HYDROGEN SULFIDE FORMATION IN *SACCHAROMYCES CEREVISIAE* AND ITS REGULATION BY ASSIMILABLE NITROGEN

BY

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DECLARATION

The work presented in this thesis is my own unless otherwise acknowledged, and has not previously been submitted to any university for the award of any degree or diploma. This thesis may be made available for loan or photocopying provided that an acknowledgement is made in the instance of any reference to this work.

Vladimir Jiranek

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Publications arising from this thesis

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Summary

An investigation was undertaken to determine the metabolic basis for the accumulation of hydrogen sulfide during fermentation by wine strains of the yeast *Saccharomyces cerevisiae* under conditions approximating those seen in oenology. A model fermentation system was established which utilised defined media and fermentation conditions with special attention given to quantitation of hydrogen sulfide formed. Preliminary screening of yeast strains under conditions of nitrogen excess allowed them to be classified according to their propensity to produce hydrogen sulfide. An especially high rate of production was subsequently demonstrated by several strains upon the depletion of assimilable nitrogen from a synthetic grape juice medium. Conversely, supplementation of the medium with ammonium ions or most individual amino acids suppressed hydrogen sulfide evolution. Maximal rates of hydrogen sulfide production were seen where nitrogen depletion occurred during exponential growth as compared with other stages of fermentation. In all cases, a high rate of hydrogen sulfide production in response to a deficiency of assimilable nitrogen was only observed in the presence of sulfite; production with sulfate as sole sulfur-source was relatively small and short-lived under the same conditions. Radiolabelling experiments confirmed sulfite to be the principal precursor for the excessive production of hydrogen sulfide.

Sulfite reductase activity was determined throughout fermentation of nitrogen-sufficient cultures. In ammonium grown cells, enzyme activity progressively increased after inoculation, peaking shortly before the stationary phase of growth. At any one point, enzyme activity reflected the rate of hydrogen sulfide production which was observed in response to a depletion of nitrogen occurring at the same point during fermentation. Growth of cultures on methionine as sole nitrogen source greatly reduced detected levels of sulfite reductase activity at all stages of growth. The characteristic level of sulfite reductase activity was a foremost factor in determining differences between strains in terms of hydrogen sulfide producing potential.

Existing sulfite reductase activity displayed a relatively long half-life following nitrogen depletion or cycloheximide addition. In the short-term, enzyme activity was not significantly affected over two cycles of nitrogen excess and limitation. Accordingly, it is suggested that repression of enzyme synthesis is not likely to be the mechanism by which nitrogen supplementation rapidly suppresses hydrogen

sulfide formation. Experimental evidence supports the notion of complete sequestration of enzyme-formed hydrogen sulfide to cellular constituents and/or enzyme inhibition.

Laboratory monitoring of industrial fermentations revealed two broad peaks of hydrogen sulfide production; a nitrogen-suppressible phase preceding complete carbon catabolism, and a nitrogen-unresponsive phase coincident with or subsequent to sugar depletion. Reductions of the latter through ergosterol and oleic acid ester supplementation could not be attributed to optimised arginine or ammonium-analogue transport capabilities. The importance of glucose depletion in preventing biosynthesis of sulfur amino acids and hence promotion of hydrogen sulfide formation was investigated.

The relative nitrogen demand of several strains of wine yeast was determined and its importance as a factor in the predisposition of certain strains to high hydrogen sulfide production examined. Strains differed in their demand for total amino acid nitrogen. Initial glucose content of the medium, the degree of aeration, starter culture preparation and ammonium supplementation influenced the quantitative and/or qualitative aspects of nitrogen utilisation. Nonetheless arginine consistently met 30-50% of the total nitrogen demands of yeast irrespective of the conditions and strain used. An ordered accumulation of individual amino acids was observed, the pattern being significantly altered by ammonium supplementation. The suggestion that high hydrogen sulfide producing strains have a correspondingly high demand for nitrogen was not completely supported. It thus appears that predisposition to high hydrogen sulfide production has principally a genetic basis and that high nitrogen demand and/or depletion during fermentation merely enable this phenotype to be manifested.

Chapter 1

Introduction

1.1 GENERAL INTRODUCTION

Hydrogen sulfide (H_2S) is a volatile sulfur-compound whose 'rotten-egg' like odour is frequently associated with sites of geological and volcanic activity. Swamps and water-logged soils are also characterised by the odour of this compound which is largely formed through the dissimilatory reduction of sulfate as part of the anaerobic respiration of obligate anaerobes such as *Desulfovibrio* and *Desulfomonas* spp., (Brock, 1979). In reality, hydrogen sulfide is produced by a broad range of organisms, though more commonly as a metabolic intermediate in the biosynthesis of the sulfur amino acids, methionine and cysteine. The amounts of hydrogen sulfide formed under these conditions are generally small and closely linked to metabolic demand. Although this is essentially the case in the yeast *Saccharomyces cerevisiae*, some combinations of yeast strain and environmental conditions can induce the formation of hydrogen sulfide in vast excess of cellular requirements, the surplus diffusing into the growth medium (Rankine, 1964; Maw, 1965; Schütz and Kunkee, 1977; Vos and Gray, 1979). The economic consequences of such an activity can be devastating where yeasts are being used in baking or the production of fermented beverages such as beer, cider or wine.

As one of the oldest processes known to man, winemaking has changed little over the centuries. Traditionally, winemaking required only that grapes be crushed and then set aside while the indigenous yeast flora present on the fruit were left to carry out the fermentation. The results of this relatively ill-defined process were not surprisingly, quite variable, with the end-product often being marred by off-odours and taints. The inate importance of the yeast in the development of these off-odours was quickly recognised by the winemaker. The

result has been a program of yeast strain selection which has continued to the present day.

With a better understanding and control of the winemaking process and access to pure-culture yeast technology, the contemporary winemaker has greater influence on the finished product. Despite this, the presence of objectionable levels of compounds such as hydrogen sulfide in the finished wine remains as one of the most commonly encountered faults afflicting winemaking today.

Hydrogen sulfide is particularly problematic due to its low aroma threshold, values of which vary according to the matrix and the method of determination. Concentrations of 5 $\mu g.L^{-1}$ (Harrison and Collins, 1968) and 0.5 $\mu g.L^{-1}$ (Jansen *et al.*, 1971) have been reported for degassed beer and beer headspace, respectively. In wines, estimates have put the threshold value at 10 $\mu g.L^{-1}$ (Amerine and Roessler, 1976), 100 $\mu g.L^{-1}$ (Acree *et al.*, 1972) and between 100 $\mu g.L^{-1}$ and 1 $mg.L^{-1}$ (Rankine, 1963).

Hydrogen sulfide which is formed early in fermentation is likely to be entrained by the evolving carbon dioxide. Residual hydrogen sulfide or that formed later in the fermentation, is more difficult to deal with. However, several protocols have been devised for its removal. Most commonly, hydrogen sulfide is removed through 'copper-fining' whereby it is precipitated as copper sulfide following addition of a copper salt. Alternatively, sulfide is oxidised through sulfite addition or aeration of the wine by transferring between fermentation tanks or by 'pumping-over'. Unfortunately, these procedures are not always successful and they may have adverse side-effects on the sensory quality of the wine. In addition, the use of the above additives is limited by legislation.

The presence of hydrogen sulfide in a finished wine *per se* may be a relatively minor problem. More serious consequences may arise if a hydrogen sulfide problem is not attended to promptly. Under these circumstances, an

unpleasant sulfidic aroma may develop often after packaging when no further treatment is possible. These sulfidic aromas purportedly arise through the formation of organo-sulfur compounds from the reaction of hydrogen sulfide with must components or the interconversion of these products (Rankine, 1963; Brenner *et al.*, 1955). Hydrogen sulfide will react with aldehydes to form ethanethiol (Rankine, 1963; Tanner, 1969), though a range of thiols have resulted from the reaction with more complex organic compounds (Täkken *et al.*, 1976; Nishimura *et al.*, 1980). The rate of these reactions in wine is not clear.

The organo-sulfur compounds most commonly encountered in fermented beverages include, ethanethiol, diethyl disulfide, diethyl sulfide, dimethyl disulfide and dimethyl sulfide. The sensory threshold values of these have been determined as 1.1, 4.3, 0.92, 29 and 25 $\mu g.L^{-1}$, respectively (Goniak and Noble, 1987). At low concentration, the presence of these compounds is considered desirable, adding complexity to wines (du Plessis and Loubser, 1974; Spedding and Raut, 1982) and being characteristic of some beer types (Sinclair *et al.*, 1970; Anderson *et al.*, 1975). However, at higher concentrations, these compounds impart odours typically suggesting rotten-cabbage, onion, garlic or rubber (Goniak and Noble, 1987).

Thus the formation of hydrogen sulfide in a wine can potentially have multiple detrimental consequences for wine organoleptic quality and therefore marketability. Until a practical solution to the problem of hydrogen sulfide production in fermentation is devised, this age old affliction of winemaking will continue to be an issue for the winemaker.

1.2 WINEMAKING

Although there are many variations of the winemaking process depending on the style that is being produced, from a biochemical viewpoint the alcoholic fermentation is essentially the same. The principle deviation concerns

the exclusion or inclusion of grape skins and grape-solid particles during white or red wine production, respectively.

The grapes are harvested, transferred promptly and crushed, with or without destemming, in a process which is now achieved almost exclusively by mechanical means. Sulfite is often added at one of these stages to limit microbial growth and the action of polyphenoloxidases in the must prior to inoculation with yeast. The amount added is dependent on fruit temperature, condition and the style of wine being made.

For white wine production, the juice is separated from skins by gravity (free-run) followed by pressing to remove the remainder. Because these juice fractions often differ with regard to organoleptic and compositional parameters, free-run and pressed juices are not always combined. Clarification of juice is carried out to varying degrees yielding suspended solid contents which range from 1 or 2% for no treatment or cold settling in the presence of pectic enzyme preparations, to between 0.5 and 0.1% or less following centrifugation or earth-filtering. The dissolved oxygen content of juice may be adjusted. A reduction is achieved by sparging nitrogen gas through the juice, whereas, oxygen sparging is employed for hyperoxidation (Guerzoni *et al.*, 1981).

Skins are not separated from the juice until the later stages or at the end of fermentation during the production of red wines. This promotes the extraction of phenolic compounds which are responsible for the colour and astringency, and to a significant extent, the mouthfeel of red wine. Extraction of phenolics is facilitated either by keeping skins submerged in the fermenting juice or by regular mixing of the two.

Both red and white ~~wines~~ musts may be routinely supplemented with vitamin mixes and ammonium ions (Monk, 1982; Monk and Costello, 1984). Typical addition rates are; inositol (100 $mg.L^{-1}$), thiamine (500 $\mu g.L^{-1}$), pantothenate (400 $\mu g.L^{-1}$), pyridoxine (200 $\mu g.L^{-1}$), nicotinic acid (200 $\mu g.L^{-1}$), biotin (12.5 $\mu g.L^{-1}$).

Diammonium hydrogen phosphate is frequently added at inoculation to between 100 and 200 $mg.L^{-1}$, subsequent additions being made especially upon the detection of hydrogen sulfide. Additives are frequently limited by legislation.

Inoculation of the must is commonly achieved through the direct addition of rehydrated active-dried wine yeasts (ADWY). Alternatively, ADWY or agar-slope cultures may be used as inocula for the propagation of a starter culture grown with vigorous aeration in diluted, vitamin and nutritionally supplemented grape juice. Inoculation rates in all cases are of the order of 5×10^6 cells mL^{-1}. It is noteworthy that some areas of the international wine industry are returning to the practice of indigenous yeast fermentations. This is achieved by permitting the indigenous grape flora to carry out the fermentation, or alternatively, through the propagation and inoculation into musts of wild yeast isolates which have undergone limited selection in small-scale trials.

Fermentations are conducted under differing degrees of temperature control. Small-volume fermentations may be carried out at ambient temperature, relying on conduction for the dissipitation of heat generated during fermentation. Generally, Australian wineries have either in-place cooling in the form of cooling-jackets or plates, or alternatively, access to heat-exchangers. In the majority of cases, cooling is effected by a secondary refrigerant chilled to -10 to -5°C, though a directly expanding refrigerant such as ammonia may sometimes be used. In Australia, white wine fermentations are usually conducted at 11 to 15°C while red wine fermentations are maintained between 14 and a maximum of 25°C.

Treatment of the wine following fermentation can vary considerably depending on the style of wine and its quality. If it has not already been done, red wines are pressed to separate the liquid from the skins. Red, and when required, white wines, may be inoculated with *Leuconostoc oenos* and allowed to undergo malo-lactic fermentation. In some cases the presence of these organisms on winery equipment may be sufficient to inoculate wines, possibly resulting in simultaneous malo-lactic and alcoholic fermentation. The metabolic activity of

this bacteria results in an increase in pH and decrease in acidity due to the conversion of the dicarboxylic acid, L-malic acid, to the monocarboxylic acid, L- or D-lactic acid. In addition, the generation of secondary metabolites, such as diacetyl, adds organoleptic complexity to the wine. Yeast and other solids are then allowed to settle or actively clarified by the addition of fining agents. The wine is 'racked-off' the lees immediately or after a period of holding, usually to encourage malolactic fermentation. A period of oak storage may follow which, in addition to imparting some oak characters, enables the wine to mature and further modify its sensory properties through controlled oxidation.

Wines may be blended before fining for clarification and/or stability, adjusted for sulfite concentration and clarified before bottling. Final clarification of the wine is achieved by centrifugation and/or sterile filtration (0.22 to 0.45 μm membrane). The wine may be made available to the consumer immediately or cellared for some time before sale.

1.3 MECHANISMS OF HYDROGEN SULFIDE PRODUCTION IN FERMENTATION

The accumulation of unacceptably high concentrations of hydrogen sulfide during fermentation has been extensively investigated over recent decades, resulting in several important mechanisms for this phenomenon being reported. These mechanisms can be categorised as chemical or biological, according to the degree to which the direct involvement of microbial metabolism is required.

1.3.1 Chemical mechanisms

The formation of hydrogen sulfide from elemental sulfur during fermentation was first observed in grape must by Nessler in 1869 and later by numerous brewing researchers in the 1950's (reviewed by Rankine, 1963). This phenomenon was subsequently investigated from an oenological perspective by

Rankine (1963, 1964, 1968) who confirmed that elemental sulfur was a potent precursor of this volatile sulfur compound. The extent of reduction of elemental sulfur was inversely correlated with particle size and pH, and increased with fermentation temperature, reductive conditions, ethanol concentration and the presence of metal ions (Acree *et al.*, 1972; Schütz and Kunkee, 1977). The influence of yeast strain is unclear (Rankine, 1963; Eschenbruch *et al.*, 1978), however, the reduction of elemental sulfur to hydrogen sulfide requires direct sulfur:yeast cell contact (Schütz and Kunkee, 1977).

The use of sulfur-containing fungicides close to harvest or the sterilisation of cooperage through the burning of sulfur candles, have been identified as primary sources of elemental sulfur residues in musts and wine (Rankine, 1963; Acree *et al.*, 1972). Due to the more cautious use of these treatments, and in particular, the strict adherence to withholding periods for sprays containing collodial sulfur, this once predominant route of hydrogen sulfide production in the fermentation, is now considered to be of minor importance in Australia.

Aside from their purported role in the reduction of elemental sulfur, metal cations, especially Cu^{2+}, Zn^{2+} and Sn^{2+}, increase the formation of hydrogen sulfide when present during fermentation (Eschenbruch, 1971; Dittrich and Staudenmayer, 1972; Brenner *et al.*, 1974; Eschenbruch and Kleynhaus, 1974). Similar observations are described for copper in beer, where, hydrogen sulfide production was increased without enzymic or microbial intervention (Jansen, 1964). Brenner and coworkers (1974) suggest that metal ions may be acting by splitting protein disulfide bridges to form sulfhydryl compounds, including hydrogen sulfide. Rankine (1963) proposed that wine acids attacking metal fixtures in the fermentation vessel may produce nascent hydrogen which directly reduces bisulfite to hydrogen sulfide. Alternatively, the presence of metal cations may be inducing or selecting for the production of hydrogen sulfide by yeasts as a protective mechanism by which these toxic ions are precipitated as sulfides (Ashida *et al.*, 1963; Hoggan and Compson, 1963). Though the exact

mechanism for the action of metal cations remains ill-defined, the approach to controlling this route of hydrogen sulfide production has been to limit the introduction of these species into the fermentation or wine.

1.3.2 Biological mechanisms

Investigations of the microbial causes of hydrogen sulfide production during fermentation have centred upon both yeasts and bacteria. There is evidence for the production of hydrogen sulfide by spoilage bacteria such as *Zymomonas* spp., *Enterobacter* spp., *Obesumbacterium* spp. (Anderson *et al.*, 1971) and *Lactobacillus* spp. (Geddes, 1986) in the wort fermentation, but their importance is regarded as secondary to that of yeasts. In light of their sensitivity to sulfite levels routinely used in winemaking, bacteria of oenological significance such as *Leuconostoc*, *Lactobacillus and Acetobacter* spp., are regarded similarly (Rankine, 1963).

One of the first factors found to contribute to the large-scale metabolic production of hydrogen sulfide by yeast was a deficiency of the vitamins, pantothenate or pyridoxine (reviewed by Maw, 1965 and Lawrence and Cole, 1968; Wainwright, 1970; Tokuyama *et al.*, 1973). A blockage of methionine synthesis was implicated as a primary consequence of these growth-limiting vitamin deficiencies since they were able to be relieved by methionine supplementation. Accordingly, hydrogen sulfide production was proposed to result from the accumulation and subsequent desulfhydration of cysteine, a proposed intermediate of the methionine biosynthetic pathway (Aida *et al.*, 1969; Tokuyama *et al.*, 1973). The formation of a modified sulfite reductase enzyme, not subject to the usual regulatory mechanisms, was also reported (Okuda and Uemura, 1965). At present, hydrogen sulfide production arising out of such deficiencies is believed due to a derepression of the pathway of sulfate and sulfite reduction to the sulfide ion. Derepression occurs as a consequence of the blocked synthesis of methionine, and hence derivatives thereof, which normally regulate the reduction sequence (see below) (Cherest *et al.*, 1971; 1973).

Pantothenate and pyridoxine contents of musts range from 0.25 to 10.5 $mg.L^{-1}$ and 0.1 to 2.9 $mg.L^{-1}$, respectively (Amerine *et al.*, 1980). While typically in excess of yeast requirements (Monk, 1982), oenological practices such as ion-exchange, bentonite fining and heating can dramatically reduce must vitamin levels (Monk, 1982, 1984; Monk and Costello, 1984). A general awareness in the wine industry of the detrimental effects on vitamin content of some must treatments, together with the widespread use of vitamin supplements, has ensured that hydrogen sulfide production due to vitamin deficiencies is rarely encountered.

Largely as a result of early brewing research which concentrated on the identification of hydrogen sulfide precursors, the presence in the fermentation medium of cysteine was put forward as a possible cause of hydrogen sulfide production (reviewed by Lawrence and Cole, 1968). The enzymic degradation of cysteine, liberating hydrogen sulfide and a nitrogen-containing moiety, is mediated by two enzymes (Aida *et al.*, 1969; Tokuyama *et al.*, 1973). Fermentations in grape juice and defined media to which cysteine is added are able to yield large amounts of hydrogen sulfide (Rankine, 1963; Eschenbruch and Bonish, 1976; Eschenbruch *et al.*, 1978). However, the levels of cysteine present in natural grape juices are insufficient to account for the quantities of hydrogen sulfide produced during grape juice fermentations (Eschenbruch, 1974a; Gallander *et al.*, 1969; Amerine *et al.*, 1980). Researchers thus proposed the release of cysteine from grape proteins and yeast cells by proteolysis (Eschenbruch and Bonish, 1976, Vos and Gray, 1979) and autolysis (Eschenbruch, 1974b), respectively.

Despite their obvious potential in controlling wine protein-haze formation, extensive efforts to identify yeast extracellular proteolytic activities have been met with only limited success (Maddox and Hough, 1970), the balance of publications arguing against the existence of an activity of consequence under oenological conditions (Rosi *et al.*, 1987; Sturley and Young, 1988). While the significance of the proteolytic activities of wild yeasts has yet to be determined

(Nelson and Young, 1986), several other observations argue against the importance of proteolysis/ autolysis. No evidence is available to confirm the autolysis mediated release of appropriate amounts of cysteine within the time frame of a typical commercial fermentation (Poux *et al.*, 1964; Feuillat and Charpentier, 1982). Maximal hydrogen sulfide production generally occurs outside of the stages of fermentation when autolysis could be expected to be peaking. Proteolytic or autolytic activities releasing cysteine would concomitantly release methionine, a demonstrated suppressant of hydrogen sulfide production (Wainwright, 1971; Eschenbruch, 1974a; Monk, 1986). Therefore, in an oenological context, cysteine degradation does not appear to be a leading candidate for a mechanism of hydrogen sulfide production.

As for cysteine, additions of threonine to media during yeast growth can result in elevated rates of hydrogen sulfide production (Wainwright, 1971; Monk, 1986). Since it lacks a sulfur component, threonine is likely to bring about its effect by influencing the biosynthesis of methionine and hence the regulatory mechanisms associated with hydrogen sulfide production (de Robichon-Szulmajster and Corrivaux, 1963). Because of the high concentrations of threonine required to elicit a response in yeast, this route of hydrogen sulfide production is purely of academic interest.

