the generation of an accurate H$_2$S production profile.

Following the method validation, its principles were implemented in a genome-wide screen for cysteine catabolism to release H$_2$S. Results of the screen revealed a surprising set of cellular factors affecting this process. The yeast vacuole, not previously associated with cysteine catabolism, emerged as a major compartment for cysteine degradation with deletants impaired in vacuole biogenesis unable to catabolise cysteine to release H$_2$S. The mechanism of vacuole acidification was identified as prominent in cysteine catabolism to release H$_2$S; deletants for each of the eight subunits of a vacuole acidification sub-complex (V$_1$ of the yeast V-ATPase) were independently classified as essential for cysteine degradation to H$_2$S.

This is the first genome-wide study aimed to elucidate the cellular factors affecting cysteine catabolism. Foundations laid here support the use of S. cerevisiae as a model organism to study cysteine catabolism and may provide insights into the underlying cause of cysteine accumulation and H$_2$S generation in eukaryotes. In conclusion, this thesis demonstrates the effective use of nutrients supplementation as a means for wine aroma management. Results and techniques obtained here significantly contribute to our understanding of volatile sulfur compounds formation and provide tools that can be implemented in winemaking for better modulation of sulfur aroma compounds in wine.
Chapter 1.

Review of the literature

1.1 Overview

Volatile sulfur compounds (VSCs) are important for the aroma of many fermented food products like wine, beer and cheese. They are considered a ‘double-edged’ sword with regard to their contribution to the food sensorial qualities, playing an attractive or repulsive role depending on their identity and concentration. Amongst the pleasant sulfur compounds are polyfunctional thiols, imparting ‘fruity’ aroma when present in moderate concentration (Swiegers et al., 2005a; Dubourdieu et al., 2006). A prominent unpleasant sulfur compound is hydrogen sulfide ($\text{H}_2\text{S}$), which possesses an odour reminiscent that of ‘rotten eggs’ (Rauhut, 1993).

The majority of volatile sulfur compounds in wine are formed during yeast fermentation of the grape juice, as yeast consume the available sulfur in the grape juice and use it for the biosynthesis of sulfur containing amino acids. Excess sulfur is then released in various forms including VSCs. Formation of VSCs is highly dependent on the nutritional composition of the grape juice. Yeast are able to produce VSCs from inorganic sulfur compounds such as sulfate and sulfite, through the sulfate assimilation pathway or through their degradation pathways of organic sulfur compounds such as cysteine and glutathione (Reviewed in: Henschke and Jiranek, 1993; Rauhut, 1993; Thomas and Surdin-Kerjan, 1997; Landaud et al., 2008; Rauhut, 2009).

This review describes current knowledge regarding the metabolism of sulfur aroma compounds in the wine yeast Saccharomyces cerevisiae and regulation of their formation by nutrient supplementation. The review first describes the formation of $\text{H}_2\text{S}$ and its oenological as well as physiological significance. The second part of the review then focuses on the formation of polyfunctional thiols during alcoholic fermentation, and regulation of genes involved.
1.2 Volatile sulfur compounds

Sulfur is the 10th most abundant element in the universe and the sixth most abundant element in microbial biomass (Klotz et al., 2011). The element sulfur occurs in a range of oxidation states, from completely reduced (sulfide, oxidation state $-2$) to completely oxidized (sulfate, oxidation state $+6$). However, only three states are abundant in nature; -2 in the form of sulphydryl and sulfide; 0 in the form of elemental sulfur and +6 in the form of sulfate (Figure 1.1). These sulfur compounds are continuously interconverted by a combination of biological, chemical and geochemical processes that are critically dependent upon microbial activity. The complex combination of these processes results in the global sulfur cycle (Cook et al., 1998; Lomans et al., 2002).

1.2.1 Assimilation and release of VSCs

Microorganisms and plants can use inorganic sulfur, mainly sulfate, to form organic sulfur compounds in an energy-dependent process referred to as assimilation. Animals on the other hand are dependent on pre-formed organic sulfur compounds for their sulfur needs. In addition to assimilation, many bacteria and archaea can use sulfur in energy-yielding reactions, referred to as dissimilation. These processes are essential for the cycling of sulfur (Cook et al., 1998; Lomans et al., 2002).

Reduction of sulfate and sulfite can be carried out either as assimilatory sulfate reduction for the synthesis of organic sulfur compounds (e.g. synthesis of sulfur containing amino acids) or as dissimilatory sulfate reduction, to obtain energy. The produced sulfide can then be either deposited as metal sulfides (e.g. FeS and FeS$_2$), or it can be chemically or biologically oxidized resulting in the formation of elemental sulfur or more oxidized sulfur compounds such as sulfate. Sulfide can also be methylated both biologically and chemically to result in volatile sulfur compounds (Zwart and Kuenen, 1992; Lomans et al., 2002).

1.2.2 Occurrence & significance of VSCs

In the global sulfur cycle VSCs effectively connect the atmospheric with the terrestrial, oceanic, estuarine and freshwater compartments. Vast amounts of VSCs such as dimethyl sulfide (DMS), H$_2$S and carbonyl sulfide/ carbon disulfide...
(COS/CS$_2$), representing 75, 15 and 10% of the total sulfur flux respectively, are released into the atmosphere (Lomans, 2001).

Besides the impact on global processes including the global sulfur cycle and temperature control (Bengtsson et al., 1999), VSCs cause environmental problems on local and regional scales. For example, the release of organic and inorganic sulfur compounds caused the acidification of large forests and lakes (Lomans et al., 2002). Locally, VSCs are notorious due to the odor problems they cause (Derikx et al., 1990). While high concentrations of VSCs are toxic, at low concentrations they affect physiological processes and determining the flavor of various types of cheese, beers and wines (Landaud et al., 2008; Albert, 2009).

1.2.2.1 VSCs physiological significance

VSCs play a significant role in human physiology (Albert, 2009). A prominent example is the formation of H$_2$S, which is synthesized enzymatically in mammalian and human tissues (Stipanuk, 1986; Stipanuk, 2004). H$_2$S has been shown to have multifaceted effects in neuromodulation, cardioprotection, smooth muscle relaxation and regulation of insulin release (Reviewed in: Kimura, 2002; Wang, 2002; Kabil and Banerjee, 2010; Kimura, 2010; Olson, 2011). In the brain, H$_2$S was shown to contribute to proper neuronal function, and abnormal H$_2$S levels are associated with pathological states. H$_2$S concentrations are considerably lower in the brains of Alzheimer's patients but higher in patients suffering from Down's syndrome, compared to normal persons (Eto et al., 2002; Kamoun et al., 2003). H$_2$S was characterised as a new gas neurotransmitter, joining carbon oxide and nitric oxide (Wang, 2002). It was also shown to confer antibiotics resistance in bacteria and to act synergistically with nitric oxide (Shatalin et al., 2011). H$_2$S has even been shown to induce a suspended animation-like state in a non-hibernating species by decreasing body temperature and metabolic rate (Blackstone et al., 2005).

While microorganisms and plants are able to assimilate sulfate to form sulfide, higher animals cannot utilise sulfate and rely on organic amino acids as their sulfur source (Nicholas, 1967). In mammals, the majority of endogenous H$_2$S is presumed to be generated through the catabolism of cysteine (Wang, 2002; Chen et al., 2004; Stipanuk, 2004; Chiku et al., 2009; Beard and Bearden, 2011; Singh and Banerjee, 2011). Aside from cysteine, sulfur compounds contained within garlic, onion,
Figure 1.1 **Sulfur redox cycle.** Significant amounts of sulfur are present in nature only in three oxidation states, -2 (sulfide, HS-, R-SH and sulfhydryl), 0 (elemental sulfur) and +6 (sulfate, SO4-2). Transformation of sulfur between these forms is critically dependent upon microbial activity.
mushrooms and various edible beans may transform in the human body to release H\textsubscript{2}S and this was suggested as a mechanism contributing to the therapeutic effects attributed to these foods (Jacob et al., 2008)

1.2.2.2 VSCs significance in food

VSCs are an important part of the aroma of many fermented foods like cheese, beer and wine. Owing to their low detection thresholds and high reactivity, these compounds contribute significantly to food quality. In cheese, VSCs primarily arise through the degradation of sulfur containing amino acids by the cheese microflora, including yeasts and bacteria (Landaud et al., 2008). Of the prominent aroma compounds in cheese are methanethiol (MTL), H\textsubscript{2}S, DMS and dimethyldisulfide (DMDS) imparting aromas of ‘rotten eggs’, ‘cooked cabbage’, ‘garlic’ and ‘cheesy’ (Dimos et al., 1996; Frank et al., 2004; Qian and Burbank, 2007). These VSCs are highly desired in certain types of cheese and extensive research has been conducted into development of cheese-ripening microorganisms and cheese-making techniques to enhance the production of VSCs (Spinnler et al., 2001; López del Castillo-Lozano et al., 2007; Lopez del Castillo Lozano et al., 2007; López del Castillo Lozano et al., 2008; Berger, 2009).

In beer, VSCs originate from malt and hops. Contribution of VSCs to beer quality is dependent upon the identity and concentration of the compound (Walker, 1992; Walker and Simpson, 1993); H\textsubscript{2}S contribute to an undesirable aroma; polyfunctional thiols derive from hop, impart ‘onion’ and ‘leek’ aromas; DMS and dimethyltrisulfide (DMTS), impart ‘fresh onion’ and ‘cooked vegetables’ aromas (Walker, 1992; Landaud et al., 2008).

In wine, certain VSCs contribute to a desirable aroma and others to unwanted aroma notes. The important VSCs in wine are summarized in Table 1. The majority of VSCs in wine arise from common sulfur-bearing precursors, such as the amino acids cysteine and methionine, glutathione (GSH) and inorganic sulfur found in the grapes (Mestres et al., 2000; Landaud et al., 2008). During fermentation and subsequent wine ageing, these precursors continuously convert to create different sulfur aroma compounds. The transformations of VSCs in wine are schematically described in figure 1.2.
Table 1.1 *Examples of volatile sulfur compounds found in wine* (Laudaud, et al. 2008; Swiegers and Pretorius 2007)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Aroma note</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{H}_2\text{S} )</td>
<td>Rotten eggs</td>
</tr>
<tr>
<td>methanethiol (MTL)</td>
<td>cooked cabbage, onion</td>
</tr>
<tr>
<td><strong>Sulfides</strong></td>
<td></td>
</tr>
<tr>
<td>* Dimethylsulfide (DMS)</td>
<td>boiled cabbage, corn</td>
</tr>
<tr>
<td>* Dimethyldisulfide (DMDS)</td>
<td>cooked cabbage, intense onion</td>
</tr>
<tr>
<td>* Dimethyltrisulfide (DMTS)</td>
<td>cooked cabbage, onion</td>
</tr>
<tr>
<td><strong>Methylthioesters</strong></td>
<td></td>
</tr>
<tr>
<td>* S-methyl thioacetate</td>
<td>rotten vegetables</td>
</tr>
<tr>
<td>* S-methyl thiopropanoate</td>
<td>Chocolate, roasted</td>
</tr>
<tr>
<td>* S-methyl thiobutanoate</td>
<td></td>
</tr>
<tr>
<td><strong>Polyfunctional thiols</strong></td>
<td></td>
</tr>
<tr>
<td>* 3-mercapto hexanol (3MH)</td>
<td>passion fruit, grape fruit</td>
</tr>
<tr>
<td>* 3-mercaptohexyl acetate (3MHA)</td>
<td>passion fruit, boxwood</td>
</tr>
<tr>
<td>* 4-Mercapto-4-methyl-pentan-2-one (4MMP)</td>
<td>box tree, cat urine</td>
</tr>
</tbody>
</table>
Figure 1.2 Volatile sulfur compounds in wine arise from common sulfur-bearing precursors. Schematic representation of sulfur aroma development during wine alcoholic fermentation and ageing; straight arrows represent microbial transformation, dashed arrows represent chemical transformation and pictures illustrate the aroma characteristics of the compounds group. The organic sulfur compounds in grapes GSH, cysteine and methionine degrade to produce \( H_2S \) and methanethiol, which further react with other compounds in wine to produce volatile sulfides and methylthioesters. \( H_2S \) is also produced from inorganic sulfur compounds for the synthesis of cysteine and methionine. Additionally, grape derived precursors such as Cys-3MH and GSH-3MH are converted by yeasts to release a free thiol, 3MH that may be further esterified by yeast activity to produce 3MHA. (Rauhut, 1993; Swiegers and Pretorius, 2007; Landaud et al., 2008)
1.3 Reductive aroma- H$_2$S

‘Reductive aroma’ is the term used to describe the presence of unwanted sulfur compounds in wine imparting aromas of ‘rotten eggs’, ‘sewage’, or ‘struck flint’; which negatively affect consumer acceptance (Godden et al., 2005). Despite its common use, from a chemical point of view the term ‘reductive’ is a misnomer, originating from the fact that under reductive conditions (such as during wine ageing when oxygen is largely excluded) sulfur compounds are susceptible to be the electron recipient in redox reactions (Mestres et al., 2000). These conditions therefore favour the development of sulfur compounds and hence the use of the term ‘reductive’. It is thus important to note that sulfur compounds can be formed even under oxidative conditions.

The two central thiol precursors for VSCs are MTL and H$_2$S (Figure 1.2, Table 1.1). MTL is the first-step degradation product of methionine. It is detectable at very low concentrations and imparts an aroma that can be described as ‘garlic’, ‘onion’ and ‘cabbage’ (Landaud et al., 2008). Once formed, MTL can be further oxidised to form other VSCs such as sulfides and thioesters. Similar to MTL, H$_2$S is detected at very low concentrations (~1µg/L), imparting aroma of ‘rotten eggs’ (Rauhut, 1993). During alcoholic fermentation and wine ageing H$_2$S combine with carbon compounds to form VSCs as described in table 1.1 (Mestres et al., 2000; Swiegers et al., 2005a; Landaud et al., 2008). The majority of H$_2$S is formed metabolically by yeasts and bacteria during the fermentation process from inorganic or organic sulfur compounds through the sulfate assimilation pathway or through the degradation of cysteine (Henschke and Jiranek, 1993; Rauhut, 1993; Rauhut, 2009).

1.3.1 H$_2$S metabolism in S. cerevisiae - Inorganic sulfur compounds

Organic sulfur compounds including cysteine, methionine, S-adenosyl methionine and GSH are essential for yeast growth. When these compounds are absent from the grape juice, yeasts must synthesise them from inorganic sulfur compounds (Acree et al., 1972). The most abundant inorganic sulfur source in grapes is sulfate (SO$_4^{2-}$) (Vos and Gray, 1979; Leske et al., 1997). Yeasts are able to assimilate sulfate and metabolise it for amino acid synthesis through ‘The sulfate assimilation pathway’, (Figure 1.3). The first step in that pathway involves transport of sulfate from the medium into the yeast cell (Park and Bakalinsky, 2000). Sulfate is then reduced to
sulfide through a series of steps using the enzymes ATP-sulfurylase and sulfite reductase (Masselot and Robichon-Szulmajster, 1975; Korch et al., 1991; Thomas and Surdin-Kerjan, 1997). The process is energy-costing and requires the use of 2 ATP and 3 NADPH molecules (Henschke and Jiranek, 1993; Rauhut, 1993; Thomas and Surdin-Kerjan, 1997). Following its formation, \( \text{H}_2\text{S} \) is sequestered into O-acetylhomoserine, an amino acid precursor, to form homocysteine that is further metabolised to produce methionine or cysteine (Yamagata et al., 1994).

Production of \( \text{H}_2\text{S} \) through this pathway is affected by genetic and environmental variables. Previous studies identified five transcription factors required for the activation of this network. These include two leucine zipper factors, Met4p and Met28p and three zinc-finger-containing transactivators, Met31p and Met32p; and Cbf1p. These proteins interact to form large multi-subunit complexes which assemble on the 5’ upstream region of the gene and vary based on the target gene (Thomas and Surdin-Kerjan, 1997; Kaiser et al., 2000; Dixon et al., 2003).

Additionally, sulfur metabolism was shown to be regulated through two ubiquitine-proteasome systems SCF\text{Met}30 and SCFSkp2 (Yoshida, et al., 2011).

The impact of underlying genetic variation on \( \text{H}_2\text{S} \) formation was demonstrated in a number of studies showing \( \text{H}_2\text{S} \) production differs between yeast strains under the same experimental conditions (Thornton & Bunker, 1989, Henschke & Jiranek, 1993, Henschke & De Kluis, 1995, Spiropoulos & Bisson, 2000, Kumar, et al., 2010). Several studies aimed to map the source for this genetic variation. A prominent enzyme displaying allele diversity is sulfite reductase. The yeast sulfite reductase enzyme complex is composed of catalytic and regulatory subunits. The catalytic subunits form an \( \alpha_2\beta_2 \) core tetramer, with the \( \alpha \) subunit encoded by the \text{MET}10 gene and the \( \beta \) subunit encoded by \text{MET}5 Activity of the complex also requires the two regulatory subunits encoded by \text{MET}1 and \text{MET}8 (Thomas & Surdin-Kerjan, 1997). Mutations in \text{MET}10 and \text{MET}5 genes lead to impaired enzyme activity and significantly less sulfide release during fermentation (Cordente, et al., 2009, Linderholm, et al., 2010).

The activity of \text{MET}17 was also examined for allele diversity (Linderholm et al., 2006). \text{MET}17 encodes a sulfhydrylase enzyme capable of using O-acetylhomoserine
Figure 1.3. **Sulfur amino acid biosynthesis in S. cerevisiae.** Yeasts are able to assimilate inorganic sulfur and convert sulfate into hydrogen sulfide. \( \text{H}_2\text{S} \) is then coupled with an amino acid precursor, derived from aspartate metabolism to produce homocysteine for the synthesis of cysteine and methionine. Cysteine may be used for the formation of glutathione or alternatively catabolise to produce \( \text{H}_2\text{S} \) (Thomas and Surdin-Kerjan, 1997).
as a substrate in vivo. It is responsible for the last step of the sulfate reduction pathway, incorporation of \( \text{H}_2\text{S} \) into amino acid precursor (Yamagata et al., 1994). However, no allelic variants of this gene were found in comparison of different wine strains (Linderholm et al., 2006). In addition, overexpression of MET17 did not result in decreased \( \text{H}_2\text{S} \) production (Spiropoulos and Bisson, 2000), suggesting Met17 level of activity does not limit reduced sulfide incorporation into amino acid precursor.

Further examination of genes involved in cysteine and methionine biosynthesis revealed allele diversity for CYS4 and MET6 as well as decreased sulfide formation when these genes were mutated, suggesting sulfide release may be regulated downstream the assimilation pathway (Linderholm et al., 2006).

Environmental factors affecting \( \text{H}_2\text{S} \) production include, above all, nutrient availability. Activation of the sulfate assimilation pathway occurs for the synthesis of cysteine and methionine and is therefore regulated by their presence (Hansen and Francke Johannesen, 2000). Additionally, this pathway includes the sequestration of \( \text{H}_2\text{S} \) into an amino acid precursor, which derives from nitrogen metabolism and is regulated by the availability of nitrogen in the media (Jiranek et al., 1995b; Bell and Henschke, 2005). A detailed review of nutrient regulation on \( \text{H}_2\text{S} \) formation is given in section 1.3.5.1.

Other environmental factors that may affect \( \text{H}_2\text{S} \) production include: high residual levels of elemental sulfur, presence of sulfur dioxide, presence of sulfur-containing organic compounds, pantothenate deficiency and relative methionine-to-ammonium concentrations (Henschke and Jiranek, 1993; Rauhut, 1993; Spiropoulos and Bisson, 2000; Rauhut, 2009). Yeast strains respond differently to these variations in environmental conditions, with some producing little or no \( \text{H}_2\text{S} \) and others producing high \( \text{H}_2\text{S} \) concentrations regardless of the conditions (Mendes-Ferreira et al., 2002; Kumar et al., 2010).

1.3.2 \( \text{H}_2\text{S} \) metabolism in \( S. \text{cerevisiae} \) - organic sulfur compounds

Formation of \( \text{H}_2\text{S} \) from organic sulfur compounds occurs through the degradation of sulfur containing amino acids, in contrast to \( \text{H}_2\text{S} \) formed through the sulfate assimilation pathway, which is for the synthesis of those amino acids. While cysteine and GSH supplementation enhance \( \text{H}_2\text{S} \) formation (Tokuyama et al., 1973; Rauhut,
2009), methionine supplementation reduces the amount of H$_2$S produced during fermentation (Jiranek et al., 1995b; Spiropoulos et al., 2000; Cordente et al., 2009). In that sense, both cysteine and GSH can be viewed as H$_2$S precursors.

1.3.2.1 Cysteine metabolism in S. cerevisiae

Cysteine is a semi-essential amino acid, it can be endogenously synthesised or consumed from the environment. Biosynthesis of cysteine is mediated through the transsulfuration pathway that yields cysteine from homocysteine through the intermediate cystathionine (Thomas and Surdin-Kerjan, 1997). Consumption of cysteine is mediated by a specific cysteine transporter (Kaur and Bachhawat, 2007) although multiple non specific amino acid permeases were also implicated in the uptake of cysteine (Ono and Naito, 1991).

Free cysteine is maintained at a low intracellular concentration (Kaur and Bachhawat, 2007). The majority of cellular cysteine is incorporated into proteins or other essential molecules such as GSH and Acetyl Co-A (Thomas and Surdin-Kerjan, 1997). It is postulated that upon sufficient concentration of GSH, synthesis of cysteine is downregulated (Hansen and Francke Johannesen, 2000) and excess cysteine is removed.

Previous studies have demonstrated that cysteine contributes to H$_2$S release during fermentation (Jiranek et al., 1995b; Jiranek et al., 1996), possibly through yeast enzymatic degradation of cysteine to release H$_2$S (Wainwright, 1971; Tokuyama et al., 1973). The biochemical pathways responsible for that degradation are described in figure 1.4. While genes implicated in cysteine degradation were characterised in both bacteria and higher eukaryotes, the mechanism responsible for cysteine degradation to release H$_2$S has not been characterised in yeasts. In various bacteria, different enzymes that participate in sulfur metabolism have shown cysteine desulphhydrase activity in vitro (Wada et al., 2002; Auger et al., 2005; Awano et al., 2005). In addition, the tryptophanase enzyme (TnaA) from Escherichia Coli, was also shown to be a cysteine desulphhydrase (Awano et al., 2003). In mammals two cytosolic, pyridoxal-5’-phosphate (PLP)–dependent, enzymes; cystathionine β-synthase (CBS, EC 4.2.1.22), and cystathionine γ-lyase (CSE, EC 4.4.1.1), were implicated in cysteine catabolism to H$_2$S (Searcy and Lee, 1998; Chen et al., 2004;
Singh and Banerjee, 2011). The regulatory mechanisms of these enzymes remain to be elucidated.

1.3.2.2 GSH metabolism in S. cerevisiae

GSH is a tri-peptide (γ-L-glutamyl-L-cysteinylglycine) that is essential for yeast growth (Grant et al., 1996; Sharma et al., 2000). The biological significance of GSH is mainly related to the free sulfydryl moiety of the cysteine residue, which confers unique redox and nucleophilic properties. In yeasts, GSH assumes a pivotal role in bioreductive reactions, transport, sulfur metabolism enzyme activity, protection against harmful oxidative species, and detoxification of xenobiotics (Reviewed in: Meister and Anderson, 1983; Penninckx, 2000; Penninckx, 2002; Pastore et al., 2003). The strong electron-donating capability of GSH and its relatively high intracellular concentration (up to millimolar levels) enable the maintenance of a reducing cellular environment. This makes GSH an important antioxidant for protecting DNA, proteins, and other molecules against oxidative damage (Pastore et al., 2003; Li et al., 2004). In an oenological context, GSH addition maintains a reductive wine environment, and protects oxidation-prone aroma compounds, such as polyfunctional thiols (Papadopoulou and Roussis, 2008; Ugliano et al., 2011).

GSH is synthesized in yeast by consecutive action of γ-GCS (γ-glutamylcysteine synthetase, EC 6.3.2.2) and L-γ-glutamylcysteine-glycine γ-ligase (glutathione synthetase, EC 6.3.2.3), encoded respectively by GSH1 and GSH2 genes (Grant and Dawes, 1996). The rate-limiting step in this pathway is Gsh1p, which is feedback-inhibited at the enzyme level by the presence of GSH (Meister and Anderson, 1983). Depletion of GSH, on the other hand, increases the expression of GSH1 in a response that is co-regulated by the Yap1p and Met4p transcription factors (Wheeler et al., 2003).

In addition to endogenous biosynthesis, yeasts can also obtain GSH from the environment. The presence of GSH transporters has been demonstrated in all three kingdom of life (Ballatori and Dutczak, 1994; Jamai et al., 1996; Miyake et al., 1998; Sherill and Fahey, 1998). The yeast GSH transporter was identified simultaneously as an oligopeptide transporter (Hauser et al., 2000) and GSH specific transporter (Bourbouloux et al., 2000). Surprisingly, it does not share homology
Figure 1.4 **Biochemical pathways for H\textsubscript{2}S formation.** Biochemical pathways for H\textsubscript{2}S production catalysed by CBS and CSE enzymes. The mechanism by which each reaction proceeds is indicated. (Adapted from: Chen et al., 2004; Chiku et al., 2009)
with GSH transporters found in organisms bacteria or mammals (Bourbouloux et al., 2000).

Inside the yeast cell, glutathione is distributed across multiple compartments, in compartment-specific concentrations. The highest glutathione accumulation was observed in mitochondria while GSH was also detected in the cytosol, nucleus, cell walls and the vacuole (Zechmann et al., 2011). GSH sub-cellular distribution dynamically changes based on environmental conditions; for example oxidative stress drives GSH accumulation to the mitochondria (Zechmann et al., 2011) while nitrogen starvation shifts GSH towards the vacuole (Mehdi and Penninckx, 1997).

Unlike GSH synthesis, its degradation has not been fully characterised to date. The enzyme $\gamma$-GT [$\gamma$-glutamyltranspeptidase, EC 2.3.2.2] encoded by the gene ECM38 was implicated in GSH degradation by Penninckx et al. (1980). Ecm38p catalyzes transfer of the $\gamma$-glutamyl moiety of GSH and other $\gamma$-glutamyl compounds to amino acids (Tate and Meister, 1981). S. cerevisiae $\gamma$-GT has been identified as a vacuolar membrane-bound glycosylated protein with an active site inside the vacuolar lumen (Klionsky and Emer, 1989). L-cysteinyl glycine dipeptidase (EC 3.4.13.6) is assumed to catalyze the last step in GSH hydrolysis. Although this hydrolytic activity has not been attributed to specific genes in S. cerevisiae, it has been demonstrated and was shown to be associated with the vacuolar membrane (Jaspers and Penninckx, 1984) thus it is likely that GSH degradation occurs in the yeast vacuole.

Recently, a cytosolic alternative pathway of GSH degradation, working independent of $\gamma$-GT in yeasts and fungi was identified (Kumar et al., 2003; Ganguli et al., 2007). GSH degradation by this pathway was found to require the participation of three genes DUG1, DUG2 and DUG3. Dug1p functions as a Cys-Gly specific dipeptidase (Kaur et al., 2009). Dug3p has a functional glutamine amidotransferase (GATase) II domain that is catalytically important for glutathione degradation and Dug2p has no peptidase activity (Kaur et al., 2012). Interactions between Dug2p and Dug3p are required for the degradation of GSH to glutamate and cys-gly. Interestingly, DUG2 and DUG3 but not DUG1 were de-repressed by sulfur limitations (Kaur et al., 2012).
One product of GSH degradation is $\text{H}_2\text{S}$. $\text{H}_2\text{S}$ release may occur through GSH degradation to individual amino acids, followed by cysteine degradation. However, direct cleavage of GSH to form $\text{H}_2\text{S}$ is also possible and further study is needed to understand the mechanisms of GSH degradation to $\text{H}_2\text{S}$. GSH metabolism is a good case study for the ‘double edge sword’-type contribution of VSCs. On one hand; GSH protects desirable aroma compounds from oxidation (Ugliano et al., 2011) while on the other hand GSH degradation generates reductive, unwanted compounds (Rauhut, 2009). This strengthens the need to better understand GSH metabolism and the genetic and environmental factors affecting it.

1.3.3 Methods for $\text{H}_2\text{S}$ detection during fermentation

Research into $\text{H}_2\text{S}$ formation necessitates accurate measurement of this highly volatile and reactive compound throughout fermentation. Typically, $\text{H}_2\text{S}$ production is measured by collection in selective traps, followed by quantification using colourimetric reactions (Ugliano and Henschke, 2010).

$\text{H}_2\text{S}$ can also be quantified by means of gas chromatography (Choi et al., 1995; Rauhut et al., 1998; Park et al., 2000) or HPLC (Mitchell et al., 1993; Gru et al., 1998), however these methods are not utilised for $\text{H}_2\text{S}$ detection during fermentation as changes in the rate of $\text{H}_2\text{S}$ formation can be very rapid (Sohn et al., 2000; Ugliano and Henschke, 2010), thus the accurate $\text{H}_2\text{S}$ profiling requires high frequency of sampling and therefore daily processing of a large number of samples.

1.3.3.1 $\text{H}_2\text{S}$ quantification using methylene blue formation

Measurement of $\text{H}_2\text{S}$ through methylene blue formation was discovered in 1883 by Fischer (Fischer, 1883); it is an extremely sensitive and quantitative method. This method utilises a reaction between sulfide and $\text{N,N,dimethyl-p-phenylenediamine}$ that results in the formation of methylene blue which can be measured spectrophotometrically and be used for the quantification of sulfide concentration. $\text{H}_2\text{S}$ absorption into a solution is typically done using a solution containing cadmium hydroxide or zinc acetate (also known as cadmium trap or zinc trap). Absorption of $\text{H}_2\text{S}$ into these solutions creates cadmium sulfide or zinc sulfide, which under acidic conditions and with the addition of oxidising agent (typically ferric ions) can react with $\text{N,N,dimethyl-p-phenylenediamine}$ to form methylene blue (Fogo and
Popowsky, 1949; Acree et al., 1971; Jiranek et al., 1995b). By replacing the cadmium/zinc traps throughout fermentation it is possible to achieve an accurate profile of H$_2$S formation during fermentation. The main disadvantage of this method is the quantification process that is very time consuming and requires specifically designed glassware and handling of various highly toxic chemicals.