The strain dependence of hydrogen sulfide production by yeast has long been recognised (Tanner, 1918) and has served as an important criterion for wine yeast strain selection. A survey of yeast strains has revealed that approximately 1% of all strains examined possess a low or undetectable hydrogen sulfide producing activity (Zambonelli *et al.*, 1984). It is suggested that these strains carry a leaky sulfite reductase mutation which is a single gene-trait (Zambonelli *et al.*, 1975). Conversely, the over-production of hydrogen sulfide by some strains may have a genetic basis, namely, the de-regulated or constitutive expression of the sulfite reductase locus (Takahashi *et al.*, 1980).

In a time when the selection and use of 'low hydrogen sulfide-producing' strains remains as one of the most successful and recommended methods for controlling hydrogen sulfide production in the winery (Rankine, 1968; Acree *et al.*, 1972; Monk, 1986), it is important to consider that the inoculated wine strain may not necessarily be the only strain present let alone that which achieves numerical dominance (Rosini, 1984; Heard and Fleet, 1985, 1986). Hence, the wine fermentation may result from a highly variable population of yeast in which the competing yeasts may be either wild species of *Saccharomyces* or non-*Saccharomyces*, or both. The often sporadic production of hydrogen sulfide, despite the use of selected wine strains, could in fact coincide with the presence of these foreign yeasts which either produce hydrogen sulfide themselves or outcompete the inoculated strain for essential nutrients (see below) inducing them to hydrogen sulfide production (Rankine, 1964; Zambonelli *et al.*, 1984). Good techniques using marked strains and selective media are now available to study the involvement of *Saccharomyces* and non-*Saccharomyces* yeasts in commercial fermentations and will enable this situation to be clarified (Vezinhet, 1985; Heard and Fleet, 1986).

Correlations have been reported between a deficiency of must assimilable nitrogen and limited yeast biomass development and/or incomplete fermentation (Cantarelli, 1957; Bizeau, 1963; Agenbach, 1977). Recent studies have confirmed this report (Ingledew and Kunkee, 1985) and demonstrated that a nutrient deficiency of this type brings about incomplete fermentation through a catabolite inactivation of glucose transport (Lagunas *et al.*, 1982; Busturia and Lagunas, 1986; Salmon, 1989). Empirical observations have also linked nitrogen deficiencies/incomplete fermentations with the production of hydrogen sulfide. Extending on earlier work (Vos, 1966), Vos and Gray (1979) were able to demonstrate an inverse correlation ($r = -0.59$) between must nitrogen content and the production of hydrogen sulfide during the fermentation of 104 grape musts. In addition, the supplementation of musts with assimilable nitrogen enabled the reduction or complete suppression of hydrogen sulfide production. Although Eschenbruch (1974a) failed to demonstrate the relationship between must

nitrogen content and hydrogen sulfide production, other groups have subsequently confirmed these findings (Monk, 1982, 1986; Stratford and Rose, 1985).

Vos and Gray (1979) proposed that the hydrogen sulfide production they observed resulted from the degradation of cysteine, released from grape protein by a proteolytic activity induced by a nitrogen-deficiency. Fermentations conducted in chemically defined, protein-free media do not support this notion (Stratford and Rose, 1985). A more likely explanation for the production of hydrogen sulfide during nitrogen limitation, follows that detailed above for vitamin deficiencies; a limited biosynthesis of methionine and, in turn, the derivatives responsible for the regulation of sulfate and sulfite reduction results in a derepression of this pathway and a nett increase in the formation of the sulfide ion.

Though these observations have yet to be confirmed under controlled oenological conditions, a deficiency of nitrogen is regarded as the prime factor leading to the production of hydrogen sulfide during fermentation and is the subject of this study.

1.4 LIMITATIONS OF EARLY STUDIES OF HYDROGEN SULFIDE PRODUCTION

The evolution and subsequent retention of hydrogen sulfide in wine during fermentation is a dynamic process which is subject to a multitude of factors. Consequently, efforts to characterise the formation of hydrogen sulfide under oenological conditions have been frustrated through the use of inappropriate methodologies for laboratory fermentation trials and sulfide-quantitation.

1.4.1 Hydrogen sulfide quantitation protocols

Qualitative methods for the measurement of hydrogen sulfide evolution from grape juice fermentations have been used until very recently (Rankine, 1963; Romano *et al.*, 1985; Abad and Gómez, 1987; Thornton and Bunker, 1989). Typically, glass tubing containing paper strips or cellulose powder impregnated with lead salt are secured over the neck of the fermentation vessel (Rankine, 1963). Hydrogen sulfide which diffuses or is swept through such devices by fermentation gases is visualised by an apparently quantitative blackening of the lead indicator. Clearly, the extent of hydrogen sulfide detection will be primarily dependent on fermentation vigour and hence rate of carbon dioxide evolution and hydrogen sulfide entrainment.

With the advent of more sensitive colourimetric and potentiometric methods for the determination of hydrogen sulfide (Delwiche, 1951; Brenner *et al.*, 1953, 1974; Gustafsson, 1960; Rees *et al.*, 1971), the adaptation of these to the wine fermentation quickly followed (Zambonelli, 1964; Acree *et al.*, 1971; MacRostie, 1968; Eschenbruch *et al.*, 1978; Kovac *et al.*, 1987). Hydrogen sulfide was commonly trapped from fermentation gases and subsequently quantified, or else, residual volatile was determined directly in the medium at either regular intervals or upon completion of fermentation (Acree *et al.*, 1972; Eschenbruch *et al.*, 1978; Schütz and Kunkee, 1977, Vos and Gray, 1979). Although some limitations of hydrogen sulfide quantitation are overcome by these more sensitive and reproducible methods, the problems of representative sampling of the fermentation are not. Again, purging of hydrogen sulfide from the medium by fermentation gases is dependent on the rate of carbon dioxide evolution. In addition, must content of complexing compounds is important, especially, where residual hydrogen sulfide is being measured at fermentation end. The potential for hydrogen sulfide to be rendered undetectable through its reaction with must components (Rankine, 1963; Täkken *et al.*, 1976; Nishimura *et al.*, 1980) will be increased with residence time of the volatile in the medium.

Some of these approaches have been central to the elucidation of mechanisms of hydrogen sulfide formation but identification of the precise biochemical basis of the phenomenon had not been possible. The method of Stratford and Rose (1985) has been instrumental in terms of the latter. Fermentations are sparged continuously with nitrogen gas and entrained hydrogen sulfide is trapped for quantitation. In this way, all hydrogen sulfide produced in the fermentation can be removed from the medium and collected. Therefore losses of hydrogen sulfide through reaction with must components are minimised as is interference from these species during subsequent sulfide quantitation. Moreover, regular replacement of traps yields kinetic information enabling hydrogen sulfide production to be correlated with physical or chemical changes during the fermentation.

1.4.2 Culture and fermentation protocols

Extensive strain screening programs (Zambonelli, 1964; Romano *et al.*, 1985; Thornton and Bunker 1989) have been made possible through the convenience of lead- or bismuth-containing solid indicator media (Nickerson, 1953). The production of hydrogen sulfide during aerobic growth on these defined media is demonstrated by the darkening of the colonies. Clearly, growth on such media bears little resemblance to growth under oenological conditions. Therefore, extrapolating from these results to the oenological fermentation must be viewed with some caution.

The use of grape juices, though more relevant, has largely been undertaken without due consideration of their specific nutritional composition, elemental sulfur residues, suspended solid content and indigenous microbial load (Rankine, 1963, 1964, 1968; Acree *et al.*, 1972; Eschenbruch *et al.*, 1973, Eschenbruch 1974a; Vos and Gray, 1979; Abad and Gómez, 1987). In addition, temperature control, agitation and the degree of aeration, particularly during sampling, are frequently overlooked. These points are likely to at least in part account for the conflicting results obtained by Rankine (1963, 1964) and

Eschenbruch (1974a) relating to the importance of yeast strain versus must composition to hydrogen sulfide production.

Whereas chemically defined synthetic media vary widely in the degree to which they reflect grape juice composition, they have nonetheless permitted important contributions to this field of research (Zambonelli, 1964; Acree *et al.*, 1972; Schütz and Kunkee, 1977; Eschenbruch *et al.*, 1978, Zambonelli *et al.*, 1984). Subsequent detailed investigations of hydrogen sulfide production under simulated oenological conditions can only be undertaken through the use of synthetic media whose composition models grape juice but can also be altered to aid the identification of regulators, effectors, precursors and their causal relationships.

1.5 YEAST SULFUR METABOLISM

Sulfur represents between 0.2 and 0.9% of total yeast cell dry weight (reviewed by Maw, 1965; Lawrence and Cole, 1968). The bulk of cellular sulfur is found in the sulfur-containing amino acids, methionine and cysteine, each accounting for 30% of total sulfur, with a further 20% being found in glutathione (Thorne, 1957, Hartnell and Spedding, 1979). Small amounts of sulfur are also located in enzyme cofactors such as acetyl coenzyme A, and the methyl group donor, *S*-adenosylmethionine, as well as in the vitamins, thiamine and biotin. The thiol ion is a transient metabolite and does not normally accumulate in the cell.

1.5.1 Sources of sulfur utilised by yeast

As for most micro-organisms, *Sacch. cerevisiae* is unable to grow without a sulfur source (Stern 1899, cited by Maw, 1965). In grape juice, however, a yeast is never faced with a shortage of this element since the minimum requirement of approximately 5 $mg.L^{-1}$ is always exceeded (Eschenbruch, 1983). Both organic and inorganic sulfur compounds present in grape juice and in must are utilisable by yeast. The quantities present in grape berries are variable, being largely

determined by vineyard conditions and viticultural practice, while that of processed must is additionally subject to oenological procedures. Of the inorganic compounds, sulfate, is the main species occurring in grape berries. Sulfite, elemental sulfur and H_2S can occur in considerable concentrations from pesticides, antimicrobials and antioxidants. The predominant organic sulfur compounds are the amino acids, methionine and cysteine, cystine, glutathione and the vitamins thiamine and biotin.

a. Inorganic sulfur sources

Sulfur assimilation by *Sacch. cerevisiae* via the Sulfate Reduction Sequence (SRS) reduces the oxidation state of the sulfur atom to 2^-, that of the sulfide ion, prior to its incorporation into the organic precursors of the sulfur-containing amino acids. Growth on hydrogen sulfide as sole sulfur source has been observed (Schultz and McManus, 1950), as has the incorporation of ^{35}S-sulfur from ^{35}S-hydrogen sulfide (O'Connor *et al.*, 1986). Although there are considerable energetic advantages associated with the utilisation of hydrogen sulfide directly, at must pH the accumulation of hydrogen sulfide through the diffusion/carrier mediated transport of HS^-, is relatively inefficient (O'Connor *et al.*, 1986). The importance of hydrogen sulfide as a source of sulfur for yeast metabolism is further reduced due to the low levels of sulfide seen in sound fruit (Shaw *et al.*, 1980), the minimal usage of vineyard and cooperage treatments containing elemental sulfur which is non-enzymatically reduced to hydrogen sulfide and the rapid loss of this compound from must through volatilisation and reaction with must components (Täkken *et al.*, 1976; Leppänen *et al.*, 1980; Nishimura *et al.*, 1980).

Sulfate (SO_4^{2-}) is by far the most abundant naturally occurring sulfur compound in grape juice, ranging in concentration from 30 to 700 $mg.L^{-1}$ (Amerine *et al.*, 1980). Active transport of sulfate by yeast is mediated by the high (k_m = 0.005 mM) and low affinity (k_m = 0.35 mM) sulfate permeases I and II in what constitutes the first step of the SRS (McCready and Din, 1974; Breton and Surdin-

Kerjan, 1977; Horák *et al.*, 1981). Maximal transport of sulfate occurs between 28 and 30°C, with permease saturation taking place at a sulfate concentration of 1 x 10^{-4} M (McCready and Din, 1974).

Although not naturally found in grape juice, sulfite (sulfur dioxide), is frequently added to suppress undesirable micro-organisms associated with the fruit, prevent oxidation and inhibit polyphenoloxidase enzymes. In the past, additions of sulfite to must ranged up to 350 $mg.L^{-1}$ (Fornachan, 1965; Rankine, 1966). However, in recent times, depending on conditions, an addition of 50 to 100 $mg.L^{-1}$ is common for white musts; often none is added to red musts. Sulfite may also accumulate in fermenting juice as a result of yeast activity (Rankine, 1968, Eschenbruch, 1974b).

Sulfur dioxide exists in solution as three species in a pH dependent equilibrium, wherein the HSO_3^- ion predominates at juice pH. In addition, 'free' and 'bound' forms occur in the must, the latter being essentially associated with carbonyl groups (Burroughs and Sparks, 1964). Accumulation of sulfite by yeast, initially reported to be an active process (Macris and Markakis, 1974), has since been demonstrated to occur by diffusion of molecular SO_2 (Stratford and Rose, 1986), bound sulfite being released from the carbonyl group prior to transport (Stratford and Rose, 1985). When present simultaneously with sulfate, sulfite is accumulated by yeast at the partial (McCready and Din, 1974; McCready *et al.*, 1979) or complete (Eschenbruch *et al.*, 1973; Stratford and Rose, 1985) exclusion of sulfate. Once in the cell, SO_2 re-equilibrates to the HSO_3^- ion due to the higher pH of the cytoplasm (*ca.* 6.5), causing its intracellular concentration to exceed that of extracellular levels by up to 60-fold.

It is of interest that the transported form of sulfite, molecular SO_2, is toxic to a variety of *Saccharomyces* spp. (King *et al.*, 1981; Stratford and Rose, 1986). Toxicity is effected by several mechanisms including reduction of cellular ATP content (Schimz and Holzer, 1979), inhibition of enzyme activity (Pfleiderer *et al.*,

1956), inhibition of glycolysis (Gancedo *et al.*, 1968) and the modification of DNA basepairs (Guerra *et al.*, 1981; Shapiro *et al.*, 1970).

b. Organic sulfur sources

Many of the organic intermediates of yeast sulfur metabolism occur in grape juices, generally in minute amounts (Amerine *et al.*, 1980), though when available, they are readily utilised by the yeast. Transport mechanisms or an ability to act as a sole sulfur source has been described for methionine, cysteine, cystine, *S*-adenosylmethionine, biotin and thiamine (Schultz and McManus, 1950; Maw, 1965; Gits and Grenson, 1967; Rogers and Lichstein, 1969; Murphy and Spence, 1972; Iwashima *et al.*, 1973). Trace compounds such as carbonyl sulfide, carbon disulfide, dimethyl sulfide, dimethyl sulfoxide, *S*-methyl methionine and a range of sulfide addition compounds have been identified in grape juice, wine and other fruits, but little is known of their transport or metabolism by yeast (Kiribuchi and Yamanishi, 1963; Wong and Carson, 1966; Spedding *et al.*, 1980; Pearson *et al.*, 1981; Eschenbruch *et al.*, 1986).

1.5.2 The sulfate reduction sequence

As already stated, the Sulfate Reduction Sequence (SRS) (Figure 1.1) begins with the uptake of sulfate through the action of specific permeases. Intracellular sulfate molecules are 'activated' by reaction with two molecules of ATP. The two step enzymic process is catalysed by ATP-sulfurylase and APS-kinase to yield adenosyl 5'-phosphosulphate (APS) and pyrophosphate (PPi), and 3'-phosphoadenosyl 5'-phosphosulfate (PAPS), respectively (Robbins and Lipmann, 1958a, 1958b; Wilson and Bandurski, 1958). The equilibrium of the first reaction is driven towards APS by the action of a pyrophosphatase which hydrolyses PPi to two molecules of inorganic phosphate. A requirement for magnesium ions in these reactions has been demonstrated (Wilson and Bandurski, 1958; Hawes and Nicholas, 1973). The enzyme PAPS-reductase (Wilson *et al.*, 1961; Schwenn *et al.*, 1988) then reduces PAPS in the presence of the co-substrate, reduced thioredoxin,

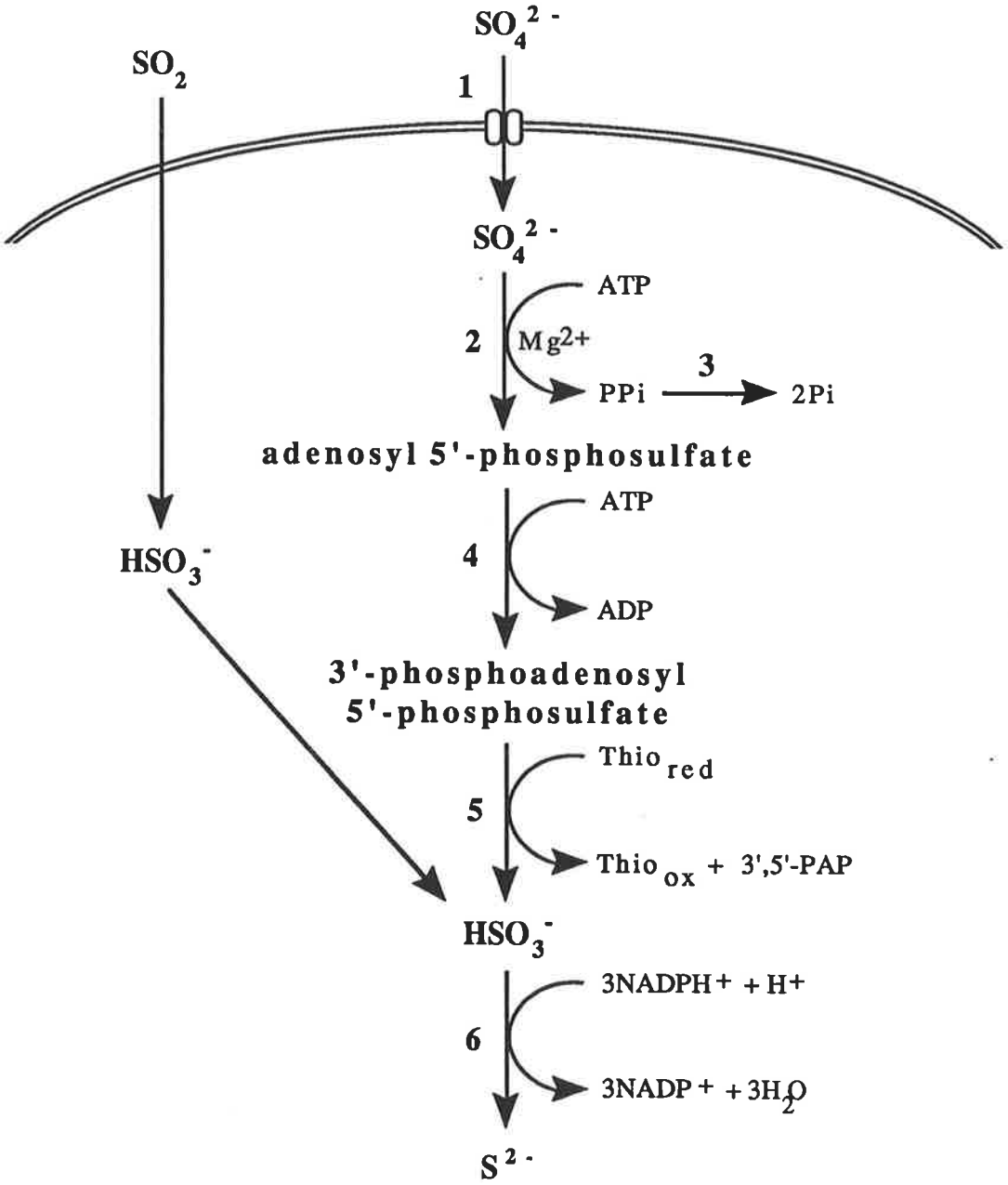


Figure 1.1 The Sulfate Reduction Sequence (SRS) of the yeast *Saccharomyces cerevisiae*.

Enzymes: (1) sulfate permeases I and II; (2) ATP-sulfurylase; (3)pyrophosphatase; (4) APS kinase; (5) PAPS reductase; (6)sulfite reductase.

Abbreviations: $Thio_{red}$, reduced thioredoxin; $Thio_{ox}$, oxidised thioredoxin; 3',5'-PAP, 3'-phosphoadenosyl 5'-phosphosulfate; APS, adenosyl 5'-phosphosulfate; PAPS reductase, 3'-phosphoadenosyl 5'-phosphosulfate reductase.

to liberate free anionic sulfite, contrary to reports of a 'bound-sulfite' intermediate being formed (Wilson and Bierer, 1976). At this point in the pathway, exogenous sulfite, which is energetically preferred by yeast, is able to enter the SRS following its diffusion into the cell. Feedback mechanisms which inhibit the accumulation and reduction of exogenous sulfate to sulfite (see Table 1.1), appear to be the principal means by which selection of sulfur sources is achieved.

The enzyme mediating the final step of the SRS has been the subject of extensive enzymological studies (Wainwright, 1961, 1962, 1967; Prabhakararao and Nicholas, 1969, 1970; Yoshimoto and Sato, 1968a, 1968b, 1970). Described as a haemoflavoprotein, sulfite reductase catalyses the 6 electron reduction of sulfite to sulfide in the presence of the hydrogen donor NADPH. Studies with enzyme extracts have demonstrated the ability of reduced cytochrome *c*, methyl- or benzyl-viologen to fulfill the *in vivo* function of NADPH (Naiki, 1965; Yoshimoto and Sato, 1968a, 1968b, 1970; Dott and Trüper, 1978).

An additional mechanism of sulfide formation, independent of the SRS, should be mentioned briefly. This mechanism involves the reduction of thiosulfite ($S_2O_3^{2-}$) to sulfite ion (SO_3^{2-}) and H_2S by thiosulfate reductase in the presence of reduced glutathione (Lawrence and Cole, 1968). Due to competitive inhibition by sulfite of the enzyme involved, the significance of this route in sulfide formation needs to be established.