A practical adaptation of this reaction was developed to perform microbial selection. This adaptation uses closed plate flasks containing a zinc-agar layer above the liquid microbial culture as a trap system where the H$_2$S can be retained and then quantified. This method was used for the quantification of H$_2$S in several cheese-ripening microorganisms (Lopez del Castillo Lozano et al., 2007).

Additionally, the same chemical reaction leading for methylene blue formation from sulfide and N,N-dimethyl-p-phenylenediamine was utilised to detect H$_2$S in yeast culture solution using a gas diffusion and flow injection analysis, coupled with a spectrophotometric detector. The gas diffusion method is based on the conversion of sulfide in a sample (donor stream) to volatile H$_2$S which diffuses across a membrane into a detection (acceptor) line (Yuan and Kuriyama, 2000). The advantage of this method is the direct measurement of H$_2$S in the fermentation culture which enhances the method sensitivity. Still, this method requires the use of specific machinery and handling of toxic chemical.

Finally, H$_2$S can be used as a reductive agent in a reaction with methylene blue, which leads to methylene blue decolourisation (Feigl and West, 1947; Mousavi and Sarlack, 1997). This reaction may be further catalysed by selenium or tellurium. A kinetic-spectrophotometric method for the determination of sulfide based on methylene blue reduction was developed to detect H$_2$S in waste. It was found to be simple, rapid and sensitive (Mousavi and Sarlack, 1997).

**1.3.3.2 H$_2$S quantification using bismuth sulfide measurement**

Conversion of sulfide into bismuth sulfide results in the formation of a dark colour that can be measured spectrophotometrically. Field and Oldach were the first to use that reaction for H$_2$S quantification (Field and Oldach, 1946). Their technique, although very sensitive (detection of 1.4 µg), is cumbersome, as it requires a very rigid control of technique and all solutions must be protected against oxygen. Further
development of this method made it more ‘user friendly’ with incorporation of bismuth sulfite into agar media which was first used for selective cultivation and differentiation of Candida spp. from pathological samples (Nickerson, 1953). In this way, colonies which produce H$_2$S become discoloured in a concentration dependent manner, ranging from off-white, through brown to near-black.

Incorporation of bismuth sulfite into agar media is a widely used technique for H$_2$S measurement in oenology. It is used for strain characterisation and large collection screening purposes (Jiranek et al., 1995a; Spiropoulos et al., 2000; Cordente et al., 2009; Kumar et al., 2010; Ugliano et al., 2010). The advantages of this method are its ease of use and also high throughput applicability; on the other hand this method is limited in sensitivity and provides a qualitative characterisation rather than a detailed profile of H$_2$S formation during fermentation.

**1.3.3.3 H$_2$S quantification using impregnated tape methods**

Collection of H$_2$S on paper tapes impregnated with silver nitrate, dicyanoargentate( I), mercuric chloride, or lead acetate, followed by optical densitometric determination of the metal sulfide is commonly used for sulfide detection during fermentation (Natusch et al., 1974). Application of the impregnated tape above the fermentation flask allows the collection of H$_2$S produced during fermentation. Probably the most widely used method involves collection of H$_2$S using a paper tape impregnated with lead acetate (Natusch et al., 1974; Rauhut, 1993; Spiropoulos et al., 2000; Cordente et al., 2009). The optical density of the resulting dark brown lead sulfide (PbS) stain is then related to the concentration of H$_2$S in the volume of air sampled. The disadvantage of this method is in the incomplete collection of H$_2$S and the instability of the sulfide stain in the presence of light, SO$_2$, or even air passing through the tape (Natusch et al., 1974).

An additional method uses silver nitrate impregnated membrane applied on top of a micro-titer plate, to collect and quantify H$_2$S formed during micro fermentation (Duan et al., 2004). While this method is suited for high throughput applications, it is limited in sensitivity and does not detect natural levels of H$_2$S produced during fermentation, requiring the addition of cysteine to the media as a sulfur source. It is therefore limited in characterisation of environmental variables affecting H$_2$S formation. Moreover, quantification of H$_2$S with this method requires removal of the
membrane from the plate and so the method provides a single-point analysis. The generation of H$_2$S profile during fermentation requires continuous membrane replacement throughout the fermentation.

1.3.3.4 H$_2$S quantification using gas collection tubes

Selective detector tubes are commonly used in environmental monitoring for the analysis of gaseous pollutants. This type of apparatus typically consists of a glass column packed with an inert material supporting a selective reagent. When the reagent is exposed to the target chemical(s), a visually detected stain is formed. The length of the coloured band is proportional to the amount of analyte passing through the tube. Detector tubes based on the selective reaction between sulfur compounds and the heavy metals lead, silver, and mercury are commercially available for their analysis in air samples. A recent study has shown that lead acetate detector tubes can be utilised for continuous monitoring of H$_2$S in fermentations when applied on top of the fermentation flask (Park, 2008). This method was compared to a reference cadmium trap procedure.

1.3.4 H$_2$S formation during fermentation

H$_2$S is formed during fermentation in concentrations up to hundreds of µg/L (Ugliano and Henschke, 2010; Ugliano et al., 2010). H$_2$S formation during fermentation usually follows the same kinetics that includes observation of two or more peaks, during the early to middle stages and last peak at towards the end of fermentation (Figure 1.5) (Thomas et al., 1993; Ugliano et al., 2008; Ugliano et al., 2010; Butzke and Park, 2011). These two phases in H$_2$S production during fermentation are associated with different fermentation factors. The majority of H$_2$S formed early in fermentation is typically attributed to yeast growth and the requirement for sulfur amino acid synthesis (as described in 1.3.1). The second peak of H$_2$S production is associated with non-proliferating or dying cells which leak nutrients into the media. These organic nutrients may include GSH and cysteine which may be further metabolised by the remaining active yeasts to produce H$_2$S (Henschke and De Kluis, 1995; Jiranek et al., 1996; Alic et al., 2004; Ugliano et al., 2009). It is postulated then that the first peak of H$_2$S is produced through the sulfate assimilation pathway, while late production is mediated through organic sulfur compounds metabolism.
Figure 1.5 Profile of H$_2$S formation during wine fermentation. H$_2$S monitoring during fermentation usually includes the observation of two or more peaks in H$_2$S production (arrows). The first peak is produced at the early stages of fermentation and includes the majority of H$_2$S produced during fermentation. The second peak is produced during the later stages of fermentation followed by methylene blue quantification (section 1.7.2) and was found to display better recoveries (Ugliano and Henschke, 2010). The advantages of this method are its ease of use as well as its accuracy; the main limitation of this method is the lack of high throughput applicability.
For wine quality, what matters is H$_2$S left in the wine at the end of fermentation (referred to as ‘residual H$_2$S’), and not necessarily the total cumulative amounts of H$_2$S produced during fermentation. Previous studies have speculated about a link between the amount of residual H$_2$S in wine and the concentration of H$_2$S produced during the later stages of fermentation (Henschke and De Kluis, 1995; Jiranek et al., 1996; Ugliano et al., 2009). Early in fermentation, most of the H$_2$S formed is purged by entrainment with the significant quantities of CO$_2$ produced, whereas later in fermentation there is much less CO$_2$ produced (Jiranek et al., 1996) and H$_2$S produced then is therefore more likely to remain in the final wine.

1.3.5 Nutrient regulation on H$_2$S formation

1.3.5.1 Yeast assimilable nitrogen

Under oenological conditions yeast are able to assimilate nitrogen in two forms, ammonium and primary amino acids (Bell and Henschke, 2005). These forms combined are referred to as YAN (Yeast Assimilable Nitrogen). YAN concentration in the grape juice is considered a key factor affecting H$_2$S formation via the sulfate assimilation pathway (section 1.3.1, Figure 1.3). Nitrogen deprivation results in insufficient amounts of the amino acid precursor, which lead to the release of excess H$_2$S (Jiranek et al., 1995b; Jiranek et al., 1996; Hallinan et al., 1999). Conversely, YAN supplementation would increase precursor availability and the sequestration of H$_2$S, which will in turn decrease the concentration of H$_2$S released.

Nitrogen supplementation as a solution for H$_2$S release during fermentation has been studied extensively. However, despite the general agreement that H$_2$S formation is influenced by YAN concentration, the nature of this regulation is not well understood. While early studies demonstrated direct negative correlation between YAN concentration and H$_2$S formation (Vos and Gray, 1979; Giudici and Kunkee, 1994b; Jiranek et al., 1995b; Wang et al., 2003; Duan et al., 2004) more recent studies suggest otherwise and find no correlation between the two (Spiropoulos et al., 2000; Ugliano et al., 2009; Ugliano et al., 2010; Bohlscheid et al., 2011; Butzke and Park, 2011). The reasons for these discrepancies include both genetic and environmental variation between experiments. Genetic variation arises from the use of different yeast strains that respond differently to nitrogen supplementation (Hallinan et al., 1999; Mendes-Ferreira et al., 2009; Ugliano et al., 2010). Still, even
for identical strains, fermentation conditions pose a great source of variation. Other factors affecting H$_2$S formation are: YAN concentration (Reviewed in Bell and Henschke, 2005), timing of YAN supplementation (Mendes-Ferreira et al., 2010a), fermentation conditions (size, temperature, agitation) and the composition of other nutrients such as vitamins (Wang et al., 2003; Bohlscheid et al., 2007; Edwards and Bohlscheid, 2007; Bohlscheid et al., 2011) or sulfur sources (Thomas et al., 1993; Jiranek et al., 1995b; Alic et al., 2004; Beltran et al., 2005; Rauhut, 2009). Additionally, the abovementioned studies used different methodologies for H$_2$S quantification, which may have contributed to the variation in their conclusions.

Nitrogen concentration of the medium regulates yeast sulfate assimilation pathway through transcription regulation. YAN supplementation was found to upregulate genes involved in the sulfate assimilation pathway (Marks et al., 2003; Mendes-Ferreira et al., 2010b), which is in line with current knowledge on yeast metabolism of nitrogen. Yeast utilize nitrogen mainly in their exponential growth and stationary phases for protein synthesis and growth (Henschke and Jiranek, 1993). An accelerated protein synthesis rate necessitates the availability of sulfur containing amino acid, hence the upregulation of the sulfate assimilation pathway as measured during the exponential and stationary phases (Marks et al., 2003; Mendes-Ferreira et al., 2010b).

1.3.5.2 Organic sulfur compounds

As described in section 1.3.2., organic sulfur compounds can function as precursors for H$_2$S formation by yeasts. Early studies focused on the effect of elemental sulfur on H$_2$S formation and found its presence to be insignificant to the amount of H$_2$S released during fermentation (Acree et al., 1972; Thomas et al., 1993). The effects of sulfur containing amino acids on H$_2$S formation have also been studied. Methionine supplementation was shown to reduce the amount of H$_2$S produced during fermentation (Jiranek et al., 1995b; Spiropoulos et al., 2000; Cordente et al., 2009). By contrast, the presence of cysteine was consistently shown to induce H$_2$S formation (Eschenbruch et al., 1973; Giudici and Kunkee, 1994a; Jiranek et al., 1995b; Nisamedtinov et al., 2010). The presence of cysteine in the fermentation medium was also shown to suppress sulfate reduction to H$_2$S (Eschenbruch et al., 1973) and to downregulate the expression of genes in the sulfate
assimilation pathway (Ono et al., 1991; Ono et al., 1996; Hansen and Francke Johannesen, 2000).

As a result of its low concentration in grape juice (Kluba et al., 1978; Pripis-Nicolau et al., 2001), free cysteine has not been considered an important factor influencing H$_2$S production during wine fermentation. However, bound-cysteine is present in grapes at considerable concentrations as part of GSH (Vos and Gray, 1979; Cheynier et al., 1989; Park and Bakalinsky, 2000; du Toit et al., 2007), and GSH was shown to enhance H$_2$S production during fermentation (Park and Bakalinsky, 2000; Rauhut, 2009). The mechanism by which GSH concentration affects H$_2$S production has not been elucidated. Comprising both nitrogen and sulfur constituents, GSH may contribute to H$_2$S production in two different ways, mediated by its different nutrient components.

1.4 Polyfunctional thiols

The polyfunctional thiols 3-mercaptohexan-1-ol (3MH), its acetylated derivative 3-mercaptohexyl acetate (3MHA) and 4-Mercapto-4-methylpentan-2-one (4MMP) impart aromas of ‘passionfruit’, ‘grapefruit’ and ‘boxwood’ (Tominaga et al. 1998b; Dubourdieu et al. 2006). These thiols have a very low detection threshold (60, 4 and 0.8 ng/L for 3MH, 3MHA and 4MMP respectively) (Tominaga et al., 1998b) and were found as highly desired in some styles of Sauvignon Blanc wines by consumers (King et al., 2011). Polyfunctional thiols are present in grapes as non-volatile precursors, conjugated to cysteine or GSH (Tominaga et al., 1998a; Peyrot des Gachons et al., 2002; Capone et al., 2010). During fermentation yeast take up these precursors and cleave them to release free volatile form into the fermentation (Swiegers and Pretorius, 2007; Grant-Preece et al., 2010). Concentration of polyfunctional thiols in wine depends on the amount of precursor cleaved during fermentation by yeast. Importantly, yeast strains vary in their abilities to release thiols and therefore selection of yeast strain is highly important to obtain high concentration of thiols in wine (Dubourdieu et al., 2006; Swiegers et al., 2009). Additionally, chemical composition of wine and storage environment affect greatly on thiols stability and their concentration in wine (Ugliano et al., 2011).

Two non-volatile thiol precursors’ forms have been identified to date. S-3-(hexan-1-ol)-L-cysteine (Cys-3MH) and S-4-(4-methylpentan-2-one)-L-cysteine (Cys-
4MMP) were identified first (Tominaga et al., 1998a) followed by the identification of GSH conjugated 3MH, (S-3-(hexan-1-ol)-glutathione, GSH-3MH) (Peyrot des Gachons et al., 2002) and later identification of GSH conjugated 4MMP (Fedrizzi et al., 2009). The nature of the relationships between the two conjugation forms and yeasts ability to metabolise them under oenological conditions have not been characterised yet.

### 1.4.1 Release of free polyfunctional thiols from bound precursors

3MH release from its cysteinylated precursor has been studied extensively. While the genes coding for the enzymes responsible for this pathway in yeast have not been fully identified, there is a general agreement regarding the biochemical processes involved. As Cys-3MH structure is similar to that of cysteine, uptake of the precursor is assumed to be mediated by amino acid transporters on the plasma membrane. Indeed, yeast deletion assays were used to demonstrate the uptake of Cys-3MH is partially mediated through the general amino acid transporter, GAP1 (Subileau et al., 2008b). Once inside the cell, Cys-3MH is thought to be cleaved by an enzyme with carbon-sulfur β-lyase activity (Tominaga et al., 1998b; Swiegers et al., 2007; Thibon et al., 2008; Holt et al., 2011). Several yeast carbon-sulfur lyase enzymes were tested for their ability to release volatile thiols, they were found to affect thiols release in a limited manner (Howell et al., 2005; Holt et al., 2011). An additional carbon sulfur lyase enzyme, Irc7p, was found to be the main contributor to 4MMP release and to a lesser extent 3MH release (Thibon et al., 2008; Roncoroni et al., 2011). Interestingly, most strains of S. cerevisiae have a 38-bp deletion that inactivates IRC7 (Roncoroni et al., 2011). This variation may account for the strain variation observed in thiol release (Swiegers et al., 2009). Overexpression of a full-length S. cerevisiae allele of IRC7 in a wine yeast increased 4MMP production in Sauvignon Blanc wine from undetectable levels to 1000 ng/L, and also increased 3MH and 3MHA concentrations (Roncoroni et al., 2011).

Cys-3MH cleavage reaction produces pyruvate, ammonia and a free volatile thiol, 3MH. Pyruvate and ammonia are further metabolised. 3MH may be further acetylated to form 3MHA (Swiegers et al., 2005b) or alternatively, it may be released into the fermentation by a mechanism still unknown, either via diffusion, similar to the release of esters (Verstrepen et al., 2003a) or through specific transporters.
Current knowledge of the Cys-3MH conversion pathway is illustrated in figure 1.5. The conversion pathway of the glutathione precursor has not been studied. It is reasonable to suppose that this conversion may occur via two separate pathways. One is through a direct, one-step cleavage of 3MH from GSH-3MH, analogous to the direct reaction observed with the cysteine precursor. A second putative pathway, inferred from studies regarding metabolism of GSH and other GSH conjugates (Wünschmann et al., 2010) includes a multi-step degradation process. In this pathway, the first steps would involve degradation of the thiol-conjugate tripeptide (Glu-Cys-3MH-Gly) into single amino acids (Glu, Cys-3MH and Gly). Based on current knowledge of GSH metabolism this degradation is likely to occur in the vacuole (Jaspers et al., 1985; Ubiyovk et al., 2006; Ganguli et al., 2007). Recent studies have focused on the analysis of 3MH precursors in various white grape juices. A study conducted in Australia reports a concentration of 10-55 µg/L Cys-3MH and a significantly higher concentration of GSH-3MH, 140-640 µg/L (Capone et al., 2010). An additional studies conducted in France reports of a similar concentration of Cys-3MH (7.9-65.2 µg/L) and a significantly lower concentration of GSH-3MH (4.4 – 7.5 µg/L) (Roland et al., 2010). These differences in GSH-3MH concentrations may be grape dependent or may be attributed to the use of different sample preparation and measurement methods.

Interestingly, Subileau et al. (2008) found that Cys-3MH was not the precursor responsible for the majority of free 3MH and 3MHA detected in their experimental wines (Subileau et al., 2008a). Deletion of the OPT1 gene, that encodes the main glutathione transporter in yeast, resulted in lower formation of 3MH (Subileau et al., 2008a). These experiments inferred GSH-3MH is the main 3MH precursor. However, that assessment requires further validations. First, the ability of yeast to metabolise GSH-3MH to 3MH and 3MHA needs to be demonstrated and then a direct comparison of the two precursors is required.

1.4.2 Nutrient regulation on polyfunctional thiol release

As thiol precursors comprised of amino acids, nutrient availability is likely to be significant influence on release of polyfunctional thiols. Indeed, a systematic screening of environmental variables influencing polyfunctional thiol release during fermentation highlighted YAN concentration as a critical factor regulating thiols
Figure 1.6 Schematic of 3MH precursors conversion pathways to 3MH and 3MHA. Straight arrows represent data that has been investigated in the context of 3MH precursors metabolism. Dashed arrows represent inferred pathways, based on studies regarding flavour metabolism, GSH and GSH conjugates metabolism.

1. Transport of GSH-3MH into the cell through the GSH transporter OPT1 (Subileau et al., 2008a)
2. Cleavage of GSH-γMH by γ-aminopeptidases present in the grape juice to form a dipeptide precursor and transport of the precursor into the cell (Ganguli et al., 2007)
3. Transport of Cys-3MH through the general amino acid transporter GAP1 (Subileau et al., 2008b)
4. Cellular metabolism of GSH-3MH. Inferred pathways include direct cleavage of GSH-3MH to produce 3MH and the tripeptide Glu-Ala-Gly, or multi step degradation of the precursor through either the cytoplasm (Ganguli et al., 2007) or vacuole (Jaspers et al., 1985; Ubivyovk et al., 2006), to single amino acids which will then be cleaved to release 3MH from its resulting cysteine conjugate
5. Release of 3MH from Cys-3MH by yeast β-lyase enzymes (Tominaga et al., 1998a; Swiegers et al., 2007; Thibon et al., 2008)
6. Acetylation of 3MH to form 3MHA (Swiegers et al., 2005b) and hydrolysis of 3MHA to 3MH (Mauricio et al., 1993)
7. Release of 3MH and 3MHA into the wine by unknown mechanisms. From (Winter et al., 2011).
release (Thibon et al., 2008). This led to the hypothesis that thiol release may be controlled by nitrogen catabolic repression (NCR). NCR is a physiological response that regulates nitrogen consumption and metabolism in order to utilise the best available nitrogen sources (ter Schure et al., 2000). It is activated in the presence of optimal sources of nitrogen (such as ammonia) to downregulate the activity of genes involved in poor nitrogen source utilization. NCR involves the transcription factors Gln3p and Gat1p (ter Schure et al., 2000; Scherens et al., 2006), which are inactivated by the Ure2 protein in the presence of nitrogen-rich sources. During wine fermentation, NCR is active (Rossignol et al., 2003) and can be silenced by deletion of the URE2 gene (Salmon and Barre, 1998) (Salmon & Barre, 1998). Thibon et al. (2008) found that a Δure2 yeast strain released higher concentrations of polyfunctional thiols (Thibon et al., 2008). This effect was mediated mainly through the enzyme activity of Irc7p and the transcription regulation of Gln3p, both downregulated when NCR is active (Thibon et al., 2008). In line with these findings, substitution of DAP (rich nitrogen source) with urea (poor nitrogen source) was found to decrease 3MH release from its cysteine precursor (Subileau et al., 2008b).

1.5 Conclusions- wine sulfur aroma management

Development of VSCs during fermentation is primarily dependent on yeast strain identity and fermentation conditions. This review described the regulation mechanisms responsible for VSCs formation and their modulation through yeast nutrients. Notably, nutrient supplementation may have contrasting effects on the formation of different sulfur compounds. DAP supplementation, for example, was found in some cases to decrease H₂S formation during fermentation (Bell and Henschke, 2005) while it was also found to decrease the concentration of 3MH in wine by prolonging NCR (Subileau et al., 2008b). As both compounds are produced through yeast activity, the effects of nutrient supplementation cannot be limited to specific compounds. This poses a challenge to the modern winemaker who needs to consider holistically the implications of nutrient supplementation in order to manage wine aroma.

1.6 Research aims

VSCs have been the focus of numerous studies. Still, the mechanisms responsible for their development during fermentation are not yet understood, particularly the
contribution of organic sulfur sources to VSCs formation. This path for sulfur compounds formation has been largely overlooked in wine research despite its importance and broad relevance. The aims of this thesis are to explore the metabolic pathways leading to the formation of volatile sulfur compounds in the wine yeast S. cerevisiae, and their modulation through nutritional supplementation.

The first aim of this research project is to characterise the effect of organic nutrient mix supplementation on the composition of the resultant wine sulfur aroma. Combining transcriptomic analysis with metabolite profiling, we aim to gain insights into individual components of the nutrient mix that affect VSCs formation. Components revealed in the first part as contributing to sulfur aroma formation will be further studied to obtain a better characterisation of the pathways, using chemical analysis of metabolites and the yeast gene deletion library.

Specific goals:

1. Modulation of aroma compounds through the use of yeast nutrients
   a. Characterise the effect of organic nutrient preparation on wine volatile composition
   b. Link metabolite changes to specific cellular pathways using parallel analysis of transcriptomics and metabolite profiling
   c. Associate the metabolite changes observed to specific component in the organic nutrient preparation

2. Formation of sulfur aroma compounds from organic sulfur sources- cysteine and GSH
   a. Explore the formation of polyfunctional thiols from cysteine and glutathione- conjugated aroma precursor
   b. Characterise the catabolic process of cysteine degradation to release H₂S
      i. Genome- wide screen of the yeast deletion collection for cysteine catabolism
1. Develop a method suitable for high throughput screen analysis of the yeast deletion collection for cysteine catabolism

2. Screen the yeast deletion collection for cysteine catabolism

ii. Perform targeted follow up experiment to validate and further explore results obtained in the screen.
Chapter 2.

Effects of rehydration nutrients on H$_2$S metabolism and formation of volatile sulfur compounds

2.1. Introduction and aims

Yeasts nutrient preparations are often added to a grape juice prior to or during alcoholic fermentation, to alleviate problems such as stuck or sluggish fermentation and to avoid the formation of undesirable off-flavours such as H$_2$S (Henschke and Jiranek, 1993; Sablayrolles et al., 1996; Blateyron and Sablayrolles, 2001; Mendes-Ferreira et al., 2009; Ugliano et al., 2010; Schmidt et al., 2011; Torrea et al., 2011). Amongst the nutrient supplements allowed by wine regulatory authorities in many countries are vitamins, inorganic nitrogen, usually in the form of DAP and organic nutrient preparations. Nutrient preparations are typically prepared from inactive or autolysed yeasts and are thus usually composed of lipids, micro- and macro-elements, amino nitrogen, mannoproteins and insoluble material (Pozo-Bayón et al., 2009). Effects of these nutrients on the formation of aroma compounds have been previously studied, particularly the effect of inorganic nitrogen supplementation which is considered a key modulator in the formation of volatile sulfur compounds, including H$_2$S (section 1.4.3). The effects of organic nutrient supplementations on the formation of volatile sulfur compounds have not been hitherto thoroughly explored.

Most studies regarding the effects of nutrients supplementations on wine volatile composition have focused on nutrient addition to the grape juice immediately prior to or during alcoholic fermentation. The common practice of using active dry yeast (ADY) for wine fermentation necessitates rehydration, since water availability in ADY is too low for yeast to maintain metabolic activity during storage (Rapoport et al., 1997). This step represents a further opportunity for nutrient supplementation. Previous studies have demonstrated the efficacy of nutrient supplementation at this point in time on both yeast viability and vitality. Supplementation of inactive dry yeast (IDY) at rehydration was found to increase fermentation rate (Soubeyrand et al., 2005). Additions of fermentable carbon source and magnesium salts were also
shown to enhance both viability and vitality of ADY following rehydration (Kraus et al., 1981; Rodríguez-Porrata et al., 2008).

Although organic rehydration nutrient supplementation is a common practice in oenology, its effect on the formation of fermentation derived aroma compounds and in particular volatile sulfur compounds, has not been explored. In this chapter we examine the effect of a proprietary rehydration organic nutrient supplement on yeast gene expression during wine fermentation and how this affects its volatile chemical composition. Using this parallel analysis consisting of transcriptomics and metabolite profiling, we aim to gain insights into which components of the organic nutrient mixture affect the formation of volatile sulfur aroma compounds and to shed light on organic nutrient regulation of sulfur metabolism in S. cerevisiae.

Work described in this chapter was published as research paper in the journal ‘AMB Express’ (Appendix A-1.1)

2.2. Materials and methods

2.2.1 Chemicals

Analytical reagents were purchased from Sigma-Aldrich unless otherwise specified. Rehydration nutrient mix was Dynastart (Laffort Australia, Woodville, SA, Australia). Cys-3MH and Cys-4MMP were synthesized and characterized as previously described (Howell et al., 2004; Pardon et al., 2008).

2.2.2 Yeast strain, treatments and fermentation conditions

Yeast strain used was a commercial active dried preparation of VL3 (Laffort Australia, Woodville, SA, Australia). ADY were rehydrated with water or water supplemented with rehydration nutrient mix (120 g/L). To examine the effect of nutrient mix components ADY were rehydrated with water containing GSH (500 mg/L). Rehydration media were mixed at 37 ºC for 30 minutes prior to addition of 10% (w/v) ADY. Yeasts were then incubated with agitation in the rehydration media for 20 minutes and then inoculated into the fermentation media to obtain concentration of 1 x 10^6 cells/ml. Fermentations were carried out in triplicate at 22 ºC with agitation (150 rpm). Fermentations were carried out in Schott bottles (SCHOTT Australia, NSW, Australia), sealed with silicone o-ring and fitted with
silver nitrate detector tubes for the quantification of H$_2$S formed in fermentation and with a sampling port. Samples were collected through the sampling port using a sterile syringe. Fermentation volume was either 2 L (for comprehensive volatile analysis) or 1 L. Fermentation progress was monitored by measurement of residual glucose and fructose using an enzymatic kit (GF2635, Randox, Crumlin, UK).

2.2.3 Fermentation media and post fermentation handling

A low nitrogen Riesling juice having a total YAN concentration of 120 mg/L (NH$_3$ = 53 mg/L; free amino nitrogen (FAN) = 90 mg/L) was used for this study. Juice analytical parameters were as follows: pH, 2.9; titratable acidity 4.6 g/L as tartaric acid; sugars, 205 g/L. To examine the effect of rehydration nutrients on thiol release, grape juice was supplemented with 5 µg/L Cys-4MMP and 200 µg/L Cys-3MH, a concentration of precursors commonly found in Sauvignon Blanc juices (Luisier et al., 2008; Capone et al., 2010). Where specified, DAP addition to the fermentation media was 0.56 g/L to increase the juice YAN concentration to 250 mg N/L. The pH of the fermentation medium was readjusted to 2.9 with 1 M HCl following DAP additions. Juice was filter sterilized with a 0.2 µm membrane filter (Sartorius Australia, Oakleigh, Victoria, Australia).

At the end of fermentation, wines were cold settled at 4 ºC and free SO$_2$ of the finished wine was adjusted to 45 mg/L by the addition of potassium metabisulfite. The wines were then carefully racked into glass bottles to avoid exposure to oxygen and were sealed with air tight caps fitted with a polytetrafluoroethylene liner. Bottles were fully filled to avoid any headspace oxygen.

2.2.4 Grape juice analyses

Titratable acidity, FAN, and ammonia were measured as previously described (Vilanova et al., 2007). Ammonia concentration was measured using the Glutamate Dehydrogenase Enzymatic Bioanalysis UV method (Roche, Mannheim, Germany). FAN was determined by using the o-phtalaldehyde/N-acetyl-L-cysteine spectrophotometric assay procedure. Both ammonia and FAN were analyzed using a Roche Cobas FARA spectrophotometric autoanalyzer (Roche, Basel, Switzerland). Amino acid analysis was carried out based on Korös et al. (2008) using a pre-column derivitisation with o-phthalaldehyde-ethanethiol-9-fluorenlymethyl chloroformate
and HPLC analysis with fluorescence detection. Reduced and oxidized glutathione were analyzed using LC-MSMS as previously described (du Toit et al., 2007).

2.2.5 Volatile compounds analyses

H₂S, MTL, DMS, methyl thioacetate (MeSAc), and ethyl thioacetate (EtSAc) were determined by static headspace injection and cool-on-column gas chromatography coupled with sulfur chemiluminescence detection (GC-SCD), as described in Siebert et al. (2010). 3MH, 3MHA and 4MMP were measured in SARCO Laboratories (Bordeaux, France) according to Tominaga et al. (2000) using a TRACE GC-MS (ThermoFisher Scientific, MA, USA). Detection limits for 3MH, 3MHA and 4MMP were 11 ng/L, 1 ng/L and 0.3 ng/L, respectively. Quantification limit is 35 ng/L ±20% for 3MH, 3 ng/L ±18% for 3MHA and 0.6 ng/L ±14% for 4MMP. Monitoring of H₂S development during fermentation was carried out using silver nitrate selective gas detector tubes (Komyo Kitagawa, Japan), as described by Ugliano and Henschke (2010).

2.2.6 RNA Extraction and cDNA synthesis

Samples for RNA analyses were collected during fermentation by filtration following the consumption of 15 g/L sugars. Cells were resuspended in RNAlater® (Ambion, Inc., Austin, TX, USA) solution at 4 °C for 24 hours. Cells were then centrifuged to remove the RNAlater® solution and were stored at -80°C. Total RNA was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad, CA) as described in Alic et al. (2004). The integrity of the RNA was analyzed using an RNA 6000 Nano LabChips on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). cDNA was synthesized from 200 ng total RNA in a total volume of 20 µl with AffinityScript QPCR cDNA synthesis kit (Statagene, Agilent Technologies, Santa Clara, CA) and oligo-dT20 primers by incubation for 5 min at 42 °C and 15 min at 55°C with heat inactivation for 5 min at 95 °C.

2.2.7 Transcription analyses

Transcription analysis was carried out at the Ramaciotti Centre for Gene Function Analysis (UNSW, Sydney, Australia). Biological duplicates were analysed using the Affymetrix GeneChip Yeast Gene 1.0 ST Array and the GeneChip® 3’ IVT Express protocol (Affymetrix, Santa Clara, CA, USA). Data were analysed using the statistical methods available in the Partek® Genomic Suite 6.5 (Partek Incorporated,
St Louis, Missouri, USA). Statistical analysis for over-representation of functional groups was performed using FunSpec (Robinson et al., 2002). Available databases were addressed by using a probability cut-off of 0.01 and the Bonferroni correction for multiple testing. To validate the results, five differentially expressed genes were further examined by quantitative real-time PCR (qRT-PCR). qRT-PCR was carried out with Brilliant II SYBR Green reagent (Statagene, Agilent Technologies) and cDNA made from 2.5 ng total RNA in a volume of 25 µl for all subsequent reactions. Primers are detailed in table 2.1. Ct values were obtained from triplicate fermentations and were normalized using the $2^{\Delta\Delta Ct}$ method (Wong and Medrano, 2005). Values were then normalized against a geometric average of two reference genes obtained from geNorm (Vandesompele et al., 2002). Selection of the reference genes was based on the microarray results using an algorithm described in Popovici et al. (2009). Each individual PCR run was normalized with an intercalibration standard.

2.2.8 Determination of glutathione

For the extraction of cellular glutathione, cells (100 mg) were washed three times with sodium-phosphate buffer (pH 7.4) and resuspended in 1 ml 8 mM HCl, 1.3% (w/v) 5-sulphosalicylic acid for 15 min at 4°C. Cells were then broken by vortexing at 4°C with 0.5 g of glass beads in four series of 1 min alternated with 1 min incubation on ice. Cell debris and proteins were pelleted in a microcentrifuge for 15

Table 2.1 qRT-PCR primers sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>GPM1</td>
<td>GCTCACGGTAACTCCTTG</td>
</tr>
<tr>
<td></td>
<td>AGATGGCTTAGATGGCTTC</td>
</tr>
<tr>
<td>TDH3</td>
<td>GCTGCCGCTGAAGGTAAG</td>
</tr>
<tr>
<td></td>
<td>CGAAGATGGAAAGTAGGAGTC</td>
</tr>
<tr>
<td>OPT1</td>
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<td>IRC7</td>
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</tr>
<tr>
<td></td>
<td>AGAACCTTTGTAGTCACGAAACC</td>
</tr>
</tbody>
</table>
min (13000 rpm at 4 °C), and supernatants were used for glutathione determination. For total GSH determination supernatant was used directly in 200 μl of total volume reaction as described in (Griffith, 1980).

2.3 Results

2.3.1 Characterisation of organic rehydration nutrients supplementation effects on volatile sulfur compounds composition

To examine the effect of organic rehydration nutrients on fermentation derived aroma compounds grape juice was fermented using ADY rehydrated in water or a commercially available rehydration nutrient mixture. Rehydration nutrient mix was prepared from IDY and contained an organic nitrogen source (mostly as amino acids) as well as other yeast constituents such as vitamins and lipids. As an additional point of reference inorganic nitrogen in the form of DAP was added directly to the fermentation medium. Resultant wines were analysed for volatile chemical composition (Figure 2.1). Concentration of 3MH and 3MHA increased with the addition of rehydration nutrient while the concentration of H₂S was significantly decreased. Other sulfur compounds including 4MMP were not affected by rehydration nutrient supplementation and we did not observe an effect on the production of other aroma compounds such as esters, higher alcohols and acids (p>0.05) (Appendix A-2.1). Rehydration nutrient supplementation also had no effect on growth rate or fermentation kinetics (Appendix A-2.2) Addition of DAP stimulated growth and fermentation rates and resulted in an increased concentration of 4MMP (Figure 2.1) and acetate esters (Appendix A-2.1), while the concentration of higher alcohols was decreased (Appendix A-2.1).

The effect of organic rehydration nutrients on the formation of volatile sulfur compounds during fermentation was monitored. Addition of rehydration nutrients resulted in an earlier onset and increased initial production of H₂S while DAP addition delayed H₂S liberation (Figure 2.1c). To test whether the rehydration nutrient effect could be attributed to YAN concentration medium YAN concentration was analysed following ADY rehydration with either water or nutrient supplementation. As demonstrated in figure 2.1d, both treatments exhibited the same YAN consumption Therefore, the increased initial production of H₂S was not correlated with available nitrogen concentration during fermentation.
2.3.2 Rehydration nutrient effects on gene expression profile

In order to gain insights into the mechanism by which rehydration nutrients affect H$_2$S formation the global transcription response for each of the nutrient treatments was analysed. RNA was extracted from samples taken after consumption of ~15 g/L of sugar from the grape juice. This sampling time corresponded with the initial increase in H$_2$S observed following rehydration nutrient supplementation (Figure 2.1c). Overall analysis revealed two principal components explaining 73% of the variation in gene expression (Figure 2.2a). This distribution indicates distinct effects of DAP and the rehydration nutrient mix upon the transcriptome. Classification of the genes to MIPS functional categories (Robinson et al., 2002) revealed that both treatments affected the same groups of genes, therefore the variation explained by the PC analysis was a result of differential effects upon the same gene networks (Figure 2.2b).

Organic rehydration nutrient mix supplementation downregulated the expression of genes involved in the biosynthesis of various amino acids and vitamin/cofactor transport (Figure 2.2b), consistent with its composition. Interestingly, amongst the downregulated genes were those involved in H$_2$S formation through the biosynthesis of the sulfur-containing amino acids and the sulfate assimilation pathway (Figure 2.2c).

Addition of DAP, in contrast, upregulated approximately 67% of the genes involved in sulfate assimilation and the synthesis of the sulfur-containing amino acids (Figure 2.2c). These results appear to conflict with the previous phenotypic observations at the sampling point where rehydration nutrients supplementation induced the formation of H$_2$S while the addition of DAP delayed it (Figure 2.1c). Nonetheless, these results support the hypothesis of distinct effects for each of the treatments and further suggest the presence of an additional organic nutrient factor regulating the formation of H$_2$S.

Confirmation of the microarray results was obtained by an independent transcription analysis using qRT-PCR for samples taken at the same point in fermentation used for the microarray analysis. GPM1 and TDH3 were selected as reference genes based on data obtained from the microarray analyses; both genes
Figure 2.1 Effects of nutrients addition on the final concentration of volatile sulfur compounds. Analysis of VSCs (A) and polyfunctional thiols (B) concentration at the end of fermentation. Nutrient treatments included supplementation of rehydration nutrients to the rehydration media (nutrient mix) or supplementation of DAP to the fermentation media (DAP) or no nutrients addition (control). Letters represent statistical significance at the 95% confidence level, as tested by Student t statistical test. C Profile of H₂S production in the headspace during fermentation. Upper panel shows a more detailed profile of H₂S formation in the early stage of a separate fermentation experiment. H₂S formation was measured using gas detection tubes D H₂S formation and YAN consumption profile during the early stages of fermentation. Fermentations were carried out in triplicate, error bars represent standard deviation.
showed high expression values and minimal variation between the different treatments. Genes related to sulfur metabolism that exhibited different trends of expressions between the treatments were chosen for validation (genes and primers are listed in Table 2.1). GPM1 and TDH3 transcript levels were similar for all treatments, as expected based on the microarray results. OPT1 was upregulated by 1.75 fold following rehydration nutrient supplementation and downregulated by 11 fold following DAP addition. MET10 was downregulated under all nutrient treatments and IRC7 was downregulated by 4.2 fold with the addition of DAP, consistent with its regulation by nitrogen catabolite repression (Scherens et al., 2006; Thibon et al., 2008) (Figure 2.3).

2.3.3 Nutrient regulation of H$_2$S formation

In addition to being affected by the general YAN concentration of the media, H$_2$S formation is regulated by specific amino acids (Jiranek et al., 1995b; Duan et al., 2004; Li et al., 2009). Rehydration of ADY in a solution containing an amino acid composition equivalent to that of the nutrient mix (Table 2.2) did not affect the kinetics of H$_2$S formation significantly (Figure 2.4a). This result suggests that amino acids were not responsible for the altered kinetics of H$_2$S formation, observed following rehydration nutrient supplementation.

Another nutrient that is a potential source for H$_2$S formation is the tripeptide GSH which can also serve as a source of organic nitrogen (sections 1.3.2.2 and 1.3.5.2). Analysis of the rehydration nutrient mixture revealed a concentration of 500 mg/L glutathione equivalent (GSH + GSSG). Furthermore, the GSH cellular content of ADY following rehydration with the nutrient mixture was approximately 1.8 fold higher than ADY rehydrated with water (Figure 2.4b). Addition of GSH as a sole nutrient during rehydration of ADY led to a significant change in H$_2$S formation kinetics and a higher cumulative amount of H$_2$S produced during fermentation (Figure 2.4c). This confirms that GSH, taken up during ADY rehydration, acts as a modulator of H$_2$S production during fermentation.
Amino Acids metabolism

- Metabolism of the cysteine - aromatic group
- Metabolism of methionine [01.01.06.05.01]
- Metabolism of arginine [01.01.06.05.01]
- Metabolism of nonprotein amino acids
- Aromate anabolism [01.05.11.04]
- Metabolism of urea (urea cycle) [01.01.05.03]
- Degradation of glycine [01.01.09.01.02]
- Biosynthesis of serine [01.01.09.02.01]
- Biosynthesis of arginine [01.01.03.05.01]
- Biosynthesis of methionine [01.01.06.05.01]

Sulfur metabolism

- Amino acid/amino acid derivatives transport
- Ribosomal proteins [12.01.01]
- Ribosome biogenesis [12.01]
- rRNA processing [11.04.01]
- Ribosomal proteins [12.01.01]
- Metabolism of energy reserves [02.19]
- rRNA processing [11.04.01]
- Translation [01.01.09.02.01]
- Amino acid/amino acid derivatives transport

MIPS functional category

- Metabolism of the cysteine - aromatic group
- Metabolism of methionine [01.01.06.05.01]
- Metabolism of nonprotein amino acids
- Aromate anabolism [01.05.11.04]
- Metabolism of urea (urea cycle) [01.01.05.03]
- Degradation of glycine [01.01.09.01.02]
- Biosynthesis of serine [01.01.09.02.01]
- Biosynthesis of arginine [01.01.03.05.01]
- Biosynthesis of methionine [01.01.06.05.01]

% of total genes in given category

- % up
- % down
Figure 2.2 Nutrient supplementation effects on gene expression. (A) Biplot of a principal component analysis performed on the interaction between the factor gene and treatment. All 10,928 probe sets from the datasets were used in the analysis. (B) Classification of the genes affected by the rehydration nutrient addition to MIPS functional categories. Bars represent percentage of affected genes out of total genes in category. (C) Schematic representation of the sulfur metabolism pathway and its regulation by the two nutrient treatments (N- rehydration nutrient addition, D- DAP addition) in comparison to the control treatment.
Figure 2.3 qRT-PCR validation of microarray results. qRT-PCR analysis of GPM1, TDH3, OPT1, MET10 and IRC7 mRNA level. Expression values were calculated using the $2^{-\Delta\Delta ct}$ method and normalised to the reference genes GPM1 and TDH3. Fermentations were carried out in triplicate, error bars represent standard deviation.
2.4 Discussion

Supplementation of rehydration nutrients has become a common practice in oenology because it generally improves yeast fermentation performance in suboptimal juices. This chapter compared the volatile composition of wines prepared from a low YAN juice by fermentation with ADY rehydrated with either an organic rehydration nutrient mixture or water. Rehydration nutrients supplementation affected the concentration of volatile sulfur compounds produced during fermentation (Figure 2.1) and the regulation of genes involved in sulfur metabolism (Figure 2.2). Importantly, the sheer nutrient contribution carried over to the fermentation medium at inoculation (following rehydration with nutrients) did not have an effect on the wine volatile composition (Appendix A-2.3).

2.4.1 Effects of organic rehydration nutrient supplementation on polyfunctional thiol release

In this study 3MH and 3MHA concentrations were increased with the addition of rehydration nutrients (Figure 2.1). Unlike 3MH and 3MHA, the concentration of 4MMP was not affected by the addition of nutrients at rehydration, while it significantly increased following DAP addition. This result suggests that different mechanisms may be regulating thiols release from each precursor. This is supported by the characterisation of Irc7p as a main contributor to 4MMP release but not 3MH (Thibon et al., 2008; Roncoroni et al., 2011). It is therefore reasonable to hypothesize that the different nutrient treatments in this study differentially regulated

Aside from bioconversion of precursors, thiols concentration in wine is strongly affected by wine chemical composition (Dubourdieu et al., 2006; Ugliano et al., 2011). It is also possible that the nutrients added to the fermentation led to an altered wine composition in a manner affecting thiols stability. In that case, the chemical difference between 3MH and 4MMP would account for their distinctive responses to each nutrient treatment.
thiol release for each precursor type. Interestingly, while transcription analyses results were consistent with previous studies showing the downregulation of IRC7 by the NCR pathway (activated under nitrogen rich conditions), we observed an increased concentration of 4MMP in response to DAP addition; although it cannot be ruled out that IRC7 expression may have changed throughout the fermentation.
Figure 2.4 **GSH uptake during rehydration and subsequent effects on H$_2$S production during fermentation.** A. Profile of H$_2$S production in the headspace during fermentation following rehydration with a laboratory-made amino acids solution equivalent to the amino acid component of the rehydration nutrient mix. B. GSH cellular content of ADY following rehydration with water or rehydration nutrient mix. Experiments were conducted in triplicates; results are presented as percentage of the control treatment. C. Profile of H$_2$S production in the headspace during fermentation following rehydration with 500 mg/L GSH. All fermentations were conducted in triplicates. H$_2$S formation was measured using gas detection tubes. Error bars represent standard deviation.
2.4.2 Effects of organic rehydration nutrient supplementation on \( \text{H}_2\text{S} \) formation

An important class of sulfur compounds include those contributing to a ‘reductive’ wine aroma (section 1.3). A prominent compound of that group is \( \text{H}_2\text{S} \). Although the subject of \( \text{H}_2\text{S} \) formation during fermentation is well studied, the factors leading to residual \( \text{H}_2\text{S} \) in the final wine remain to be elucidated. Previous studies have linked \( \text{H}_2\text{S} \) formation kinetics with the amount of residual \( \text{H}_2\text{S} \) in wine (section 1.3.4). Results from this study show that rehydration nutrient supplementation decreased the amount of residual \( \text{H}_2\text{S} \) and affected \( \text{H}_2\text{S} \) kinetics during fermentation. It can be speculated that the decreased residual \( \text{H}_2\text{S} \) in the final wine may result from this altered kinetics. Nonetheless, further study is needed in order to link between the two effects and to understand the factors affecting \( \text{H}_2\text{S} \) during fermentation.

\( \text{H}_2\text{S} \) formation during fermentation is considered to be mainly regulated by YAN concentration in the fermentation medium (section 1.3.5.1). Monitoring of \( \text{H}_2\text{S} \) formation following organic nutrient supplementation revealed non-YAN mediated effect on \( \text{H}_2\text{S} \) kinetics during fermentation (Figure 2.1d). This suggests that nitrogen deficiency is not a sole regulator of \( \text{H}_2\text{S} \) formation, which is in agreement with recent studies (Moreira et al., 2002; Linderholm et al., 2008; Ugliano et al., 2010). Subsequent transcription analyses supported this observation and confirmed that regulation of \( \text{H}_2\text{S} \) formation by rehydration nutrients did not involve the sulfate assimilation pathway as this pathway was down-regulated in response to rehydration nutrient supplementation (Figure 2.2c). In contrast, the same pathway was upregulated following DAP addition to the fermentation medium, in accordance with previous reports in the literature (Marks et al., 2003; Mendes-Ferreira et al., 2010b). Together, these results suggest that \( \text{H}_2\text{S} \) produced following the organic nutrient supplementation was formed via an alternative biochemical route.

2.4.3 GSH contribution to \( \text{H}_2\text{S} \) formation

A potential activator of that route would be GSH (section 1.3.2.2). The nutrient mixture contained a substantial component of GSH, demonstrated to be uptaken up by yeasts during rehydration (Figure 2.4b). Additionally, supplementation of GSH to the rehydration medium altered the kinetics of \( \text{H}_2\text{S} \) formation during fermentation (Figure 2.4c). Interestingly, other components of the nutrient mixture may have had a
significant effect on yeast metabolic responses to GSH supplementation during rehydration. When GSH was added as a component of the nutrient mix, changes in H$_2$S kinetics occurred during the early stage of fermentation but did not affect the final amount of H$_2$S produced during fermentation (Figure 2.1c). By contrast, rehydration with GSH supplemented in isolation resulted in a change in H$_2$S kinetics throughout the fermentation process and led to a higher cumulative production of H$_2$S. This variation may be associated with differences in the uptake of GSH from each medium, or reactivity of GSH with other substances of the nutrient mixture.

Nonetheless, these experiments are first to demonstrate the effect of GSH supplementation at rehydration on H$_2$S formation during fermentation. It is worth noting that previous studies indicated a concentration of ~50 mg/L GSH in the grape juice is required to detect H$_2$S formation from GSH (Rauhut, 2009). In this study, the concentration of GSH carried over from the rehydration media to the grape juice was less than 1 µg/L, highlighting the importance of glutathione uptake during rehydration.

The mechanism of GSH contribution to H$_2$S formation during alcoholic fermentation has not been elucidated. GSH contains both nitrogen and sulfur constituents, who may regulate the formation of H$_2$S in different manners (section 1.3.2). Addition of organic nitrogen to the rehydration medium as an amino acid mixture did not result in changes in H$_2$S kinetics during fermentation (Figure 2.4a), suggesting that organic nitrogen by itself did not contribute to H$_2$S formation, when added at rehydration. This result points to the sulfur constituent of GSH, cysteine, as a contributor to H$_2$S formation. Direct production of H$_2$S from cysteine has been demonstrated previously for S. cerevisiae (section 1.3.2.1). Accordingly, the mechanism suggested here for H$_2$S formation from GSH requires GSH degradation to its individual amino acids, followed by degradation of cysteine to H$_2$S by an enzyme having a cysteine desulfuhydrase activity. This mechanism is in accordance with our phenotypic and transcriptomic results as it describes non-YAN mediated regulation on H$_2$S formation, not through the sulfate assimilation pathway.

2.5 Conclusions and future directions

As wine quality can be greatly affected by the composition of sulfur compounds, this study demonstrates a potential approach for sulfur aroma management by
optimising yeast rehydration conditions and providing nutrients at rehydration. This study also highlights the important role of organic sulfur compounds present in the fermentation medium and their modulation of VSCs in the final wine. These factors have been largely overlooked in the study of sulfur aroma development and will be the focus of the next chapters of this thesis. Chapter 3 will focus on polyfunctional thiols and the availability of different thiol conjugate precursors towards yeasts. Based on the results obtained here, suggesting GSH contributes to H$_2$S formation through its cysteine component, chapter 4 and 5 will further investigate this metabolic pathway and aim to characterise the cellular processes involved in cysteine degradation to H$_2$S.
Chapter 3.

The contribution of cysteine and glutathione conjugates in the formation of the volatile thiols 3MH and 3MHA during fermentation by Saccharomyces cerevisiae

3.1 Introduction and aims

The polyfunctional thiols 3MH and 3MHA have been identified as major contributors to wine aroma (Tominaga et al. 1998b; Dubourdieu et al. 2006). 3MH is present in grape juice as a non-volatile cysteine-conjugated precursor (Tominaga et al. 1998a). During fermentation, the precursor is cleaved and the free thiol is released as a result of yeast metabolic activity (section 1.3). Chapter 2 demonstrated an increase in 3MH concentration as a result of organic nutrient supplementation and raised the hypothesis this increase may be due to increased yeast thiol-release activity, following nutrient supplementation.

An important unresolved question concerning thiol-precursors conversion and its regulation by nutritional factors regards the types of precursors available in the grape juice and yeasts’ ability to metabolise them during fermentation. Two non-volatile 3MH conjugates have been identified; Cys-3MH (Tominaga et al., 1998a) and GSH-3MH (Peyrot des Gachons et al., 2002). Additionally, an alternative biogenetic pathway from (E)-hex-2-enal leading to 3MH was shown (Schneider et al., 2006). Subileau et al. (2008) found that neither (E)-hex-2-enal nor Cys-3MH were responsible for the majority of 3MH and 3MHA in wine and they suggested GSH-3MH as the main contributor. Nevertheless this evidence regarding GSH-3MH contribution was obtained indirectly and a direct comparison of the two precursors is needed in order to understand the relations between them. Moreover, as the ability of yeast to generate 3MH from GSH-3MH has been recently shown (Roland et al. 2010; Grant-Preece et al. 2010), the importance of this precursors to the formation of the powerful odorant 3MHA remains to be established. Having an odour threshold of 4 ng/L, 3MHA is one of the most powerful odorants known, and is a major contributor to the distinct aroma characters of many red and white wines (Mateo-Vivaracho et al. 2009).
This chapter examines the ability of yeast to metabolise GSH-3MH and compare the potential of this process to yield 3MH and 3MHA with the conversion of Cys-3MH. Understanding yeast preference towards each of the precursor will provide knowledge towards better modulation of thiols concentration in wine.

Work described in this chapter was published as research paper in the journal ‘Australian Journal for Grape and Wine Research’ (Appendix A-1.2)

3.2 Materials and methods

3.2.1 Chemicals

Analytical reagents have been purchased from Sigma-Aldrich unless otherwise specified. Cys-3MH synthesis is described in Pardon et al. (2008) and GSH-3MH was synthesized according to Capone et al. (2010).

3.2.2 Culture media, yeast strain, and fermentation conditions

Fermentations were conducted in triplicate, with a working volume of 1 L under isothermal conditions (20 °C) and agitation (150 rpm). A chemically defined grape juice based on Henschke and Jiranek (2003) was used with the following modifications: 1.5 g/L magnesium sulphate heptahydrate, 1 mg/L pyroxidine HCl, 1 mg/L nicotinic acid, 0.5 mg/L inositol, 0.05 mg/L biotin and 0.05 mg/L folic acid. Nitrogen component of the media was 13.3 mg/L alanine, 9.1 mg/L γ-amino butyrate, 34.2 mg/L arginine, 0.5 mg/L asparagine, 3.8 mg/L aspartate, 0.5 mg/L citrulline, 7.6 mg/L glutamate, 10.6 mg/L glutamine, 0.5 mg/L glycine, 1.5 mg/L histidine, 1.5 mg/L isoleucine, 1.5 mg/L leucine, 0.5 mg/L lysine, 0.5 mg/L methionine, 0.5 mg/L ornithine, 1.1 mg/L phenylalanine, 6.8 mg/L serine, 7.6 mg/L threonine, 0.5 mg/L tryptophan, 0.5 mg/L tyrosine, 2.7 mg/L valine, 1.5 mg/L cysteine, 82.1 mg/L proline and 11.6 mg/L ammonia solution. YAN value of the media was 155 mg N/L (68 mg FAN/L and 106 mg NH₃/L). For the different precursor treatments, media was supplemented with either Cys-3MH or GSH-3MH as specified in Table 3.1. The yeast strain used in this study was Saccharomyces cerevisiae VL3 from Laffort Australia. Active dry yeasts were rehydrated as recommended by the manufacturer (http://www.laffort.com/) and then inoculated into the fermentation media to give a cell concentration of 1 x 10⁶ cells/ml. Fermentation progress was monitored by measuring sugar consumption using a
commercial kit by Randox (catalogue number GF320, http://www.randox.com/food%20and%20wine.php). Free SO₂ value of the resultant wine was adjusted by the addition of potassium metabisulfite to 45 mg/L as measured by Rankine and Pocock (1970).

### 3.2.3 Analysis of 3MH and 3MHA

3MH and 3MHA were measured by SARCO Laboratories (Bordeaux, France) according to Tominaga et al. (2000). Molar conversion yields were calculated as the total concentration of 3MH and 3MHA (nM) measured at the end of fermentation divided by the concentration of potential 3MH (the relative part of 3MH from its conjugate, nM) added to the media. 3MH esterification ratios were calculated as 3MHA concentration (nM) divided by the total concentration of 3MH and 3MHA (nM).

**Table 3.1: 3MH amino acid conjugates supplementation**

<table>
<thead>
<tr>
<th>Precursor Type</th>
<th>Molecular Weight (g/mol)</th>
<th>Supplemented amount (µg/L)</th>
<th>Potential 3MH (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-3-(hexan-1-ol)-L-cysteine</td>
<td>221.32</td>
<td>500</td>
<td>303.5</td>
</tr>
<tr>
<td>S-3-(hexan-1-ol)-glutathione</td>
<td>409.1</td>
<td>925</td>
<td>303.5</td>
</tr>
</tbody>
</table>

### 3.2.4 Carbon sulfur β-lyase assay

The assay mixture contained 110 µg Cys-3MH or 378 µg GSH-3MH, 20 µl 1 mM pyridoxal 5’-phosphate and 500 µl assay buffer (50 mM phosphate buffer, pH 7.5, 1 mM EDTA). Distilled H₂O was added to obtain a volume of 1 ml per reaction. Twenty µl of tryptophanase enzyme (Aportryptophanase from Escherichia coli, 10
µg/µl) was added and the reaction was incubated at 37 °C for 60 minutes. Following incubation, 100 µl 1 mM Ellman’s reagent [5,5-dithiobis-(2-nitrobenzoic acid)] was added and absorbance was measured at 412 nm to give an indication of the formation of thiols during the reaction. Experiments were conducted in 3 technical triplicates.

3.3 Results

3.3.1 Molar conversion rate of Cys-3MH and GSH-3MH

To assess whether GSH-3MH is metabolised by yeast under oenological conditions, chemically-pure synthetic GSH-3MH was supplemented to a chemically-defined grape juice medium, which was then fermented by yeast. A separate fermentation was supplemented with Cys-3MH in equimolar concentrations of potential 3MH (Table 3.1), to compare the conversion rates of GSH-3MH with those of Cys-3MH. Figure 3.1 shows fermentation kinetics for the different fermentation conditions of this study. Precursor supplementation had no effect on biomass formation or fermentation rate, despite each 3MH conjugate contributing yeast nutrients in the form of GSH and cysteine.

To assess the conversion yields for the two precursors, concentrations of 3MH and 3MHA were measured at the end of fermentation. Molar conversion yields were calculated under the assumption that both precursors were fully consumed from the medium and products (i.e. 3MH, 3MHA) were not (partially) converted to other (unknown) products that were not measured. Importantly, no 3MH or 3MHA were detected in control fermentations that were not supplemented with 3MH precursors. Figure 3.2 shows a molar conversion yield of ca. 1% for Cys-3MH and a value of ca. 0.5% for GSH-3MH. This result indicates that Cys-3MH is a more favourable substrate for the formation of 3MH under the fermentation conditions used. Interestingly, no significant differences were observed in 3MHA concentrations obtained using the two precursors (Figure 3.2).
Figure 3.1 **Fermentation kinetics following 3MH precursor supplementation.** Alcoholic fermentation parameters, sugar consumption (A) and biomass formation (B). Fermentations were supplemented with Cys-3MH (white diamond), GSH-3MH (black diamond) or a control with no additions (black triangle). Fermentations were carried out in triplicate, error bars represent standard deviation.
3.3.2 In vitro assessment of 3MH direct release from GSH-3MH

The difference in conversion rate for the two precursors may be attributed to differences in the conversion of each precursor. Conversion of Cys-3MH is detailed in section 1.4.2. In short, following its uptake, the cysteine conjugated precursor is cleaved by an enzyme having a carbon-sulfur \( \beta \)-lyase activity to release the free volatile thiol in a one-step reaction. The pathway for GSH conjugate thiol release has not been characterised and there are two possible pathways that can be reasonably hypothesised. One is through a direct, one-step cleavage of 3MH from GSH-3MH, analogous to the direct reaction observed with the cysteine precursor. A second putative pathway includes a multi-step degradation process, first step releases the cysteine conjugate and second step includes release of the free thiol (section 1.4.2).