1.5.3 Biosynthesis of methionine and cysteine

The nitrogen-containing carbon precursors, *O*-acetylserine (OAS) and *O*-acetylhomoserine (OAH), each combine with a sulfide ion to generate cysteine and the methionine precursor, homocysteine, in a reaction catalysed by the enzyme, OAS-OAH sulfhydrylase [formerly homocysteine synthetase] (Yamagata *et al.*, 1974, 1975) (Figure 1.2). The transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine by homocysteine methyltransferase

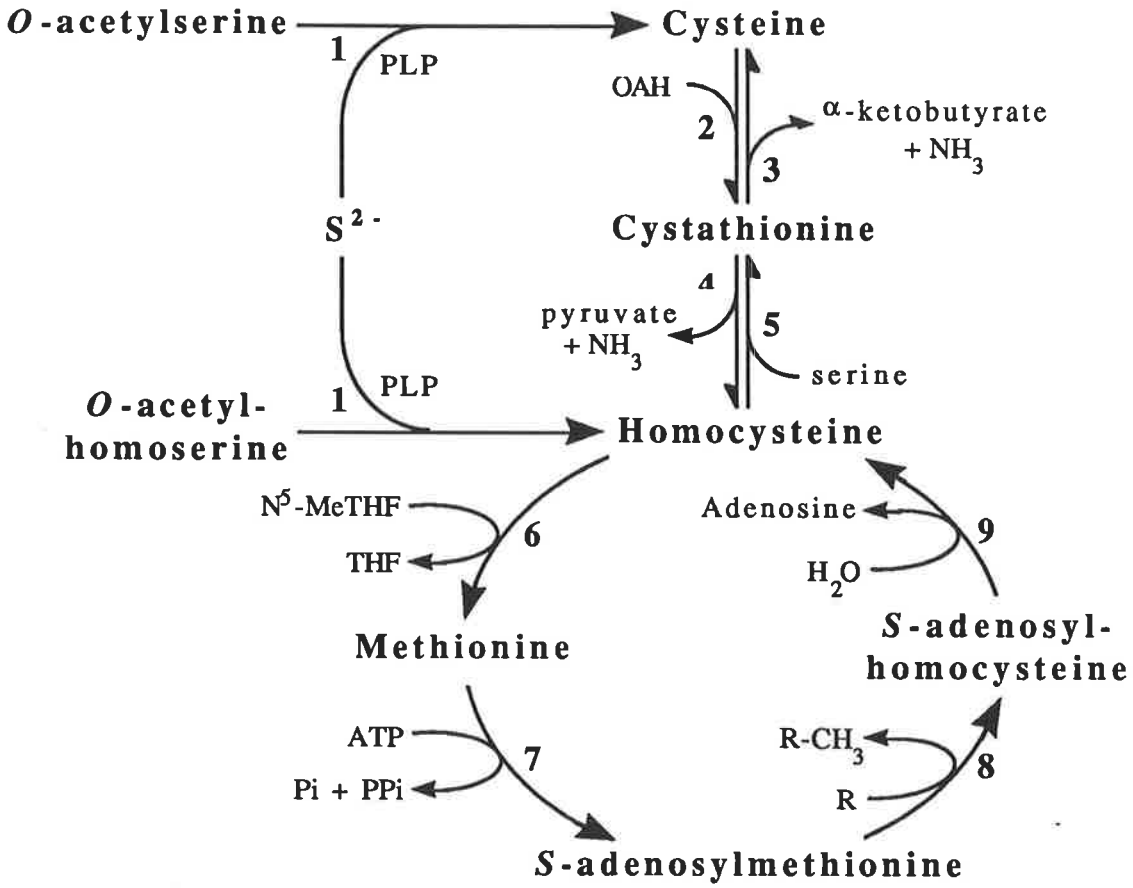


Figure 1.2 Biosynthesis and interconversion of methionine and cysteine in the yeast *Saccharomyces cerevisiae*.

Enzymes: (1) OAS-OAH sulfhydrylase; (2) γ-cystathionine synthase; (3) γ-cystathionase; (4) β-cystathionase; (5) β-cystathionine synthase; (6) homocysteine methyltransferase; (7) methionine *S*-adenoslytransferase; (8) SAM demethylase; (9) adenosylhomocysteinase.

Abbreviations: PLP, pyridoxal phosphate; OAH, *O*-acetylhomoserine; N^5-MeTHF, N^5-methyl-tetrahydrofolate; THF, tetrahydrofolate; R, methyl group acceptor; SAM, *S*-adenosylmethionine.

concludes the biosynthesis of methionine. Pyridoxal phosphate and hence the vitamin pyridoxine, are essential to the optimal functioning of OAS-OAH sulfhydrylase (Yamagata and Takeshima, 1976).

Studies carried out with methionine and cysteine auxotrophs have identified additional mechanisms for the formation of both amino acids. Since methionine auxotrophs are able to grow in media supplemented with cysteine, it is postulated that homocysteine, and hence methionine, is synthesised via cystathionine from cysteine (Yamagata *et al.*, 1975; Masselot and de Robichon-Szulmajster, 1975). Ono and coworkers (1984, 1988) subsequently provided evidence to indicate the existence of the reverse reaction, that is, for the synthesis of cysteine from homocysteine via cystathionine (Figure 1.2). Thus, it appears that the sulfur amino acids, methionine and cysteine, are essentially interchangeable via the intermediates cystathionine and homocysteine.

1.5.4 Fate of sulfur-containing amino acids and hydrogen sulfide

Both methionine and cysteine are formed by yeast to supplement exogenous sources of these amino acids, required primarily for protein synthesis. In addition, methionine is adenylated to yield *S*-adenosylmethionine, a methyl group donor central to yeast metabolism. Cysteine is a substrate for the synthesis of glutathione for which a possible role in thiosulfite reduction has already been discussed. The degradation of cysteine to yield hydrogen sulfide, catalysed by cysteine desulfhydrase or the reverse reaction of cysteine synthetase, has been demonstrated in yeast (Tokuyama *et al.*, 1973). This reaction is apparently inhibited by ATP and *S*-adenosylmethionine.

The involvement of methionine, cysteine and hydrogen sulfide in the formation of methane thiol, dimethyl sulfide, dimethyl disulfide and ethyl methyl sulfide was proposed some time ago (Brenner *et al.*, 1955) though little work has been conducted in this area since. Other than a possible participation in non-

enzymic addition reactions, such as that with acetaldehyde to yield ethane thiol (Rankine, 1963), hydrogen sulfide, which does not take part in sulfur-amino acid synthesis, is likely to simply diffuse from the cell into the growth medium. The possibility of hydrogen sulfide subsequently being reabsorbed has been confirmed (O'Connor *et al.*, 1986).

1.5.5 Control mechanisms of the SRS and methionine/cysteine biosynthetic pathways

The enzymes of the SRS and their key allosteric inhibitors are listed in Table 1.1. Feedback mechanisms exist primarily for enzymes preceeding the ATP-requiring steps of the SRS, presumably to minimise the unnecessary commitment of energy by yeast.

Supplementary to these feedback inhibitions are a complex array of repressive mechanisms which effect control over sulfate reduction and methionine/cysteine biosynthetic enzymes. Adenosine trinucleotide phosphate sulfurylase and sulfite reductase display sensitivity to cysteine (de Vito and Dreyfuss, 1964; Heinzel and Trüper, 1976, 1978; Dott and Trüper, 1978). Additional repression is mediated by methionine on these enzymes (de Vito and Dreyfuss, 1964; Okuda and Uemura, 1965; Cherest *et al.*, 1969, 1971, 1973; Colombani *et al.*, 1975; Dott and Trüper, 1978; Heinzel and Trüper, 1978) together with sulfate permease (Breton and Surdin-Kerjan, 1977; Horák *et al.*, 1981), APS kinase (Jones and Fink, 1982), homocysteine synthetase [OAS-OAH sulfhydrylase] (Dott and Trüper, 1979), aspartate kinase, homoserine dehydrogenase (Cherest *et al.*, 1971, 1973) and homoserine acetyltransferase (de Robichon-Szulmajster and Cherest, 1967) (Figure 1.3). Although described as such, methionine repression is not mediated by methionine exclusively, but largely by the metabolites, *S*-adenosylmethionine and methionyl t-RNA (Cherest *et al.*, 1971, 1973).

Due to the co-ordinated nature of their repression and insensitivity to methionine in methionyl t-RNA synthetase (ts-296) and ethionine resistant

Table 1.1 Inhibitors of enzymes of the sulfate reduction sequence.

Enzyme	Inhibitor						Reference
	SO_4^{2-}	APS	PAPS	$S_2O_3^{2-}$	SO_2	S^{2-}	
sulfate permease	+[a]	+		+	+		1,2
ATP-sulfurylase[wt]	+	+	+	-	-	+	3,4,5
ATP-sulfurylase[hs]		+/-					5
sulfite reductase[wt]					+/-	+	6, 7
sulfite reductase[hs]					+	+	6,7

References: (1) McCready and Din, 1974; (2) Breton and Surdin-Kerjan, 1977; (3) de Vito and Dreyfuss, 1964; (4) Heinzel and Trüper, 1976; (5) Hawes and Nicholas, 1973; (6) Yoshimoto and Sato, 1968a; (7) Dott and Trüper, 1976.

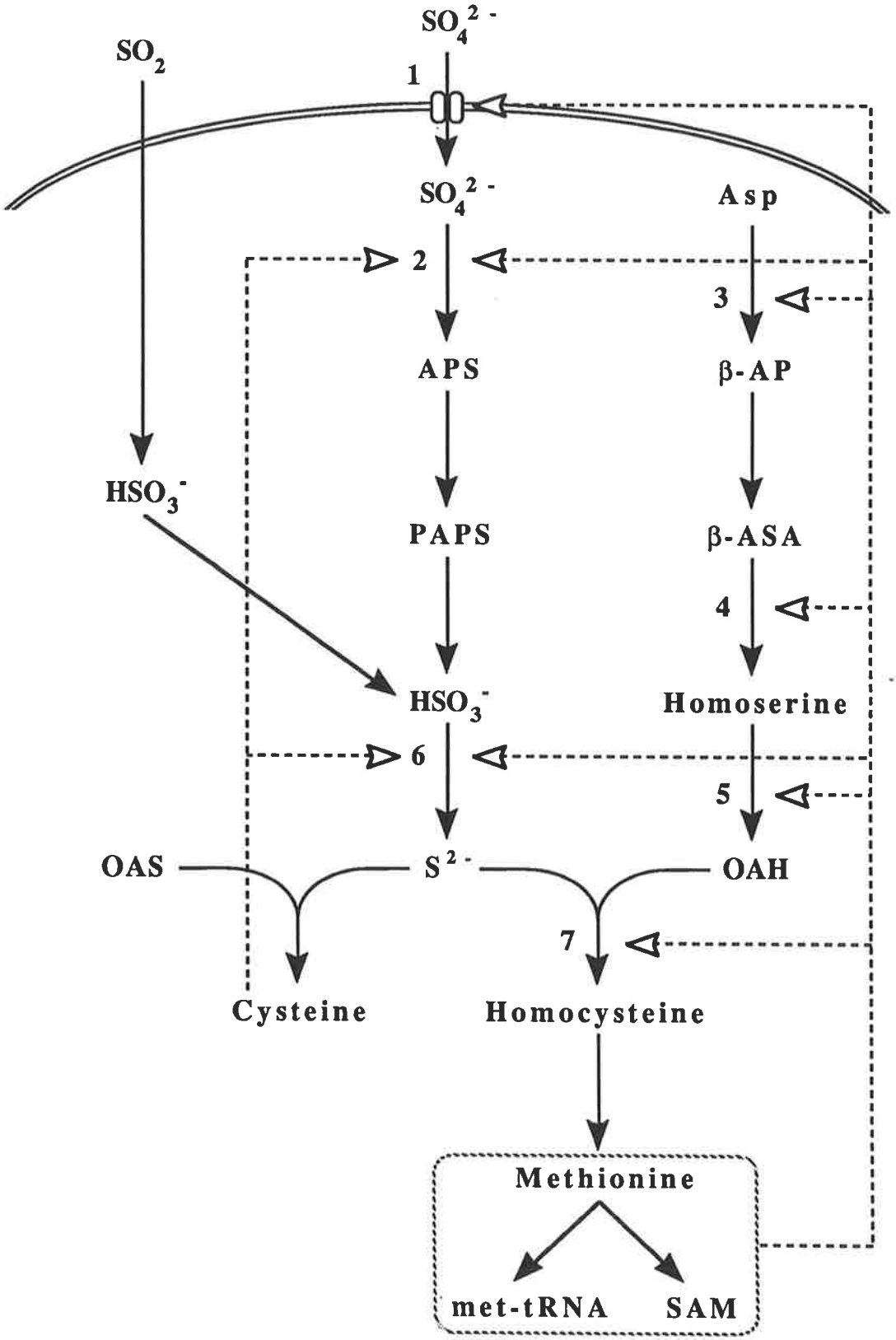
[a] +, inhibition; -, no inhibition; +/-, partial inhibition; no entry, effects not determined.
[hs] high sulfite-producing strains.
[wt] wild-type strains.

Figure 1.3 Repressive regulation of the Sulfate Reduction Sequence and methionine/cysteine biosynthetic pathways.

Repression of enzyme synthesis ---------▷

Enzymes: (1) sulfate permease; (2) ATP-sulfurylase; (3) aspartate kinase; (4) homoserine dehydrogenase; (5) homoserine acetyltransferase; (6) sulfite reductase; (7) homocysteine synthetase [OAS-OAH sulfhydrylase].

Abbreviations: Asp, aspartate; APS, adenosyl 5'-phosphosulfate; β-AP, β-aspartyl phosphate; PAPS, 3'-phosphoadenosyl 5'-phosphosulfate; β-ASA, β-aspartate semialdehyde; OAS, *O*-acetylserine; OAH, *O*-acetylhomoserine; SAM, *S*-adenosylmethionine; met-tRNA, Methionyl-tRNA.


SO_4^{2-}
SO_2
1
SO_4^{2-}
Asp
2
3
APS
β-AP
HSO_3^-
PAPS
β-ASA
4
HSO_3^-
Homoserine
6
5
OAS
S^{2-}
OAH
7
Cysteine
Homocysteine
Methionine
met-tRNA
SAM

(*eth2r*) mutants, sulfate permease, ATP-sulfurylase, sulfite reductase, homocysteine synthetase [OAS-OAH sulfhydrylase] and homoserine acetyltransferase are referred to as the methionine group I enzymes (Cherest *et al.*, 1969, 1971, 1973). Co-ordinated repression implies that the genes for these enzymes exist on the same locus or, less likely, are subjected to the same post-transcriptional regulatory mechanism or species. The remaining enzymes, which not only fail to show resistance to methionine repression in the above cited mutants, but also share an absence of co-ordinacy with the group I enzymes, are referred to as the methionine group II enzymes.

Given that the formation of the regulatory derivatives of methionine is able to take place, it is convenient to regard methionine as the principal regulatory molecule of the SRS and sulfur-amino acid biosynthesis. For this reason, a reduced cellular content of methionine can be expected to derepress these pathways and increase the flux of inorganic sulfur compounds, including the sulfide ion. The fate of sulfide is then subject to availability of the appropriate reactive nitrogen-containing carbon compounds arising from nitrogen metabolism. When these compounds are present, hydrogen sulfide will be sequestered into methionine and cysteine biosynthesis, thus preventing its loss to the medium.

1.5.6 Biosynthesis of acetylated precursors of methionine and cysteine

As suggested by the preceeding discussion, the synthesis of *O*-acetlyhomoserine, the precursor of methionine biosynthesis, is essential for the ultimate regulation of sulfide ion formation. In addition, evidence supporting the interconversion of methionine and cysteine, suggests that the biosynthesis of the cysteine precursor, *O*-acetlyserine, is similarly important.

The nitrogen component of *O*-acetylhomoserine is ultimately derived from glutamate by way of a multi-step reaction pathway (Figure 1.4). As a precursor of threonine, isoleucine and also methionine synthesis, homoserine

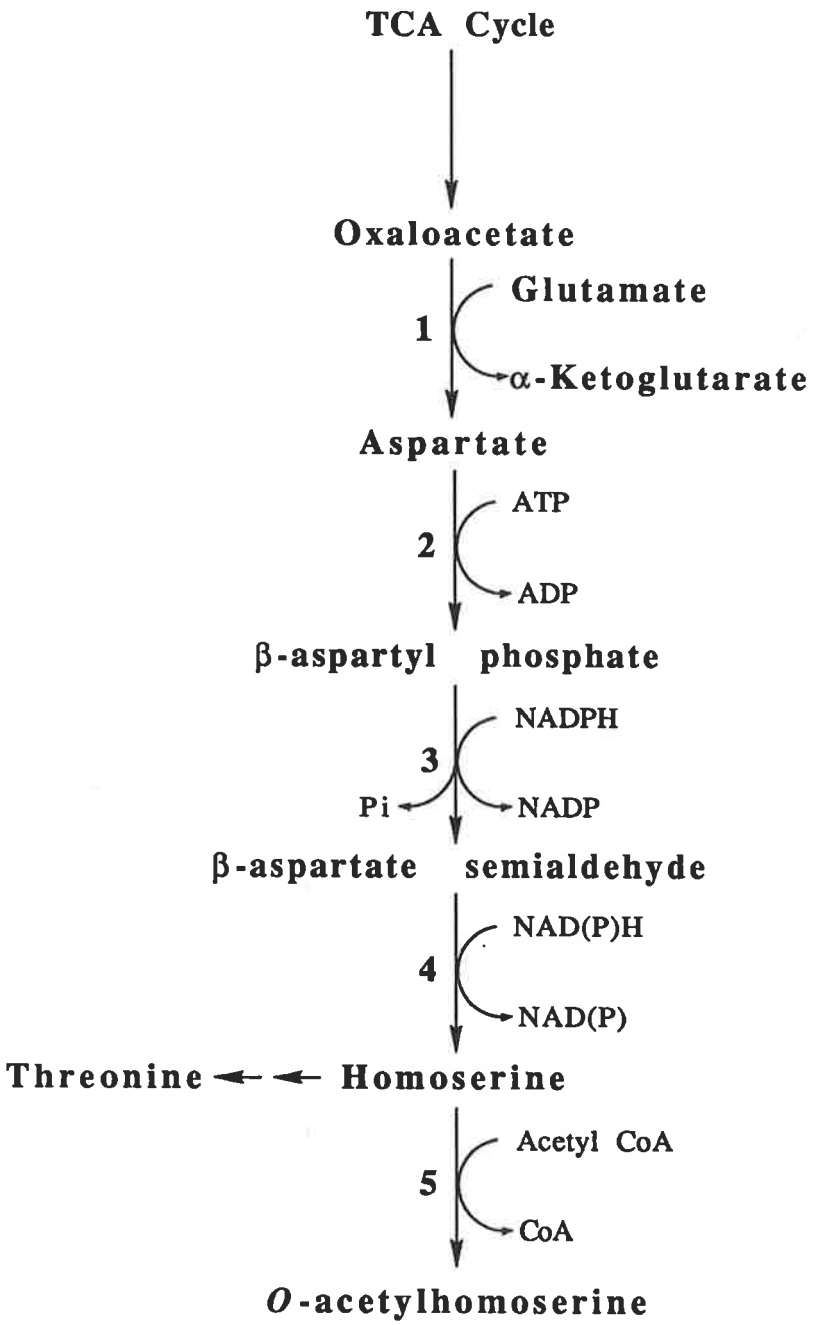


Figure 1.4 Biosynthesis of *O*-acetylhomoserine, nitrogen-containing carbon precursor of methionine.

Enzymes: (1) aspartate aminotransferase; (2) aspartate kinase; (3) aspartate-semialdehyde dehydrogenase; (4) homoserine dehydrogenase; (5) homoserine acetyltransferase.

forms an important branching point in this pathway. The first committed step to methionine biosynthesis is the formation of *O*-acetylhomoserine from homoserine, as mediated by homoserine acetlytransferase in the presence of acetyl Co-enzyme A (de Robichon-Szulmajster and Cherest, 1967). As a subunit of the Co-enzyme A, pantothenate, is essential to the formation of *O*-acetyl-homoserine and hence the ultimate repression of enzymes of the SRS. This fact accounts for earlier observations of high rates of hydrogen sulfide production by yeast grown in pantothenate deficient media (Wainwright, 1970; Tokuyama *et al.*, 1973).

As for *O*-acetylhomoserine, the amino group of the cysteine precursor, *O*-acetylserine, is also derived from glutamate by transamination (Figure 1.5). The glycolysis product 3-phosphoglycerate is converted to 3-phosphohydroxypyruvate in a NAD-dependent reduction, and in turn transaminated to yield 3-phosphoserine. A dephosphorylation produces the amino acid serine, which is subsequently acetylated to *O*-acetylserine in a reaction catalysed by serine acetyltransferase. Presumably serine may also be derived directly from exogenous supplies of this amino acid.

Glutamate exists within the cytoplasmic pool of free amino acids in equilibrium with the ammonium ion and α-ketoglutarate. These species are interconverted by the enzymes, NADP-dependent glutamate dehydrogenase, and NAD-dependent glutamate dehydrogenase (Holzer and Schneider, 1957; Heirholzer and Holzer, 1963).