To test whether 3MH may be released from its glutathione conjugate via a one-step reaction, an in vitro enzymatic assay was performed using a carbon sulphur \( \beta \)-lyase enzyme, previously shown to have high efficacy towards the cysteine conjugate of 3MH (Wakabayashi et al. 2003; Swiegers et al. 2007). Figure 3.3 shows that while the enzyme cleaves the cysteine precursor, it has no activity towards the glutathione precursor each 3MH precursor contributing different amino acids to the medium, it can be postulated that it is the nitrogen fraction of each precursor that affects 3MHA formation. It is important to note though that in this experiment, precursor supplementation was less than 1 mg/L (Table 3.1). While this extent of nutrient supplementation is probably too minor to account for any differences in fermentation progress (measured in g/L of sugar consumption, Figure 3.1), it may account for the more attenuated differences in 3MH esterification ratio (measured in ng/L). Further studies are needed to understand the factors affecting 3MHA formation and the degree of nutrient regulation on this process.
Figure 3.2. **Cys-3MH and GSH-3MH molar conversion yields.** 3MH (sparse bars) and 3MHA (dense bars) concentration at the end of alcoholic fermentation supplemented with either Cys-3MH or GSH-3MH. Molar conversion yields of 3MH+3MHA from Cys-3MH or GSH-3MH (black star) and 3MH esterification ratios (white stars). fermentations were conducted in triplicates, error bars represent standard deviation.
3.4 Discussion

3.4.1 Comparison between molar conversion yields of Cys and GSH-3MH

This chapter included the direct comparison of conversion yields for the two 3MH precursors Cys-3MH and GSH-3MH. Conversion yield obtained here for Cys-3MH is in agreement with data previously published (Murat et al., 2001; Subileau et al., 2008a) whereas this is the first time the conversion yield of GSH-3MH to 3MH and 3MHA is estimated. This result clearly demonstrates the metabolism of GSH-3MH by yeast to release 3MH during fermentation. Nonetheless, when comparing conversion yields for the two precursors, the amount of 3MH obtained from Cys-3MH is considerably higher than that obtained from GSH-3MH. This result indicates that Cys-3MH is a preferable substrate for the formation of 3MH under the fermentation conditions used.

As 3MH concentration was significantly different for each treatment, the similarity in 3MHA concentration indicates a difference in the esterification ratios (3MHA concentration / total 3MH + 3MHA concentration) of 3MH for each precursor. In this case, opposite to the conversion yields for 3MH release, 3MH esterification ratios were higher for GSH-3MH (~2.3%) than Cys-3MH (~1.4%). This implies that the formation of 3MHA is not regulated by 3MH concentration.

Indeed, acetate ester formation is generally agreed to be mainly influenced by the expression levels of the esterifying enzymes ATF1 and ATF2, rather than by substrate availability (Lilly et al., 2000; Verstrepen et al., 2003b). Genetic regulation of these enzymes is mediated through nutritional factors including YAN concentration (Yoshimoto et al., 2002; Vilanova et al., 2007). Thus with
Figure 3.3. **Enzymatic release of 3MH from Cys-3MH and GSH-3MH using Cysteine β-lyase assay.** Ellman’s reagent reaction with free thiols forms a yellow complex (absorbance measured at 412 nm, white bars). The same letters in parentheses indicate homogeneous groups at the 95% confidence level, according to the Tukey honestly significant difference test.
3.4.2 Differences in conversion pathways may account for differences in conversion yields for the two precursors

While the conversion pathway for cysteine conjugated thiols is under general agreement, the pathway for GSH conjugated precursor remains to be elucidated. An in vitro assessment of thiol release from this precursor showed no direct activity of a carbon-sulfur β-lyase enzyme on glutathionylated thiol precursor. This result supports the hypothesis of a multi-step process for the release of 3MH from GSH-3MH. Indeed, this view is in agreement with reports regarding catabolism of xenobiotic glutathione conjugates by yeast, that have been shown to involve degradation of the tripeptide to individual amino acids (Ubiyvovk et al. 2006; Wünschmann et al. 2010). We can therefore hypothesise that the conversion pathway for GSH conjugate thiols is longer and more complex and therefore the cysteine precursor seems to be more favourable for yeast during fermentation.

In addition to different conversion pathways, other factors may account for the observed difference in 3MH conversion yields. An important one to consider involves differences in the uptake for the two precursors, as each precursor is being uptaken by different transporters (detailed in 1.3.1). Differences in transport efficiency are likely to carry different amount of precursors which may affect the final conversion rate. Additionally, each transporter is subjected to a different regulation mechanism which may affect the timing of precursor uptake. This is of particular importance to the final conversion rate as 3MH release and subsequently esterification are reported to occur to a higher extent during the early stages of fermentation (Tominaga et al. 1998b; Tominaga et al. 2006).

Another aspect to consider is 3MH metabolism. Aside from esterification, 3MH may participate in other, unknown reactions. These reactions may well be affected by the precursor type as we observed here for the esterification ratio (Figure 3.2). Finally, the different conversion rates may be attributed to differences in chemical stability of the precursors in the fermentation medium or reactivity of each precursor with other substances present in the medium or inside the cell. In order to determine which factors ultimately affect precursor conversion yields further studies are needed.
3.5 Conclusions and future directions

This chapter demonstrated that yeast metabolic conversion of the GSH conjugate 3MH can generate 3MH and subsequently 3MHA. Interestingly, the conversion process of GSH-3MH was found to be significantly less efficient than that of Cys-3MH. Noteworthy, during wine fermentation, the low molecular yield observed for GSH-3MH may be compensated by its high abundance, which in some grape juices has been reported to be up to 35 times higher than that of Cys-3MH (Capone et al. 2010).

Most importantly, the experiments in this chapter were carried out using chemically defined medium. The nutrient composition of the grape juice may have a critical role in the uptake and metabolism of each precursor, and thus change yeast preference and relative precursor contribution. For example, in chapter 2, supplementation with a GSH-containing complex nutrient led to an upregulation of GSH transporters in the plasma membrane (section 2.3.2). If GSH-3MH is being taken up by the same transporters then it is possible that GSH supplementation will enhance GSH-3MH uptake and lead to a greater thiol release from the GSH precursor.

Finally, while the results of this study demonstrate that, under model conditions resembling those of wine fermentation, GSH-3MH is a possible precursor for 3MH and 3MHA, the question remains as to which is the main precursor for 3MH in wine fermentations. To answer that, further studies are required, to investigate the amount of individual precursor in the grape juice, the efficiency of precursor conversion to the free volatile form and the existence of other types of 3MH precursors in the grape.
Chapter 4- Preface

Organic sulfur compounds such as GSH and cysteine can also function as precursors to the formation of H$_2$S (section 1.3.2). In chapter 2 we demonstrated the contribution of GSH to H$_2$S formation during fermentation, even when supplemented during ADY rehydration. Our results inferred that the cysteine component of GSH was the sulfur source responsible for H$_2$S release. Direct degradation of cysteine to H$_2$S has been previously shown in yeasts (Wainwright, 1971; Tokuyama et al., 1973). Still, the cellular mechanisms involved in this process remain to be characterized. We therefore aimed to identify the cellular processes affecting cysteine degradation to H$_2$S using a genomic screen of the yeast genome-wine deletion collection (Winzeler et al., 1999).

A genomic screen for cysteine degradation to H$_2$S requires the use of a high throughput method for H$_2$S detection, able to differentiate whether H$_2$S is produced from cysteine or other sulfur sources in the media. Current methods to assess H$_2$S formation during fermentation are limited in their ability to meet these requirements (section 1.5). Thus a new methodology for H$_2$S detection was developed, suitable for high throughput assays and able to differentiate H$_2$S formation from different sulfur sources. The method was filed by The University of Western Sydney for Australian provisional patent protection (Appendix A-1.3) and was published in “Journal of Microbiological Methods” (Appendix A-1.4). Development of the method is described in chapter 4 and its implementation in a genomic screen for cysteine degradation to H$_2$S is described in chapter 5.
Chapter 4.

In situ high throughput method for H$_2$S detection during fermentation

4.1 Introduction and aims

As a major contributor to the aroma of fermented foods H$_2$S has been the focus of a considerable amount of research. However, due to its volatile nature and reactivity, available methods for H$_2$S detection are limited. Current methods employed to estimate H$_2$S production potential, or detect H$_2$S formation during a single fermentation are reviewed in section 1.3.3. In short, Qualitative methods include the use of bismuth sulfite agar plates (Giudici and Kunkee, 1994b) or lead acetate strips (Giudici and Kunkee, 1994b), while quantitative methods involve capture of released H$_2$S in a cadmium or zinc trap (Cd(OH)$_2$, ZnO) followed by quantification with methylene blue colorimetric reaction (Brenner et al., 1954; Acree et al., 1971; Jiranek et al., 1995b; Lopez del Castillo Lozano et al., 2007), or capture and quantification in lead acetate detection tubes (Park, 2008).

While these methods suffice for a single fermentation analysis, they do not display high throughput capabilities, suitable for an accurate screen of a large microbial strain collection such as the yeast deletion collection. A current high throughput method for sulfide detection uses a membrane impregnated with silver nitrate (Duan et al., 2004). However, that method is limited in sensitivity and automaticity and does not detect natural levels of H$_2$S produced during micro-scale fermentation, requiring the addition of the sulfur containing amino acid cysteine to the media (section 1.3.3).

In this chapter we aim to develop a new screening methodology for H$_2$S detection that would be compatible with robotic handling systems for screening large numbers of microbial strains. We sought to develop a method applicable to the study of H$_2$S produced from organic sulfur sources such as cysteine and GSH, able to differentiate and between organic inorganic sources leading to H$_2$S production in various fermentation systems.

4.2 Materials and Methods
4.2.1 Reagents

Chemical reagents were obtained from Sigma-Aldrich. Detection tubes for $\text{H}_2\text{S}$ detection were obtained from Komyo Kitagawa (Tokyo, Japan).

4.2.2 Strains and growth conditions

$\text{S. cerevisiae}$ strain AWRI 1631 was used for assay development. Assay validation was carried out using strains: $\text{S. cerevisiae}$ (AWRI 796, AWRI 1483, AWRI 1616, AWRI 1639 and BY4742). Medium used was chemically defined model grape juice as described in section 2.2.3. Cysteine supplementation was at 500 mg/L and DAP supplementation was 940 mg/L.

4.2.3 Development of $\text{H}_2\text{S}$ detection assay

Cells were pre-grown to stationary phase in 10 ml YPD (1% w/v yeast extract, 2% w/v peptone and 2% w/v glucose) or LB with shaking (150 rpm) prior to inoculation. Fermentations were conducted in a microtiter plate at a total volume of 200 µl per well. Each well contained 170 µl of medium, 10 µl of microbial cell culture to give a final optical density of 0.3-0.5 at 600 nm wavelength and 20 µl of a methylene blue reaction mix containing 5 ml of 1 mg/ml methylene blue, and 5 ml of 100 mM citric acid buffer at pH 4.5. Fermentations were carried out in quadruplicate. Duplicate fermentations were also performed without the reaction mix, to monitor growth rate as measured by the absorbance at 600 nm. Un-inoculated wells containing fermentation media with or without reaction mix were monitored to detect medium contamination and spontaneous methylene blue decolourisation, respectively.

4.2.4 Data analysis

Biomass formation and methylene blue decolourisation data were normalised to create $\text{H}_2\text{S}$ generation profile using the formula \[ \frac{(\text{OD}_{663 t_0} - \text{OD}_{600 t_0}) - (\text{OD}_{663 t} - \text{OD}_{600 t})}{\text{OD}_{600 t_0}, \text{no reaction mix control}}. \] Extraction of kinetic parameters was done via a locally weighted regression (loess) algorithm using a custom R script (Appendix A2.4) (R Development Core Team, 2008).

4.3 Results
4.3.1 Method principles

The method employs a well characterised redox reaction between methylene blue and a sulfide ion, which can be further catalysed by traces amounts of tellurium or selenium (Feigl and West, 1947; Fogo and Popowsky, 1949; Mousavi and Sarlack, 1997). This reaction leads to decolourisation of methylene blue (Figure 4.1a) (Mousavi and Sarlack, 1997). Under acidic conditions, sulfide ions are present as H$_2$S. Incorporation of the methylene blue reaction mix (which includes methylene blue and citric acid buffer, to maintain acidic conditions) into fermentation media allows the immediate in situ detection of H$_2$S produced during fermentation, without affecting fermentation performance (Figure 4.1b). Simultaneous kinetic measurements for H$_2$S generation (indicated by methylene blue decolourisation) and biomass formation (measured for control wells, not supplemented with methylene blue) allow the generation of an H$_2$S production profile. Kinetic parameters extracted from this profile allow the comparison of factors affecting H$_2$S formation.

4.3.2 Development of the assay

The use of micro-scale fermentation was recently found to be a meaningful simulation of wine fermentation (Liccioli et al., 2010). To demonstrate high throughput methodology, fermentations were conducted in a microtiter plate at a total volume of 200 µl per well and included both medium and H$_2$S detection mix. Whilst inclusion of a catalyst (0.01 mg/ml of tellurium dioxide or selenium dioxide) enhanced the sensitivity of H$_2$S detection; during yeast fermentation of synthetic grape juice the relatively high concentrations of H$_2$S produced sufficed for the generation of an accurate H$_2$S formation profile (Figure A-2.5).

Kinetic spectrophotometric measurements throughout fermentation allowed generation of H$_2$S formation profiles, inferred from methylene blue decolourisation. These profiles represent methylene blue decolourisation rate normalized to biomass formation. Comparison of the methylene blue reduction (MBR) method profile with an established quantitative H$_2$S production profile display similar trends for H$_2$S formation during fermentation (Figure 4.2). Both profiles show an initial lag followed by increased rate of formation, an H$_2$S maxima, and then production of smaller amounts of H$_2$S (Figure 4.2). Maximum rate of increase and maximum value were extracted from the MBR method profile via a locally weighted regression
(loess) algorithm using a custom R script (R Development Core Team, 2008) (Figure 4.2). Lag time was defined as the crossing point of H$_2$S profile (A663/A600) with zero, indicative of the start of H$_2$S formation. From that point H$_2$S formation profile is mainly modulated by H$_2$S formation rather than biomass formation. Table 4.1 shows reproducibility of kinetic parameters extracted from inter-plate, triplicate fermentations with the S. cerevisiae wine yeast AWRI 1631. Fermentations were conducted independently, using different inoculation cultures and medium batches. As the chemical reaction equilibrated and H$_2$S production slowed, H$_2$S evaporation (or possible oxidation) altered the rate of the reaction and methylene blue re-colourised within the fermentation media (Figure 4.2). As a consequence, precision of kinetic parameters extracted following the point of maximum H$_2$S detection is limited.

4.3.3 Detection threshold and linearity.

To construct a calibration curve, known volumes of sodium hydrosulfide hydrate solution (final H$_2$S concentration of 1 mg/ml) were added to water supplemented with the methylene blue reaction mix, and were monitored at 663 nm for 30 minutes, to fit the reading intervals of the experimental data presented here. The MBR method displayed linearity between the ranges of 0-50 µg H$_2$S (Figure 4.3). Samples spiked with H$_2$S plus potassium bisulfite (final SO$_2$ concentration of 50 mg/L), dimethyl sulfide (1 mg/ml) or methyl mercapto acetate (1 mg/ml) were also tested under the same conditions. No interference in H$_2$S detection was observed following these additions.

4.3.4 Method application

The use of methylene blue decolourisation as an indicator for sulfide formed during fermentation was verified using different nutritional conditions known to modulate H$_2$S production and using yeast strains previously characterised for their H$_2$S formation capacities. Nutritional conditions included modulation of YAN concentration (Jiranek et al., 1995b) and cysteine concentration (Tokuyama et al., 1973). Quantitative determination of H$_2$S produced during fermentation, using detection tubes (Park, 2008) demonstrated that increasing YAN concentration from 150 mg N/L to 350 mg N/L through the addition of DAP decreased the amount of H$_2$S produced. Addition of freshly made cysteine solution decreased the time of .
Figure 4.1 Incorporation of methylene blue into the fermentation media enables \( \text{H}_2\text{S} \) detection without affecting fermentation rate. **A** Redox reaction between methylene blue and sulfide ion, leading to decolourisation of methylene blue. **B** Fermentation performance measured by CO2 production rate. Triplicate fermentations of AWRI 1631 with (dashed line) or without methylene blue reaction mix (solid line). Error bars represent standard deviation.
Figure 4.2 Analysis of H$_2$S formation during fermentation using MBR method. A Demonstration of methylene blue colour change during micro scale fermentation. Cells from an overnight culture grown in YPD were inoculated into a 96 well plate filled with model grape juice with or without the reaction mix. B H2S formation profile generated using the methylene blue reduction method (left) or using lead acetate detection tubes (right).
Figure 4.3 Method linearity. Known amounts of H2S were added into a 96 well plate filled with model grape juice and the reaction mix. Reactions were carried out in triplicates. Error bars represents standard deviation.
onset for H$_2$S production and increased the amount produced (Figure 4.4a). Concurrent micro-scale fermentations using the MBR method displayed the same trends. DAP addition decreased H$_2$S production maxima while cysteine addition decreased lag time and increased both estimated H$_2$S production rate and maximum value (Figure 4.4b).

MBR H$_2$S formation profiles were also generated for known high and low H$_2$S producing S. cerevisiae wine strains, AWRI 1483 and AWRI 796 (Ugliano et al., 2009), in comparison to AWRI 1631. From 5 hours post-inoculation AWRI 1483 produced H$_2$S at a greater rate and reached a greater maximum value than AWRI 1631 and AWRI 796 (Figure 4.5a), in agreement with data obtained utilising reference quantitative methods (Figure 4.4a and (Ugliano et al., 2009)). Additionally, figure 4.5b demonstrates low production of H$_2$S by a mutant strain impaired in H$_2$S release through the sulfate assimilation pathway (Cordente et al., 2009), compared to the wild type strain. The rapid in situ detection of H$_2$S using the MBR method was further demonstrated by directly supplementing H$_2$S solution to an ongoing fermentation. Addition of H$_2$S after 25 hours of fermentation immediately led to decolourisation of the methylene blue dye (Figure 4.5a), as demonstrated by the sudden peak in H$_2$S production profiles.

### 4.4 Discussion

This chapter demonstrated an effective novel use for a well known chemical reaction between methylene blue and sulfide ion. The MBR method point of uniqueness is in the incorporation of methylene blue to the fermentation medium,

<table>
<thead>
<tr>
<th></th>
<th>Lag time (h)</th>
<th>Increase rate (*AU h$^{-1}$)</th>
<th>Maximum value (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>9.5</td>
<td>0.09</td>
<td>0.83</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>10.92</td>
<td>0.12</td>
<td>0.69</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>9.42</td>
<td>0.096</td>
<td>0.9</td>
</tr>
<tr>
<td>Average (AU)</td>
<td>9.95</td>
<td>0.10</td>
<td>0.81</td>
</tr>
<tr>
<td>CV (%)</td>
<td>8.48</td>
<td>15.56</td>
<td>13.26</td>
</tr>
</tbody>
</table>

*Absorbance Units
Figure 4.4. Detection of differential H2S formation in response to environmental factors. Different media composition including model grape juice (black diamonds) supplemented with 200 mg N/L (grey squares) or 500 mg/L cysteine (grey triangles), were fermented with strain AWRI 1631. A H2S formation profile generated using lead acetate detection tubes. Fermentations were carried out in triplicates. B H2S formation profile generated on a micro-scale fermentation using the methylene blue reduction method. Fermentations were carried out in quadruplicates. Error bars represent standard deviation.
Figure 4.5. **Detection of yeast-strain dependent H$_2$S production.** A H$_2$S formation profile generated using the methylene blue reduction (MBR) method for AWRI 1631 (black diamonds), known high H$_2$S producer, AWRI 1483 (grey squares) and low H$_2$S producer, AWRI 796 (grey triangles). A separate fermentation of AWRI 161 (dashed black line) was supplemented with 50 μg H$_2$S at 25 hours of fermentation (indicated by arrow). B MBR H$_2$S generation profile under nitrogen rich conditions (400 mg YAN/L) for wild type (AWRI1616) and mutant strain (AWRI 1639) impaired in the ability to reduce sulfate to sulfide. Fermentations were carried out in quadruplicates, error bars represent standard deviation.
which allows for in situ detection of \( \text{H}_2\text{S} \) produced. Methylene blue dye is widely used in microbial research mainly as a means to assess microbial viability without affecting it (Trevors et al., 1983; Kucsera et al., 2000). Indeed, its incorporation to the fermentation medium did not affect fermentation rate (Figure 4.1) and was found to be a meaningful indication of \( \text{H}_2\text{S} \) produced.

Strengths of the MBR method are in high throughput applicability and the ability to differentiate different sulfur sources leading to \( \text{H}_2\text{S} \) formation. This method is likely to be more sensitive than other methods measuring \( \text{H}_2\text{S} \) accumulation in the head space. Here the reactant is present in the medium where it can react immediately with \( \text{H}_2\text{S} \) as it is produced, thus the reaction is not dependent on other factors, such as \( \text{CO}_2 \) production (that varies within and between fermentations) to purge \( \text{H}_2\text{S} \) from the medium to the head space. The main limitation of this method is in the re-colourisation of methylene blue (Figure 4.2a), observed once \( \text{H}_2\text{S} \) production slows, which may be due to evaporation of \( \text{H}_2\text{S} \) or possible oxidation of the dye. However, with further characterization of the recolourisation phenomena, the MBR method may be applicable to studies of chemical reactions in wine that occur post-fermentation, that may influence residual \( \text{H}_2\text{S} \).

### 4.5 Conclusions and future directions

This chapter describes an in situ method for \( \text{H}_2\text{S} \) detection during fermentation. All processes in the method can be carried out using robotic systems with liquid handling and multi-well spectrophotometric reading capabilities; therefore the assay is suitable for screening large collections of microorganisms. Kinetic parameters obtained using this method can be successfully used for profiling \( \text{H}_2\text{S} \) formation. Most importantly, the method is able to differentiate between organic and inorganic sulfur sources leading to \( \text{H}_2\text{S} \) formation, based upon \( \text{H}_2\text{S} \) formation kinetics. It is thus well suited for the purpose of a genomic screen to identify genes involved in cysteine degradation to release \( \text{H}_2\text{S} \).
Chapter 5- Preface

This thesis has focused on the formation of H\textsubscript{2}S through yeast GSH and cysteine catabolism, highlighting the relevance of this metabolic pathway to wine and how its modulation may affect wine volatile composition. Cysteine catabolism to release H\textsubscript{2}S carries a broader relevance through the known cytotoxic effects of cysteine accumulation and the prominence H\textsubscript{2}S is gaining as a regulator of many aspects of cell physiology (Reviewed in: Kimura, 2002; Wang, 2002; Stipanuk, 2004).

The yeast S. cerevisiae has been long used as a model organism of the eukaryotic cell. Consequently, yeast genome-wide deletion libraries have been widely used for the study of fundamental cellular processes. The development of a suitable high throughput methodology (described in chapter 4) made it possible to conduct a genome-wide screen of the yeast deletion collection to shed light on the mechanisms involved in cysteine catabolism to release H\textsubscript{2}S. The screening process and results are described in this chapter. Considering the fundamental nature of the process of cysteine catabolism it is presented and discussed here in a broad cell biology context rather than wine fermentation. The applications of yeast cysteine catabolism to the development of wine aroma compounds will be described in an addendum to this chapter.
Chapter 5.  

Formation of hydrogen sulfide from cysteine in  
Saccharomyces cerevisiae:  
Genome wide screen reveals a central role of the vacuole

5.1 Introduction and aims

The concentration of free intracellular cysteine is tightly regulated in eukaryote and prokaryote cells, serving two opposing homeostatic requirements. Cysteine concentration must meet the need for protein synthesis and other essential sulfur containing molecules such as GSH and coenzyme A, while on the other hand cysteine concentration must be kept below the threshold of cytotoxicity (Stipanuk, 2004). The toxicity of excess cysteine was demonstrated in yeasts (Ono et al., 1991; Kumar et al., 2006) and animal models (Lee et al., 2004), and is associated with growth inhibition (Ono et al., 1991), endoplasmic reticulum (ER) stress (Kumar et al., 2006) and various diseases including Parkinson’s disease and Alzheimer’s disease (Heafield et al., 1990). A key product of cysteine catabolism is H$_2$S (Stipanuk, 2004; Chiku et al., 2009; Kabil and Banerjee, 2010), increasingly recognised to be a powerful regulator of many aspects of cell physiology (section 1.2.2.1). Still, despite the increasing interest in H$_2$S generated from cysteine, fundamental questions regarding regulation of cysteine homeostasis remain to be answered.

H$_2$S generation is evolutionarily conserved in all three kingdoms of life (Kabil and Banerjee, 2010). In mammals, endogenous H$_2$S is presumed to be generated through the catabolism of cysteine by two cytosolic enzymes cystathionine $\beta$-lyase (CBS) and cystathionine $\gamma$-lyase (CSE) (section 1.3.2.1) (Wang, 2002; Chen et al., 2004; Stipanuk, 2004; Chiku et al., 2009; Beard and Bearden, 2011; Singh and Banerjee, 2011). Regulation of expression and activity of these enzymes still remain to be elucidated. Consistent with the evolutionary conservation of cysteine catabolism, mammalian CBS- and CSE-encoding genes are similar to those of yeast and bacteria (Barton et al., 1993; Kruger and Cox, 1994; Kruger and Cox, 1995; Shan and Kruger, 1998). Predicted yeast and human CBS proteins share 72% similarity and these have
significant similarity to the predicted rat CBS protein and bacterial cysteine synthase (Kruger and Cox, 1994). Expression of the gene encoding a human CBS in yeast was able to recover cysteine-auxotrophy caused by deletion of the yeast native CBS (Kruger and Cox, 1994). The predicted yeast CSE product was also found to be closely related to rat and E. coli CSE (Barton et al., 1993). These similarities reinforce the utility of S. cerevisiae as a model eukaryotic system to explore cellular homeostasis of cysteine and the catabolism of cysteine to release H₂S.

This chapter’s aim is to screen the S. cerevisiae genome-wide deletion library, to shed light on cellular processes influencing cysteine catabolism. Cysteine catabolism will be estimated through H₂S release using the MBR method developed in chapter 4.

5.2 Materials and methods

5.2.1 Reagents, yeast strains and media composition

Chemical reagents were obtained from Sigma-Aldrich. The S. cerevisiae strains used in this study are derivatives of BY4742 (MATα/his3Δ1/ leu2Δ0/ lys2Δ0/ ura3Δ0) (Brachmann et al., 1998), which were homozygous for the relevant gene deletion. Yeast genome deletion library, constructed as described in (Winzeler et al., 1999) was purchased from Euroscarf (Frankfurt, Germany). Strains were grown in YPD medium and inoculated into chemically defined medium; this medium was based on section 2.2.3 and included the following modifications: 100 g/L sugars (50 g/L glucose, 50 g/L fructose), addition of auxotrophic requirements (20 mg/L uracil, 20 mg/L histidine, 60 mg/L leucine and 30 mg/L lysin), and exclusion of cysteine from the media. Where specified cysteine was supplemented at 500 mg/L, adenine supplementation was 20 mg/L.

5.2.2 H₂S detection

Detection of H₂S was carried out as described in section 4.2.3. For large scale experiments H₂S was detected using silver nitrate selective gas detector tubes (Komyo Kitagawa, Japan), as described in section 2.2.5.

5.2.3 Genome-wide screening for genes involved in cysteine catabolism to H₂S
AWRI1522 cells ($\Delta his3$, $\Delta leu2$, $\Delta trp1$, $\Delta lys2$ mat $\alpha$) were grown in YPD with shaking (100 rpm) at 25 ºC. Mitochondrial mutants (AWRI1520, mat $\alpha$, $\Delta his3$, $\Delta leu2$, $\Delta trp1$, $\Delta lys2$, rho –ve) were isolated by treating cells for 8 hours in synthetic complete medium (0.17% (wt/vol) yeast nitrogen base without amino acids, 0.5% (wt/vol) ammonium sulfate, 2% (wt/vol) d-glucose) containing 10 µg/ml ethidium bromide. Cells were then diluted in water and, due to their inability to perform aerobic metabolism and utilise glycerol as a carbohydrate source, mitochondrial mutants were revealed by petite colony growth on YPDG (1% w/v yeast extract, 2% w/v peptone, 3% w/v glycerol and 0.1% w/v glucose) (Sherman et al., 1986). Loss of respiratory function in these petites was then confirmed by their inability to grow on YPG media (1% w/v yeast extract, 2% w/v peptone, 3% w/v glycerol).

5.2.5 In-vivo V-ATPase downregulation

Cells were grown at 28°C with agitation (150 rpm) in chemically defined medium supplemented with cysteine until mid-log phase (OD 0.5). While producing H$_2$S, cells were centrifuged and washed with distilled water prior to transfer into chemically defined medium containing 0.112 M glucose or 0.112 M galactose as carbon source, with or without cysteine supplementation. Cells were then incubated with shaking (150 rpm) at 28°C. Following 15 hours of incubation glucose was added to medium containing galactose as sole carbon source to a final concentration of 0.112 M. Cysteine-generated H$_2$S was measured throughout the assay using H$_2$S detection tubes. Biomass formation was monitored by measuring the absorbance at 600 nm wavelength.

5.3 Results

5.3.1 Screen background and methodology

While developing a novel assay for H$_2$S detection (Chapter 4), we noted that S. cerevisiae laboratory strain BY4742 did not produce detectable concentrations of H$_2$S when grown in a chemically defined medium. After supplementation of the medium with cysteine, we detected high concentrations of H$_2$S, suggesting this H$_2$S was generated solely through cysteine catabolism (Figure 5.1). Figure 5.1b shows the discolouration of H$_2$S detection-dye when cysteine was added to the medium, indicative of H$_2$S formation. Notably, H$_2$S formation occurred during yeast
logarithmic phase of growth and cysteine addition did not affect biomass formation as measured by optical density at 600 nm wavelength (Figure 5.1b).

Because detectable H$_2$S excreted by strain BY4742 is derived from cysteine catabolism, it provides a useful marker to identify genetic factors influencing this cellular process. It was therefore used in a genome-wide screen of S. cerevisiae deletion strains (Winzeler et al., 1999) to identify cellular processes involved in cysteine catabolism and H$_2$S formation. H$_2$S production was normalised against biomass formation by plotting the minima for each strain in the assay plate, supplemented with H$_2$S detection dye (indicative of maximum H$_2$S production) against the maxima for each strain in the growth plate (indicative of maximum biomass formation) (Figure 5.1c). Deletants able to grow under the experimental conditions were classified as low or high producers of H$_2$S from cysteine based upon their H$_2$S minima value in the assay plate, in comparison to the wild-type strain. Figure 5.1c shows an example of a strain classified as low producer of H$_2$S from cysteine (well number A3, circled).

### 5.3.2 Genome-wide classification of mutants that lead to low or high H$_2$S production from cysteine

A total of 226 deletion strains were found that displayed differences in their H$_2$S accumulation profiles compared to wild-type. They were classified as low (188 strains) and high (38 strains) producers of H$_2$S from cysteine. Strains were grouped according to the gene ontology description of the encoded gene product, as defined in the Saccharomyces Genome Database ([www.yeastgenome.org](http://www.yeastgenome.org)) using Gorilla (Eden et al., 2009). Most deletions that impacted on H$_2$S production were for genes that fell into nine functional groups (Table 5.1, a comprehensive list is available at appendix A-2.6). Interestingly, in most cases genes within a group displayed the same trend with respect to H$_2$S production (where deletions led to either low or high H$_2$S production), indicative of the specificity of the cellular processes involved in cysteine catabolism to H$_2$S. Functional groups of deletions causing high H$_2$S production included mitochondrial iron-sulfur homeostasis, mitochondrial translation and cellular response to ionic iron. Deletions causing low H$_2$S production were associated with purine base metabolism process, cellular aromatic compound metabolic process, vacuole acidification, vesicle trafficking to the vacuole, and.
translation and RNA processing functional groups. This provides a clear insight into metabolic and regulatory networks that influence cysteine catabolism to H$_2$S. A schematic representation of the data is provided in figure 5.2.

5.3.3 Role of the mitochondrion in cysteine catabolism to H$_2$S

A large number of deletants affected in mitochondrial function were shown to overproduce H$_2$S from cysteine relative to the wild-type strain. Importantly, these deletants did not excrete H$_2$S at detectable concentrations in the absence of cysteine (similar to the wild-type strain, A-2.6). Amongst these were strains deleted in genes involved in mitochondrial maintenance of iron-sulfur (Fe-S) homeostasis (Figure 5.3a). ‘Maintenance of Fe-S homeostasis’ includes genes involved in Fe-S cluster synthesis, iron transport into the mitochondria, and a cytosolic protein complex involved in regulating transcription of the iron regulon in response to mitochondrial Fe-S cluster synthesis. Whilst for most genes involved in these processes null mutation leads to inviability (Giaever et al., 2002), viable deletants from each sub-group led to over production of H$_2$S from cysteine (Figure 5.3a), highlighting the role of Fe-S homeostasis.

5.3.4 Role of the mitochondrion in cysteine catabolism to H$_2$S

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Table 5.1. Cellular processes influencing cysteine catabolism identified by H$_2$S production phenotype

<table>
<thead>
<tr>
<th>Functional group/ cellular process</th>
<th>No. of genes</th>
<th>H$_2$S production phenotype*</th>
<th>Example mutants</th>
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<tbody>
<tr>
<td>Vacuole related</td>
<td>37</td>
<td>Low</td>
<td>VAM7, CVT16, PEP12, VMA3, VMA8, VMA10</td>
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<tr>
<td>Purine base metabolic process</td>
<td>19</td>
<td>Low</td>
<td>AAH1, ADE5,7, ADE6, ADE8, ADE1, ADO1</td>
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<tr>
<td>Cellular aromatic compound metabolic process</td>
<td>11</td>
<td>Low</td>
<td>APT2, ARO7, BAS1, TRP2, AAH1, ADO1</td>
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<tr>
<td>Translation</td>
<td>32</td>
<td>Low</td>
<td>RPL29, DBP7, RPS10B, YDR417C, YVH1, RPL9A</td>
</tr>
<tr>
<td>RNA Processing</td>
<td>22</td>
<td>Low</td>
<td>LEO1, TAF14, RRP8, RIF1, IR1, SKY1</td>
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<td>Mitochondria related</td>
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<td>High/Low</td>
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<tr>
<td>Mitochondrion translation</td>
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<td>High</td>
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<tr>
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<tr>
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<td>22</td>
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<td>YLR225C</td>
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<tr>
<td>Miscellaneous</td>
<td>64</td>
<td>High/Low</td>
<td>BDF1, RGS2, BRP1, YDL199C, ASF1, MNN10</td>
</tr>
</tbody>
</table>

*Amount of H$_2$S produced relative to background strain BY4742
Figure 5.2. **Overview of the cellular processes influencing cysteine catabolism to release H$_2$S in S. cerevisiae.** The figure depicts cysteine catabolism cellular pathway with known and putative transport routes that may mediate the cysteine flux. Boxed annotations indicate some of the cellular processes identified as important for cysteine catabolism to H$_2$S and evaluated for statistical significance (P-value < 0.01) with the GO Term Finder program of the Saccharomyces Genome Database (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl).
Figure 5.3 **Influence of mitochondrial gene deletions on cysteine catabolism to release H$_2$S.** A Representation of genes involved in cellular iron-sulfur homeostasis with indication of inviable mutants (grey), mutants that lead to ever-secretion of H$_2$S (red) and mutants that do not affect H$_2$S release from cysteine (black). B Extracellular release of cysteine-generated H$_2$S (upper graph) and growth curves (lower graph) for wt and petite cells lacking respiratory function, with and without cysteine supplementation. H$_2$S formation was measured at 663 nm using the MBR method growth was measured at 600 nm. Experiments were carried out in a microtiter plate. Error bars represent the standard deviation for quadruplicate experiments.
An additional class of deletants overproducing \( \text{H}_2\text{S} \) from cysteine were those impaired in mitochondrial translation (\( \Delta\text{img2, Arml2, Amrps35, Amrpl20, Amrpl4, Amrpl24, Amrpl10} \)), implying that de novo synthesis of mitochondrial proteins is important for cysteine catabolism. The connection between mitochondrial function and \( \text{H}_2\text{S} \) generation from cysteine was further examined using respiratory deficient petite mutants. These strains were shown to produce considerably higher concentration of \( \text{H}_2\text{S} \) relatively to the wild-type strain in the presence of cysteine, and to produce detectable amounts of \( \text{H}_2\text{S} \) even in the absence of cysteine (Figure 5.3b). These results reinforce a mitochondrial role in catabolism of endogenously synthesised and supplemented cysteine to \( \text{H}_2\text{S} \).

5.3.5 Low production of \( \text{H}_2\text{S} \) from cysteine

A total of 188 deletants were shown to excrete less \( \text{H}_2\text{S} \) than the wild-type strain when supplemented with cysteine. Included in this number were genes involved in purine base metabolism and more specifically in the de novo inosine monophosphate (IMP) biosynthesis (\( \Delta\text{ade1, Ade5,7, Ade6, Ade8, Ado1, Aah1, Apt2} \)). Aside from minimal (undetectable in most cases) degradation of cysteine to \( \text{H}_2\text{S} \) we observed that, under our experimental conditions, these mutants grew significantly slower than the wild-type, with or without cysteine addition. It is worth noting that mutants for purine metabolism have an auxotrophic requirement for adenine (Roberts et al., 2003). The medium used for the screen described in this chapter did not contain adenine, however mutants were still able to reach late logarithmic phase of growth (Figure 5.4). Upon supplementation of adenine, mutants were able to grow and catabolise cysteine to produce \( \text{H}_2\text{S} \) at a rate similar to the wild-type (Figure 5.4). Purine synthesis is linked to folate one-carbon-group metabolism and the metabolism of methionine (Cossins and Chen, 1997). Interestingly, deletion of genes involved in these pathways (\( \Delta\text{shm2, Met6} \)) resulted in low \( \text{H}_2\text{S} \) release from cysteine, although these strains were able to grow at a rate similar to the wild-type (Figure 5.4).

5.3.6 Mutants affected in vacuolar function

A large group of 39 deletants impaired in the release of \( \text{H}_2\text{S} \) when supplemented with cysteine included those affected in vacuolar function and vesicle transport to the vacuole. Amongst these were deletions in genes involved in vacuole acidification
Figure 5.4 Influences of mutants in purine metabolism on cysteine catabolism to H$_2$S. A Extracellular release of cysteine-generated H$_2$S (left panel) and growth curves (right panel) from wt and mutants in purine biosynthesis pathway, with and without adenine supplementation. B Extracellular release of cysteine-generated H$_2$S (left panel) and growth curves (right panel) from wt and mutants in folate derived one-carbon metabolism. H$_2$S formation was measured at 663 nm using detection-dye; discolouration of the dye represents release of H$_2$S. Experiments were carried out in a microtiter plate. Error bars represent the standard deviation for quadruplicate experiments.
(Δvps3, Δvma3, Δvps45, Δvps16, Δvps34, Δvma22, Δvph2, Δmeh1, Δvma13, Δvma1, Δvma2, Δvma4, Δvma7, Δvma10, Δvma13, Δvma21), transport to the vacuole (Δccz1, Δypt10, Δvps52, Δvma3, Δvma8, Δvps45, Δmon1, Δcog1, Δgr2, Δavt7, Δmeh1, Δvts1, Δvma13, Δshp1, Δsym2, Δatg18) and vesicle fusion (Δvam7, Δpep12, Δvps45, Δvts1, Δyck3, Δcog1, Δmon1, Δccz1, Δpep7). Cells deficient in vacuole biogenesis (Δvam1) did not produce H$_2$S when supplemented with cysteine. Moreover, cysteine supplementation resulted in growth inhibition for this deletants (Figure 5.5a). Notably, low H$_2$S production was not correlated with the slow growth; that is, a comparison of the wild type strain and the mutant at the same growth stage (as indicated by OD measurements) shows H$_2$S production by the wild type strain and not by the deletant.

An important aspect of vacuole function is its acidic pH. Vacuole acidification is regulated by a vacuolar-type ATPase (V-ATPase) protein complex. This protein complex is comprised of two multi subunits domains, a membrane integral V$_0$ domain and a peripheral V$_1$ domain (Kane, 2006). The interaction between the two domains is essential for ATP-driven transport to maintain acidic vacuolar pH. Deletion of each of the eight subunits comprising the V$_1$ complex were identified in this screen as defective in cysteine catabolism to H$_2$S (Figure 5.5b) although these strains were able to grow under the experiment conditions (Appendix A-2.8). Furthermore, deletion of genes encoding each of the three proteins responsible for V-ATPase assembly in the endoplasmic reticulum led to a similar phenotype of minimal cysteine degradation to H$_2$S (Appendix A-2.6). Interestingly, deletants of V$_0$ subunits were able to degrade cysteine to H$_2$S at a rate similar to the wild-type strain.
Figure 5.5 Central role of the vacuole in cysteine catabolism to H$_2$S. A Extracellular release of cysteine-generated H$_2$S (upper panel) and biomass formation (lower panel) profiles of wt and mutant in vacuole biogenesis (Δvam1). Experiments were carried out in quadruplicates. B Extracellular release of cysteine-generated H$_2$S for mutants deleted in each of the sub-units comprising V$_1$ of the V-ATPase complex. Experiments were carried out in quadruplicates. C in-vivo downregulation of the yeast V-ATPase complex through nutrient limitation. H$_2$S generation profile measured with H$_2$S detection tubes (left panel) and biomass formation profile (right panel) for BY4742 cells grown with glucose or galactose as carbon source. An addition of glucose to a final concentration of 0.112 M was made to the galactose medium at the time indicated by arrows.
The activity of yeast V-ATPase can be manipulated in vivo through growth medium composition (Kane and Smardon, 2003). When cells are transferred to a poor carbon source, up to 75% of existing V-ATPase complexes are disassembled into cytoplasmic (V₁) and membrane-bound (Vₒ) sectors, and this disassembly is completely reversible. We used this in vivo down-regulation mechanism to test whether V-ATPase assembly and activity is essential for cysteine degradation to H₂S or whether the individual sectors have an effect on that process when disassembled. H₂S-producing cells grown on glucose were transferred into a growth medium containing glucose or galactose as sole carbon source. Figure 5.5c shows that upon transfer to galactose-containing medium cells did not degrade cysteine to H₂S, whereas those transferred to glucose-containing medium continued to produce H₂S. Furthermore, transfer into galactose-containing medium resulted in growth inhibition irrespective of cysteine supplementation, although to a lesser extent. Addition of 0.112 M glucose to the galactose medium restored H₂S formation (Figure 5.5c); consistent with a poor carbon source and consequent disassembly of the V-ATPase complex inhibiting cysteine catabolism to release H₂S.

5.4 Discussion

Cysteine is maintained at a low intracellular concentration by regulation of its production and, when in excess, its efficient removal. Amongst cysteine catabolic pathways is cysteine desulfhydration in which sulfur is cleaved from cysteine to produce H₂S. In mammals, desulfhydration of cysteine is responsible for the production of reduced sulfur and may act on cellular function through H₂S formation (Stipanuk, 2004). This chapter links cellular compartments and functions to the release of H₂S following cysteine supplementation using the Euroscarf S. cerevisiae genome-wide deletion library.

The S. cerevisiae deletion library has been an effective resource to study a broad range of biological processes including cellular response to Na⁺ (Warringer et al., 2003), metal toxicity (Ruotolo et al., 2008), oxidative stress (Thorpe et al., 2004), glutathione homeostasis (Perrone et al., 2005) and H₂S formation through the sulfate assimilation pathway (Linderholm, et al., 2008). The latter utilised BiGGy agar (section 1.3.3.1) as an indication for sulfite reductase activity with follow up fermentation experiments on selected strains using gas collection tubes (chapter
1.3.3.4). Screening of the yeast deletion library for cysteine catabolism revealed a large number of genes (226) whose deletion altered cysteine degradation to release H₂S. The caveat to this genetic approach is the high base level of H₂S production from cysteine observed for the wild-type strain, which is close to the method upper detection limit (Figure 5.1). That is, the MBR method is based on spectrophotometric measurements of methylene blue discolouration, indicative of H₂S production. The method maximum H₂S detection is limited by the dye concentration in the medium, which is limited by the linearity of spectrophotometric absorbance measurements. In some screening runs we observed the wild type strain produced high amounts of H₂S leading to full discolouration of the medium, therefore in these runs over-secreting strains may have gone undetected. For that reason this screen is aimed more at the discovery of mutants impaired in cysteine catabolism to H₂S. Discoveries made here regarding high H₂S producers require further investigations using conditions more suited to monitor high H₂S production as well as including additional H₂S detection methods.

Two PLP-dependent enzymes, well conserved between bacteria, yeast and higher eukaryotes, were previously implicated in cysteine catabolism to H₂S: CBS and CSE (Stipanuk and Beck, 1982; Chen et al., 2004; Stipanuk, 2004; Singh and Banerjee, 2011). The yeast genes encoding these enzymes are CY S4 and CY S3, respectively. Surprisingly, deletion of CY S4 did not affect H₂S production from cysteine (Appendix A-2.6). Growth of a Δcys3 strain was delayed under cysteine supplementation, however cells still reached stationary phase of growth and were able to catabolise cysteine to release H₂S (Appendix A-2.6). The regulatory mechanism and relative contribution of the two enzymes to cysteine catabolism is not yet understood (Stipanuk and Beck, 1982; Singh et al., 2009). A better characterised role of these two enzymes is in cysteine synthesis (Thomas and Surdin-Kerjan, 1997), converting homoserine to cystathionine (Cys4p) which is then converted to cysteine (Cys3p). Considering that role, it is not surprising that deletion of CY S4 was previously shown to increase H₂S production through the sulfate assimilation pathway (Linderholm, et al., 2008). Under cysteine limiting conditions H₂S is produced as an intermediate in the pathway for cysteine synthesis and thus deletion of CY S4 would lead to accumulation of H₂S and finally its release from the cell. That effect was not observed here as the experimental conditions included ample amounts of cysteine in the
medium which would be expected to downregulate cysteine synthesis (section 1.3.2.1).

5.4.1 Cysteine role in Fe-S cluster biogenesis

An additional PLP-dependent enzyme, Nfs1p, produces sulfur from cysteine for incorporation into Fe-S proteins, which is similar to its bacterial orthologs NifS and IscS (Mühlenhoff et al., 2002; Mühlenhoff et al., 2004). In eukaryotes, including S. cerevisiae, the biosynthesis of Fe-S clusters is mostly carried out in the mitochondrion (Lill and Kispal, 2000; Balk and Lill, 2004; Rouault and Tong, 2005). Amongst other physiological roles, the synthesis of Fe-S clusters serves to maintain mitochondrial iron homeostasis (Kispal et al., 1999; Li et al., 1999; Rouault and Tong, 2005). Disruption of this process leads to mitochondrial iron overload, which is highly detrimental to the cell (Rouault and Tong, 2005). Accordingly, null mutation of many of the genes involved in Fe-S cluster biogenesis, including NFS1, results in cell death (Giaever et al., 2002).

In this study mutants involved in all aspects of iron-sulfur homeostasis maintenance produced high amounts of H\textsubscript{2}S in comparison to the wild type strain (Figure 5.3a). Overproduction of H\textsubscript{2}S was also observed in a respiratory deficient petite mutant (Figure 5.3b), although this mutant produced more H\textsubscript{2}S than the wild-type cells even in the absence of exogenous cysteine. The link between mitochondrial function and H\textsubscript{2}S formation was observed in a previous study, where deletion of gene YIA6, involved in NAD+ transport to the mitochondria, resulted in high H\textsubscript{2}S production when cells were grown on BiGGy agar. The fact that deletants involved in Fe-S cluster assembly were not identified as H\textsubscript{2}S over-producers in that study suggests that this phenotype appears only when cysteine is in excess, and reinforces the connection between iron-sulfur homeostasis maintenance and cysteine catabolism. Taken together these results lend weight to the hypothesis that if assembly of Fe-S clusters is disrupted, the sulfur component (cleaved from cysteine) is secreted from the cell.

Still, the observation that respiratory deficient petite mutants were shown to secrete high amounts of H\textsubscript{2}S raises a question regarding specific deletants that have been known to confer such respiratory deficient phenotype. Currently 38 deletants are listed in the Saccharomyces Genome Database (http://www.yeastgenome.org/) as
having increased frequency of rho- or rho0 phenotype, however none of these strains was classified in the screen described here as high H₂S producer. This may be due to the method’s limitations in detection of high H₂S producing strains. Alternatively, this link may be obscured by the high sugar content of the medium (0.28 M glucose, 0.28 M fructose), supporting fermentative rather than respiratory mode of metabolism (Deken et al 1969) and lowering the frequency of this respiratory deficient phenotypes. The link between respiratory deficient phenotype and cysteine catabolism to H₂S requires further examination using different growth conditions to promote respiratory metabolism.

5.4.2 Cysteine link to purine biosynthesis and folate derived one-carbon metabolism

Acting as a sulfur source for Fe-S biogenesis is one of the many cellular functions of cysteine. Cysteine serves as a precursor for synthesis of proteins and several other essential molecules including GSH, coenzyme A, taurine, and inorganic sulfur (Stipanuk, 2004). Additionally, cysteine concentration regulates transcription of genes associated with yeast sulfur assimilation and metabolism (section 1.3.2.1). A link between purine metabolism and H₂S production was previously found (Linderholm, et al., 2008). In that study deletants involved in purine metabolism produced darker colour colonies when grown on BiGGY agar, indicative of H₂S over-production. Here, the same deletants produced less H₂S than wild-type cells upon cysteine supplementation during fermentation. The difference between the two results may reflect the two pathways examined under each experiment, as Linderholm et al. (2008) studied H₂S formation through the SRS pathway while here the mechanism of H₂S release from cysteine was studied. Alternatively, as the BiGGY agar results were not verified in fermentation environment this discrepancy may be a result of the known poor correlation of H₂S measurements in plate and fermentation environments (Linderholm, et al., 2008, Spiropoulos, et al., 2000). Finally, differences in the medium composition between the two experiments could have led to these contrasting results. In this study addition of adenine resulted in increased H₂S production, at a similar level to the wild-type strain (Figure 5.4), suggesting the observed low H₂S production was due to auxotrophic requirements of the deletants. Though this avenue was not tested in Linderholm, et al., (2008) further studies are needed to identify the extent of a link between sulfur and purine metabolism.
A possible connection between the two is through folate derived one-carbon metabolism, necessary for synthesis of purines and methionine (Cossins and Chen, 1997). We report here, somewhat surprisingly, that disruption of methionine biosynthesis through the methyl cycle, results in decreased catabolism of cysteine to release $H_2S$, without affecting growth rate (Figure 5.4b). Under these conditions one pathway for cysteine catabolism (conversion to methionine) is inhibited, thus the fact that cysteine was not catabolised to produce $H_2S$ under these conditions requires further investigation.

**5.4.3 Central role of the vacuole in cysteine catabolism**

Cysteine accumulation was previously shown to have a cytotoxic effect (Ono et al., 1991; Kumar et al., 2006). From that point of view the central role discovered here for the vacuole in cysteine catabolism to release $H_2S$ is reasonable. The yeast vacuole is an acidic compartment that shares a great deal of functional and morphological similarities with the mammalian lysosome and supports detoxification, protein degradation and ion and metabolite storage (Klionsky et al., 1990; Li and Kane, 2009). The genome-wide screen here identified a strong link between the vacuole and cysteine degradation to release $H_2S$ (Figure 5.5). Of particular importance was maintenance of the vacuole acidic pH. Deletion of each of the subunits comprising the $V_1$ sub complex of V-ATPase, responsible for vacuolar acidic pH, resulted in low (to undetected) $H_2S$ production from cysteine. V-ATPases are evolutionary conserved multisubunit enzymes responsible for acidification of the vacuole in yeast and the lysosome in mammals (Li and Kane, 2009). Study of the V-ATPase system is particularly valuable in yeasts as deletions of ubiquitously expressed V-ATPase subunits appear to be lethal in all organisms except fungi (Li and Kane, 2009). It was previously shown that a change in the medium composition from rich to poor carbon source leads to the disassembly of V-ATPase complex, which is immediately restored once a rich carbon source is added (Kane and Smardon, 2003). Here, switching the medium composition from glucose to galactose resulted in impaired $H_2S$ production, which was restored upon the addition of glucose to the galactose medium (Figure 5.5). Taken together, our results infer a major role of V-ATPase in the catabolism of excess cysteine to $H_2S$ and highlight the peripheral $V_1$ sub-complex as the active unit.
Most interestingly, these deletants were previously classified as high H\textsubscript{2}S producers when evaluated for sulfate reductase activity using BiGGy agar (Linderholm, et al., 2008). While their classification was not validated in a fermentation assay, Linderholm’s observations may differ from those described in this study due to the multifaceted effects of the V-ATPase complex. Impaired V-ATPase activity is identified in many genomic screens including drugs sensitivity, metal ion sensitivity, sensitivity to multiple forms of oxidative stress and more (these findings are well summarised in (Kane, 2007). This over-representation suggests multiple roles for the V-ATPase complex, depending on the yeast growth conditions. It is thus plausible that activation of the SRS pathway in these deletants could promote high H\textsubscript{2}S production through a mechanism unrelated to cysteine catabolism. As previously demonstrated (section 4.3.4), the use of different environmental conditions leads to different H\textsubscript{2}S production patterns, therefore the role of yeast V-ATPase should be evaluated under these environmental conditions using a yeast strain that is able to produce high amounts of H\textsubscript{2}S through both SRS activation pathway and cysteine catabolism. A good candidate for these experiments would be the model haploid wine yeast AWRI1631, carrying a mutation causing impaired V-ATPase activity.

The nature of the role V-ATPase plays in cysteine catabolism requires further elucidation. V-ATPase generates a proton gradient across the vacuole membrane that drives transport of ions and small molecules into the vacuole (Ohsumi and Anraku, 1981; Arai et al., 1989). It is possible that cysteine transport into the vacuole is facilitated by this proton gradient, which would explain our identification of the proton-generating ATP hydrolysis sub-complex (V\textsubscript{1}) as the active contributor to cysteine degradation. Supporting this, GSH transport to the vacuole is also partially mediated through V-ATPase coupled system (Penninckx, 2002). Alternatively, the observation that deletants impaired in vesicle formation and fusion with the vacuole (Table 5.1) are impaired in cysteine catabolism to release H\textsubscript{2}S, suggests that V-ATPase activity may facilitate cysteine transport to the vacuole in a vesicle-mediated manner. This is in line with recent studies demonstrating vesicle fusion with the vacuole requires the activity of the V-ATPase complex (Baars et al., 2007; Li and Kane, 2009). Interestingly, cysteine metabolism was indirectly linked with V-ATPase through the discovery that \textit{Δcys4} mutants display an in vivo loss of vacuole acidification, due to inactivation of the V-ATPase complex (Oluwatosin and Kane,
It was then suggested that V-ATPase activity is regulated by cytosolic redox state; a concept supported by other studies suggesting that the thiol/disulphide ratio may serve as a “third messenger” (Walters and Gilbert, 1986). These findings indicate that, in addition to being regulated by redox state, V-ATPase may operate to maintain cytosolic redox balance by removing excess cysteine from the cytosolic pool.

5.4.4 Cysteine catabolism as a detoxification mechanism

While discussing cysteine exclusion from the cytosolic pool cysteine incorporation into glutathione (GSH) must be considered as a mechanism. GSH synthesis facilitates cysteine homeostasis by acting as the cellular reservoir for this amino acid. However, GSH homeostasis is tightly regulated (Perrone et al., 2005) and the reservoir capacity is limited, thus it is unlikely to be the main destination in cysteine exclusion from the cytosolic pool when cysteine is present at toxic concentrations. Supporting this view we observed cysteine catabolism to release H$_2$S in mutants impaired in GSH synthesis (Δgsh1, Δgsh2), at a similar concentration to the wild-type.

Two genome-wide studies have explored GSH homeostasis, analysing both intra- and extracellular concentration of GSH (Perrone et al., 2005; Suzuki et al., 2011). Our findings suggest that deletants with perturbed cysteine catabolism generally differed from those affecting GSH homeostasis. Most notably, there was a high degree of similarity between our findings and results from genome-wide screens for metal tolerance (Ruotolo et al., 2008; Arita et al., 2009). A prominent point of resemblance was for deletants impaired in V-ATPase activity, conferring both metal sensitivity and low cysteine catabolism leading to release of H$_2$S. In addition, mutants reported to confer metal resistance were similar to those conferring high H$_2$S production (Appendix A-5.2). These points of resemblance reinforce that degradation of excess cysteine to H$_2$S is a detoxification mechanism, similar to that functioning in response to toxic metal exposure.

5.5 Conclusions and future directions

Cysteine catabolism provides reduced sulfur and maintains cysteine homeostasis in eukaryotes (Stipanuk, 2004). There is a substantial body of evidence supporting the physiological roles of cysteine degradation to release H$_2$S that raises the need for a better understanding of the factors influencing this process. In this chapter we
identified genes not previously linked with cysteine catabolism, and opened new
directions for future studies to advance our understanding of this process in higher
eukaryotes. A great deal of research is still needed for the full characterisation of this
process however the main contribution of this chapter is in offering a new prospect for
the study of cysteine catabolism in Eukaryotes by using S. cerevisiae as a model
organism.
Chapter 5- Addendum

The concentration of free cysteine in grapes is low (Kluba et al., 1978; Pripis-Nicolau et al., 2001). It was therefore never considered as a major contributor to the development of VSCs during wine fermentation. However, cysteine plays an important role in VSCs formation as a constituent of GSH and as amino acid conjugate of polyfunctional thiols (Chapter 1). This chapter has focused on the formation of H$_2$S from cysteine. While this study has clear implications to the formation of H$_2$S from GSH it may also support the understanding of the mechanisms involved in polyfunctional thiol release.

Polyfunctional thiol release involves the release of the sulfur component from a cysteine conjugated precursor. The process is mediated by the activity of a carbon sulfur β-lyase enzyme that cleaves the sulfur residue from the β-carbon of cysteine. A similar enzyme activity is needed to release H$_2$S from cysteine. The similarities between the two chemical reactions are displayed in figure 5.6a. To further explore the connection between the two reactions cysteine catabolism to H$_2$S was monitored during a micro-fermentation of yeast overexpressing the geneSTR3 and an E. coli tryptophanase gene tnaA; known to release high amounts of thiols from their precursors (Swiegers et al., 2007; Holt et al., 2011). Overexpression of the endogenous carbon sulfur β-lyase enzyme, STR3, enhanced cysteine catabolism to release H$_2$S relative to the wild-type strain, while overexpression of the E. Coli tryptophanase enzyme further enhanced H$_2$S formation from cysteine. This data is consistent with previous report on these strains contribution to 3MH release from Cys-3MH, showing an increased 3MH concentration using strain overexpressing STR3 and a much greater increase in 3MH concentration.
Figure 5.6 **Similarities between polyfunctional thiol release and cysteine catabolism to release H$_2$S.** A The release of 3MH and H$_2$S from cysteine involves cleavage of the same carbon-sulfur bond (indicated by arrows). B Extracellular release of cysteine-generated H$_2$S for strains overexpressing S. cerevisiae (STR3) and E. Coli (TRP) carbon sulfur β-lyase enzymes previously demonstrated to release high concentrations of 3MH from Cys-3MH.
following the overexpression of the E. Coli tryptophanase enzyme (Holt et al., 2011). Together the results here provide preliminary evidence suggesting the two processes may be mediated by similar enzymes however further study is needed to establish this hypothesis, particularly considering that free cysteine and cysteine conjugates significantly differ in their steric structure and so may require the activity of different enzymes.

In this chapter we revealed a central role of the yeast vacuole in H$_2$S release from cysteine. The yeast vacuole was also shown to play a major role in the degradation of GSH (Jaspers et al., 1985; Ubiyovk et al., 2006; Ganguli et al., 2007). Understanding the mechanisms involved in vacuolar degradation of cysteine and GSH is important for an educated manipulation of fermentation conditions to obtain a more desirable sulfur aroma profile. For example, identification of the vacuole V-ATPase complex’ role in cysteine degradation allowed the downregulation of this complex through nutrient modulation which impeded H$_2$S formation from cysteine (section 5.3.5).
Chapter 6.

Concluding remarks and future perspectives

The overall aim of this thesis was to study the formation of volatile sulfur compounds during fermentation and their modulation through nutrients supplementation. Thesis was focused on organic sulfur compounds contributing to H$_2$S, 3MH, and 3MHA. Chapter 2 demonstrated the modulation of these compounds as a result of organic nutrient supplementation. GSH was then inferred to contribute to H$_2$S formation through the degradation of cysteine (Chapter 2). Additionally, GSH conjugated 3MH was found to be metabolised by yeast during fermentation, to release 3MH and 3MHA. This release was hypothesised to occur in two steps including GSH degradation to individual amino acids followed by 3MH release from Cys-3MH (Chapter 3). A mechanism similar to that we inferred regarding H$_2$S release from GSH. Next chapters of this thesis focused on understanding the cellular pathways activating the release of cysteine sulfur residue using H$_2$S as a marker for cysteine catabolism. First, a method suitable for high throughput detection of cysteine-generated H$_2$S was developed (Chapter 4). The method was then used in a genome-wide screen of the yeast deletion collection for cysteine catabolism to release H$_2$S (Chapter 5). Results of this screen support our understanding of this pathway and provide valuable insights into cysteine catabolism and the release of VSCs from cysteine and GSH.