As an essential component of the organic precursors of methionine and cysteine biosynthesis, nitrogen in the form of glutamate therefore fulfils an important function in the ultimate regulation of the SRS. Accordingly, there

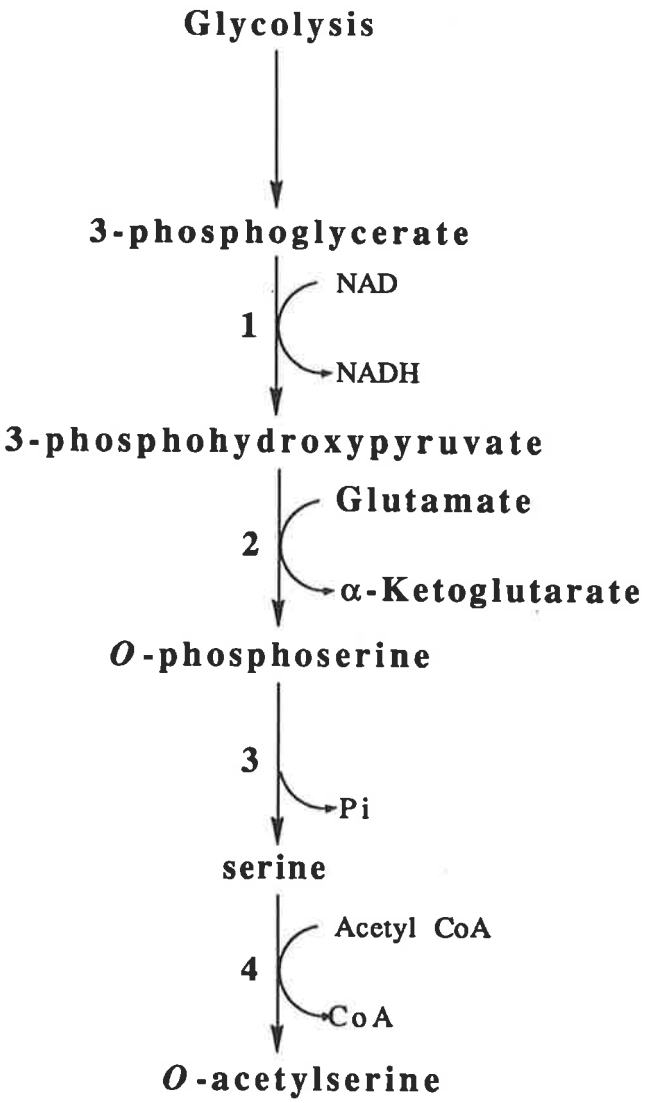


Figure 1.5 Biosynthesis of *O*-acetylserine, nitrogen-containing carbon precursor of cysteine.

Enzymes: (1) phosphoglycerate dehydrogenase; (2) phosphoserine aminotransferase; (3) phosphoserine phosphatase; (4) serine acetyltransferase.

exists considerable potential for nitrogen metabolism and intracellular nitrogen-pool dynamics to dramatically influence hydrogen sulfide production.

1.6 YEAST NITROGEN METABOLISM

The importance of nitrogen to the growth and metabolism of yeasts through its involvement in cell structure, metabolic activity and cell replication has long been appreciated. Glutamate specifically is vital in this regard due to its role as precursor of essentially all anabolic nitrogen metabolism, a fact attributing to the general predominance of this amino acid in the yeast cytoplasm (Watson, 1976; Messenguy *et al.*, 1980). Although exogenous supplies of glutamate may be exploited to sustain the cytoplasmic pool of this amino acid, these sources are likely to be finite, compelling the yeast to resort to the degradation of other nitrogen compounds (Jones *et al.*, 1969). Numerous recent studies (reviewed by Cooper, 1982a and Large, 1986) have identified a considerable range of nitrogen compounds which are degraded by one of two pathways to yield glutamate, either directly, or via the ammonium ion, through its reaction with α-ketoglutarate. As both end products and biosynthetic precursors, glutamate and ammonium ions through their interconversion, effectively form the interface between catabolic and anabolic processes of nitrogen metabolism in the yeast cell.

1.6.1 Nitrogen sources in fermentation

a. Grape juice and must composition

Grape must contains the full complement of nutrients necessary to support yeast growth during fermentation. The main source of carbon and energy for growth is provided by the grape sugars usually present in large excess (often exceeding 20% w/v) to that needed for maximal growth. By contrast, the

total nitrogen content of grape juice ranges 40 fold from 60 to 2400 mg $N.L^{-1}$ (Amerine *et al.*, 1980; Ough and Amerine, 1988) and can therefore be growth limiting. Furthermore, nitrogen is present in a complex range of compounds (Table 1.2). Trace amounts of nitrogen compounds such as vitamins, nucleotides (Amerine *et al.*, 1980) and nitrates (Ough and Crowell, 1980) are present. Various amines have also been detected in musts. The involatile amines, histamine and tyramine are thought to result from microbial activity, although the origin of volatile amines is unknown (Daudt and Ough, 1980). Ammonium, amino acids, peptides and proteins are the quantitatively important nitrogen compounds.

Ammonium ion concentrations range between 24 and 309 $mg.L^{-1}$ (average 123 $mg.L^{-1}$) in Californian grapes (Ough, 1969), between 55 and 148 $mg.L^{-1}$ in Stellenbosch varieties (Ough and Kriel, 1985) and 27 and 311 $mg.L^{-1}$ (average 89 $mg.L^{-1}$) in Australian fruit (Gockowiak and Henschke, unpublished data).

By accounting for between 60 and 90% of total juice nitrogen (Kliewer, 1969), the amino acids alone represent the main source of nitrogen for yeast growth. Surveys of amino acid content of grape juices from several viticultural regions of the world have revealed immense qualitative and quantitative variation in the amino acids present (Table 1.3). Despite these differences, proline, arginine, alanine, glutamate, glutamine, serine, threonine and γ-amino butyric acid frequently predominate.

For methodological reasons few reliable reports are available on the peptide and protein content of grape must. Cordonnier (1966) estimated peptides at 20.8% of total nitrogen. Protein content has variously been reported between 2 and 13.1% total nitrogen (Koch and Sajak, 1959, Tarantola, 1970, Anelli, 1977, Amerine *et al.*, 1980, Correa *et al.*, 1988).

Table 1.2 Nitrogenous fractions of grape juice.

Nitrogen fraction	Grape juice content ($mg.L^{-1}$)									
	1[a]		2		3		4		5	
Ammonia	10-120	(2)	0-146	(25)	45-99	(6)	45-89	(4)	7-127	(15)
Amino Acid	170-1120[b]	(2)	-		-		704-1070[c]	(4)	19-144	(10)
Amino	-		15-182	(22)	101-168	(6)	46-81	(4)	14-176	(15)
Amide[d]	10-40	(2)	-		-		-		-	
Humin[e]	5-20	(2)	-		-		-		-	
Polypeptide	-		38-132	(5)	-		-		10-70	(10)
Hexosamine	-		18-29	(2)	-		-		-	
Protein	10-100	(2)	28-97	(5)	-		-		-	
Residual	100-200	(2)	-		-		-		-	
TOTAL	305-1600	(2)	98-1130	(28)	358-570	(6)	322-490	(4)	98-618	(15)

Numbers in parentheses indicate the number of samples analysed.

[a] References: (1) Hennig, 1945 cited by Koch and Sajak, 1959; (2) Cordonnier, 1966; (3) Lafon-Lafourcade and Peynaud, 1959; (4) Lafon-Lafourcade and Guimberteau, 1962; (5) Bizeau, 1963.

[b] Dipeptides included.

[c] Arginine, proline, serine and threonine.

[d] Asparagine and glutamine.

[e] Tryptophan and tyrosine.

Table 1.3 Mean amino acid composition of grape juices.

[a] Values quoted were calculated from published data except for those sourced from references 1,2,3 and 8

[b] References: (1) Castor, 1953; (2) Récolte, 1957 cited by Lafon-Lafourcade and Peynaud, 1959; (3) Gallander *et al.*, 1969; (4) Dittrich and Sponholz, 1975; (5) Kluba *et al.*, 1978; (6) Ooghe and Kastelijn, 1988; (7) Huang and Ough, 1989; (8) Gockowiak and Henschke, unpublished data 1991.

[c] Asparagine and glutamine.

[d] Asparagine and glutamate.

[e] Serine and amides.

[f] Serine and threonine.

Amino acid	Grape juice content ($mg.L^{-1}$)[a]							
	1[b]	2	3	4	5	6	7	8
Origin	CA, USA	France	OH, USA	Germany	NY, USA	France	CA, USA	Australia
Alanine	-	-	304	181	596	164	192	79
γ-Amino butyric acid	-	-	-	-	81	44	-	62
Arginine	403	327	176	820	402	481	339	179
Asparagine	-	-	-	-	194[c]	-	170[d]	3
Aspartate	52	2	61	62	37	119	33	18
Citrulline	-	-	-	-	2	-	7	2
Cysteine	-	-	-	-	-	7	-	-
Cystine	4	0	8	-	-	-	-	-
Glutamate	687	173	144	132	71	352	170[d]	60
Glutamine	-	-	-	-	194[c]	-	198	61
Glycine	7	22	8	11	8	62	-	3
Histidine	92	11	17	62	20	42	35	11
Isoleucine	66	7	7	79	33	47	26	13
Leucine	62	20	14	108	43	78	33	15
Lysine	16	16	14	37	1	64	7	3
Methionine	14	1	7	24	12	7	12	2
Ornithine	-	-	-	-	4	7	10	3
Phenylalanine	51	5	-	103	58	55	37	11
Proline	-	266	198	484	85	383	1427	-
Serine	-	69	302	385[e]	33	103	133[f]	38
Threonine	-	258	66	152	76	122	133[f]	39
Tryptophan	47	1	-	1	17	-	-	4
Tyrosine	20	0	-	25	16	37	19	2
Valine	60	6	21	79	49	66	43	20
TOTAL $mg.L^{-1}$	1581	1184	1347	2745	1838	2240	2721	628
$mg\ N.L^{-1}$	264.7	208.0	211.8	522.4	339.8	373.6	426.8	120.0
Nº samples analysed	7	8	8	30	3	80	45	735

b. Factors affecting composition

In the past, most investigations on the viticultural aspects of nitrogen have been directed towards vine vigour, crop yield and sugar/acid content. Only recently has interest turned towards grape nitrogen composition and its relationship to fermentation and wine composition. Nitrogen concentration and composition of berries prior to crushing may be subject to various viticultural factors. The amino acids in particular have been studied in this regard and can vary according to variety (Castor, 1953; Récolte, 1957 cited by Lafon-Lafourcade and Peynaud, 1959; Gallander *et al.*, 1969; Anelli, 1977; Etiévant *et al.*, 1988; Huang and Ough, 1989), berry maturation (Lafon-Lafourcade and Guimberteau, 1962; Kliewer, 1968; Kluba *et al.*, 1978), nitrogen fertilisation (Agenbach, 1977; Bell *et al.*, 1979), rootstock (Ough and Tabacman, 1979; Monk *et al.*, 1987), fungal infection of either the black rot or 'Noble Rot' type (Dittrich and Sponholz, 1975; Dittrich *et al.*, 1975), soil type (Rankine *et al.*, 1971; Etiévant *et al.*, 1988; Huang and Ough, 1989) and 'Carbonic Maceration' (Flanzy, 1982).

Following crushing, the must is immediately inoculated with a selected yeast culture, or alternatively it may be processed further. Examples of these processes include pressing (Bergner and Haller, 1969; Amerine and Joslyn, 1970), enzymic treatment, clarification by 'cold-settling' (Vos and Gray, 1979; Houtman and Du Plessis, 1981), floatation, centrifugation or filtration (Houtman and du Plessis, 1981), fining with various adsorbants (Colagrande *et al.*, 1973; Houtman and du Plessis, 1981), ion-exchange (du Plessis, 1963; Dittrich *et al.*, 1975) and juice preservation and storage (Grechko and Emel'yanova, 1984; Monk, 1984; Monk and Costello, 1984). Must nitrogen composition may be altered directly by these treatments or indirectly because the time and temperature regimes employed may permit considerable growth of various microorganisms with concomitant utilisation of nitrogen. Enzymic treatment, cold-settling, and juice storage are especially important in this regard. A recent trend towards low or nil addition of sulfites to musts could also be expected to encourage the growth of microorganisms. Little or no quantitative data exists on the effects of many of

these treatments on microbial growth or nitrogen depletion. The nitrogen content of grapes at harvest will, therefore, only serve as a guide for that available at inoculation.

In view of the data of Heard and Fleet (1986) it has become apparent that wild yeast can play a much greater role in juice processing and fermentation than was previously appreciated. In this respect, the potential proteolytic activity of these yeasts (Nelson and Young, 1986) may be important in providing an additional source of assimilable nitrogen. This warrants further investigation particularly in light of the benefit of such activities in the later, nitrogen-depleted, stages of fermentation.

c. Nitrogen supplements

Analysis of must nitrogen is not a routine commercial practice. Nevertheless, must is frequently supplemented with assimilable nitrogen largely as a preventative measure against the development of nitrogen-related fermentation problems. Salts of ammonium, generally diammonium hydrogen phosphate (DAP), are widely permitted for use. Under European law, additions of DAP up to 300 $mg.L^{-1}$ are permissible, in the U.S.A. up to 960 $mg.L^{-1}$, whereas, in Australia the maximum addition is indirectly limited by a maximum wine phosphate concentration of 400 mg Pi (inorganic phosphate).L^{-1} (Sneyd, 1989). Generally, DAP (200 $mg.L^{-1}$) is added to must prior to inoculation with yeast, with further additions (100 $mg.L^{-1}$) in response to hydrogen sulfide formation.

Urea and several commercial 'yeast foods' are alternative nitrogen supplements. The apparent involvement of urea in ethyl carbamate formation in wine (Ough *et al.*, 1988; Montiero *et al.*, 1989) has led to its use being prohibited in most wine-producing countries. The value of yeast foods is also questionable since their assimilable nitrogen content is highly variable ~~in assimilable nitrogen content~~ and some contain relatively high concentrations of heavy metals (Ingledew *et al.*, 1986), restricted by legislation in some countries.

Yeast hull (ghost) preparations, widely recognised to stimulate fermentation, are proposed to function by providing trace amounts of assimilable nitrogen and by adsorbing toxic medium-chain fatty acids from the medium (Lafon-Lafourcade *et al.*, 1984; Munoz and Ingledew, 1989a, 1989b).

d. Assay methods: detection of assimilable nitrogen

The diversity of nitrogen compounds present in grape must presents a practical analytical problem. Accurate quantitation can be achieved by specific methods but these are not suited to rapid, routine analysis. Commonly used methods involve the chemical determination of the free alpha (primary) amino nitrogen (FAN) group using either the ninhydrin (Lie, 1973) or 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Bateson, 1970; Crowell *et al.*, 1985) procedure. While these methods conveniently give a 'chemical' value for nitrogen this does not necessarily equate with the 'biological value' which is a measure of the assimilable nitrogen. Discrepancies arise from non-alpha amino nitrogen present in the nitrogen rich amino acids, arginine, lysine, glutamine and asparagine, which will result in an underestimate of nitrogen content. The secondary amino acid, proline, although not usually utilised under oenological conditions (Pekur *et al.*, 1981; Ingledew *et al.*, 1987), is also underestimated. The presence of large peptides and protein, which are not assimilated by most species of yeast (Becker *et al.*, 1973), will inflate estimates of nitrogen content by these methods.

Several attempts have been made to devise a method to measure assimilable nitrogen. A bioassay approach, somewhat like that employed by Castor (1953) in an unrelated study, may be of value. Ideally a yeast growth or fermentation parameter is correlated with must assimilable nitrogen content. However, this method is likely to have serious practical limitations. As will be discussed, the uptake and metabolism of nitrogen compounds by *Saccharomyces cerevisiae* depends on the strain of yeast, its physiological condition and the

immediate environment. In this context, it is the unique chemical and highly variable nutrient conditions provided by grape must that profoundly affects the metabolic behaviour of yeast. This condition-dependence of nitrogen utilisation, therefore, dictates the use of test conditions as close as possible to actual fermentation conditions. Consequently, the incubation times involved will make such a method inconvenient. Nevertheless, this approach shows promise and warrants further development.

1.6.2 Utilisation of assimilable nitrogen

Much of our knowledge about the fundamental metabolic activities and interaction with the environment of fermentation yeasts is derived from studies on laboratory and brewers' yeasts under the respective conditions; information about the specific influence of oenological conditions on favoured wine yeast strains is rapidly increasing, but is still far from complete.

Saccharomyces cerevisiae is capable of growth on a diverse range of nitrogen compounds, including ammonium, urea, amino acids, small peptides, purine- and pyrimidine-based compounds (Cooper, 1982a; Large, 1986). Grape must contains varying proportions of all these compounds, which are, therefore, potentially utilisable by the cell. Before utilisation can proceed, the nitrogen compound must be transported into the cell by a membrane bound permease protein. Depending on the type of nitrogen compound to be accumulated, it may be utilised:

i) without modification, e.g. an amino acid is incorporated directly into protein.

ii) as a source of nitrogen, e.g. an amino acid is degraded to liberate nitrogen for the biosynthesis of other nitrogen cell constituents; the carbon skeleton may be excreted from the cell.

iii) as a source of carbon, e.g. the carbon component of an amino acid is released and used for the biosynthesis of other carbon cell constituents (Large, 1986).

The metabolic efficiency of a nitrogen source is dependent on the expression, regulation and efficiency of transport systems as well as the regulation and energetics of subsequent catabolic and anabolic processes. Consequently, growth and fermentation rates and biomass yield will be a manifestation of not only the quantity but also the nature of the nitrogen source(s) available.

The majority of the nitrogen compounds found in grape must are utilisable by yeast as sole sources of nitrogen. Of the amino acids, lysine, cysteine, proline and to a variable extent, histidine and glycine (Watson, 1976; Pekur *et al.*, 1981; Cooper, 1982a; Large, 1986; Ingledew *et al.*, 1987), are notable exceptions. Through direct incorporation they nonetheless fulfill a role in protein synthesis. Large peptides and proteins do not support growth since *Sacch. cerevisiae* is neither capable of hydrolysing or accumulating these macromolecules. Their exploitation may, however, be facilitated by other yeast species present in fermenting must which possess extracellular proteolytic activity (Nelson and Young, 1986).

The failure of certain amino acids to meet biosynthetic nitrogen requirements is a consequence of conditional or permanent metabolic blocks preventing the partial or complete degradation of these compounds. Proline is utilised only to a limited extent under oenological conditions due to nitrogen catabolite inhibition of the proline permease (Lasko and Brandriss, 1981; Horák and Ríhová, 1982) and a limited supply of molecular oxygen essential for the activity of proline oxidase (Duteurtre *et al.*, 1971; Mantachian, 1984), the first step in proline catabolism. The inability of *Sacch. cerevisiae* to grow solely on lysine is suggested to result from toxicity of α-amino adipate semialdehyde, a catabolite of lysine which accumulates in the absence of better nitrogen sources (Zaret and

Sherman, cited by Cooper, 1982a). This property has been employed as a basic selection criterion for *Sacch. cerevisiae* (Morris and Eddy, 1957) in grape must fermentation (Heard and Fleet, 1986). Yeast is able to degrade cysteine (Aida *et al.*, 1969; Tokuyama, *et al.*, 1973) and utilise the nitrogen component. However, at levels needed for appreciable growth, cysteine shows toxic effects similar to other sulfhydryl compounds (Maw, 1965). Histidine, although nitrogen rich, with three atoms of nitrogen, is apparently not degraded by many yeasts (see reviews by Cooper, 1982a; Large, 1986).

Grape juice contains concentrations of nucleic acid derivatives apparently too low to support significant growth, although these compounds, thymine or thymidine excepted (Wickner, 1975), can be utilised by yeast as a nitrogen source (Lee, 1987).

a. Order of amino acid and ammonium uptake

When present in a mixture, a selective accumulation of various amino acids and ammonium has been repeatedly observed (Table 1.4). In an early quantitative kinetic study of the fermentation of grape must, Castor (1953) observed differences in the timing, rate and extent of uptake of individual amino acids. The important amino acids, as evident by their uptake coinciding with cell growth, were arginine, glutamate, valine, isoleucine, leucine, histidine, aspartate, tryptophan, phenylalanine and methionine, 70-95% removal of these being achieved by the cessation of growth. Arginine and histidine formed the greatest residual concentrations, however, arginine was initially present in the must at 5-10 times greater concentration than the other amino acids studied. Initiation of the uptake of valine and tyrosine was delayed and small amounts of these amino acids together with isoleucine, leucine and tryptophan were released back into the medium during the final stage of fermentation. Glycine, lysine and cysteine were not significantly utilised. Overall, the accumulation of amino acids and ammonium (Castor and Archer, 1959), albeit at different rates, was restricted to

Table 1.4 Order of absorption of nitrogen compounds from the medium during fermentation.

Test medium	1[a] Grape juice		2 Wort			3 Wort			4 Defined maltose	
Nitrogen compound	Hours to 50% depletion	Relative position	Hours to 50% depletion	Relative position	Group	Hours to 50% depletion	Relative position	Group	Hours to 50% depletion	Relative position
Alanine	-	-	32	9	C	21	10	C	11	7
Ammonium	-	-	30	8	C	17	7	C	16	11
Arginine	33	8	17	2	A	14	5	A	3	1
Asparagine	-	-	-	-	A	-	-	A	-	-
Aspartate	24	5	20	4	A	15	6	A	8	4
Glutamate	27	7	17	2	A	13	4	A	7	3
Glutamine	-	-	-	-	A	-	-	A	-	-
Glycine	>130	12	37	11	C	27	13	C	13	9
Histidine	34	9	27	7	B	18	8	B	-	-
Ileucine	15	1	27	7	B	18	8	B	10	6
Leucine	20	2	26	6	B	19	9	B	9	5
Lysine	120	11	17	2	A	8	1	A	3	1
Methionine	21	3	21	5	B	11	2	B	7	3
Phenylalanine	25	6	32	9	C	23	12	C	12	8
Serine	-	-	19	3	A	12	3	A	6	2
Threonine	-	-	16	1	A	12	3	A	6	2
Tryptophan	22	4	43	13	C	32	14	C	18	12
Tyrosine	35	10	38	12	C	32	14	C	>21	13
Valine	25	6	33	10	B	22	11	B	14	10

Values quoted were calculated or derived from published data in all cases except those sourced from 4.