6.1 Relevancy of cysteine and GSH catabolism to wine sulfur aroma composition

H$_2$S is a central metabolite in yeast sulfur metabolism in addition to being a precursor for the development of other volatile sulfur compounds (section 1.2). The majority of studies on H$_2$S formation during fermentation have focused on H$_2$S produced through the sulfate assimilation pathway, in which H$_2$S is formed prior to sequestration in sulfur-containing amino acids. This thesis studies an alternative pathway for H$_2$S formation during fermentation, looking at sulfur-containing amino acids as precursors and focusing on the contribution of their degradation pathway (rather than their synthesis) to the formation of H$_2$S and other volatile sulfur compounds. Although the degradation of both cysteine and GSH was previously
identified to contribute to $H_2S$ formation (Jiranek et al., 1995b; Park et al., 2000; Rauhut, 2009), this issue has been largely overlooked in wine research and both cysteine and GSH have not been considered as significant contributors to $H_2S$ production during wine fermentation.

Several factors are responsible for this. For the most part, cysteine concentration in grapes is low (Kluba et al., 1978; Pripis-Nicolau et al., 2001) while sulfate concentration is relatively high (Leske et al., 1997), therefore it has been concluded that the majority of $H_2S$ produced during wine fermentation is through the sulfate assimilation pathway. Nevertheless, wine quality is not necessarily affected by the amount of $H_2S$ produced during fermentation, as most is purged by entrainment with the high rate of $CO_2$ produced; it is only the $H_2S$ that remains in the resultant wine (residual $H_2S$) that affects its quality. Previous studies have pointed out a link between the concentration of residual $H_2S$ in wine and the concentration of $H_2S$ produced at the later stages of fermentation (Henschke and De Kluis, 1995; Jiranek et al., 1996; Ugliano et al., 2009). Production of $H_2S$ at the later stages of fermentation has not been correlated with the sulfate assimilation pathway. It is probable that $H_2S$ produced at that stage arrives from organic sulfur amino acids, released to the medium during yeast autolysis. In that case GSH would be the main candidate to contribute to $H_2S$ production, considering the high volume it occupies in the yeast cell (0.1-1% of dry cell weight) (Li et al., 2004). In this thesis we presented preliminary evidence that GSH degradation to $H_2S$ (Chapter 2) as well as GSH conjugate 3MH release to 3MH (Chapter 3) occurs through GSH degradation to individual amino acids followed by degradation of cysteine to release the sulfur residue. This hypothesis however, requires further studies and validation.

As a result of its low abundance, cysteine- or GSH-generated $H_2S$ is usually masked by the higher amounts of $H_2S$ produced from sulfate metabolism. While differentiation of the pathways can be achieved through the use of mutant strains it is difficult to differentiate the two in commercial wine strains. By monitoring $H_2S$ production during fermentation under conditions modulating each pathway and with the use of mutant strains to validate our findings, we identified distinct kinetic profiles for $H_2S$ formation through cysteine catabolism or sulfate assimilation (Chapter 4). These kinetic profiles are simple to generate and can successfully be implemented in
future research of various industrial strains, to draw conclusions regarding H₂S generated from cysteine.

In this thesis we further highlight the importance of cysteine and GSH to sulfur aroma formation by correlating cysteine and GSH catabolism to release H₂S with polyfunctional thiol cysteine and GSH conjugate release. The two share several points of resemblance: first, release of both H₂S and Cys-3MH is mediated by the activity of a carbon sulfur β-lyase enzyme (Figure 5.6). Second, we identified the yeast vacuole as a major compartment important for cysteine catabolism to release H₂S (Chapter 5). While the role of the vacuole in polyfunctional thiol release has not been examined, it is known to facilitate the degradation of GSH sulfur conjugates (Jaspers and Penninckx, 1984; Mehdi et al., 2001; Penninckx, 2002) and therefore is likely to be involved in 3MH conjugate release as well. Furthermore, transport of GSH and GSH conjugates is mediated in part by V-ATPase coupled system (Penninckx, 2002). Our genome-wide screen found the same V-ATPase complex to be essential for cysteine catabolism to release H₂S (Chapter 5). Finally, we examined this link using genetically modified yeast strains known to release polyfunctional thiols from their cysteine conjugate (Figure 5-6). These strains produced more H₂S following cysteine supplementation in comparison to the wild-type strain, similar to their 3MH production patterns (Holt et al., 2011). Taken together, these preliminary evidences support the connection between thiols precursor conversion and cysteine and GSH catabolism to release H₂S, and merit further studies of that link. If confirmed, this may advance polyfunctional thiol research significantly. Currently, polyfunctional thiol research is held back due to thiols analysis techniques that are complicated to operate and require special skills and equipment (Tominaga et al., 1998b; Capone et al., 2011). The potential use of the MBR assay for cysteine catabolism to release H₂S as an indicator for thiol release capacity would simplify the analyses process and allow for simpler data collection.

### 6.2 Research contribution

This thesis integrates both industry-related and fundamental research interests. Fundamental knowledge of yeast metabolism is essential to develop new strategies for nutrient modulation of aroma compounds. Examples for that are given throughout the thesis. The indication that GSH supplementation may enhance GSH thiol precursor
uptake, and subsequent conversion, requires further studies and may represent a valuable strategy to increase thiols concentration in wine. Additionally the identification of the yeast vacuole acidification complex, V-ATPas, as essential for cysteine degradation to release H$_2$S allows its strategic downregulation through the use of nutrients to alleviate H$_2$S production from cysteine during fermentation (example in Chapter 5, Figure 5-5).

An unexpected contribution of this thesis to the fermented food industry was the development of a high throughput method for detection of H$_2$S. While this method was developed as a means to overcome technical difficulties, it provides a potentially useful technology for the development of various microorganism specialized in H$_2$S production, either low H$_2$S producers (suitable for wine and beer production) or high (suitable for cheese production). The method was filed for provisional patent by UWS, prior to further commercialization.

Finally, an important finding of this thesis is the elucidation of the cellular factors involved in cysteine catabolism to H$_2$S. The process of cysteine catabolism to release H$_2$S has mainly been studied for mammalian cells, originating from the physiological importance of H$_2$S. The identification of S. cerevisiae as a suitable model organism to study that process expands the research possibilities in that field, with the vast knowledge obtained for S. cerevisiae and the ease of use of genetic manipulation. Our genome wide screen for cysteine catabolism has laid the grounds for further research in that field, mainly focused on the vacuole as the major compartment for cysteine degradation.

In conclusion, this thesis demonstrates the importance of understanding yeast metabolism for an educated manipulation of fermentation conditions to modulate the formation of sulfur aroma compounds. Results and techniques obtained here can successfully be implemented in the wine industry as well as contribute to the scientific community in the fundamental research of cysteine and GSH catabolism.
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Appendices

A-1 Publications and patents

A-1.1 Effects of rehydration nutrients on H₂S metabolism and formation of volatile sulfur compounds by the wine yeast VL3

A-1.1.1 Statements of authorship

Gal Winter
Contributed to experimental design, collected and analysed the data, wrote manuscript.
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Contributed to the research idea and experimental design and assisted with preparation and editing of the manuscript.
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Contributed to the research idea and experimental design, supervised the work and assisted with preparation and editing of the manuscript.
Sign

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Sign

Chris D. Curtin
Contributed to the research idea and experimental design, supervised the work, assisted with preparation and editing of the manuscript and acted as corresponding author.
Sign
Effects of rehydration nutrients on H₂S metabolism and formation of volatile sulfur compounds by the wine yeast VL3

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Abstract

In winemaking, nutrient supplementation is a common practice for optimising fermentation and producing quality wines. Nutritional suboptimal grape juices are often enriched with nutrients in order to manipulate the production of yeast aroma compounds. Nutrients are also added to active dry yeast (ADY) rehydration media to enhance subsequent fermentation performance. In this study we demonstrate that nutrient supplementation at rehydration also has a significant effect on the formation of volatile sulfur compounds during wine fermentations. The concentration of the 'fruity' aroma compounds, the polyfunctional thiols 3-mercaptobutan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), was increased while the concentration of the 'rotten egg' aroma compound, hydrogen sulfide (H₂S), was decreased. Nutrient supplementation of the rehydration media also changed the kinetics of H₂S production during fermentation by advancing onset of H₂S production. Microarray analysis revealed that this was not due to expression changes within the sulfate assimilation pathway, which is known to be a major contributor to H₂S production. To gain insight into possible mechanisms responsible for this effect, a component of the rehydration nutrient mix, the tri-peptide glutathione (GSH) was added at rehydration and studied for its subsequent effects on H₂S formation. GSH was found to be taken up during rehydration and to act as a source for H₂S during the following fermentation. These findings represent a potential approach for managing sulfur aroma production through the use of rehydration nutrients.

Keywords: Rehydration, yeast, nutrients, H₂S, hydrogen-sulfide, GSH, glutathione

Introduction

In many viticultural regions the natural nutrient composition of grape juice is considered suboptimal and may lead to a variety of fermentation problems including slow or stuck fermentations and formation of undesirable off-flavours (Blateau and Sablayrolles 2001; Henschke and Hirane 1993; Mendes-Ferreira et al. 2009; Sablayrolles et al. 1996; Schmidt et al. 2011; Torrea et al. 2011; Ugliano et al. 2010). To alleviate these deficiencies, various yeast nutrient preparations are often added to the juice prior to or during alcoholic fermentation, to contribute to the production of a quality wine. Among the nutrient supplements allowed by wine regulatory authorities in many countries are vitamins, inorganic nitrogen, usually in the form of diammonium phosphate (DAP) and organic nutrient preparations. The latter are typically prepared from inactive or autolysed yeast and are therefore usually composed of lipids, micro- and macro-elements, amino nitrogen, mannoproteins and insoluble material (for example see Pozo-Bayón (2009).). Effects of these nutrients on the formation of key aroma groups in wine have been studied widely. The concentration of esters and higher alcohols, which impart fruity and fusel aromas respectively, were found to be influenced mostly by nitrogen availability (reviewed by Bell and Henschke (2005). Nitrogen is also considered a key modulator in the formation of volatile sulfur compounds, including H₂S, a highly potent compound which possesses an odour reminiscent of rotten egg (Rashut 1993).

The majority of studies regarding the effect of nutrients on yeast derived aroma compounds have focused
on nutrient addition to the grape juice immediately prior to or during alcoholic fermentation. The common oenological practice of using active dry yeast (ADY) for wine fermentation necessitates rehydration, since water availability in ADY is too low for yeast to maintain metabolic activity during storage (Rapoport et al. 1997). This step represents a further opportunity for nutrient supplementation. Previous studies have demonstrated the efficacy of nutrient supplementation at this point in time on yeast viability and vitality. Supplementation of organic nutrient in the form of inactive dry yeast (IDY) was found to increase fermentation rate, supposedly due to an incorporation of solubilised sterol present in IDY (Soubeiran et al. 2005). Additions of fermentable carbon source and magnesium salts were also shown to enhance both viability and vitality of dehydrated yeast following rehydration (Kraus et al. 1981; Rodriguez-Porrata et al. 2008).

Although rehydration nutrient supplementation is a common practice in winemaking, its effect on the formation of fermentation derived aroma compounds has not been explored. In this paper we examine the effect of a proprietary rehydration nutrient supplement on yeast gene expression during wine fermentation and how this affects its volatile chemical composition. This parallel analysis consisting of transcriptomics and metabolite profiling provided insights into which components of the rehydration nutrient mixture affect the formation of aroma compounds.

Materials and methods

Chemicals

Analytical reagents were purchased from Sigma-Aldrich unless otherwise specified. Rehydration nutrient mix was Dynastart (Laffort Australia, Woodville, SA, Australia). S-3-(hexan-1-0y)-L-cysteine (Cys-3MH) and S-4-(4-methylpentan-2-0y)-L-cysteine (Cys-4MMMP) were synthesized and characterized as previously described (Howell et al. 2004; Pardon et al. 2008).

Yeast strain, treatments and fermentation conditions

The yeast strain used was a commercial active dried preparation of VL3 (Laffort Australia, Woodville, SA, Australia). ADY were rehydrated with water or water supplemented with rehydration nutrient mix (120 g/L). To examine the effect of nutrient mix components ADY were rehydrated with water containing GSH (500 mg/L). Rehydration media were thoroughly mixed at 37°C for 30 minutes prior to addition of 10% (v/v) ADY. ADY were incubated with agitation in the rehydration media for 20 minutes and then inoculated into the fermentation media to give a cell concentration of 1 × 10⁶ cells/ml. Fermentations were carried out in triplicate under isothermal conditions at 22°C with agitation. Fermentations were carried out in Schott bottles (SCHOTT Australia, NSW, Australia), silled with silicone o-ring and fitted with silver nitrate detector tubes for the quantification of H₂S formed in fermentation and a sampling port. Samples were collected through the sampling port using a sterile syringe. Fermentation volume was either 2 L (for comprehensive volatile analysis) or 1 L. Fermentation progress was monitored by measurement of residual glucose and fructose using an enzymatic kit (GF2635, Randox, Crumlin, UK).

Fermentation media

A low nitrogen Riesling juice with a total yeast assimilable nitrogen (YAN) concentration of 120 mg/L (NH₃ = 53 mg/L; free amino nitrogen (FAN) = 90 mg/L) was used for this study. Juice analytical parameters were as follows: pH 2.8; titratable acidity 4.6 g/L as tartaric acid; sugars, 205 g/L. To examine the effect of rehydration nutrients on polyfunctional thiol release, juice was supplemented with 5 µg/L Cys-4MMMP and 200 µg/L Cys-3MH, a concentration of precursors commonly found in Sauvignon Blanc juices (Capone et al. 2010; Lusier et al. 2008). Where specified, DAP addition to the fermentation media was 0.56 g/L to increase the juice YAN value to 250 mg N/L. The pH of the fermentation medium was readjusted to 2.9 with 1 M HCl following DAP additions. Juice was filter sterilized with a 0.2 µm membrane filter (Sartorius Australia, Oakleigh, Victoria, Australia).

Post fermentation handling

At the end of grape juice fermentation, wines were cold settled at 4°C and free SO₂ of the finished wine was adjusted to 45 mg/L by the addition of potassium metabisulfite. The wines were then carefully racked into glass bottles to avoid exposure to oxygen and were stored with air tight caps fitted with a polytetrafluoroethylene liner. Bottles were fully filled to avoid any headspace oxygen.

Grape juice analyses

Titratable acidity, FAN, and ammonia were measured as previously described (Vilanova et al. 2007). Ammonia concentration was measured using the Glutamate Dehydrogenase Enzymatic Bioanalysis UV method (Roche, Mannheim, Germany). FAN was determined by using the o-phthalaldehyde/N-acetyl-L-cysteine spectrophotometric assay procedure. Both ammonia and FAN were analyzed using a Roche Cobas FARA spectrophotometric autoanalyzer (Roche, Basel, Switzerland). Amino acid analysis was carried out based on Körös et al. (2008), using a pre-column derivatization with o-phthalaldehyde-ethanethiol-9-fluorenylethylmethyl chloroformate and HPLC analysis with fluorescence detection. Reduced
and oxidized glutathione were analyzed using LC-MSMS as previously described (du Toit et al. 2007).

**Volatile compounds analyses**

H$_2$S, methanethiol (MeSH), dimethyl sulfide (DMS), methyl thioacetate (MeSAc), and ethyl thioacetate (EtSAC) were determined by static headspace injection and cool-on-column gas chromatography coupled with sulfur chemiluminescence detection (GC-SCD), as described in Siebert et al. (2010). 3MH, 3MHA and 4-Mercapto-4-methylpentan-2-one (4MMP) were measured in SARCO Laboratories (Bordeaux, France) according to Tominaga et al. (2000) using a TRACE GC-MS (ThermoFisher Scientific, MA, USA). Detection limits for 3MH, 3MHA and 4MMP were 11 ng/L, 1 ng/L and 0.3 ng/L, respectively. Quantification limit is 35 ng/L ± 20% for 3MH, 3 ng/L ± 18% for 3MHA and 0.6 ng/L ± 14% for 4MMP. Monitoring of H$_2$S development during fermentation was carried out using silver nitrate selective gas detector tubes (Komyo Kitagawa, Japan), as described by Ugiano and Henschke (2010).

**RNA Extraction and cDNA synthesis**

Samples for RNA analyses were collected by filtration during fermentation after consumption of 15 g/L sugars. Cells were resuspended in RNAlater® (Ambion, Inc., Austin, TX, USA) solution at 4°C for 24 hours. Cells were then centrifuged to remove the RNAlater® solution and were stored at -80°C. Total RNA was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad, CA) as described in Alic et al. (2004). The integrity of the RNA was confirmed using an RNA 6000 Nano LabChips on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). cDNA was synthesized from 200 ng total RNA in a total volume of 20 μL with AffinityScript QPCR cDNA synthesis kit (Agilent Technologies, Santa Clara, CA) and oligo-dT20 primers by incubation for 5 min at 42°C and 15 min at 55°C with heat inactivation for 5 min at 95°C.

**Transcription analyses**

Transcription analysis was carried out at the Ramaciotti Centre for Gene Function Analysis (UNSW, Sydney, Australia). Biological duplicates were analysed using the Affymetrix GeneChip Yeast Gene 1.0 ST Array and the GeneChip® 3′ IVT Express protocol (Affymetrix, Santa Clara, CA, USA). Data were analysed using the statistical methods available in the Partek® Genomic Suite 6.5 (Partek Incorporated, St Louis, Missouri, USA). Statistical analysis for over-representation of functional groups was performed using FunSpec (Robinson et al. 2002). Available databases were addressed by using a probability cutoff of 0.01 and the Bonferroni correction for multiple testing. To validate the results, five differentially expressed genes were further examined by quantitative real-time PCR (qPCR). qPCR was carried out with Brilliant II SYBR Green reagent (Stategene, Agilent Technologies) and cDNA made from 2.5 ng total RNA in a volume of 25 μL for all subsequent reactions. Primers are detailed in Table 1. Ct values were obtained from triplicate fermentations and were normalized using the 2$^{-\Delta\Delta Ct}$ method (Wong and Mediano 2005). Values were then normalized against a geometric average of two reference genes obtained from geNorm (Vandesompele et al. 2002). Selection of the reference genes was based on the microarray results using an algorithm described in Popovic et al. (2009). Each individual PCR run was normalized with an intercalibration standard.

**Determination of glutathione**

For the extraction of cellular glutathione, cells (100 mg) were washed three times with sodium-phosphate buffer (PBS, pH 7.4) and resuspended in 1 ml 8 mM HCl, 1.3% (w/v) 5-sulphosalicylic acid for 15 min at 4°C. Cells were then broken by vortexing at 4°C with 0.5 g of glass beads in four series of 1 min alternated with 1 min incubation on ice. Cell debris and proteins were pelleted in a microcentrifuge for 15 min (13000 rpm at 4°C), and supernatants were used for glutathione determination. For total GSH determination supernatant was used directly in 200 μl of total volume reaction as described in (Griffith 1980).

**Results**

**Rehydration nutrient effect on wine volatile composition**

To assess the effect of rehydration nutrients on fermentation derived aroma compounds we fermented grape juice using ADY rehydrated in either water or a commercially available rehydration nutrient mixture. Rehydration nutrient mix was prepared from inactivated yeast and contained an organic nitrogen source (mostly as amino acids) in addition to other yeast constituents including vitamins and lipids. As an additional point of

**Table 1 qRT-PCR primers sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPM1</td>
<td>GCTACCCGTTAAGTCTCAGT</td>
</tr>
<tr>
<td>TDH3</td>
<td>GCTGCGCCAAGAGTGTCAGT</td>
</tr>
<tr>
<td>OPT1</td>
<td>GTCGCCCAGTGGGTGTATTT</td>
</tr>
<tr>
<td>MET10</td>
<td>GCTGACGCTCAGGCTACCC</td>
</tr>
<tr>
<td>IRC7</td>
<td>CCTGAGTTTGCTGTCGTCG</td>
</tr>
</tbody>
</table>


reference we included inorganic nitrogen in the form of DAP added directly to the fermentation media. DAP addition to the fermentation media is a common practice among winemakers and its effects on wine aroma composition have been studied widely (Bell and Henschke 2005). Resultant wines were analysed for volatile chemical composition (Figure 1). The concentration of the polyfunctional thiols 3MH and 3MHA increased with the addition of rehydration nutrient while the concentration of hydrogen sulfide was significantly decreased. Other sulfur compounds including 4MMP were not affected by addition of nutrients to the rehydration media and we did not observe an effect on production of esters, higher alcohols and acids (p > 0.05) (Additional file 1). Rehydration nutrient supplementation also had no effect on growth rate or fermentation
kinetics (data not shown). Addition of DAP stimulated growth and fermentation rates and resulted in an increased concentration of the polyfunctional thiol 4MMP (Figure 1) and acetate esters (Additional file 1), while the concentration of higher alcohols was decreased (Additional file 1). Further characterisation of the effect of rehydration nutrients on the formation of volatile sulfur compounds was obtained by monitoring H₂S production throughout fermentation. Addition of rehydration nutrients resulted in an earlier onset and increased initial production of H₂S while DAP addition delayed the liberation of H₂S (Figure 1c). To test whether the rehydration nutrient effect could be attributed to YAN availability we compared the fermentation YAN concentration following ADY rehydration with either water or nutrient supplementation. As shown in Figure 1d, both treatments exhibited the same YAN consumption rate. Therefore, the increased initial production of H₂S was not correlated with available nitrogen concentration during fermentation.

Rehydration nutrient effect on gene expression profile

To gain insight into how rehydration nutrients affect H₂S formation we performed a global transcription analysis for each of the treatments. RNA was extracted from yeast samples taken after consumption of approximately 15 g/L of sugar from the grape juice. This sampling time corresponded with the initial increase in H₂S due to addition of rehydration nutrient (Figure 1c). Overall analysis of the data revealed two principal components explaining 73% of the variation in gene expression (Figure 2a). This distribution is indicative that DAP and the rehydration nutrient mix had distinct effects upon the transcriptome. Classification of the genes to MIPS functional categories (Robinson et al. 2002) revealed that both treatments affected the same groups of genes, therefore the variation explained by the PC analysis was due to differential effects upon the same metabolic pathways (Figure 2b).

Addition of the rehydration nutrient mix downregulated the expression of genes involved in the biosynthesis of different amino acids and vitamin/cofactor transport (Figure 2b), consistent with its composition in these nutrients. Interestingly, amongst the downregulated genes were those involved in H₂S production through the biosynthesis of the sulfur-containing amino acids and the sulfate assimilation pathway (Figure 2c). Addition of DAP, on the other hand, upregulated approximately 67% of the genes involved in sulfate assimilation and the synthesis of the sulfur-containing amino acids (Figure 2c). This appears to conflict with our phenotypic observations at the sampling point where the addition of rehydration nutrients induced the formation of H₂S while the addition of DAP delayed it (Figure 1c). Nonetheless, these results support our previous hypothesis of distinct effects for each of the treatments and further suggest the presence of an additional nutrient factor regulating the formation of H₂S.

Confirmation of the microarray results was obtained by an independent transcription analysis using qRT-PCR for samples taken at the same point in time used for the microarray analysis. GPM1 and TDH3 were selected as reference genes based on data obtained from the microarray analyses where both genes were shown to have high expression values and minimal variation between the different treatments. Genes related to sulfur metabolism that exhibited different trends of expressions between the treatments were chosen for validation (genes and primers are listed in Table 1). Consistent with transcriptomic data, GPM1 and TDH3 transcript levels were similar for all treatments. OPT1 was upregulated by 1.75 fold with the addition of rehydration nutrient mix and downregulated by 1.1 fold following DAP addition. MET10 was downregulated under all nutrient treatments and IRC7 was downregulated by 4.2 fold with the addition of DAP, consistent with its regulation by nitrogen catabolite repression (Scherens et al. 2006.; Thibon et al. 2008) (Figure 3).

Nutrient regulation of H₂S formation

Aside from being affected by the general YAN concentration of the media, H₂S formation is regulated by the presence of specific amino acids (Du et al. 2004.; Iranek et al. 1995.; Li et al. 2009). We therefore evaluated whether the source for the initial increase in H₂S production, which was observed following rehydration with nutrients, was the amino acid component of the mixture (detailed in Table 2). Rehydration in a solution containing an amino acid composition equivalent to the nutrient mix did not significantly affect the kinetics of H₂S formation (Figure 4a). This result suggests that amino acids were not responsible for altered H₂S formation kinetics following rehydration nutrient supplementation.

Another nutrient that is a potential source for H₂S formation is the tripeptide glutathione (GSH) (Hallman et al. 1999.; Raubelt 2008.; Søhn and Kuriyama 2001.; Vos and Gray 1979.), which can also serve as a source of organic nitrogen (Mehdi and Penninckx 1997). Analysis of the rehydration nutrient mixture revealed it contained a concentration of 500 mg/L glutathione equivalent (GSH + GSSG). Furthermore, GSH cellular content of ADY following rehydration with the nutrient mixture was ca. 1.8 fold higher than those rehydrated with water (Figure 4b). Addition of GSH as a sole nutrient during rehydration led to a significant change in H₂S formation kinetics and a higher cumulative concentration of H₂S produced during fermentation (Figure 4c). This confirms that GSH, taken up during
Figure 2 Effect of rehydration nutrient and nitrogen supplementation upon the transcriptome. (A) Biplot of a principal component analysis performed on the interaction between the factor gene and treatment. All 10,028 probe sets from the datasets were used in the analysis. (B) Classification of the genes affected by the rehydration nutrient addition to MIPS functional categories. Bars represent percentage of affected genes out of total genes in category. (C) Schematic representation of the sulfur metabolism pathway and its regulation by the two nutrient treatments: (N) rehydration nutrient addition, (D): DAP addition) in comparison to the control treatment.
rehydration, acts as a modulator of H$_2$S production during fermentation.

**Discussion**

Supplementation of ADY rehydration mixture with nutrients has become a common practice amongst winemakers because it generally improves yeast fermentation performance in suboptimal juices. In this study we compared the volatile composition of wines prepared from a low YAN juice by fermentation with ADY rehydrated with either a commercially available rehydration nutrient mixture or water. We found that the presence of rehydration nutrients affected the concentration of volatile sulfur compounds produced during fermentation (Figure 1) and the regulation of genes involved in sulfur metabolism (Figure 3). Importantly, the sheer nutrient contribution of the rehydration mix that was added with the ADY at inoculation did not have an effect on the wine volatile composition (data not shown).

Sulfur compounds exert a strong influence on wine aroma, due to their low detection threshold. These compounds can be classified into two groups based on their contribution to the sensorial properties of wine. Amongst the positive contributors are the polyfunctional thiolos, imparting fruity aroma to wine when present at moderate concentrations (Dubourdieu et al. 2006.). 3MH, its acetylated derivative 3MHA, and 4MMP are present in grapes in their precursor form, conjugated to cysteine or glutathione (Capone et al. 2010.; Peyrot des Gachons et al. 2002.; Tomina et al. 1998.). During fermentation yeast take up these precursors and cleave them to release free volatile thiolos into the media (Grant-Preece et al. 2010.; Swiegers et al. 2007.; Winter et al. 2011.). This process is affected by environmental conditions such as temperature and media composition (Masneuf Pomarède et al. 2006.; Subileau et al. 2008.). Concentration of polyfunctional thiolos in wine depends on the amount of precursor cleaved during fermentation and the resultant wine composition (Dubourdieu et al. 2006.; Ugliano et al. 2011.). In this study 3MH and 3MHA concentrations were increased with the addition of rehydration nutrients (Figure 1). Unlike 3MH and 3MHA, the concentration of 4MMP was not affected by the addition of nutrients at rehydration, while it significantly increased in fermentations where DAP was added. This result suggests that bioconversion of each thiol precursor may be driven by different regulatory mechanisms. Recently, a gene encoding a β-lyase enzyme, IRC7, was found to be the key determinant of 4MMP release. 3MH release, on the other hand, appears to be mediated by more than one gene (Roncoroni et al. 2011.; Thibon et al. 2008.), therefore it is reasonable to speculate that the treatments in our study have differentially regulated release of these thiolos. Interestingly,
Figure 4 Amino acid and GSH supplementation during rehydration. A. Profile of H$_2$S production in the headspace during fermentation following rehydration with a laboratory-made amino acids solution equivalent to the amino-acid component of the rehydration nutrient mix. B. GSH cellular content of ADY following rehydration with water or rehydration nutrient mix. Experiments were conducted in triplicates, results are presented as percentage of the control treatment. C. Profile of H$_2$S production in the headspace during fermentation following rehydration with 500 mg/L GSH. All fermentations were conducted in triplicates. H$_2$S formation was measured using gas detection tubes. Error bars represent standard deviation.
while our transcription analyses were consistent with previous studies showing the downregulation of IRC7 by the nitrogen catabolite repression (NCR) pathway, we observed an increased concentration of 4MMP in response to DAP addition. We cannot rule out that IRC7 expression may have changed throughout the fermentation; nonetheless our results support the notion that thiol release is a complex process involving multiple enzymes.

Aside from bioconversion of precursors, thiol concentrations in wine are highly affected by wine composition (Dubourdieu et al. 2006; Ugliano et al. 2011). Nutrients addition to the fermentation may have altered the final wine composition in a manner affecting thiol stability. In that case, the chemical difference between 3MH and 4MMP would account for their distinctive responses to each nutrient treatment.

A second class of sulfur compounds include those that impart unwanted odours and contribute negatively to wine quality (Swiegers and Pretorius 2007). An important compound of that group is H$_2$S, which imparts a rotten egg aroma. H$_2$S presence in wine is regarded as a sensory fault. Although the subject of H$_2$S formation during fermentation is well studied, the factors leading to residual H$_2$S in the final wine remain to be elucidated. Previous studies have pointed out a link between the kinetics of H$_2$S formation during fermentation and amount of residual H$_2$S in wine (Hiranek et al. 1996; Ugliano et al. 2009). Ugliano et al. 2009. In the study we found the supplementation of rehydration nutrients decreases the amount of residual H$_2$S and affects H$_2$S kinetics during fermentation. We can speculate that the decreased residual H$_2$S in the final wine may be due to this altered H$_2$S production kinetics, still, further study is needed in order to link between the two effects and to understand the factors affecting H$_2$S during fermentation.

H$_2$S is formed during fermentation as an intermediate in the biosynthesis of the sulfur-containing amino acids (pathway is illustrated in Figure 2c). This pathway involves reduction of sulfate: the most abundant sulfur source in grape must, into sulfide through the sulfate assimilation pathway and incorporation of sulfide into an amino acid precursor. Insufficient amounts of the amino acid precursor lead to accumulation and liberation of H$_2$S into the media. As precursor availability derives from nitrogen metabolism, YAN concentration of the media is regarded as a key regulator of H$_2$S formation (Hiranek et al. 1995).

When hydrogen sulfide formation was monitored during fermentation, we observed non-nitrogen mediated effect on H$_2$S kinetics following rehydration nutrient supplementation (Figure 1d). This suggests that nitrogen deficiency is not the sole regulator of H$_2$S production, in agreement with recent studies (Linderholm et al. 2008; Moreira et al. 2002; Ugliano et al. 2010), and that other nutrients may be involved. Subsequent transcription analyses supported this observation and demonstrated that regulation of H$_2$S formation by rehydration nutrients did not involve the sulfate assimilation pathway (Figure 2a). This pathway was downregulated in response to rehydration nutrient supplementation. On the contrary, the same pathway was upregulated following DAP addition to the fermentation medium, in accordance with previous results in the literature (Marks et al. 2003; Mendes-Ferreira et al. 2010). Together, our results suggest that H$_2$S produced under these conditions was formed via an alternative biochemical route. A potential activator of that route would be the tri-peptide glutathione, which was previously implicated as a source for H$_2$S (Rauhut 2008; Vos and Gray 1979). The nutrient mixture contained a considerable component of GSH that was taken up by yeasts during rehydration (Figure 4b) and we also observed an upregulation of genes involved in GSH metabolism following rehydration with nutrients (Figure 4c). Supplementation of the rehydration medium with GSH altered H$_2$S kinetics during fermentation (Figure 4c). Interestingly, other components of the commercial rehydration nutrient studied had a significant effect on yeast metabolic responses to GSH supplementation during this process. When GSH was added as a component of the rehydration nutrient mixture, the early stage of fermentation but did not affect the final cumulative amount of H$_2$S produced during fermentation (Figure 1c). On the other hand, rehydration in the presence of GSH alone resulted in a change in H$_2$S kinetics throughout the fermentation process and led to a higher cumulative production of H$_2$S. This difference may be associated with differences in the uptake of GSH from each medium, or reactivity of GSH with other substances of the rehydration nutrient mixture. Nonetheless, these experiments are first to demonstrate a clear effect of GSH supplementation at rehydration on the kinetics of H$_2$S formation during fermentation. It is worth noting in that regard that previous studies indicated the concentration of -50 mg/L glutathione in the grape juice is required to detect H$_2$S formation from GSH (Rauhut 2008). In this study the concentration of glutathione that was carried over from the rehydration media to the grape juice was less than 1 µg/L, highlighting the importance of glutathione uptake during rehydration.

The mechanism of GSH contribution to H$_2$S formation during the wine fermentation has not been elucidated. GSH is composed of the three amino acids: glutamate-cysteine-glycine. As such it contains both nitrogen and sulfur constituents, which may regulate the
formation of H₂S in different manners. When organic nitrogen was added to the rehydration medium as an amino acid mixture we did not observe changes in H₂S kinetics during fermentation (Figure 4a), suggesting that organic nitrogen by itself did not contribute to or regulate H₂S formation, when added at rehydration. This result points to the sulfur constituent of GSH, cysteine, as a contributor to H₂S formation. Direct production of H₂S from cysteine has been demonstrated previously for S. cerevisiae (Irani et al. 1995; Ruft et al. 2008; Tokuyama et al. 1973). Accordingly, the mechanism suggested here for H₂S production from GSH requires sulfur degradation to the individual constituent amino acids, followed by degradation of cysteine to H₂S by an enzyme having a cysteine desulphhydrase activity (EC 4.4.1.15, EC 4.4.1.11). This mechanism is in accordance with our phenotypic and transcriptomic results as it describes non-nitrogen mediated regulation on H₂S formation, which is not via the sulfate assimilation pathway. In conclusion, wine quality can be greatly affected by the composition of sulfur compounds, this study demonstrates a potential approach for sulfur aroma management by optimising yeast rehydration conditions and providing nutrients at rehydration.

Additional material

Additional file 1: Concentration of wine acids, acetate esters and higher alcohol following nutrient supplementation. Concentration of acids, acetate esters and volatile alcohols following two nutrient treatments, addition of rehydration nutrients to the rehydration media and addition of DAP to the fermentation media.

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Competing interests

The authors declare that they have no competing interests.

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A-1.2  Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) during fermentation by Saccharomyces cerevisiae

A-1.2.1  Statements of authorship

Gal Winter
Contributed to experimental design, collected and analysed the data, wrote manuscript.
Sign

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Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptophexyl acetate (3MHA) during fermentation by Saccharomyces cerevisiae

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Abstract

Background and Aims: 3-Mercaptohexan-1-ol (3MH) and its ester 3-mercaptophexyl acetate (3MHA) are potent aromatic thiols that substantially contribute to varietal wine aroma. During fermentation, non-volatile 3MH conjugates are converted by yeast to volatile 3MH and 3MHA. Two types of 3MH conjugates have been identified, 3-(3-hexan-1-yl)-L-cysteine (Cys-3MH) and 3-(3-hexan-1-yl)-glutathione (GSH-3MH). Yeast-driven formation of 3MH from these precursors has been previously demonstrated, while the relationship between 3MHA and GSH-3MH remains to be established. This paper aims to investigate yeast conversion of GSH-3MH to 3MH and 3MHA, and to assess the relative contribution of each individual conjugate to the 3MH/3MHA pool of finished wines.

Methods and Results: Fermentation experiments were carried out in model grape juice containing Cys-3MH and GSH-3MH. We found 3MH formation from GSH-3MH to be significantly less efficient than that of Cys-3MH. Conversely, esterification of 3MHA to 3MHA was higher when 3MH was formed from GSH-3MH. Additional in vitro assays for measuring enzyme deavage activity suggest the involvement of a different mechanism in 3MH conversion for the two precursors.

Conclusions: These results indicate that although both 3MH conjugates can be converted by yeast, the type of precursor affects the rate of formation of 3MH and 3MHA during fermentation.

Significance of the Study: Management of the pool of aromatic thiols during fermentation can depend on relative proportions of different 3MH conjugates.

Keywords: 3MH, 3MHA, Cys-3MH, fermentation, GSH-3MH, thiol precursor

Introduction

The volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptophexyl acetate (3MHA) have been identified as major contributors to wine aroma and are associated with aroma characteristics of fruits such as passion fruit and grapefruit (Tominaiga et al. 1998b, Dubourdieu et al. 2006). 3MH is present in grape juice as a non-volatile cysteine-conjugated precursor (Tominaiga et al. 1998a). During fermentation, the precursor is cleaved, and the free thiol is released as a result of yeast metabolic activity. Further metabolism of 3MH results in the formation of the acetate ester 3MHA (reviewed by Dubourdieu et al. 2006, Swiegen and Pretorius 2007). The conversion process of 3MH-conjugated precursors has been the focus of a considerable amount of research, though many aspects remain to be elucidated. One important unresolved question concerns the types of precursors present in the grape juice and the ability of yeast to metabolise them during fermentation.

Two non-volatile 3MH conjugates have been identified to date: 3-(3-hexan-1-yl)-L-cysteine (Cys-3MH) (Tominaiga et al. 1998b) and 3-(3-hexan-1-yl)-glutathione (GSH-3MH) (Peyron des Gachons et al. 2002). Additionally, an alternative biogenetic pathway from (E)-hex-2-enal leading to 3MH was demonstrated (Schnieder et al. 2006). Subtle et al. (2008a) found that neither (E)-hex-2-enal nor Cys-3MH was responsible for the majority of free 3MH and 3MHA detected in wine. The same authors found that deletion of the GPT1 gene that encodes the main glutathione transporter resulted in lower formation of 3MH, suggesting that GSH-3MH could be the major 3MH precursor. Nonetheless, current data regarding the conversion of 3MH are limited to Cys-3MH, while the role of the glutathionylated 3MH conjugate still needs to be examined. In particular, while the ability of yeast to generate 3MH from GSH-3MH has been recently shown (Grand-Prece et al. 2010, Roland et al. 2010), the importance of this precursor for the formation of the powerful odorant 3MHA remains to be established. Having an
odour threshold of 4 mg/L. 3MHA is one of the most powerful odourants known and is a major contributor to the distinct aroma characters of many red and white wines (Mateo-Vivacho et al. 2009). Moreover, a direct comparison of the ability of the two precursors hitherto identified, namely Cys-3MH and GSH-3MH, to give 3MH and 3MHA has not been carried out. In this study, using chemically pure synthetic 3MH conjugates, we examined the ability of yeast to metabolise GSH-3MH and compared the potential of this process to yield 3MH and 3MHA in comparison to the conversion of Cys-3MH.

Materials and methods

Chemicals

Analytical reagents have been purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise specified. GSH-3MH and Cys-3MH were synthesised and characterised in our laboratories (Pardon et al. 2008, Grant-Preece et al. 2010). Both precursors were found to be stable at wine pH as well as in storage at −20°C (Capone et al. 2010)

Culture media, yeast strain and fermentation conditions

Fermentations were conducted in triplicate, with a working volume of 1 L under isothermal conditions (20°C) and agitation (150 rpm). A chemically defined grape juice medium based on Henschke and Jiranek (1995) was used with the following modifications: 1.5 g/L magnesium sulphate heptahydrate, 1 mg/L pyridoxine HCl, 1 mg/L nicotinic acid, 0.5 mg/L L-lysine, 0.05 mg/L biotin and 0.05 mg/L L-ascorbic acid. Nitrogen component of the media was 13.5 mg/L alanine, 9.1 mg/L γ-aminobutyrate, 34.2 mg/L arginine, 0.5 mg/L asparagine, 3.8 mg/L aspartate, 0.5 mg/L citrulline, 7.6 mg/L glutamate, 10.6 mg/L glutamine, 0.5 mg/L glycine, 1.5 mg/L histidine, 1.5 mg/L isoleucine, 1.5 mg/L leucine, 0.5 mg/L L-lysine, 0.5 mg/L methionine, 0.5 mg/L ornithine, 1.1 mg/L phenylalanine, 6.8 mg/L serine, 7.6 mg/L threonine, 0.5 mg/L tryptophan, 0.5 mg/L tyrosine, 2.7 mg/L valine, 1.5 mg/L cysteine, 82.1 mg/L proline and 11.6 mg/L ammonia solution. Yeast assimilable nitrogen value of this medium was 155 mg N/L, composed of 68 mg/L free alpha amino nitrogen and 106 mg NH₄/L. For the different precursor treatments, medium was supplemented with either Cys-3MH or GSH-3MH as specified in Table 1. Yeast strain used in this study was Saccharomyces cerevisiae VLM from LaForte Australia (Woodville North, South Australia, Australia). Active dry yeasts were rehydrated as recommended by the manufacturer and then inoculated into the fermentation media to give a cell concentration of 1 × 10⁶ cells/mL. Fermentation progress was monitored using sugar consumption using a commercial kit by Randox (GF2635, Randox, Crumlin, UK). Biomass formation was monitored by measuring at 600 nm. At the end of fermentation, the wines were cold settled at 4°C, and free SO₂ of the finished wine was adjusted to 45 mg/L by the addition of potassium metabisulphite. The wines were then carefully racked into glass bottles, which were sealed with airtight caps fitted with a Polytetrafluoroethylene (PTFE) liner. Bottles were fully filled to avoid any headspace oxygen.

Analysis of 3MH and 3MHA

Samples of the finished wines were air sent to SARCO Laboratories (Bordeaux, France) for 3MH and 3MHA analysis. 3MH and 3MHA were measured according to Tomimaga et al. (2000) using a TRACE GC-MS (ThermoFisher Scientific, Waltham, Massachusetts, USA) equipped with a BP-20 capillary column (SGE, 50 m, 0.22 mm internal diameter, film thickness 0.25 µm). Injector temperature was 250°C. The column was heated at 35°C for 10 min, and then the temperature was raised to 230°C at 3°C/min. The volatile thiol 3MH and 3MHA were analysed in selected ion monitoring (SIM) mode using the ions m/z 134 and m/z 116 for 3MH and 3MHA, respectively. 4-Methoxy-2,3-dihydro-2-methoxybutane was used as internal standard (m/z 134). Detection limits for 3MH and 3MHA using this method were 11 ng/L and 1 ng/L, respectively. Quantification limit is 35 ng/L ± 20% for 3MH and 3 ng/L ± 18% for 3MHA. Molar conversion yields were calculated as the total concentration of 3MH and 3MHA (nM) measured at the end of fermentation divided by the concentration of potential 3MH (the relative part of 3MH from its conjugate, nM) added to the media ([(134) + [3MHA] + [potential 3MH])]. 3MH esterification ratios were calculated as 3MHA final concentration (nM) divided by the total concentration of 3MH and 3MHA (nM) ([(134) + [3MHA] + [3MH])].

Cystine β-hexosase assay

The assay mixture contained 110-µg Cys-3MH or 378-µg GSH-3MH, 20-µL 1 mM pyridoxal 5′-phosphate and 500-µL assay buffer (50 mM phosphate buffer, pH 7.5, 1 mM Ethylenediaminetetraacetic acid (EDTA)). Distilled H₂O was added to obtain a

<table>
<thead>
<tr>
<th>Precursor type</th>
<th>Molecular weight (g/mol)</th>
<th>Supplemented amount (µg/L)</th>
<th>Potential 3MH (µg/L)</th>
</tr>
</thead>
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<td><img src="image" alt="Chemical structure" /></td>
<td>221.32</td>
<td>500</td>
<td>303.5</td>
</tr>
<tr>
<td><img src="image" alt="Chemical structure" /></td>
<td>409.1</td>
<td>925</td>
<td>303.5</td>
</tr>
</tbody>
</table>

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volume of 1 mL per reaction. Twenty microlitres of tryptophanase enzyme (apoptryptophanase from Escherichia coli, 10 µg/µL) was added, and the reaction was incubated at 37°C for 60 min. Following incubation, 100-µL 1 mM Ellman’s reagent (5,5-dithiobis-(2-nitrobenzoic acid)) was added, and absorbance was measured at 412 nm to give an indication of the formation of thiols during the reaction. Experiments were conducted in triplicates.

Results and discussion

To test whether GSH-3MH is metabolised by yeast during fermentation, we supplemented chemically pure synthetic GSH-3MH to a chemically defined grape juice medium, which was then fermented by yeast. Separate fermentations supplemented with Cys-3MH in equimolar amounts of potential 3MH (Table 1) were carried out to compare the conversion rates of GSH-3MH and Cys-3MH. Figure 1 shows fermentation kinetics under the different fermentation conditions of this study. All fermentations achieved dryness, and supplementation with 3MH conjugates had no effect on biomass formation or fermentation rate, despite each 3MH conjugate contributing yeast nutrients in the form of glutathione and cysteine.

To assess the conversion rate for the two 3MH conjugates, concentrations of 3MH and 3MHA were measured at the end of fermentation. Molar conversion yields were calculated under the assumption that both precursors were fully taken up, and products (i.e. 3MH and 3MHA) were not converted to other (unknown) products that were not measured. No 3MH or 3MHA were detected in fermentations that were not supplemented with 3MH precursors. Figure 2 shows a molar conversion yield of approximately 1% for Cys-3MH and a value of approximately 0.5% for GSH-3MH. The conversion yield of Cys-3MH is in agreement with data previously published (Murat et al. 2001, Subileau et al. 2004a), whereas this is the first time that the conversion yield of GSH-3MH to 3MH and 3MHA has been estimated. This result demonstrates that GSH-3MH is metabolised by yeast to release the volatile thiol 3MH during fermentation. Nevertheless, when comparing conversion yields for the two precursors, the amount of 3MH obtained from Cys-3MH is significantly higher than that obtained from GSH-3MH. This result indicates that Cys-3MH is a more favourable substrate for the formation of 3MH under the fermentation conditions used.

Interestingly, no significant differences were observed in 3MHA concentrations obtained using the two precursors (Figure 2). As the concentration of 3MH was significantly different for each precursor treatment, this result indicates a difference in the esterification ratios (3MHA concentration/total 3MH + 3MHA concentration) of 3MH for the two precursors.
Figure 3. Schematic of 3-mercaptohexan-1-ol (3MH) precursor conversion pathways to 3MH and 3-mercaptohexyl acetate (3MHA). Straight arrows represent data that have been investigated in the context of 3MH precursors’ metabolism. Dashed arrows represent inferred pathways, based on studies regarding flavour metabolism. S-3-(hexan-1-ol)-glutathione (GSH) and GSH conjugates’ metabolism. (1) Transport of GSH-3MH into the cell through the GSH transporter OPT1 (Subileau et al. 2008a); (2) cleavage of GSH-3MH by γ-glutamyltransferases present in the grape juice to form a dipeptide precursor and transport of the precursor into the cell (Ganguly et al. 2007); (3) transport of S-3-(hexan-1-ol)-L-cysteine (Cys-3MH) through the general amino acid transporter GAPI (Subileau et al. 2008b); (4) cellular metabolism of GSH-3MH. Inferred pathways include direct cleavage of GSH-3MH to produce 3MH and the tripeptide Glu-Ala-Gly, or multi-step degradation of the precursor through either the cytoplasm (Ganguly et al. 2007) or vacuole (Jaspers et al. 1985, Ubybov et al. 2006) to single amino acids, which will then be cleaved to release 3MH from its resulting cysteine conjugate; (5) release of 3MH from Cys-3MH by yeast β-lactase enzymes (Tominaga et al. 1998a, Swiegers et al. 2007, Thibon et al. 2008); (6) acetylation of 3MH to form 3MHA (Swiegers et al. 2005) and hydrolysis of 3MHA to 3MH (Mauricio et al. 1993); (7) release of 3MH and 3MHA into the wine by unknown mechanisms.

In this case, contrary to the conversion yields for 3MH release, 3MH esterification ratios were higher for GSH-3MH (1.23%) than Cys-3MH (1.14%). This suggests that the formation of 3MHA is not regulated by the concentration of 3MH. Indeed, it is generally agreed that acetate ester formation is mainly influenced by the expression levels of the esterifying enzymes ATE1 and ATE2, rather than by substrate availability (Lilly et al. 2006, Verstrepen et al. 2001b). Genetic regulation of these enzymes is mediated through nutritional factors including available nitrogen concentration (Yoshimoto et al. 2002, Verstrepen et al. 2003a, Vilanova et al. 2007). Consequently, with each 3MH precursor contributing different amino acids to the media, it is possible that it is the nitrogen fraction of each precursor addition that affects 3MHA formation. Notably, in this experiment, precursor supplementation was less than 1 mg/L (Table 1). While this degree of nutrient supplementation is probably too marginal to account for any differences in fermentation progress (measured in g/L of sugar consumption, Figure 1), it may account for the more attenuated differences in 3MH esterification ratio (measured in ng/L). Further research is needed to understand the factors affecting 3MHA formation and the degree of nutrient regulation on this process.

The difference in 3MH conversion yields for the two precursors (Figure 2) may be attributed to a number of factors. An obvious one to consider regards the metabolic pathway responsible for the conversion of 3MH amino acid precursors into the free volatile thiol. The process of 3MH release from its cysteinylated precursor has been studied extensively. While the genes coding for the enzymes responsible for this pathway in yeast have not been fully identified, there is a general agreement regarding the biochemical processes involved. As the structure of Cys-3MH is similar to that of cysteine, uptake of the precursor is assumed to be driven by amino acid transporters. Indeed, yeast deletion assays have found the uptake of Cys-3MH to be partially mediated through the general amino acid transporter, GAPI (Subileau et al. 2008b). Once inside the cell, Cys-3MH is thought to be cleaved by an enzyme with carbon-sulfur β-lactase activity (Tominaga et al. 1998a, Swiegers et al. 2007, Thibon et al. 2008). The products of that reaction are pyruvate, ammonia and the free volatile thiol, 3MH. Pyruvate and ammonia are known participants in numerous cellular reactions and are further metabolised. 3MH may be further acetylated to form 3MHA (Swiegers et al. 2005), or alternatively, it may be released into the culture medium by a mechanism still unknown. Current knowledge on the Cys-3MH conversion pathway is illustrated in Figure 3.

The conversion pathway of the glutathione precursor has not been studied. It is reasonable to suppose that this conversion may occur via two separate pathways. One is through a direct, one-step cleavage of 3MH from GSH-3MH, analogous to the direct reaction observed with the cysteine precursor. This direct reaction may be replicated in vitro using a β-lactase enzyme. While this enzyme cleaves cysteine conjugate 3MH with high efficiency (Wakabayashi et al. 2003, Swiegers et al. 2007), we have found it to have no activity towards the glutathione precursor (result not shown), thus supporting the possibility of a different degradation pathway.

A second putative pathway, inferred from studies regarding GSH metabolism, includes a multi-step degradation process. In this pathway, the first steps would involve degradation of the thiol conjugate tripeptide (Glu-Cys-3MH-Gly) into single amino acids (Glu, Cys-3MH and Gly) (Jaspers et al. 1985, Ubybov et al. 2006).
et al. 2006, Ganguli et al. 2007), followed by cleavage of Cys-3MH to release free 3MH (Figure 3). Degradation of GSH-3MH through this pathway would be in agreement with reports regarding catabolism of xenobiotic glutathione conjugates by yeast that have been shown to involve degradation of the tripeptide to individual amino acids (Ubilovik et al. 2006, Wünschmann et al. 2010).

In addition to different conversion pathways, other factors may be responsible for the observed difference in 3MH conversion yields. An important one to consider involves differences in the uptake for the two precursors, as each precursor is being taken up by different transporters (detailed in Figure 3). Differences in transport efficiency are likely to deliver different amounts of precursors and may affect the final conversion rate. Additionally, each transporter is subjected to a different regulation mechanism, which may affect the timing of precursor uptake. This is of particular importance to the final conversion rate as 3MH release and, subsequently, esterification are reported to occur at a higher extent during the early stages of fermentation (Tominaiga et al. 1998b, 2006). Another aspect to consider is 3MH metabolism. Aside from esterification, 3MH may participate in other, unknown reactions. These reactions may be affected by the precursor type as we have observed for the esterification ratio (Figure 2). Finally, the different conversion efficiency of GSH-3MH may be compensated by its high abundance, which in some grape juices has been reported to be up to 35 times higher than that of Cys-3MH (Capone et al. 2010). More extensive investigations are needed to establish the nature of the main precursor for 3MH in wine fermentations.

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References


Contribution of precursors to JMHB and JMHA release


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Invention Title: Method for hydrogen sulfide detection

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University of Western Sydney

PROVISIONAL SPECIFICATION

Invention Title:

Method for hydrogen sulfide detection

The invention is described in the following statement:
Technical Field
The present invention relates to methods for detecting hydrogen sulfide (H₂S), particularly H₂S produced by microorganisms during cell culture.

Background Art
Sulfur compounds exert a strong influence on the aroma of food and fermented beverages, due to their low detection threshold and high reactivity. Sulfur compounds are produced during microbial cell growth as a result of the microorganism metabolism. Amongst the positive contributors to foods and beverages are the polyfunctional thiols, imparting fruity aroma while a noted compound of the negative contributors is H₂S, imparting ‘rotten eggs’ aroma.

Hydrogen sulfide may be formed metabolically by microorganisms from either inorganic sulfur compounds, sulfate and sulfite, or organic sulfur compounds, cysteine and glutathione present in culture. Hydrogen sulfide, a highly volatile compound which imparts a ‘rotten egg’ aroma, is considered a major off-flavour in fermented food and beverage products. The nature of the sulfur source affects greatly on the timing for H₂S production and the final aroma of food and fermented beverages.

In microorganisms, the amount of H₂S produced during cell culture is dependent upon genetic factors of the microorganism and cell culture conditions, such as the nutrient composition of the cell growth media.

Several techniques are currently employed for the detection of H₂S after the completion of the cell culture or growth. Known qualitative methods routinely use bismuth sulfite agar plates or lead acetate strips. Known quantitative methods commonly utilise colorimetric assays. The quantitative methods commonly utilise Cd(OH)₂ and methylene blue colorimetric reaction or lead acetate detection tubes. A known high throughput method uses a membrane impregnated with silver nitrate. However, the known methods are limited in either high throughput applicability or sensitivity and do not detect natural levels of H₂S produced during cell culture. The known methods often require the addition of cysteine to the cell culture media as a sulphur source. All known methods cannot characterise genetic and cell culture factors affecting H₂S production during microbial cell culture.

There is a need for a high throughput method for the characterisation of H₂S production during microbial culture. A further need is to better understand the factors affecting H₂S
production during microbial culture. There is an additional need for a method which is able to the detection of both genetic and cell culture factors affecting H₂S production during microbial culture. Another need is for high throughput analysis of H₂S production during microbial cell growth which is capable of distinguishing between the different sulfur sources.

The present inventors have developed a new method for detecting H₂S produced by a microorganism that is suitable for high throughput analysis of microbial H₂S production.

Disclosure of Invention

The present inventors have surprisingly found that the addition of a thiazine dye to a culture medium does not adversely effect microbial cell growth or metabolism and the dye can be used to determine H₂S production during microbial culture.

A group of dyes called “thiazine dyes” includes Methylene blue, Azure A, Methylene green, New methylene blue, Tolonium chloride, and chemical variations or modifications thereof. These dyes have the potential to act similarly to methylene blue. Also, chemical variation on methylene blue or other thiazine dyes such as ethylene blue may also be suitable for the present invention. Preferably, the thiazine dye is methylene blue.

In a general aspect, the present invention relates to culturing a microorganism in the presence of a thiazine dye to determine H₂S production by the microorganism.

In a first aspect, the present invention provides a method for detecting H₂S produced by a microorganism comprising:

- providing a thiazine dye to a culture medium;
- culturing a microorganism in the culture medium; and
- detecting H₂S produced by the microorganism by determining a colour change of the culture medium from an interaction of the thiazine dye with H₂S.

The microorganism may be a bacterium or yeast. Preferably, the bacterium or yeast is suitable for, or used in, food or beverage production.

The thiazine dye may be added to the culture medium in an amount from about 1 μg/ml to about 1 mg/ml or more. Preferably, the thiazine dye is added to the culture medium at about 50 μg/ml. Typically, there needs to be sufficient dye present to provide a colour detectable by any suitable means.
In order to assist the thiazine dye to react with H₂S, a catalyst may also be added to the culture medium. Preferably, the catalyst is a metal cation. Preferably, the metal cation is at an oxidation number of IV selected from Se(IV), Te(IV), Ti(IV). More preferably, the transitional metal is titanium oxide. Titanium oxide is typically added to the culture medium at about 1 μg/ml to 1 mg/ml or more.

The thiazine dye may be added to the culture medium as a mix containing a thiazine dye, catalyst and any other co-factors or buffers. An example of a suitable mix includes 5 ml of 1 mg/ml methylene blue, 1 ml of 1 mg/ml titanium oxide and 4 ml of 100 mM citric acid buffer at pH 4.5.

The culture medium may be liquid or semi solid or solid. Preferably, the culture medium is liquid or broth. The semi solid or solid culture medium may be an agar medium provided as a petri dish or a tube containing an agar slope.

The culturing a microorganism in the culture medium may be carried out in a microtitre plate having multiple wells or an array of test tubes or an array of suitable culture vessels. The culture vessels may be flasks or schott bottles or plastic centrifuge tube or glass tube or cuvette.

The microorganisms may be cultured under suitable conditions for cell growth. The culturing may occur in an incubator with or without agitation. The culturing may occur for any suitable time and temperature. Typical culture times range from about 1-5 hours and to about 60 or more hours. The culturing may occur at temperatures from about 14°C to about 40°C. Incubation temperatures of about 21°C, or about 22°C, or about 23°C, or about 24°C, or about 25°C, or about 26°C, or about 27°C, or about 28°C, or about 29°C, or about 30°C, or about 31°C, or about 32°C, or about 33°C, or about 34°C, or about 35°C, or about 36°C, or about 37°C have been found to be suitable for a range of different microorganisms. It will be appreciated that the incubation times and temperatures may vary depending on the microorganism being cultured.

The microorganisms may be cultured aerobically or anaerobically.

The colour change of the culture medium can be measured optically. Preferably, any colour change is monitored by a spectrophotometer, a plate reader, or image editing software. Preferably, the measurement occurs at a visible wavelength in the range of about 380 nm to about 750 nm. Preferably, the wavelength is between about 600 nm to 663 nm. More preferably, the wavelength is 663 nm.

Determining the colour change may be through a single measurement, multiple measurements or continuous measurement during the microbial culture.
If H₂S is produced, there will be a decolourisation of the medium. The present inventors have found that there is a quantitative relationship with the colour of the medium and H₂S present.

In a preferred form, a plurality of microorganisms are cultured in a plurality of culture media containing a thiazine dye and H₂S production is compared between the plurality of microorganisms. For example, microorganisms cultured in a microtitre plate containing multiple wells of culture media can be used as a high throughput assay to compare H₂S produced by different microorganisms. Similarly, the same microorganism can be tested for H₂S production in a plurality of different media.

An H₂S profile for a given microorganism may be obtained by multiple measurements or continuous measurement during the microbial culture. Preferably, continuous measurement during culture is used to obtain an H₂S profile.

It will be appreciated that the present invention maybe used to compare H₂S production from strains of a given microorganism to compare H₂S production. The strains may contain one or more mutations and mutant genotypes maybe studied for their affect on H₂S production.

The present invention is particularly useful to select suitable microorganisms for starter cultures or for microbial fermentation for the food and beverage industry. Industries that utilize such microorganisms include dairy, fermented beverages. For example, starter cultures or microbial strains may be used in the production of cheese, yoghurt, wine, beer, vinegar, fermented meat, or fermented yogurt.