[a] References: (1) Castor, 1953; (2) Jones and Pierce, 1964; (3) Maule *et al.*, 1966; (4) Egbosimba *et al.*, 1988.

the growth phase, after which no important changes occurred except for a small efflux of some amino acids near the end of fermentation.

The observations of Castor have in general terms been confirmed by subsequent empirical investigations (Pekur *et al.*, 1981; Ingledew and Kunkee, 1985; Monk *et al.*, 1987), but these also have been limited by the fact that grape juice is difficult to define or control adequately in chemical terms. This strategy demands the study of a large number of juices before generalisations can be drawn; there are few instances of this in the literature. This restriction may be overcome by employing model media which permits precise and easy definition of media composition and experimental conditions; it would then be necessary to test the findings in grape juice as a confirmatory measure. Model media have been in use for some time. Yeast Carbon Base (Difco, USA), for example, supplemented with amino acids as required, has proven convenient but is a poor model for grape juice. A Synthetic Grape Juice medium based on typical grape juice analysis may represent a better alternative (Monk and Cowley, 1984).

Despite differences in medium composition and fermentation conditions, there are a surprising number of similarities between observations made in grape juice and those made for the wort fermentation (Table 1.4). The purported stability of the sequence of amino acid and ammonium utilisation, almost independent of the culture conditions employed, allowed these compounds to be classified into four groups according to the kinetics of their disappearance from the medium (Jones and Pierce, 1964). Group A compounds were characterised by a rapid onset of uptake and near complete depletion within 20 hours. A pronounced lag preceded the uptake of group B amino acids. The initiation of uptake of group C compounds coincided with the depletion of those of group A. Proline, in Group D, was little utilised.

A major difference relates to the timing of ammonium accumulation, which was rapid under oenological conditions (Monk *et al.*, 1987) but fell into Group C under brewing conditions. The delayed uptake of ammonium has been

attributed to the influence of maltose on the ammonium permease (Egbosimba *et al.*, 1988). Contrary to the observations of Jones and Pierce (1964) at least one factor, ammonium supplementation, clearly influenced the utilisation of the two amino acids examined, arginine and γ-amino butyric acid, by delaying their removal from grape juice (Monk *et al.*, 1987). Ammonium is well known to exert an inhibitory effect on the transport of many amino acids (refer to Table 1.6; Grenson and Hou, 1972; Wiame *et al.*, 1985). These observations have important implications for the use of ammonium based supplements for adjusting total nitrogen content of musts in relation to the evolution of urea and aroma compounds (see below).

b. Amino acid utilisation profile

Nitrogen utilisation and efflux is a dynamic and continuous process which proceeds throughout fermentation and beyond until the wine is microbiologically stabilised (Feuillat and Charpentier, 1982). From the preceeding section, it is clear that once growth has ceased and the fermentation has become 'dry', changes in nitrogen composition are small. The nitrogen balance at the end of fermentation, when related to initial must composition, can provide both qualitative and quantitative information on yeast nitrogen demand during fermentation. This area has not been investigated in the past and obtaining this type of information from published fermentation data is difficult for two reasons. Grape juices are most commonly the test medium, thus the contribution and influence made by other nitrogen and non-nitrogenous compounds remains undefined, and all 20 amino acids and ammonium are not always present in excess of yeast requirements. Therefore, although grape juices may have total amino nitrogen which represent an excess, individual amino acids may be limiting. Consequently, utilisation patterns for many amino acids essentially reflect the available quantities of these compounds.

The data of two groups which prove more useful in this respect is shown in Figure 1.6. On average, all amino acids were present in excess to yeast demand.

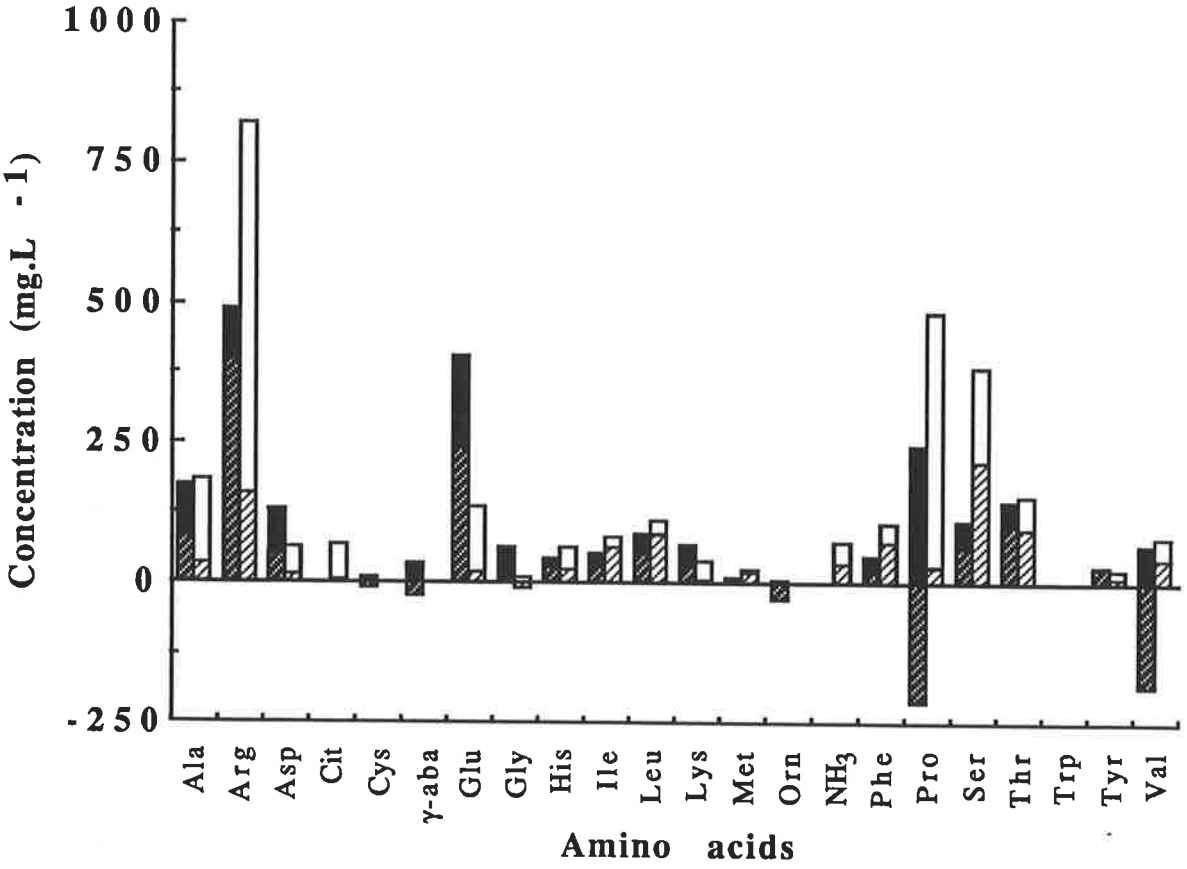


Figure 1.6 Amino acid utilisation profile of yeast during grape juice fermentation.

Initial amino acid content of grape juice (solid colours) is compared with the extent of amino acid utilisation or excretion (crosshatching).Values are derived from the published data of Ooghe and Kastelijn, 1988 (black bars) and Dittrich and Sponholz, 1975 (white bars). In the latter, serine was determined together with the amides.

The most preferred nitrogen sources were typically arginine, serine, glutamate, threonine, leucine and alanine.

c. Total nitrogen demand

Information regarding the total nitrogen requirements of yeast is more readily available. Table 1.5 presents several estimates of yeast nitrogen requirements under a variety of fermentation conditions. Little work has been done to investigate the influence of physical, chemical fermentation parameters and yeast strain on both total nitrogen demands and specific amino acid utilisation profiles. The impact of these parameters on wine organoleptic properties, as a consequence of their influence over yeast nitrogen metabolism, has been only partly examined (Ough *et al.*, 1966; Vos, 1981; Tromp, 1984; Castino and di Stefano, 1990; Rosi *et al.*, 1990).

1.6.3 Transport

a. Ammonia and urea

The accumulation of the ammonium ion by *Sacch. cerevisiae* has been investigated through the use of the non-metabolisable ammonium analogue, methylamine (Roon *et al.*, 1975b; Dubois and Grenson, 1979). Two specific permeases sensitive to temperature and pH have been identified with substrate affinities (K_m) of 0.25 and 2 mM and maximal accumulation rates (V_{max}) of 50 and 20 nmol . mg protein^{-1} min^{-1}, respectively (Dubois and Grenson, 1979). Transport activity is non-competetively inhibited by several amino acids and requires the presence of a fermentable or oxidizable energy source (Roon *et al.*, 1975b; Egbosimba and Slaughter, 1987).

Urea is transported via two mechanisms in yeast. The first is an active transport system which has an apparent K_m of 14 μM. It is repressed by nitrogen and dependent on both energy metabolism and exposure to oxaluric acid, a

Table 1.5 Nitrogen utilisation by yeast during grape juice and wort fermentations.

Nitrogen utilised for complete fermentation				
Total		Minimum		
mg.L^{-1}	mg N.L^{-1}	mg N.L^{-1}	Medium sugar content (g.L^{-1})	Reference[a]
-	-	150[bc]	112-124	1
916	174	-	190	2
615[d]	144[d]	-	221	2
-	-	130[c]	208-233	3
-	-	70[c]	183	3
283	-	-	232	4
-	878[ce]	-	216	5
5297	795	-	220	6
-	26-495[c]	-	220	6
-	-	113-190[bc]	112	7
654	159	-	-	8

[a] References: (1) Enari, 1974; (2) Dittrich and Sponholz, 1975; (3) Agenbach, 1977; (4) Bell *et al.*, 1979; (5) Vos *et al.*, 1979; (6) Ingledew and Kunkee, 1985; (7) Pickerell, 1986; (8) Ooghe and Kastelijn, 1988.

[b] Wort fermentation.

[c] mg Free Amino Nitrogen.L^{-1}.

[d] Juice from *Botrytis* infected fruit.

[e] Level required for maximal fermentation rate.

gratuitous inducer of allantoin degradative enzymes (Cooper and Sumrada, 1975). The second system is one of passive or facilitated diffusion which operates at external urea concentrations of greater than 0.5 mM.

b. Amino acids

Comprehensive reviews on the amino acid transport mechanisms of *Sacch. cerevisiae* have been published by Eddy (1982), Wiame and coworkers (1985), Horák (1986) and Cartwright and coworkers (1989). Briefly, a number of transport systems specific for a single or a small related group of L-amino acids have been identified from kinetic studies. Their substrate affinities and accumulation rates are given in Table 1.6. In addition to these systems both D- and L-isomers of the neutral and basic amino acids, and proline to a limited extent, are transported by the group-specific transport system, the 'general amino acid permease' (GAP) (Grenson *et al.*, 1970). The GAP appears to operate as a 'nitrogen scavenger' system, being expressed only when the readily assimilated nitrogen sources have been utilised.

It has been suggested that the kinetics of utilisation and the described grouping of amino acids may be partly explained by the appearance and activity of amino acid transport systems (Rose and Keenan, 1981). During the initial stage of fermentation, permeases specific for a small number of amino acids mediate the accumulation of most of the Group A and B species. Differences in transport kinetics of elements of these groups are possibly attributable to competition for permease sites by the transported amino acids and permease characteristics namely, timing of expression, substrate affinities (K_m), maximal transport rates (V_{max}) and transinhibition (Roon *et al.*, 1975; Woodward and Cirillo, 1977; Rose and Keenan, 1981; Eddy, 1982; Knatchbull and Slaughter, 1987).

The GAP is the principal transport mechanism for amino acids during the later stage of the fermentation. Inhibited and repressed by ammonium (Grenson and Hou, 1972; Wiame *et al.*, 1985), GAP activation appears to coincide

Table 1.6 Amino acid transport mechanisms of *Saccharomyces* yeasts.

References: (1) Grenson *et al.*, 1970; (2) Grenson *et al.*, 1987; (3) McKelvey *et al.*, 1990; (4), Grenson *et al.*, 1966; (5), Chan and Cossins, 1976; (6) Gregory *et al.*, 1982; (7) Darte and Grenson, 1975; (8) Joiris and Grenson, 1969; (9) Grenson and Dubois, 1982; (10) Ballarin-Denti *et al.*, 1984; (11) Crabeel and Grenson, 1970; (12) Bussey and Umbarger, 1970; (13) Gits and Grenson, 1969; (14) Ramos *et al.*, 1980; (15) Grenson, 1966; (16) Gits and Grenson, 1967; (17) Horák and Ríhová, 1982; (18) Magaña-Schwencke and Schwencke, 1969; (19) Magaña-Schwencke *et al.*, 1973; (20) Lasko and Brandriss, 1981; (21) Shukla *et al.*, 1982; (22) Verma *et al.*, 1984; (23) Wiame *et al.*, 1985; (24) Greasham and Moat, 1973; (25) Kotyk and Dvoráková, 1990.

[a] Activity in ammonium grown cells.
[b] nmol.mg protein^{-1}.min^{-1}.
[c] nmol.g dry weight^{-1}.min^{-1}.
[d] nmol.g dry weight^{-}.s^{-1}.

Amino Acid	Transport System[e]	Ammonium sensitive[a]	K_m (μM)	V_{max}[b]	Reference
L-Alanine	GAP	Yes			1
γ-Amino butyric acid	Proline permease	Yes	12		2,3
	GABA permease	Yes	100		2,3
	GAP	Yes			2
L-Arginine	Arginine permease	No	10	12	4,5
	GAP	Yes	7.6	23	1,5
L-Asparagine	Glutamine permease	No	350	33[c]	6,7
	GAP	Yes			6,7
L-Citrulline	GAP	Yes	80		1
L-Glutamic acid	Dicarboxylic amino-acid permease 1	No	17		7,8
	Dicarboxylic amino-acid permease 2	Yes			7,8
	GAP	Yes	1000	20[c]	7
L-Glutamine	Glutamine permease 1	No			9
	Glutamine permease 2	No			9
	GAP	Yes	40		1,9
L-Glycine	Glycine permease	No	400	2	10
	GAP	Yes	14000	2	1,10
L-Histidine	Histidine permease 1	No	17	11	11
	Histidine permease 2	No	4000	28	11
	GAP	Yes	25	16	1
L-Leucine	Leucine permease	No	1000		12,13,14
	GAP	Yes			12,14
L-Lysine	Lysine permease	No	25	8.1	15
	Arginine permease	No	200	17	15
	GAP	Yes	3.1		1
L-Methionine	Methionine permease 1	No	12	13	16
	Methionine permease 2	No	770	13	16
	GAP	Yes			1
L-Proline	Proline-imino acid permease	Yes	31	40[d]	17,18,19
	GAP	Yes	>2500	160[d]	17,20
L-Serine	specific permease	Partial	250		21,22
	GAP	Yes	500		1,21,22
L-Threonine	specific permease	No			13
	GAP	Yes			23
L-Tryptophan	Arom permease	No	410	1.2[c]	23,24,25
	GAP	Yes	10		1,23
L-Valine	GAP	Yes			1

with the depletion of ammonium and/or the close derivatives glutamate and glutamine, whether of extracellular origin or a product of the catabolic transport of Group A and B amino acids (Rose, 1987). Accumulation of Group C amino acids is believed to be achieved entirely via the GAP.

c. Peptides and proteins

No information is available on the removal of peptides from grape musts, although analogous work carried out in wort (Calderbank *et al.*, 1985) suggests that yeasts may utilise these species during fermentation. The transport of di- and tripeptides only is thought to be accomplished by a general peptide-transport system (Marder *et al.*, 1977; Moneton *et al.*, 1986a; 1986b), although the utilisation of peptides of up to five residues has been reported (Becker *et al.*, 1973; Naider *et al.*, 1974). The importance of peptides to yeast metabolism remains to be clarified. The utilisation of large peptides or proteins is dependent on the ability of yeast either to transport these compounds or degrade them extracellularly. Neither of these mechanisms have been reported in *Sacch. cerevisiae* (Rosi *et al.*, 1987; Sturley and Young, 1988), however, some non-*Saccharomyces* species isolated from fermenting must possess significant extracellular acidic protease activity (Nelson and Young, 1986).

d. Other nitrogen compounds

As discussed in a recent review (Large, 1986) a broad range of nitrogen compounds can be metabolised by yeast. Presumably, the ability to transport these compounds will be the single most important criterion to permit their utilisation by this organism. D-amino acids (Rytka, 1975), *S*-adenosylmethionine (Murphy and Spence, 1972), nucleotides and nucleic acid derivatives (Chen *et al.*, 1973; Lee, 1987) and vitamins including thiamin, riboflavin and biotin (Iwashima *et al.*, 1973; Perl *et al.*, 1976; Rogers and Lichstein, 1969) have been shown to be accumulated from the medium by *Sacch. cerevisiae*.

e. Regulation

The activity of transport systems can be regulated by a number of mechanisms which vary in their specificity, response time and level of influence (i.e., transcription, translation or precursor/active enzyme). The four that will be discussed here are feedback inhibition, transinhibition, inactivation/reactivation and repression.

In the context of transport, feedback inhibition is the process by which an accumulated substrate inhibits further accumulation by the same transport carrier. Experimentally, feedback inhibition manifests itself as a progressive decrease of substrate accumulation rate. According to a simplified model of this mechanism, the substrate carrier is prevented from returning to the external plasma membrane surface by continuing to be occupied by substrate which is present in high intracellular concentrations. Where internal concentrations of substrate are kept low by catabolism or compartmentation into subcellular organelles, feedback inhibition is considered to be of little consequence.

The phenomenon of 'transinhibition' refers to the competition for uptake exhibited between substrates which do not share a common transport mechanism. Cooper and Sumrada (see Cooper, 1982b) provide the example of asparagine inhibiting the accumulation of allantoin, a structurally and metabolically unrelated nitrogen compound. Although repression may play a role, the rapidity of the inhibition seems to relegate repression to being of importance only in the long term. Instead, it is suggested that inhibition arises out of competition for a component common to both transport mechanisms (Roon *et al.*, 1977; Cooper, 1982b).

In contrast with glucose and fructose which are principally accumulated into the cell by facilitated diffusion (Cirillo, 1961; McClellan *et al.*, 1989), nitrogen compounds are actively transported against a concentration gradient. This difference in approaches to solute accumulation is likely to reflect the relatively

lower availability of nitrogen compounds from natural sources. Solute influx *per se* by active transport is a purely physical process which is achieved by coupling the carrier-mediated uptake of solute molecules to the movement of ions, typically protons, down a concentration gradient across the plasma membrane. An input of energy, in the form of ATP hydrolysis, is required by active transport to fuel the plasma membrane ATPases which excrete protons from the cell to prevent acidification and ultimate cell death. It seems conceptually feasible that the plasma membrane ATPases have a finite capacity to excrete protons. Thus any factors which challenge this capacity will be likely to influence transport systems as a whole. It is possible that the common component being competed for in transinhibition, is this finite capacity of the ATPases.

Transinhibition may account for the delayed accumulation of ammonium in the wort fermentation relative to the wine fermentation. Unlike grape sugars, maltose, the principal carbon source for yeasts in brewing, is accumulated by active transport, resulting in the influx of one proton per molecule of maltose (Serrano, 1977). Therefore, a nett increase is observed in proton influx for a given amount of nitrogen during growth on this carbohydrate (Seaston, 1973). Consequently, accumulation of nitrogen compounds, such as ammonium, may be reduced because a portion of the ATPases normally employed by it are required to deal with the proton influx associated with maltose uptake. Although, such a proposal may explain the delayed accumulation of ammonium from a defined maltose medium when it is sole nitrogen source (Egbosimba *et al.*, 1987, 1988), extrapolation to the multiple nitrogen-source wort fermentation is not appropriate. The apparently specific influence on ammonium transport by what would appear to be a non-specific effect, is not explained.

Transinhibition may also go part of the way to explain why yeast will simultaneously accumulate a range of nitrogen compounds when a mixture is available in the medium. Potentially, transinhibition could represent an important mechanism through which growth is optimised by ensuring a diversity of nitrogen accumulation.

The ratio of solute:proton absorption varies not only between carbon or nitrogen sources but also across transport systems for the same compound. It is of interest to note that uptake of glycine, methionine, lysine, citrulline, phenylalanine and leucine via the GAP results in the absorption of two protons per molecule of substrate, whereas, proton influx is halved by the majority of the specific permeases (Eddy, 1978, 1982). Accordingly, these differences in stoichiometry may be important in transport selectivity by which the most energetically favourable compounds are accumulated via the most efficient mechanism, even at changing substrate concentrations.

Inactivation-reactivation processes have been described for several ammonium-sensitive permeases of *Sacch. cerevisiae*, including the general amino-acid permease (GAP) (Grenson and Acheroy, 1982; Grenson, 1983a, 1983b). During growth on a poor nitrogen source such as proline, the permease is synthesised through expression of the structural gene, *GAP1*. Permease activity is subsequently determined according to the nett dominance of reactivator and inactivator proteins. Thus in proline grown cells, the product of the constitutively expressed *NPR1* [nitrogen permease reactivator] gene will dominate over the *NPI1* and *NPI2* [nitrogen permease inactivator; formerly *MUT2* and *MUT4*] gene products to yield GAP activation. In the presence of ammonium ions, the balance is shifted towards the inactivator proteins resulting in permease inactivation through stoichiometric binding to the PGR site of the molecule (Vandenbrol *et al.*, 1987). Thus, when present, good nitrogen sources such as ammonium ions are preferentially accumulated over poorer ones. Efficient nitrogen sources such as ammonium, or derivatives thereof, are thought to favour the dominance of the negative effect by either inactivating the NPR1 protein, repressing its synthesis or escalating the NPI1-NPI2 system.

In addition to the above mechanism, both ammonium-sensitive permeases and degradative enzymes have been reported to be regulated by nitrogen catabolite repression (NCR). Repression may be acting at the level of

transcription, translation or processing of mRNA or precursor proteins. In their recent review, Wiame, Grenson and Arst (1985) concluded that experimental evidence points to the product of the *gdhCR* gene, GDHCR, as a candidate for 'general repressor' exerting negative control on synthesis of all but one of the enzymes, permeases and regulatory proteins which are subject to NCR. Activation of the GDHCR molecule to the 'activated GDHCR repressor' required glutamine as an effector. Failure of NCR to operate in *gdhA*$^-$ ($NADP^+$-linked glutamate dehydrogenase lacking) mutants even upon glutamate supplementation (Grenson and Hou, 1972) suggested that a complex formed from this protein together with the ammonium ion and α-ketoglutarate might also act as an effector of GDHCR in wild-type cells.

1.6.4 Factors affecting nitrogen accumulation

a. General aspects

Many factors have been described that affect the extent of assimilation of nitrogen compounds from a mixture. These include culture conditions, medium composition with regard to types and concentrations of nitrogen and carbon compounds, and yeast strain.

b. pH

Depending on membrane permeability, a passive influx of protons into the cell will inevitably occur. As the exogenous concentration of protons becomes higher with lower pH, a greater proportion of ATPase activity must be devoted to maintaining the near-neutral cytosolic pH. In addition, increasing protonation of substrates and the cell surface will increase electrostatic repulsion between the two. Consequently, active transport systems will suffer at lower pH, a fact attested to by reports in the literature of the pH optima of several permeases generally being well above the pH of grape juice (Greasham and Moat, 1973; Dubois and Grenson, 1979; Gregory *et al.*, 1982; Horák and Rihová, 1982; Ballarin-Denti *et al.*,

1984). There is no information available regarding the influence of pH on the selective uptake of amino acids.

c. Ethanol toxicity

Yeast tolerance to ethanol is a complex phenomena, the fundamentals of which are discussed by Jones (1989) and van Uden (1989) and the oenological significance reviewed by Larue and Lafon-Lafourcade (1989). Ethanol has long been recognised to be toxic to a range of microorganisms including *Sacch. cerevisiae*, but the latter organism is distinguished by both tolerating and producing a high concentration of ethanol. Cell growth is generally observed to be more sensitive than fermentation to ethanol toxicity. Only the influence of ethanol on the ability of yeast to accumulate nutrients will be briefly considered here.

By increasing membrane permeability to protons (Leão and van Uden, 1984a; Cartwright *et al.*, 1986; Petrov and Okorokov, 1986) and/or inhibiting the proton-pumping membrane ATPases (van Uden, 1989), ethanol dissipates the proton-motive force across the plasma membrane. Active transport mechanisms are, therefore, diminished or rendered inoperative (Thomas and Rose, 1979; Leão and van Uden, 1983, 1984b; Ferreras *et al.*, 1989). Inefficient uptake of amino acids is exacerbated by leakage of accumulated nitrogen compounds due to ethanol-modified permeability of cell membranes (Vas, 1953). Ethanol may also affect active transport by a mechanism involving a reduction in the number of carriers located in the plasma membrane (Ferreras *et al.*, 1989). This interpretation of kinetic data needs to be substantiated. These observations, together with the report that facilitated transport systems for (glucose), fructose, mannose are not significantly deactivated by ethanol (van Uden, 1989) goes some way towards explaining the relatively greater ethanol-sensitivity of yeast growth compared with fermentation. A further effect of ethanol is the inhibition of sterol biosynthesis (Gal'tsova and Liapunova, 1968), the significance of which is discussed below.

d. Temperature

Because carrier-mediated active transport is a temperature dependent process, the rate of accumulation of amino acids is reduced at lower temperatures. In general, the quantitative demand for amino acids has been shown to be similarly affected. Data relating to the influence of temperature on the sequence of amino acid accumulation are scant (Castor and Archer, 1959). Ammonium accumulation, however, seemed to be largely independent of temperature.

e. Carbon dioxide pressure

Carbon dioxide pressure reduces the rate and extent of amino acid absorption from wort (Kumada *et al.*, 1975) or grape juice (Pekur *et al.*, 1981), in the latter by *ca.* 50%. Specifically, rates of valine, leucine and isoleucine absorption from wort were progressively reduced with increasing CO_2 pressure (Knatchbull and Slaughter, 1987). It is noteworthy that the α-keto derivatives of these amino acids are commonly excreted by the yeast cell during fermentation indicating that they are in excess (see below). The ability of yeast to fix CO_2 provides an avenue through which exogenous carbon dioxide can enter carbon metabolism (Slaughter, 1989), possibly impinging on α-keto acid formation. Thus at higher CO_2 pressures, cellular pools of these α-keto acids and hence their corresponding amino acids, may become elevated resulting in the inhibition of the respective transport mechanisms and in turn reduced uptake of exogenous amino acid.

f. Degree of aeration

As previously mentioned, the utilisation of proline is affected by the degree of aeration of the medium (Pekur *et al.*, 1981; Ingledew *et al.*, 1987). In addition, under anaerobic conditions, proline is sometimes observed to be released into the medium, possibly as a result of arginine catabolism (Brandriss and Magasanik, 1980).

g. Membrane lipid composition

Growth of yeast under strictly anaerobic conditions induces an absolute requirement for the membrane structural lipids, sterols and unsaturated fatty acids (Andreason and Stier, 1953, 1954). Molecular oxygen is required for; the induction of mevalonic acid synthesis mediated by 3-hydroxy-3-methylglutaryl-CoA reductase (Kawaguchi, 1970), oxidase activity required to cyclise squalene to yield the ergosterol precursor (Klein, 1955), and for desaturation reactions to form the unsaturated fatty-acyl residues of phospholipids (Aries and Kirsop, 1977). Aerobically propagated yeast may accumulate over 2% of its dry mass as sterols. During anaerobic growth, these aerobically-synthesised membrane lipids become greatly diluted through cell division (Haukeli and Lie, 1976; Aries and Kirsop, 1977). Growth is limited to several generations, unless small amounts are supplied exogenously (David and Kirsop, 1973). Around 0.02% yeast dry mass appears to be growth-limiting for wine yeasts (Strydom *et al.*, 1982). It has been suggested that changes in membrane composition under fermentative growth, especially in the presence of ethanol, reduce the efficiency of transport systems (Beavan *et al.*, 1984), and cell survival is compromised (Lafon, 1978; Lafon-Lafourcade, 1986).

The observation that anaerobically grown yeasts can satisfy their growth requirement for sterols by a range of structurally distinct sterol species (Proudlock *et al.*, 1968), has been taken to indicate that sterols largely fulfill a non-specific structural role in the membrane of yeast (Nes, 1973; Rattray *et al.*, 1975). Consequently, yeast membrane ergosterol may be comparable to cholesterol in mammalian cell membranes whose presence influences phospholipid/protein interactions and in turn, the activity of membrane-bound enzymes such as permeases (Papahadgopoulos *et al.*, 1973). One of the few investigations into the relationship between plasma membrane sterol composition and the uptake of nitrogenous compounds (FAN) found a positive correlation (Haukeli and Lie, 1976). A more widely accepted explanation (Proudlock *et al.*, 1969) proposes that sterols merely stabilise membrane structure, thereby maintaining high

permeability integrity. Analogous studies of mammalian membranes support this (Papahadgopoulos *et al.*, 1973).

Extensive reviews of the role of phospholipid fatty-acyl residues in membrane structure and function have been prepared (Rattray *et al.*, 1975; Prasad and Rose, 1986). As for sterols, the growth requirement for fatty acids by yeasts appears fairly non-specific (Light *et al.*, 1962). However, at the level of transport efficiencies, the degree of unsaturation of the fatty-acyl residues, an oxygen dependent process, is important. By using the rate of L-alanine accumulation as a measure of GAP activity, Calderbank and coworkers (1984) observed that anaerobically grown yeasts more rapidly acquired GAP activity when enriched in the doubly unsaturated linoleyl rather than the singly unsaturated oleyl fatty-acyl residues. Similarly, enrichment with linoleyl residues lead to elevated rates of L-arginine (Keenan and Rose, 1979) and peptide (Calderbank *et al.*, 1985) accumulation, and also offered some protection against the effects of ethanol (Thomas and Rose, 1979). The influences on transport were attributed to changes in membrane fluidity in the vicinity of permease proteins.

Whether all transport systems are equally affected by changes in membrane lipid composition is not known. Nonetheless, the importance of aerobic starter-culture propagation for ensuring optimal nitrogen accumulation and retention will be evident.

h. Strain of yeast

The precedent set by the degree of strain differences with respect to physiological and oenological attributes, predicts a similar diversity for nitrogen transport characteristics. This area has not been investigated to date. Given that these differences exist, they will be manifested as strain differences with regard to the quantitative and qualitative demands for nitrogen.

i. Wild yeast growth

The smaller degradation products of large peptides and proteins can be assimilated by yeast. As already mentioned, the growth of some non-*Saccharomyces* species in the fermentation can result in the elaboration of proteolytic activity (Nelson and Young, 1986) which may bring about such degradation. The inoculated yeast may, therefore, benefit by direct utilisation of the released assimilable nitrogen. Alternatively, should this fail to occur, a second model takes into account the generally lower ethanol-tolerance of wild yeast populations. It is proposed the products of proteolysis may be released back into the medium given a suitable rate and extent of autolysis of wild yeast populations, which die off early in fermentation (Heard and Fleet, 1985), with increasing concentrations of ethanol.

1.6.5 Catabolism

a. General aspects

The accumulation of several amino acids is solely mediated by permeases, such as the GAP, which are not necessarily active through all stages of fermentation (Grenson *et al.*, 1970; Grenson and Hou, 1972; Courchesne and Magasanik, 1983). This fact, together with the existence of a specific order of amino acid uptake, makes it apparent that the full complement of amino acids required, mainly for protein synthesis will not always be available from exogenous sources. The endogenous biosynthesis of the deficient amino acids will, therefore, be necessary despite the availability of exogenous supplies. Jones, Pragnell and Pierce (1969) confirmed this by tracing the fate of ^{15}N-labelled amino acids following their absorption from a wort containing all amino acids. Label was found to be randomly distributed throughout all cellular amino acids. Furthermore, the continued synthesis of amino acids, despite their accumulation from the medium in amounts exceeding metabolic requirements, was also reported. Thus, of the three possible fates of an accumulated nitrogen compound,

that is, direct incorporation, use as a nitrogen source or use as a carbon source, the second option, degradation of the compound and use of the nitrogen component as a biosynthetic precursor, is the more important outcome.

Labelling of the carbon skeletons of amino acids with ^{14}C revealed little interconversion between amino acids, the majority of carbon skeletons found in yeast proteins being derived either from the exogenous amino acid or from α-keto acids arising from sugar metabolism (Jones *et al.*, 1969). As a consequence of the contribution of carbon skeletons from sugar metabolism, a considerable proportion of the deaminated derivatives of accumulated amino acids are excreted into the medium. In contrast, during nitrogen starvation, the carbon skeletons of only hydrophobic amino acids are excreted, whereas those of basic amino acids are retained (Woodward and Cirillo, 1977). Decarboxylation and an NADH-linked reduction of the deaminated derivatives of amino acids prior to their excretion, is an important route for the formation of the flavour active higher alcohols (Sentheshanmuganathan, 1960; reviewed by Äyräpää, 1971 and Nykänen, 1986), as will be discussed.

The recent review by Large (1986) conveniently summarises the nitrogen catabolic pathways of yeast, but also serves to highlight the range of compounds that can be utilised by this organism. Essentially all nitrogen compounds that are degraded, yield one of two endproducts, ammonium or glutamate. Through the action of NADP-dependent glutamate dehydrogenase (NADP-GDH), catalysing the formation of glutamate from ammonium and α-ketoglutarate, and NAD-dependent glutamate dehydrogenase (NAD-GDH) catalysing the reverse reaction, these end-products of nitrogen catabolism are interconverted (Holzer and Schneider, 1957; Heirholzer and Holzer, 1963). An alternative pathway has been reported in *Saccharomyces cerevisiae* (Roon *et al.*, 1974), *Saccharomycodes ludwigii* and *Schizosaccharomyces* spp. (Johnson and Brown, 1974). Based on the low activities of the enzymes, glutamate synthase and glutamine synthetase, the system is considered to fulfill an auxiliary role.

b. Relative efficiencies of nitrogen sources

A good nitrogen source is one which is (1) rapidly and economically transported; (2) able to be readily degraded in as few steps as possible to ammonium and/or glutamate; and (3) one which is without toxic effects on the cell (Cooper, 1982a). The wide use of ammonium in industrial fermentations is a reflection of not only its cost effectiveness but also its metabolic efficiency. Determinations of growth rate (μ) in a chemically defined laboratory medium have identified ammonium, glutamine, arginine, glutamate and asparagine (μ = 0.37 - 0.47 h^{-1}) as good sources of nitrogen, whereas, glycine, threonine, isoleucine, valine and tryptophan (μ = 0.08 - 0.12 h^{-1}) are poorer ones (Watson, 1976). Cooper (1982a) reports similar findings for five laboratory strains of *Sacch. cerevisiae* as summarised in Table 1.7.

Clearly, ammonium and glutamate are good nitrogen sources because they are able to directly enter the intracellular pool of biosynthetic precursors. The ability of arginine to support high growth rates presumably stems from the fact that it is rich in nitrogen and requires the symport of only one proton, making it economic to transport. The oenological importance of arginine is made evident by observations which show this amino acid often to be the most predominant in Australian juices (Table 1.3). Nevertheless, the degradation of arginine, which proceeds via proline formation, must be energetically taxing, a fact likely to contribute to its delayed and reduced utilisation from ammonium supplemented fermentations (Monk *et al.*, 1987).

Typically, the 'preferred' amino acids, that is those largely taken up early in the fermentation, supported high growth rates when supplied as sole nitrogen sources. It is, therefore, proposed that the sequence of amino acid accumulation and the extent to which individual amino acids contribute to total nitrogen requirements is a function of the closeness with which amino acids match the criteria for a good nitrogen source.

Table 1.7 Mean doubling times for the growth of five laboratory strains of yeast on various compounds as sole source of nitrogen.

Nitrogen compound	Doubling time (min)
Glutamine	137
Ammonia	142
Asparagine	146
Urea	158
Glutamate	159
Serine	163
Arginine	169
Aspartate	170
Alanine	187
Phenylalanine	190
Ornithine	203
Citrulline	208
Allantoin	208
Valine	225
Leucine	257
Proline	260
Tyrosine	265
Isoleucine	280
Threonine[1]	298
Tryptophan	298
Allantoate	322
Methionine[a]	349
Histidine[2]	475
Glycine[3b]	608
Lysine	no growth
Cysteine	no growth

Values were calculated from the data of Cooper (1982a). Organisms were cultured in Wickerham's medium. Superscripted numbers indicate the number of yeasts which failed to grow on the respective nitrogen compound.

[a] Mean of four strains only.
[b] No further growth seen.

It must be noted that although studies involving compounds as sole sources of nitrogen have helped to clarify the roles of individual amino acids in yeast metabolism, in nature and in the alcoholic beverage industries, single sources of nitrogen are not normally encountered. Discrepancies will, therefore, arise when comparing relative efficiencies of amino acids when present alone or as part of a mixture with other sources of nitrogen. Mixtures of amino acids generally support faster rates of growth and fermentation than do single compounds. The functioning of a number of transport systems, increases the rate and diversity of nitrogen accumulation and reduces the need for amino acid catabolism for biosynthesis of deficient compounds.

1.6.6 Integration and regulation

a. Compartmentation

In the ever-changing environment of the wine fermentation, the yeast cell is continually forced to select between nitrogen sources of varying efficiency. While nitrogen catabolite repression offers a means of suppressing the transport, degradation and hence, utilisation of poorer nitrogen sources in the presence of superior ones, this process is a long-term one. Only the *de novo* synthesis of enzymes is controlled. Existing activities are unaffected by nitrogen catabolite repression, their activity only being dissipated according to enzyme half-life or dilution through cell division. In addition, no amount of enzyme regulation will enable the degradation of a nitrogen compound to occur simultaneously with its storage or synthesis in the cytosol. Thus the most potent method of partially or completely preventing the degradation of a substrate by an induced enzyme is to restrict the supply of substrate. This can be achieved either by regulating the flow of substrate from preceeding enzymic steps of a pathway or alternatively, at the level of transport, by limiting access of the compound to the enzyme environment. The latter case is achieved by extracellular/intracellular compartmentation.

The selective degradation and assimilation of nitrogen compounds is accomplished through their selective accumulation. The availability of good nitrogen sources rapidly inhibits the transport systems for poorer sources (Grenson and Hou, 1972; Roon *et al.*, 1975a; Courchesne and Magasanik, 1983). The failure of substrate to enter the cell will not only prevent its degradation but also the needless induction of further enzymic activity. Proline oxidase and ornithine transaminase are almost completely regulated through inducer/substrate exclusion (Brandriss and Magasanik, 1979; Deschamps *et al.*, 1979).

The amino acid/H^+ antiport of 10 amino acids into the vacuole is mediated by at least seven transport systems (Sato *et al.*, 1984). Sequestration of amino acids into the vacuole enables these compounds to be accumulated in excess of immediate metabolic requirements and also affords these reserves protection from the degradative machinery which is induced as a result of their initial accumulation into the cytosol. A similar subcellular partitioning of metabolic processes is reported for arginine degradation. Catabolism of this amino acid ordinarily proceeds via the formation of proline which in turn is catabolised to yield glutamate (Brandriss and Magasanik, 1980). The isolation of the synthetic pathway in the cytosol and the degradative pathway in the mitochondrion, circumvents the potential for futile cycling and results in the nett catabolism of arginine (see review by Cooper, 1982a).

b. Homeostasis

The immediate metabolic demands for nitrogen are catered for by the cytoplasmic nitrogen pool, central to which is the glutamate and ammonium/α-ketoglutarate interconversion. Fulfilling the requirement for these biosynthetic precursors remains a principal concern of the yeast cell under all conditions. Depending upon where medium composition lies between the two extremes of nitrogen availability, there are essentially two ways in which these requirements can be met. During nitrogen excess, accumulation of exogenous assimilable nitrogen via numerous transport systems should not only satisfy metabolic

demands, but also enable the establishment and maintenance of cellular amino acid reserves. Conversely, under conditions of limited nitrogen, mobilisation of these reserves and the induction of degradative enzymes will be the main mechanisms by which the cytoplasmic nitrogen pool is sustained.

Therefore, as the supply of exogenous nitrogen sources begins to diminish, a corresponding decline in cytoplasmic nitrogen levels will lead to a lifting of nitrogen catabolite repression. The induction and reactivation of the high affinity general amino acid permease, along with other ammonia-sensitive permeases (see Table 1.6) and intracellular proteolytic (Betz, 1976; Achstetter and Wolf, 1985) and allantoin- or arginine-degrading enzymes, will result (Whitney and Magasanik, 1973, Bossinger *et al.*, 1974, Sumrada and Cooper, 1978). While some of the permeases or degradative enzymes may already be present, others will need to be synthesised. Vacuolar nitrogen may be mobilised to fuel this *de novo* synthesis associated with the escalation of the 'nitrogen-acquiring' response of the yeast cell. Nitrogen mobilisation is typically pre-empted by an elevation in arginase activity (Whitney and Magasanik, 1973; Sumrada and Cooper, 1978).

A failure of induced transport and catabolic processes to yield sufficient nitrogen from extracellular sources or the intracellular turn-over of proteins will lead to a critical nitrogen limitation within the cell. Under these conditions, the role of the vacuolar nitrogen supply will shift from one of a source of nitrogen for the induced nitrogen-acquiring processes to one of a nitrogen source for the sustenance of metabolism *per se* (see reviews by Cooper, 1982a; Large, 1986).

Differential extraction of cellular amino acid pools has revealed that the proportion of these compounds located within the vacuole can be substantial (Wiemken and Durr, 1974; Messenguy *et al.*, 1980; Kitamoto *et al.*, 1988). In fact, with the exception of glutamate, aspartate and leucine, the proportion of free cellular amino acids sequestered to the vacuolar pool is typically greater than 50%, or of the order of 90% for arginine, histidine, lysine and ornithine. Also

present in the vacuole are the products of adenine and guanine catabolism, allantoin and allantoate (Zacharski and Cooper, 1978) as well as *S*-adenosyl methionine (Schlenk *et al.*, 1970), polyphosphates (Indge, 1968) and several enzymes associated with the degradation or salvage of cellular constituents (reviewed by Achstetter and Wolf, 1985).

The ability of a growing culture of yeast to continue cell division for one or two generations after transfer from a rich medium to distilled water is well known, yet the time period for which intracellular nitrogen pools are able to sustain yeast metabolism upon depletion of exogenous supplies has not been fully characterised. Yeast strain, growth phase, growth conditions and medium composition prior to depletion are likely to be key factors in this regard. Upon depletion of nitrogen from the medium, Bezenger and Navarro (1988) observed a reduction of between 20 and 65% in the nitrogen content of cells. Watson (1976) reported a reduction of approximately 45% in the total cellular nitrogen pool after only 15 minutes incubation in a nitrogen-free medium. Incubation of *Sacch. cerevisiae* X2180-1A cells in nitrogen-free medium leads to a similar reduction of the vacuolar arginine pool after one hour (Kitamoto *et al.*, 1988).