In a second aspect, the present invention provides a method for determining an affect of an agent on H₂S production by a microorganism, the method comprising:

- providing a thiazine dye to a culture medium;
- providing an agent to the culture medium;
- culturing a microorganism in the culture medium;
- detecting H₂S production by the microorganism by determining a colour change of the culture medium from an interaction of the thiazine dye with H₂S; and
- comparing H₂S production by the microorganism under similar culture conditions in the absence of the agent to determine any effect of the agent on H₂S production by the microorganism.
The agent maybe a nutrient, co-factor, food additive, food component, substrate, amino acid, peptide, protein, metal, vitamin, element or the like.

The present invention is particularly suitable for the food and beverage industry where H₂S may be a problem of spoilage or unwanted organoleptic qualities.

In a third aspect, the present invention provides a high throughput method for detecting H₂S produced by microorganisms comprising:

- providing a thiazine dye to a plurality of vessels containing culture media;
- culturing microorganisms in the plurality of vessels; and
- detecting H₂S produced by the microorganisms by determining colour change of the culture media from an interaction of the thiazine dye with H₂S in the plurality of vessels.

The microorganisms may be different microorganisms or strains and the plurality of vessels contain the same culture media. In this form, H₂S production by different microorganisms can be compared under the same culture conditions.

Alternatively, the microorganisms may be the same microorganism or strain and the plurality of vessels contain different culture media. In this form, H₂S production from different media by the same microorganism can be compared.

The plurality of vessels may be a microtitre plate or the like having multiple wells. A preferred array of vessels is a 96 well microtitre plate but other plates with a lower or higher number of wells would also be suitable. An advantage of such an arrangement is that small volume cultures can be carried out and handled easily with established laboratory equipment.

The plurality of vessels may be an array of test tubes or an array of culture vessels. The culture vessels may be tubes, flasks, schott bottles or the like, or plastic centrifuge tube or glass tube or cuvette.

The vessels may incubated on a rocking platform, shaking tray, water bath or any suitable incubator.

Preferably, the plurality of vessels are exposed to the same incubation conditions at substantially the same time.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or
steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this specification.

In order that the present invention may be more clearly understood, preferred embodiments will be described with reference to the following drawings and examples.

**Brief Description of the Drawings**

Figure 1: A Reduction reaction between methylene blue and sulfide, catalysed by traces of titanium dioxide, leading to decolourisation of methylene blue. B Cell culture performance measured by CO₂ production rate.

Figure 2: Analysis of H₂S production during cell culture using methylene blue reduction method. A Cells from an overnight culture grown on YPD were inoculated into a 96 well plate filled with model grape juice with or without methylene blue added to the cell culture medium. B H₂S production profile generated using methylene blue added to the cell culture medium (left) or using lead acetate detection tubes (right). C Method linearity, known amounts of H₂S added into a 96 well plate filled with model grape juice and methylene blue added to the cell culture medium.

Figure 3: Detection of genetic factors affecting H₂S production. H₂S profile generated using methylene blue added to the cell culture medium for a known high H₂S producer, AWRI1483 and a low H₂S producer, AWRI796.

Figure 4: Detection of cell culture factors affecting H₂S profile. A H₂S profile using lead acetate detection tubes. B H₂S profile produced for a microbial cell culture using methylene blue added to the cell culture medium.

Figure 5: H₂S profile kinetic parameters for various microorganisms.
Mode(s) for Carrying Out the Invention

Method

A preferred method employed a catalytic-reduction reaction between methylene blue and sulfide ions, catalysed by traces amounts of transitional metal. The reaction leads to decolourisation of methylene blue (Figure 1a). Under cell culture conditions, sulfide ions are present as H₂S. Incorporation of the methylene blue into the cell culture media allows the immediate in situ detection of H₂S produced during cell culture, without affecting cell culture performance (Figure 1b). A H₂S production profile may then be generated by kinetic spectrophotometric measurements at 663 nm.

Procedure

To demonstrate high throughput methodology, cell cultures were conducted in 96 well plate at a total volume of 200 µl per well. Each well contained 170 µl of cell culture media (model grape juice), 10 µl of microbial cells culture to give a final optical density of 0.3-0.5 at 600 nm wavelength and 20 µl of the methylene blue reaction mix. Reaction mix contained 5 ml of 1 mg/ml methylene blue, 1 ml of 1 mg/ml titanium oxide and 4 ml of 100 mM citric acid buffer at pH 4.5. The 96-well plate was covered with a Breathe easy membrane (Astral Scientific, Australia). Cell cultures were monitored spectrophotometrically at 663 nm and 600 nm using a 96 wells plate reader. Cell cultures were carried out in quadruplicate. Duplicate cell cultures were performed without the reaction mix to monitor microbial cell growth rate as measured by the absorbance at 600 nm. Un-inoculated wells containing cell culture media with or without reaction mix were monitored to detect media contamination, and spontaneous production of H₂S, respectively.

Data Analysis

Kinetic spectrophotometric measurements throughout cell culture allowed the generation of H₂S production profiles. These profiles represent methylene blue decolourisation rate normalized to biomass formation, and is calculated as

\[
\left[ \frac{(OD_{663} - OD_{600}) - (OD_{6631} - OD_{6001})}{OD_{600}, no reaction mix control} \right]
\]

Comparison of the methylene blue reduction (MBR) method profile with a quantitative H₂S production profile, obtained using H₂S detection tubes, shows similar trends (Figure 2). Both profiles show an initial lag followed by increased rate of H₂S production,
an H₂S maxima, and then production of smaller amounts of H₂S (Figure 2). Kinetic parameters can be extracted from the MBR method profile using a locally weighted regression (loess) algorithm (supplementary data) (Figure 2). Table 1 shows reproducibility of kinetic parameters extracted from inter-plate, triplicate cell cultures of the yeast AWRI1631. Due to equilibrium of the chemical reaction, once H₂S production slowed, H₂S evaporation altered the rate of the reaction and methylene blue re-colourised within the cell culture media (Figure 2). As a consequence, precision of kinetic parameters extracted following the point of maximum H₂S detection is limited.

Table 1 – Kinetic parameters extracted from AWRI1631 micro cell culture

<table>
<thead>
<tr>
<th></th>
<th>Lag time (AU)</th>
<th>Increase rate (AU)</th>
<th>Maximum value (AU)</th>
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<tr>
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<tr>
<td>CV (%)</td>
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<td>15.56</td>
<td>13.26</td>
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</table>

Detection threshold and linearity

To construct a calibration curve, known volumes of aqueous solution of Na₂S-9H₂O (final H₂S concentration of 1 mg/ml) previously standardized by means of iodine-thiosulfate titration were added to a cell culture media containing methylene blue reaction mix and were monitored at 663 nm for 30 minutes. The MBR method displayed linearity between the ranges of 0-50 μg H₂S (Figure 2). Samples spiked with Na₂S-9H₂O plus potassium bisulphate (final SO₂ concentration of 50 mg/L), dimethyl sulfide (1 mg/ml) and methyl mercapto acetate (1 mg/ml) were also tested under the same conditions. No interference in H₂S detection was observed following the additions.

Method Validation

Method validation was carried out by measuring H₂S profiles for known high and low H₂S producing strains, AWRI1483 and AWRI796, respectively. From 5 hrs post-inoculation AWRI1483 produced H₂S at a greater rate than, and reached a greater
maximum value, in comparison to AWRI796 (Figure 3), in agreement with previous work utilising quantitative methods. The MBR method was also tested for its ability to detect different environmental factors affecting H$_2$S production, including media yeast assimilable nitrogen (YAN) and cysteine concentration. Quantitative determination of H$_2$S produced during cell culture, using detection tubes, demonstrated that increasing YAN concentration of the media from 150 mg N/L to 350 mg N/L through addition of diammonium phosphate (DAP) decreased the amount of H$_2$S produced. Addition of freshly made cysteine solution, at a concentration of 500 mg/L, decreased the lag time for H$_2$S production and increased both the rate and amount produced (Figure 4a).

Concurrent cell cultures evaluated on a micro-scale cell culture using the MBR method displayed the same trends. DAP addition decreased the H$_2$S production maxima while cysteine addition decreased lag time, increased both H$_2$S production rate and maximum value (Figure 4b).

Method Applicability

H$_2$S production is an evolutionary conserved phenomenon. Applicability of the MBR method to various cell culture systems was tested using micro-scale cell cultures of various microorganisms (Table 2). H$_2$S production was detected in all cell cultures. Moreover, differences in H$_2$S production due to cysteine addition were detected using this method (Table 2).

Conclusions

This *in situ* method for H$_2$S detection during cell culture. Kinetic parameters obtained using this method can be successfully used for profiling H$_2$S production in various cell culture systems, enabling detection of different environmental sources for H$_2$S production. The method is suited for high throughput screening purposes by virtue of its simplicity and ability to detect H$_2$S during cell culture.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.
<table>
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<th>Lag Time (Hours)</th>
<th>Increase Rate (AU)</th>
<th>Maximum Point (AU)</th>
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<td>Cysteine addition caused growth inhibition</td>
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<td>Cysteine addition caused growth inhibition</td>
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<tr>
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<td>0.005</td>
<td>0.398</td>
<td>3.77</td>
<td>0.44</td>
<td>1.65</td>
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<td>Model grape juice with 10% sugars and the auxotrophic amino acids</td>
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<tr>
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<td>17.05</td>
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<td></td>
<td></td>
<td></td>
<td>Model grape juice</td>
<td>Cysteine addition caused growth inhibition</td>
</tr>
</tbody>
</table>
Claims:

1. A method for detecting H₂S produced by a microorganism comprising:
   providing a thiazine dye to a culture medium;
   culturing a microorganism in the culture medium; and
   detecting H₂S produced by the microorganism by determining a colour change of
   the culture medium from an interaction of the thiazine dye with H₂S.

2. A method for determining an effect of an agent on H₂S production by a
   microorganism, the method comprising:
   providing a thiazine dye to a culture medium;
   providing an agent to the culture medium;
   culturing a microorganism in the culture medium;
   detecting H₂S production by the microorganism by determining a colour change
   of the culture medium from an interaction of the thiazine dye with H₂S; and
   comparing H₂S production by the microorganism under similar culture conditions
   in the absence of the agent to determine any effect of the agent on H₂S production by
   the microorganism.

3. A high throughput method for detecting H₂S produced by microorganisms
   comprising:
   providing a thiazine dye to a plurality of vessels containing culture media;
   culturing microorganisms in the plurality of vessels; and
   detecting H₂S produced by the microorganisms by determining colour change of
   the culture media from an interaction of the thiazine dye with H₂S in the plurality of
   vessels.

4. The method according to any one of claims 1 to 3 wherein the thiazine dye is
   selected from Methylene blue, Azure A, Methylene green, New methylene blue,
   Tolonium chloride, or chemical variations thereof.

5. The method according to claim 4 wherein the thiazine dye is methylene blue.
A

B

Figure 1

---

**Figure 1**

- **A**: Chemical reaction diagram showing a chemical reaction with blue and colorless states.
- **B**: Graph showing the production of O₂ over time with and without methylene blue.
Figure 2
Figure 4
A-1.4  In situ high throughput method for H$_2$S detection during micro-scale wine fermentation

A-1.4.1 Statements of authorship

Gal Winter
Contributed to experimental design, collected and analysed the data, wrote manuscript.
Sign

Chris D. Curtin
Contributed to the research idea and experimental design, supervised the work, assisted with preparation and editing of the manuscript and acted as corresponding author.
Sign
A-1.4.2 Research paper

In situ high throughput method for H₂S detection during micro-scale wine fermentation

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ABSTRACT

An in situ high throughput method for the detection of H₂S during fermentation was developed. The method utilizes a redox reaction in which sulfide ion reduces methylene blue, leading to its depolarization. Incorporation of methylene blue into the fermentation media allows real-time detection of H₂S during fermentation and the generation of an H₂S production profile. Kinetic parameters extracted from the H₂S production profile can be used to characterise genetic factors affecting H₂S production and differentiate between environmental conditions affecting it. The method, validated here for Hedosophia salome, is suited for high throughput screening purposes by virtue of its simplicity and the ability to detect H₂S in micro-scale fermentations.

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1. Introduction

Hydrogen sulfide (H₂S) is a major component in the aroma of fermented food products, including wine, beer and cheese, in which it can play an attractive or repulsive role (Kadota and Ishida, 1972; Landuad et al., 2008). In isolation, H₂S possesses an odor reminiscent of 'roasted egg' and can be perceived sensorially at very low concentrations (~1 μg/L) (Ratzen, 1995). Furthermore, it is highly reactive and functions as a precursor for the development of other sulfur aroma compounds such as dimethyl sulfide, diethyl sulfide and dimethyl disulfide, which impart aromas of 'soiled cabbage', 'cooked vegetables', 'onion', 'garlic' and 'burnt rubber' (Landuad et al., 2008; Metres et al., 2009; Swofford et al., 2009). In fermented foods, H₂S is formed by microbial metabolic activity during the fermentation process. Microorganisms are able to produce H₂S from inorganic sulfur compounds, sulfate and sulfides, through the sulfate assimilation pathway (Thomas and Sardis-Keenan, 1987) or other organic sulfur compounds, through the catabolism of cysteine and glutathione (Heinrichs and Jirasek, 1993; Landuad et al., 2008; Ratzen, 1993, 2008).

Having a central role in the final sensory qualities of food, the fermented food industries have invested extensively in the development of specialized microorganisms to produce high (for the purposes of cheese production) or low (for the purposes of wine and beer production) amounts of H₂S. Several techniques are currently employed to estimate H₂S production potential, or detect H₂S during a single fermentation. Qualitative methods include the use of thiosulfate sulfide agar plates (Giudici and Runkel, 1994) or lead acetate strips (Giudici and Runkel, 1994). Quantitative methods involve capture of released H₂S in acidimetric or anion trap (Cd(OH), ZnO) and subsequent quantification with the methylene blue colorimetric reaction (Alcini et al., 1971; Breuer et al., 1964; Jirasek et al., 1995; Lopez del Castillo, 2005; Landuad et al., 2007), or more recently, capture and quantification in lead acetate detection tubes (Hart, 2008). However, the development of specialized microorganisms requires a high throughput detection method applicable to screening of large microbial strain collections. A current high throughput method for sulfide detection uses a membrane impregnated with silver nitrate (Duan et al., 2004) to measure H₂S produced during micro-scale fermentation. Due to limited sensitivity, the addition of the sulfur-containing amino and cysteine is required, limiting the method’s application.

The aim of this study was to develop a new screening methodology for H₂S detection that would be compatible with robotic handling systems for screening large numbers of microbial strains. We further sought to develop a method applicable to study of H₂S production from organic and inorganic sources, in various fermentation systems. The method was developed here for the yeast Hedosophia salome, a major microorganism in the fermented food industry primarily used in beer and wine fermentations (Jirasek and Jirasek, 2005; Protorius and Rauer, 2002).

2. Materials and methods

2.1. Reagents

Chemical reagents were obtained from Sigma-Aldrich. Detection tubes for H₂S detection were obtained from Komura Kikagawa (Tokyo, Japan).

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2.2. Strains and growth conditions

5. cerevisiae strain AWR 1031 was used for assay development. Assay validation was carried out using strains 5. cerevisiae (AWR 706, AWR 1033, AWR 1036, AWR 1039 and R1432), Candida stellata (AWR 1153), Kluyveromyces thermotolerans (AWR 318), Schizosaccharomyces pombe (AWR 1141), Hansenula polymorpha (AWR 861) and Oospora oenii (AWR 855). Media used was chemically defined model grape juice and described in (Winter et al., 2011). Cysteine supplementation where indicated was at 100 mg/L and diammonium phosphate (DAP) supplementation was 340 mg/L.

2.3. Development of H2S detection assay

Cells were pre-grown to stationary phase in 10 ml YPD (1% yeast extract, 2% peptone, 2% glucose) or LB (1% tryptone, 0.5% yeast extract, 1% sodium chloride) with shaking at 150 rpm prior to inoculation. Fermentations were conducted in a microtiter plate at a total volume of 200 μL per well. Each well contained 170 μL of medium, 10 μL of microbial cell culture to give a final optical density of 0.3-0.5 at 600 nm wavelength and 20 μL of a methylene blue reaction mix containing 5 ml of 1 mg/ml methylene blue, and 5 μl of 100 mM citric acid and buffer at pH 4.5. Fermentations were carried out in quadruplicate. Duplicate fermentations were also performed without the reaction mix, to monitor growth rate as measured by the absorbance at 600 nm. Uninoculated wells containing fermentation media with or without reaction mix were monitored to detect media contamination and spontaneous methylene blue decolourisation, respectively.

2.4. Data analysis

Methylene blue decolourisation and biomass data were normalised to generate H2S generation profiles using the formula [(OD₅₆₂₅ (n) - OD₅₆₂₅ (t))/OD₅₆₂₅ (t)]/OD₅₆₂₅ (no reaction mix control). Extraction of kinetic parameters was done via a locally weighted regression (loess) algorithm using a custom R script (R Development Core Team, 2010).

3. Results and discussion

3.1. Method principles

The methylene blue reduction method (MBR) employs a well characterised redox reaction between methylene blue and a sulfide ion which can be further catalysed by traces amounts of tellurium or selenium (Hils and Vester, 1947; Pigno and Popovkow, 1949; Moussavi and Sahl, 1997). This reaction leads to decolourisation of methylene blue (Fig. 1A) (Moussavi and Sahl, 1997). Under acidic conditions sulfide ions are present as H₂S. Incorporation of H₂S detection mix (which includes methylene blue and citric acid buffer) into fermentation media allows the immediate detection of H₂S produced during fermentation without affecting fermentation performance (Fig. 1B). Simultaneous kinetic measurements for H₂S generation (indicated by methylene blue decolourisation) and biomass formation (measured for control wells, not supplemented with methylene blue) allow the determination of an H₂S production profile. Kinetic parameters extracted from this profile allow the comparison of factors affecting H₂S formation.

2.2. Development of the assay

The use of micro-scale fermentation was recently found to be a meaningful surrogate for wine fermentation (Lucchiari et al., 2010). To demonstrate high throughput methodology, fermentations were conducted in a microtiter plate at a total volume of 200 μL per well and included both medium and H₂S detection mix. Whilst addition of a catalyst (0.01 mg/L of potassium or tellurium) enhanced the sensitivity of H₂S detection, we found that during yeast fermentation of synthetic grape juice the high concentrations of H₂S produced sufficed for the generation of an accurate H₂S formation profile (data not shown). Kinetic spectrophotometric measurements throughout fermentation allowed generation of H₂S formation profiles, inferred from methylene blue decolourisation. These profiles represent methylene blue decolourisation rate normalised to biomass formation. Comparison of the MBR method profile with an established quantitative H₂S production profile show similar trends for H₂S formation during fermentation (Fig. 2). Both profiles display an initial

![Fig. 1. Incorporation of methylene blue into the fermentation media results H₂S detection without affecting fermentation rate. A: Reduction of methylene blue and sulfide ion leading to decolourisation methylene blue. B: Fermentation performance measured by Cl₃H₅O production. With methylene blue (dashed line) or without methylene blue (solid line). Error bars represent standard deviation.](image-url)
Fig. 2. Analysis of H$_2$S formation during fermentation using methylene blue reduction method. A. Demonstration of methylene blue colour change during micro-scale fermentation. Cells from an overnight culture grown in WYP were inoculated into a 96-well plate filled with model grape juice with or without the methionine. H$_2$S formation profile generated using the methylene blue reduction method (left) or using yeast extract detection rates (right).

lag, followed by increased rate of formation, an H$_2$S maximum, and then production of smaller amounts of H$_2$S (Fig. 2). Maximum rate of increase and maximum value were extracted from the MBR method profile via a locally weighted regression (loess) algorithm (Fig. 2). Lag time was defined as the crossing point of the H$_2$S profile (A665/A600), with zero, indicative of the start of H$_2$S formation. Table 1 shows reproducibility of kinetic parameters extracted from inter-plated triplicate fermentations with the S. cerevisiae wine yeast (AWRI 1631). Fermentations were conducted independently using different inoculation cultures and medium batches. As the chemical reaction equilibrated and H$_2$S production slowed, H$_2$S vaporisation (or possible oxidation) altered the rate of the reaction and methylene blue re-entrained within the fermentation media (Fig. 2). As a consequence, precision of kinetic parameters extracted following the point of maximum H$_2$S detection is limited.

2.3. Detection threshold and linearity

To construct a calibration curve, known volumes of sodium hydrosulphide hydrate solution (final H$_2$S concentration of 1 mg/L) were added to water supplemented with the H$_2$S detection mix, and were monitored at 665 nm for 30 min, consistent with the reading intervals of the experimental data presented here. The MBR method displayed linearity between the ranges of 0-50 µg H$_2$S (Fig. 3). Samples spiked with H$_2$S plus potassium thiosulphate (final SO$_2$ concentration of 50 mg/L), dimethyl sulphone (1 mg/mL) or methyl mercaptoacetate (1 mg/mL) were also tested under the same conditions. No interference in H$_2$S detection was observed following these additions (data not shown).

2.4. Method application

The use of methylene blue decolorisation as an indicator for sulfide formed during fermentation was verified using different nutritional conditions known to modulate H$_2$S production, and using yeast strains previously characterised for their H$_2$S formation capacities. Nutritional conditions included modification of yeast assimilable nitrogen (YAN) (Janek et al., 1995) and cysteine concentrations (Todorov et al., 1997). While YAN concentration largely affects H$_2$S formation from an inorganic sulfur source such as sulfur, cysteine represents an organic sulfur source whose degradation results in H$_2$S formation independently of YAN concentration.
Fig. 3. Method linearity. Known amounts of H$_2$S were added into a 96-well plate filled with model grape juice and the reaction mix. Reactions were carried out in triplicates from the same enzymatic solution.

(Jirados et al., 1995). In fact, cysteine supplementation was previously used as means to increase H$_2$S concentration to allow for H$_2$S detection in a less sensitive method (Suan et al., 2004), which limited that method application to study H$_2$S formed from an inorganic source only.

Quantitative determination of H$_2$S produced during fermentation, using detection units (Park, 2005) demonstrated that increasing YAN concentration of the media from 150 mg N/L to 350 mg N/L, through the addition of diammonium phosphate (DAP) decreased the amount of H$_2$S produced. Addition of freshly made cysteine solution decreased the time of onset for H$_2$S production and increased the amount produced (Fig. 4A). Consistent micro-scale fermentations using the MBR method displayed the same trends. DAP addition decreased H$_2$S production maximum while cysteine addition decreased lag time and increased both estimated H$_2$S production rate and maximum value (Fig. 4B).

MBR H$_2$S formation profiles were also generated for known high and low H$_2$S producing S. cerevisiae wine strains, AWRI 1483 and AWRI 706 (Ugliano et al., 2009), in comparison to AWRI 1923. From 5 hours post-inoculation AWRI 1483 produced H$_2$S at a greater rate and reached a greater maximum value than AWRI 1923 and AWRI 706 (Fig. 5A), in agreement with data obtained utilizing reference quantitative methods (Fig. 5B and (Ugliano et al., 2009)). The rapid increase in H$_2$S formation by the MBR method was further demonstrated by directly supplementing H$_2$S solution into an ongoing fermentation. Addition of H$_2$S after 25 hours of fermentation immediately led to de-colorisation of the methylene blue dye (Fig. 5A), as demonstrated by the sudden peak in the H$_2$S production profile. Finally, the MBR method was validated using a MIT10 mutant strain impaired in H$_2$S release through the sulfate assimilation pathway (Cordero et al., 2003) (Fig. 5B).

4. Conclusions

This study describes a novel in situ method for H$_2$S detection, validated for yeast fermentation. Kinetic parameters obtained using
this method can be successfully used for profiling H2S formation, enabling comparison of different factors affecting H2S production. H2S production is an evolutionary conserved phenomenon (Kadota and Koda, 1972) and so the method presented here can be adapted in various fermentation systems, where H2S is formed.

Strengths of the MIBR method are in high throughput applicability and the ability to differentiate different sulfur sources leading to H2S formation. All processes in the method can be carried out using robotic systems with liquid handling and multiwell spectrophotometric reading capabilities; therefore, the method is suitable for screening large collections of microorganisms. Moreover, this method is likely to be more sensitive than other methods measuring H2S accumulation in the head space. Here the reagent is present in the medium where it reacts immediately with H2S as it is produced, thus the reaction is not dependent on factors, such as CO2 production (that varies within and between fermentations) to purge H2S from the head space. The method is fully automated and suited for high throughput screening purposes by virtue of its simplicity and ability to detect H2S in micro-scale fermentations.

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We thank Laffont Australia and in particular Dr. Tertius Van der Westhoven for financial support. Dr. Paul Henschke and Guy Black are thanked for critical review of the manuscript. We thank Prof. Salome Pretorius, Dr. Paul Smith, Dr. Mark Smith and other colleagues at the Australian Wine Research Institute for useful discussions. John Field is acknowledged for commenting on kinetic parameters extraction. Chaira Berzas is thanked for her help with H2S assessment in mutant yeast strains. This research was supported by an Industry Partnership grant and Gil Winter is a recipient of a PhD scholarship from the University of Western Sydney. Research at the Australian Wine Research Institute is supported by Australia’s grape growers and winemakers through their investment agency the Grape and Wine Research and Development Corporation with matching funds from the Australian Government. The Australian Wine Research Institute is a member of the Wine Innovation Cluster.

References


Conflict of interest

A Patent application concerning the method described in this manuscript has been submitted to the Australian Patent Office (Provisional Patent Application Number: 20119003516) by the University of Western Sydney.


### A-2 Supplementary material

#### A-2.1 Concentration of wine acids, acetate esters and higher alcohol following nutrient supplementation

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A-2.2  Fermentation progress following nutrient supplementation.

Fermentation progress measured by sugar consumption following rehydration nutrient supplementation (grey triangles), DAP addition to the medium to increase YAN concentration to 250 mg N/L (grey squares) or no nutrient supplementation (black diamonds). Fermentations were conducted in triplicates. Error bars represent standard deviation.
### A-2.3 VSCs concentration in wine following rehydration nutrient added at inoculation

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*Addition of rehydration nutrients to the fermentation medium, equivalent to the amount carried over with yeast inoculation following rehydration with nutrients.*
A2.4 R function to extract kinetic parameters from MBR H₂S profile

# R function h2s, written for Gal Winter, AWRI, 20/11/2010 vn2 7/12/2010
# by John Field Consulting Pty Ltd
# johnfield@ozemail.com.au, phone 0409 097 586
#
# Purpose: Provide estimates for various aspects of H₂S production over time:
# Lag time, Rate of increase, Maximum, Rate of decrease
# Optionally plots data, fitted loess line and estimates.
#
# Usage: h2s(y, x, title='", span=0.2, plot=TRUE)
#
# Inputs: Required arguments:
# y: H₂S production at successive times (a numeric vector)
# x: times at which y is measured (a numeric vector the same length as y)
#
# Optional arguments:
# title: a title for the plot (character variable)
# span: smoothing parameter for loess fit (a number < 1; default=0.2;)
# larger values will give greater smoothing )
# plot: logical: TRUE (default): plot graph, FALSE: omit graph
#
# Outputs: prints Lag time, Rate of increase, Maximum, Rate of decrease, Validity of fit
# and returns these values as LagTime, RateIncrease, Max, RateDecrease, Validity.

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# span: smoothing parameter for loess fit (a number < 1; default=0.2;)
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#
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# Optional arguments:
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#
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#
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# Optionally plots data, fitted loess line and estimates.
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# x: times at which y is measured (a numeric vector the same length as y)
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# by John Field Consulting Pty Ltd
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# plot: logical: TRUE (default): plot graph, FALSE: omit graph
#
# Outputs: prints Lag time, Rate of increase, Maximum, Rate of decrease, Validity of fit
# and returns these values as LagTime, RateIncrease, Max, RateDecrease, Validity.
A-2.5  Supplementation of catalyst to MBR - H₂S detection mix

MBR H₂S formation profile for strain AWRI 1631 with or without supplementation of a catalyst (0.01 mg/ml of tellurium dioxide). Micro-fermentations were conducted in quadruplicates. Error bars represent standard deviation.
A-2.6 Genes classified as low or high H$_2$S producers in a genome-wide screen for cysteine catabolism

A-2.6.1 List of genes

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A-2.6.2 GO terms associated with low H$_2$S producers
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Strains were grouped according to the gene ontology description of the encoded gene product, as defined in the Saccharomyces Genome Database (www.yeastgenome.org) using Gorilla (Eden et al., 2009).
A-2.6.3 GO terms associated with high H$_2$S producers
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**Strains were grouped according to the gene ontology description of the encoded gene product, as defined in the Saccharomyces Genome Database ([www.yeastgenome.org](http://www.yeastgenome.org)) using Gorilla (Eden et al., 2009).**
A-2.7  R script for genomic screen data analysis

The aim of this script is to identify wells that show exceptionally low or high rate of discoloration (measured on the MB plate) and are able to grow well on this media (measured on the GR plates). Data was collected robotically. Plates were read in the spectrophotometer at two wavelengths 663 and 600 nm. Each read generated a separate XML file (A-2.6.1); the file is identified by plate number (read form a barcode tape on the plate), date and time.

This script extracts data from multiple XML files (OD663 for MB plates and OD 600 for GR plates) and generates data frame with the time of read and the data. It then summarises plate statistics for the four replicates and save it as a separate text file, chooses minimum value for the MB plates (to look for the max discolouration) and maximum value for the GR plate (look for max growth) and rank them within the plate, it then deduct the maxima value of the GR plate from the minima value of the MB plate, for each strain, and print the results as a separate file.

A-2.7.1 An example of XML output file

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A-2.7.2 R script

# R script for the analysis of genomic screen for H2S detection
# from cysteine
#
# Written by- Gal Winter
#
# set working directory

##Main Script
## load packages XML, timeDate and ggplot2
library(XML)
library(timeDate)
library(ggplot2)

## set working directory
setwd("\\\Lister\users\Gal.Winter\My Documents\Experiments\screen h2s\screen h2s 9.5.11")

# 5 plates
A-2.8  Growth data of mutants deleted in individual subunits comprising V₁ of the V-ATPase complex

Growth was measured as optical density at 600 nm. Experiments were carried out in quadruplicates, error bars represent standard deviation.