Cellular reserves appear to have a variable ability to obviate metabolic imbalances of oenological importance upon depletion of exogenous nitrogen supplies. Thus while glucose catabolism is sustained for several days following nitrogen starvation though at an ever decreasing rate (Busturia and Lagunas, 1986; Salmon, 1989), the excessive formation of hydrogen sulfide is observed within *ca.* 30-60 minutes after nitrogen depletion (Stratford and Rose, 1985). The relative demands for nitrogen and the turn-over time of the key enzymes and permeases associated with these processes, is suggested to be an important factor in their long-term maintenance.

1.6.7 Intracellular nitrogen deficiencies: possible causes

The development of an intracellular deficiency of nitrogen may be attributable to two factors; must composition and efficiency of yeast nitrogen transport mechanisms. The preceeding discussion of must composition has highlighted the existence of great variability with respect to must assimilable nitrogen content. Hence, yeasts fermenting a low-nitrogen must will rapidly deplete available assimilable nitrogen resulting in a critical depletion of their intracellular nitrogen pools. Alternatively, an intracellular nitrogen deficiency may occur irrespective of the presence of nitrogenous nutrients in the medium. This latter scenario is proposed to arise out of a diminution of nitrogen transport, resulting in an inability of the yeast to accumulate nitrogenous species at a rate sufficient to meet the metabolic demand for these compounds.

These transport disorders have not yet been ascribed causes, however, currently favoured hypotheses implicate many of the factors already discussed with respect to their influence on nitrogen accumulation. Of particular importance is membrane lipid composition and ethanol concentration.

1.7 PROJECT AIMS

The aims of this project are to identify the principal metabolic route(s) of excessive hydrogen sulfide production by wine yeast during the oenological fermentation and to determine the influence of yeast strain, and physical and chemical must parameters on the regulation of this process. The development of a model fermentation system and a suitable quantitative method for hydrogen sulfide will be important criteria for establishing the relative importance of the above parameters. Based on the importance of assimilable nitrogen to this problem that is suggested by previous studies, an initial characterisation of the quantitative and qualitative demands for this nutrient will be undertaken and the specific role of individual nitrogen compounds defined.

Chapter 2

Materials and Methods

2.1 YEAST STRAINS

The yeasts used in this study were the *Saccharomyces cerevisiae* strains listed in Table 2.1. Except for strains 6523, 6842, 7070 and 10278 which were kindly supplied by Dr C. Zambonelli of the Department of Microbiology, University of Bologna, all strains were obtained as freeze-dried cultures from the Australian Wine Research Institute culture collection. Yeast cultures were maintained at 4°C upon slopes of MYPG medium (malt extract, 3 $g.L^{-1}$; yeast extract, 3 $g.L^{-1}$; peptone, 5 $g.L^{-1}$; glucose, 10 $g.L^{-1}$; agar, 20 $g.L^{-1}$) and were subcultured every four months.

2.2 CULTURE MEDIA

2.2.1 Grape juice

The grape juice used in this study was produced from Sultana grapes and was kindly supplied by Lindeman's Wine Company Pty. Ltd., Mildura, Victoria. Grape juice (10 Bé) was clarified by sparging with nitrogen gas ~~nitrogen floatation~~ and pumped over *ca.* 24 hours into fermentation tanks (600 hL) containing *Saccharomyces cerevisiae* strain 77 at a cell density sufficient to yield 5 x 10^6 $cells.mL^{-1}$ in the final tank volume. Samples were collected upon completion of the filling procedure and maintained in an anaerobic atmosphere at 15°C prior to being dispensed into anaerobic fermentation flasks in the laboratory.

2.2.2 Yeast carbon-base medium

Yeast carbon-based medium contained per litre; glucose (125 g), $(NH_4)_2SO_4$ (0.5 g), yeast carbon-base (Difco, 11.7 g), pyruvic acid (200 mg),

Table 2.1 *Saccharomyces cerevisiae* strains used in this study.

Strain number	Synonyms	Description and/or characterisics[a]
59	-	high H_2S producer
64	AWRI 6A; 348	wine yeast, SO_2 resistant
65	AWRI 1A; 350	wine yeast, flocculant, H_2S non-producer
70	AWRI 2A, NCYC 816; 729	wine yeast, low H_2S producer
72	R93, WE 14	wine yeast, high H_2S producer
73	R92, WE 1	wine yeast, high H_2S producer
77	AWRI 3A, R 107; 796	wine yeast, low H_2S and SO_2 producer, K_2 killer
78	AWRI 4A, R 108	wine yeast, K_2 killer
81	AWRI 10A; 835	wine yeast, low H_2S producer, K_2 killer
92F	AWRI 11A, R2, ATCC 46273	wine yeast, K_2 killer
94	AWRI 7A; 833	wine yeast; low SO_2 producer
95	AWRI 8A; 834	wine yeast, K_2 killer
108	-	wine spoilage yeast, high H_2S producer
109	-	wine yeast, high H_2S producer
115	AWRI 5A; 138	flocculant wine yeast, low SO_2 tolerance
6523	-	wine yeast, non-H_2S producer
6842		wine yeast, non-H_2S producer
7070		wine yeast, non-H_2S producer
10278		wine yeast, non-H_2S producer

[a] Abstracted from The Australian Wine Research Institute culture collection records.

Thiamin•HCl (1.12 mg) and trisodium citrate/citric acid buffer (30 mM, pH 4.0). Freshly prepared sodium metabisulfite was added to 50 $mg.L^{-1}$ following sterilisation of the medium by autoclaving at 121°C for 10 min.

2.2.3 Synthetic grape juice medium (SGJM)

Synthetic grape juice medium approximating the composition of typical grape juice contained per litre; glucose (200 g), amino acid stock solution (50 mL), L-malic acid (3 g), potassium hydrogen tartrate (2.5 g), $MgSO_4 \cdot 7H_2O$ (1.23 g), K_2HPO_4 (1.14 g), $CaCl_2 \cdot 2H_2O$ (0.44 g), citric acid (0.2 g), myo-inositol (100 mg), pyridoxine•HCl (2 mg), nicotinic acid (2 mg), calcium pantothenate (1 mg), thiamin•HCl (1 mg), *p*-amino benzoic acid (0.2 mg), riboflavin (0.2 mg), folic acid (0.2 mg), biotin (0.125 mg), $MnCl_2 \cdot 4H_2O$ (198.2 µg), $ZnCl_2$ (135.5 µg), $FeCl_2$ (31.96 µg); $Co(NO_3)_2 \cdot 6H_2O$ (29.1 µg), $NaMoO_4 \cdot 2H_2O$ (24.2 µg), $CuCl_2$ (13.6 µg), KIO_3 (10.8 µg), H_3BO_3 (5.7 µg). The medium was adjusted to pH 3.5 with KOH and filter sterilized through a 0.45 µm membrane.

Amino acid stock solution (20 x) contained per litre; arginine•HCl (18.14 g), glutamate (10 g), proline (10 g), serine (8 g), aspartate (7 g), threonine (7 g), lysine•HCl (6.24 g), leucine (6 g), glutamine (4 g), isoleucine (4 g), valine (4 g), asparagine (3 g), histidine (3 g), methionine (3 g), phenylalanine (3 g), alanine (2 g), tryptophan (2 g), glycine (1 g) and tyrosine (0.4 g). Where indicated in the text, amino acids were substituted for by NH_4Cl.

The synthetic grape juice starter medium used for the propagation of yeast starter cultures, was prepared as for SGJM, however, glucose was present at only 100 $g.L^{-1}$. Following sterilisation, the medium was supplemented with ergosterol and Tween 80 (Sigma) through the addition of 2 mL of a solution prepared by dispersing 100 mg of ergosterol into 5 mL of Tween 80 (Sigma) and diluting to 20 mL with hot ethanol.

2.2.4 Synthetic grape juice agar (SGJA)

Synthetic grape juice agar was prepared as for SGJM with the addition of agar (20 $g.L^{-1}$) and either bismuth sulfite (8 g.L-1) or bismuth citrate (11 $g.L^{-1}$). The medium was not filter sterilised but instead heated only sufficiently to completely dissolve the agar and then dispensed into petri dishes.

2.3 FERMENTATION PROTOCOL

A pinhead amount of yeast maintained on MYPG slopes was seeded into 25 mL of MYPG broth in a 50 mL Erlenmeyer flask loosely fitted with a screwcap lid and incubated overnight with shaking (180 rpm) at 25°C. Organisms were subcultured at 2 x 10^6 $cells.mL^{-1}$ into 25 mL of synthetic grape juice starter medium in 250 mL baffled Erlenmeyer flasks loosely plugged with cotton wool and incubated as above until the culture reached early stationary phase (*ca.* 24 h). The latter culture formed the inoculum for fermentation trials and unless otherwise indicated was used at a cell density of 5 x 10^6 $cells.mL^{-1}$.

Fermentations were conducted in 250 mL Erlenmeyer flasks modified to enable both the trapping of hydrogen sulfide entrained in fermentation/ sparging gases, and the anaerobic sampling of the fermentation or introduction of supplements (Figure 2.1). When monitoring the formation of hydrogen sulfide, fermentations were sparged at a rate of 1 $L.L^{-1}.min^{-1}$ with oxygen-free inert gas by way of a hypodermic needle (18 G) inserted through the sample-port septum. Larger volume fermentations were carried out in similarly modified 2 L round-bottomed flasks and were maintained in a temperature controlled room. Cultures were agitated by way of a magnetic stirrer. Growth was monitored by the absorbance (650 nm, Varian model 635) of samples of the fermentation culture diluted to fall in the range 0.1 to 0.3 absorbance units. Dry weight of the organisms was determined from washed, freeze-dried culture samples or by interpolation from a calibration curve of optical density versus dry weight.

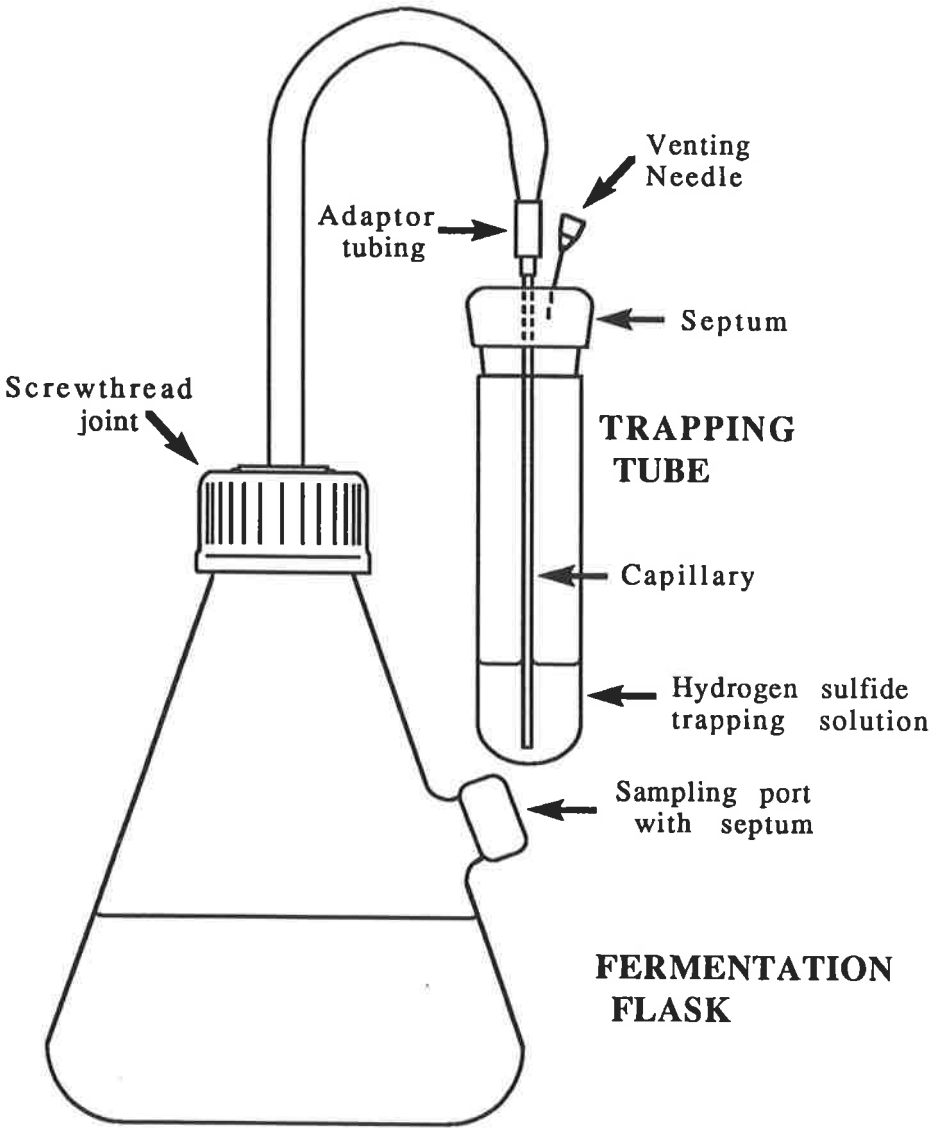


Figure 2.1 Configuration of the 250 mL fermentation flask fitted with a trap for the collection of hydrogen sulfide.

The trap was held in place by way of a clip attached to the neck of the fermentation flask.

Fermentation progress was monitored by refractive index of the cell-free fermentation medium and related to glucose concentration by standard curve. Fermentations were considered 'dry' at glucose concentrations of ≤ 2.5 $g.L^{-1}$ as estimated from reaction with $CuSO_4$/NaOH *Clinitest* tablets (Ames). Residual glucose at refractive indices below 80 was quantified using enzymic test kits (Boehringer) according to the manufacturers instructions.

2.4 ANALYSIS

The disappearance of ammonium ions from the fermentation medium was monitored through the use of enzymic test kits (Boehringer) according to the manufacturers recommendations. An ammonia specific ion-selective electrode (Orion model 9512) enabled rapid determinations of residual ammonia levels (McWilliam and Ough, 1974). Total sulfur dioxide was measured by the method of Rankine and Pocock (1972). Protein quantitation was achieved by a modification of the method of Lowry (Stauffer, 1975).

2.4.1 Hydrogen sulfide

The growth medium was sparged with a stream (1 $L.L^{-1}.min^{-1}$) of oxygen-free inert gas throughout the fermentation. Entrained hydrogen sulfide was precipitated as a sulfide salt by bubbling the gas exiting the fermentation flask through a trapping solution. Traps and capillary tubes were replaced upon the collection of large quantities of sulfide as indicated by the appropriate discolouration of the trapping solution or alternatively, replacement occurred at regular intervals during kinetic studies.

a. Determination by sulfide-specific ion-selective electrode

A sulfide-specific ion-selective electrode (ISE) (model IAB120) and a calomel double-junction reference electrode (model BJ121, Activon Scientific Products Co.) were used together with a model PHM85 precision pH meter (Radiometer). A stock sulfide solution was prepared by dissolving 100 g $Na_2S \cdot 9H_2O$ in 100 mL deionised water and kept in a tightly stoppered flask. A diluted sulfide solution was prepared weekly by diluting the stock solution 1:500 in sulfide anti-oxidant buffer (SAOB) which contained per litre in degassed, deionised water, NaOH (40 g), Na_2•EDTA (33.5 g) and ascorbic acid (17.5 g). All SAOB solutions were stored under anaerobic conditions. Diluted sulfide solution (20 mL) was titrated with 0.1 M lead perchlorate solution (Orion) diluted 1:10 in SAOB, using the ISE for endpoint detection. A standard curve of electrode potential versus sulfide concentration was determined using further dilutions of the sulfide stock in SAOB. Measurements were made at constant temperature (25°C) under a headspace of oxygen-free inert gas using a water-jacketed sample chamber (Figure 2.2).

Hydrogen sulfide, for quantitation by ISE, was collected from fermentations as described in 10 mL aliquots of a trapping solution containing lead acetate (20 $g.L^{-1}$) and ascorbic acid (10 $g.L^{-1}$). For quantitation, sulfide containing traps were attached to a sparging apparatus (Figure 2.3), acidified through the addition of 5 mL of concentrated H_2SO_4, and the liberated hydrogen sulfide carried into 20 mL of SAOB by a stream of oxygen-free nitrogen gas (300 $mL.min^{-1}$ for 10 min). Subsequently, the sample chamber was water-jacketed (25°C), the ISE introduced into the stirred sample and an inert headspace established with nitrogen gas. Readings of electrode potential were taken after an equilibration time of 9 min, during which the next sample was being acidified and sparged in a duplicate sparging device. Hydrogen sulfide concentrations were calculated from a standard curve which was re-determined at 1-2 hourly intervals.

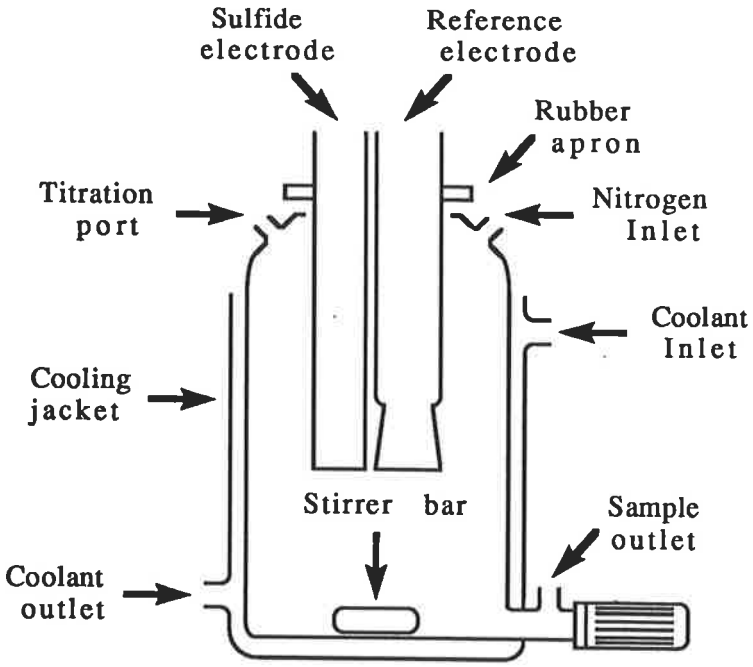


Figure 2.2 Sample chamber used during quantitation by sulfide-specific ion-selective electrode of sulfide-containing samples in sulfide anti-oxidant buffer.

Sample withdrawal and chamber rinsing were facilitated by the application of a vacuum to the sample outlet.

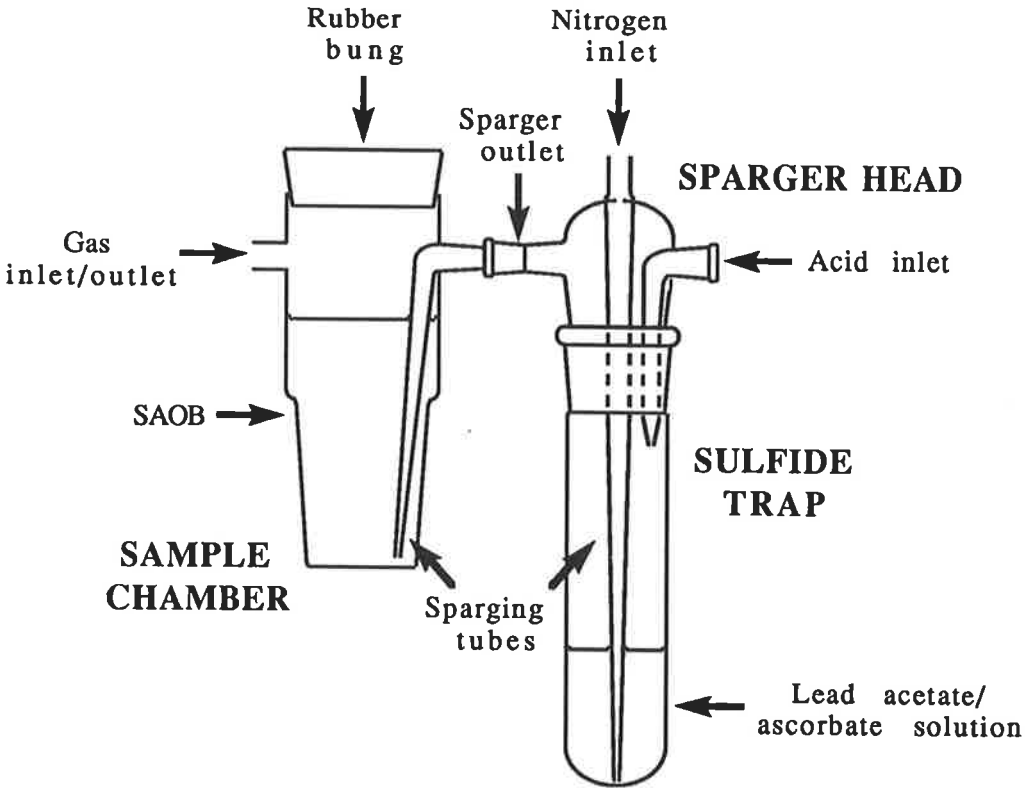


Figure 2.3 Configuration of the sulfide-trap sparging apparatus.

Connections between components were made via ground-glass joints. The syringe for the addition of acid was left in place after acid delivery to seal the acid inlet.

b. Colourimetric determination

Hydrogen sulfide to be quantified colourimetrically was sparged from fermentation flasks and trapped as described in 10 mL aliquots of trapping solution containing $3CdSO_4 \cdot 8H_2O$ (4.3 $g.L^{-1}$) and NaOH (0.6 $g.L^{-1}$). Sulfide-containing traps were protected from light due to the sensitivity of CdS to photooxidation after extended light exposure (Acree *et al.*, 1971).

Samples which showed a strong yellow discolouration were diluted prior to quantitation. A stock amine reagent was prepared by dissolving 12 g of *N,N*-diethyl-p-phenylene diamine•HCl in 80 mL of 11.3 M H_2SO_4 and was stored in the dark at 4°C. A working strength amine reagent was prepared by diluting the stock reagent 1:20 in 9 M H_2SO_4 and adding an $FeCl_3$ solution (60% w/w) at a rate of 25 µL per 300 µL immediately prior use. Undiluted traps or 10 mL aliquots of diluted trapping solution in 30 mL screw-cap bottles were dosed with 325 µL of working strength amine reagent, closed immediately and vigorously shaken for 30 seconds. After incubation for at least 30 minutes at ambient temperature the optical density of samples was measured at their absorption maxima which had been pre-determined for the spectrophotometer in use (672 nm, Beckman DU-64). Sulfide content of samples was calculated from a standard curve prepared by measuring the optical densities of reacted standard samples containing hydrogen sulfide in the range of 0 to 12.1 µg.

2.4.2. Amino acids

Amino acids in SGJM, grape juice fermentation and cell extract samples were quantified by high performance liquid chromatography (HPLC) (Gockowiak and Henschke, unpublished results). The amino acids were derivatised with *o*-phthaldialdehyde, separated on a C18 reverse-phase column and detected fluorometrically.

a. Reagents and buffers

Derivatisation reagent was made by dissolving 25 mg of *o*-phthaldialdehyde in 2 mL of 0.4 M potassium borate buffer (pH 10.4), to which was added 0.5 mL of methanol, 25 µL of *Brij* 35 (30%, Pierce) and 13 µL of 2-mercapto-ethanol. This reagent was stored at 4°C and was diluted 1:3 with 0.4 M potassium borate buffer (pH 10.4) before use. Internal standard solution contained ethanolamine•HCl (172.6 $mg.L^{-1}$) in 0.1 M HCl.

Buffer A contained 25 mM sodium acetate (pH 6.8), 9% methanol, 1% tetrahydrofuran and 0.1 mM EDTA in reagent grade water. Buffer B contained 95% methanol. Both buffers were filtered (0.45 µm), degassed by vacuum sonication and kept under a headspace of helium.

b. Sample preparation

Samples were clarified by centrifugation in a microcentrifuge at 12,000 x g for 3 min. A 400 µL aliquot of the supernatant was transferred to a fresh microcentrifuge tube, diluted with an equal volume of ethanolamine internal standard and 240 µL of 30% sulphosalicylic acid, vortexed and centrifuged as before. The resultant supernatant was diluted with an equal volume of 0.4 M potassium borate buffer (pH 10.4), and filtered through a 0.45 µm membrane into a fresh microcentrifuge tube and stored at 4°C until analysed. External standard was prepared from uninoculated SGJM by the same protocol as above.

c. Chromatographic equipment and conditions

An HPLC consisting of an *ETP-Kortec* K45 gradient controller, K25M pump, K35M pump, K65 autosampler, a *Waters* CX4-2 column heater and 420-AC fluorescence detector and *Delta Chromatography Data System* software (Digital Solutions, Brisbane) run on a *Clarke* PC-88-3 computer, was used. Separation of

amino acid derivatives was effected on an *HPLC Technologies* Techsphere ODS column (4.6 mm x 100 mm) with 3 µm particle size preceded by a *Brownlee* RP18 guard column. The column chamber was kept at 30°C. Buffer flow rates were 1 $mL.min^{-1}$.

Pre-column derivatisation was carried out by mixing 25 µL of diluted derivatisation reagent with 5 µL aliquots of the sample and allowed a reaction time of 1 min before injection of 10 µL of the sample onto the column. Elution of the sample then proceeded according to the gradient program shown in Table 2.2.

2.5 MEASUREMENT OF RATE OF SOLUTE ACCUMULATION

2.5.1 ^{35}S- sulfate

The rate of accumulation of ^{35}S-sulfate by yeast cells was determined by a method based on that described by Stratford (1983). Radioactive tracer (Amersham) (20 $\mu Ci.L^{-1}$, specific activity 4 $\mu Ci.mmol^{-1}$) was added to the fermentation medium before inoculation. Two mL samples of the fermentation were taken via the sample port at regular intervals throughout the fermentation. Cells were rapidly harvested from 1 mL aliquots of the fermentation sample by filtration through a nitrocellulose membrane filter (0.45 µM, 25 mm) and were washed with 12 mL of cold citrate buffer (20 mM, pH 3.5) containing the unlabelled sulfate at the concentration present in synthetic grape juice medium (5 mM). Filters were then placed into 1.5 mL micro-centrifuge tubes and dosed with 1.1 mL of scintillation fluid (ASC II, Amersham). Radioactivity was quantified in a Beckman model 3600 liquid scintillation spectrometer.

The extent of ^{35}S-hydrogen sulfide formation from ^{35}S-sulfate was determined by quantitation of radioactivity collected in cadmium hydroxide sulfide-traps. Fermentations were supplemented as above with ^{35}S-tracer and monitored for hydrogen sulfide production as already described. Before

Table 2.2 Gradient program for the resolution and quantitation of amino acids by the HPLC protocol described in the text.

Time (min)	Buffer B as proportion of Buffer A (percent)
0	3
5	18
25	18
30	47
47	100
55	100
58	3
62	3

colourimetric quantitation of hydrogen sulfide, a sample of trapping solution (250 µL) was withdrawn from sulfide traps and transferred into a 1.5 mL microcentrifuge tube and diluted with 1.1 mL of scintillation fluid (ASC II, Amersham). Radioactivity was quantified as above.

2.5.2 ^{14}C- arginine and methylamine

The rate of accumulation of nitrogen compounds was determined essentially by the method reported by Roon and coworkers (1975b) used for the study of methylamine transport. Cells were harvested, washed with and resuspended in ice-cold 20 mM phosphate buffer (pH 7.0) containing 2% glucose to an absorbance$_{650nm}$ of 1.5. Cells were stored on ice until used. Aliquots (5.4 mL) were introduced into a 50 mL screw capped flask and allowed to equilibrate for 45 min at 30°C with shaking (150 rpm). The experiment was initiated with the addition of either ^{14}C-methylamine or ^{14}C-arginine tracer (0.7 µCi) in 600 µL of phosphate buffer to yield a final specific activity of 0.23 µCi.mmol^{-1}. At regular intervals 1 mL of the cell suspension was removed and the cells quickly collected onto a nitrocellulose membrane filter (0.45 µM, 25 mm) under vacuum and washed with 10 mL ice-cold water containing unlabelled solute at 5 times the concentration of the label. Membranes were counted as described.

2.6 ESTIMATION OF SULFITE REDUCTASE ENZYME ACTIVITY

The method used in this study is an adaptation of that described by Prabhakararao and Nicholas (1969). Culture samples were cooled on ice while a microscopic cell count was carried out. Where possible all subsequent steps were conducted below 2°C. The equivalent of 200 mg dry weight of cells (*ca.* 1 x 10^{10} cells) was collected by centrifugation, washed twice with an equal volume of 0.25 M phosphate buffer (pH 7.3) containing 0.1 mM EGTA and resuspended in 5 mL of the same clarified (0.45 µm membrane) buffer containing glycerol at 20% (w/w). After the addition of 10 g of glass beads (Braun, Ø = 0.45-0.5 mm), cells were

shaken in a cell homogeniser (Braun, model MSK) with CO_2 cooling at 2000 rpm until >99% disruption was achieved (determined by microscopic examination) after *ca.* 5 min. Glass beads were removed by filtration through a glass sinter and cellular debris was removed from the filtrate by centrifugation at 35-50K x g for 30 min. Dialysis was performed for two periods of 75 min, each against 1000 volumes of fresh resuspension buffer. The supernatant was stored on ice before being assayed.

The reaction mixture was prepared in the resuspension buffer and contained glucose-6-phosphate (1.7 mM), $MgCl_2$ (1 mM), Na_2SO_3 (0.1 mM) NADP (0.1 mM; Boehringer) and glucose-6-phosphate dehydrogenase (166 $U.L^{-1}$; Boehringer). For convenience, separate stock solutions of glucose-6-phosphate, $MgCl_2$ and NADP were prepared in resuspension buffer and stored at -20°C or 2°C (NADP). A stock solution of Na_2SO_3 was prepared fresh before use.

For each sample to be assayed, 3 mL of reaction mixture was dispensed into 10 mL disposable tubes in triplicate. Cell-free extract (routinely 400 µL) was added to the tubes and the total volume adjusted to 4 mL with resuspension buffer. Tubes were quickly stoppered with septa (Suba-seal, Nº 29) and gently inverted several times before being incubated at 30°C for 1 h. Working strength amine reagent (300 µL; see above) was immediately injected into the tubes, the contents mixed and incubated at ambient temperature for at least 1 h. Samples were routinely centrifuged at 12,000 x g for 5 min to remove any precipitate which may have formed. The extinction at 672 nm of clarified samples was measured. Blanks and standards were identical to samples in all respects except that cell-free extract were boiled for 3 minutes or substituted for with known amounts of hydrogen sulfide, respectively. Hydrogen sulfide formation was calculated from a standard curve prepared from the latter.

Chapter 3

Methodological development

3.1 INTRODUCTION

As discussed in Chapter 1, the majority of earlier investigations of hydrogen sulfide production by *Saccharomyces cerevisiae* yeasts have been subject to a number of methodological limitations. The oenological emphasis of this study dictates the use of a method capable of accurately and reproducibly quantifying hydrogen sulfide in the nanomole range seen near the sensory threshold of this compound in wine. In addition, as part of an attempt to describe the metabolic basis for hydrogen sulfide production, such a method must enable the collection of kinetic information while minimally impinging on yeast growth and fermentation characteristics. Thus, simple reliance on fermentation gases to entrain hydrogen sulfide from the media into a trapping solution for quantitation, or the need for large-volume aerative sampling of fermentations, is inappropriate.

The practical aspects of the method reported by Stratford (1983) whereby hydrogen sulfide is actively sparged from fermentations, collected as a non-volatile sulfide precipitate and then quantified in isolation from the fermentation medium, made this approach potentially useful in the present study. However, initial evaluation of the associated colourimetric method for hydrogen sulfide quantitation (Gustafsson, 1960) revealed it to be poorly reproducible, particularly at the sulfide concentration range encountered during fermentation of nitrogen-sufficient, sulfate-containing media. An alternative potentiometric protocol was devised which was suitable for use with small sample numbers, while an optimised colourimetric procedure proved more convenient for use with fermentations which generated large numbers of samples, typically high in hydrogen sulfide.

The need to develop a procedure for the quantitation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-linked sulfite reductase activity of cell-free extracts of *Saccharomyces cerevisiae* yeasts was apparent since exisiting protocols proved unreliable for the detection of this activity in the studied strains of yeast.

The experimental rationale behind the modifications made to existing protocols giving rise to the methods detailed in the previous chapter for the determination of both hydrogen sulfide and NADPH-linked sulfite reductase activity under the experimental conditions employed in this study are briefly described.

3.2 RESULTS

3.2.1 Potentiometric quantitation of hydrogen sulfide

Previous workers have utilised the ion-selective electrode for hydrogen sulfide quantitation directly in wines and fermentation media (MacRostie, 1968; Schütz and Kunkee, 1977; Kovac *et al.*, 1987). This approach was found to be unsatisfactory, due to sample instability, and subject to interference, primarily from bubbles of carbon dioxide gas which collected on the electrode membrane surface. Thus in this study, all measurements of hydrogen sulfide were made after its transfer to a sulfide anti-oxidant buffer (SAOB).

a. Ion-selective electrode protocol and sensitivity

The *Activon* sulfide-specific ion-selective electrode, model IAB120, was evaluated for determining hydrogen sulfide evolved in the model fermentation system employed. Experiments with a standard hydrogen sulfide solution showed that electrode potential stabilised with time but was influenced by sample temperature and the ingress of air (Figure 3.1). These sources of error between

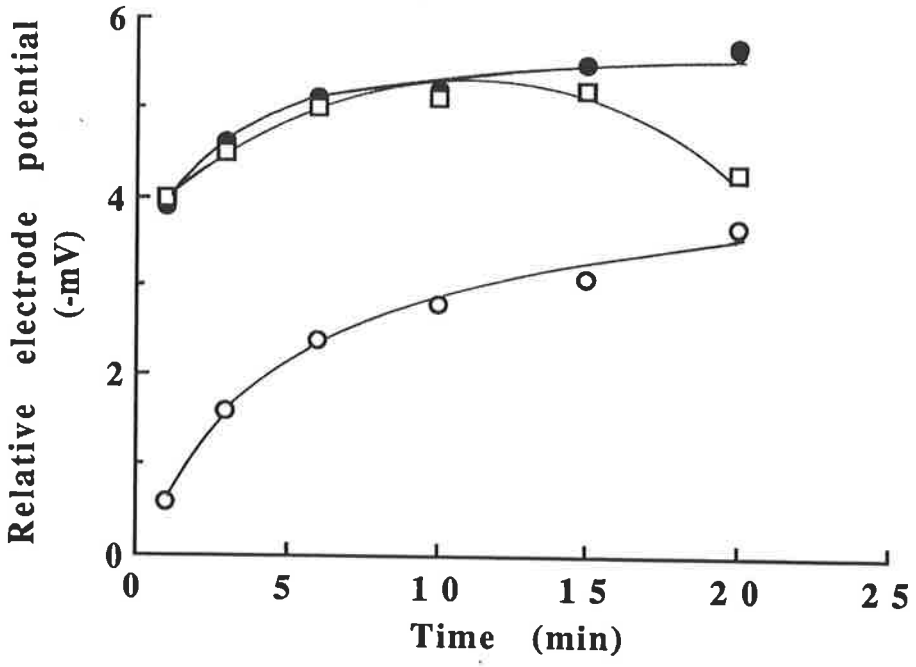


Figure 3.1 The effect of temperature and air exposure upon electrode potential of a standard hydrogen sulfide solution.

Samples of a 3.5 μM solution of hydrogen sulfide in sulfide anti-oxidant buffer were measured potentiometrically while being maintained under oxygen-free inert gas at 21°C (O) and 25°C (●), or 25°C with exposure to air (□).

samples were eliminated by maintaining samples at 25°C in an anaerobic atmosphere through the use of a water-jacketed sample chamber assembly (Figure 2.2) and recording electrode potential after a set equilibration time (9 min).

The sensitivity of the sulfide electrode was sufficient for use in this study (Figure 3.2). The standard error for a minimum of 5 successive readings ranged between ± 0.4 % at a hydrogen sulfide concentration of 380 µM and ± 5.3 % at a hydrogen sulfide concentration of 500 nM.

b. Trapping and recovery of sulfide from lead acetate solution

Mock fermentations (1 L) were dosed with gram amounts of $Na_2S \cdot 9H_2O$, fitted with lead acetate sulfide traps and sparged with inert gas as for the described fermentation protocol. The complete trapping of entrained hydrogen sulfide by the 2% lead acetate trapping solution was qualitatively demonstrated by an absence of blackening of lead acetate impregnated indicator wicks connected to trap outlets. Traps efficiently removed entrained hydrogen sulfide from sparging gases in amounts several orders of magnitude greater than those seen in actual fermentation samples, up to the theoretical trap capacity of *ca.* 528 µmoles.

As detailed elsewhere, hydrogen sulfide present in fermentation gases was trapped as lead sulfide, subsequently liberated by acidification of the trapping solution and sparged into sulfide anti-oxidant buffer to be quantified. Because lead sulfide accumulated on the walls of the trap vessel, maximal recoveries of hydrogen sulfide from lead acetate trapping solution were obtained when the traps were attached directly to the sparger head, enabling *in situ* acidification and sparging (Table 3.1). Sparging of hydrogen sulfide from acidified trapping solution to sulfide anti-oxidant buffer for electrode quantitation required at least 10 min under the described conditions. The above points considered, quantitative trapping and recovery of sulfide from lead acetate

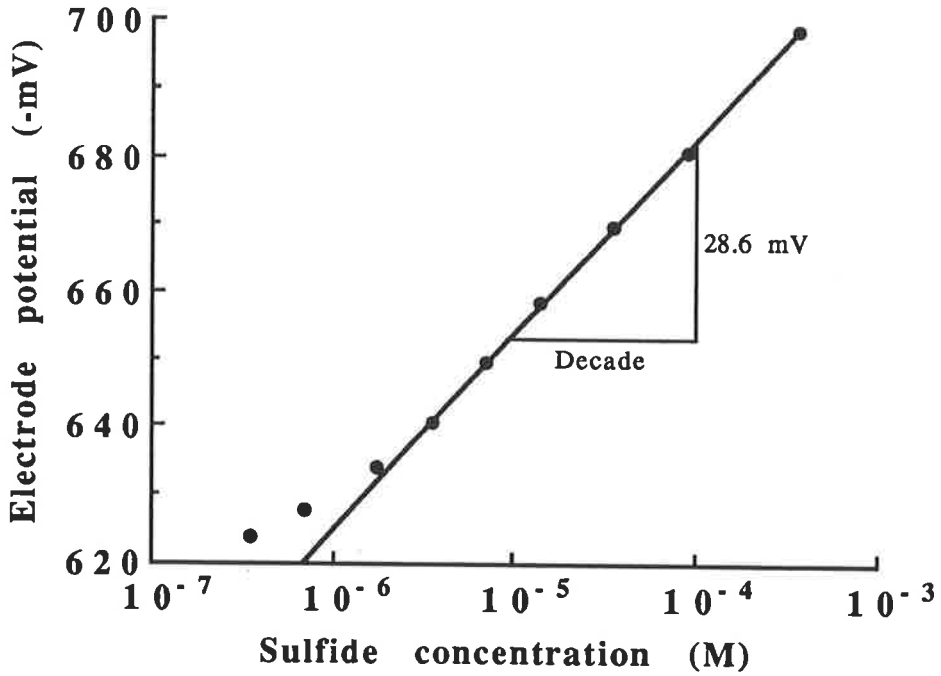


Figure 3.2 Response curve for the *Activon* sulfide-specific ion-selective electrode model IAB120.

Readings of electrode potential are the mean of at least 5 determinations and were taken after an equilibration time of 9 minutes. Samples were maintained at 25°C under oxygen-free inert gas.

Table 3.1 The effect of treatment of lead acetate trapping solution and sparging duration upon recovery of hydrogen sulfide.

Acidified in situ and sparged from trap vessel	Duration of sparging (min)	Relative recovery of hydrogen sulfide (percent)
Yes	10	100.0 ± 1.2^{2}
No	10	87.6 ± 7.5^{2}
Yes	10	100.0 ± 2.1^{7}
Yes	9	87.8 ± 5.2^{7}

Hydrogen sulfide (0.2 nmoles in 100 µL of sulfide anti-oxidant buffer) was added to 10 mL trapping solution, traps were acidified and sparged as described. Recovery of hydrogen sulfide in 20 mL sulfide anti-oxidant buffer was estimated from electrode potential of the solution. Values are the mean of the superscripted number of determinations.

traps was attained for amounts of hydrogen sulfide typically seen in preliminary fermentations trials (Table 3.2).

3.2.2 Colourimetric quantitation of hydrogen sulfide: optimisation of existing protocols

For fermentation trials with a large number of samples, a rapid and reliable, colourimetric method is essential for the quantitation of hydrogen sulfide. As already indicated, a recent adaptation (Stratford, 1983) of Gustafsson's (1960) quantitative method gave poor colour development and relatively large standard errors (*ca.* 8.4 %) at lower levels of hydrogen sulfide (*ca.* 5.9 nmoles). Replacing the *N,N*-dimethyl-p-phenylene diamine reagent with its more sensitive (Rees *et al.*, 1971) diethyl derivative (reaction product absorption maximum: 672 nm) adversely affected colour development but reduced observed standard errors by three-fold (Figure 3.3). Conversely, while similar limitations were seen with a second protocol (Acree *et al.*, 1971) employing the original dimethyl- compound (data not shown), substitution with *N,N*-diethyl-p-phenylene diamine produced the greatest overall colour development and reproducibility between replicates (Figure 3.3). A further benefit of the method of Acree and colleagues (1971) was the use of a cadmium hydroxide rather than zinc acetate-based trapping solution, which largely eliminated interference from sulfite.

3.2.3 Estimation of sulfite reductase enzyme activity

a. Optimal extraction- and assay-buffer composition

The apparently routine detection of sulfite reductase activity in cell-free extracts of *Saccharomyces cerevisiae* cultures (de Vito and Dreyfuss, 1964; Okuda and Uemura, 1965; Yoshimota and Sato, 1968a, 1968b, 1970; Prabhakararoa and Nicholas, 1969, 1970; Cherest *et al.*, 1971; Dott and Trüper, 1976, 1978; Stratford and Rose, 1985) was at first difficult to reproduce in this laboratory. Early efforts focused on the preparation and assay of cell-free extracts in 0.1 to 0.2 M

Table 3.2 Efficiency of hydrogen sulfide recovery from lead acetate trapping solution.

Amount of hydrogen sulfide added (µmoles)		
SAOB	Trapping solution (sparged to SAOB)	Hydrogen sulfide recovered (percent)
0.2	-	100.0 ± 4.3
-	0.2	99.6 ± 3.8
2	-	100.0 ± 5.7
-	2	101.2 ± 3.4

Hydrogen sulfide was added in 100 µL of sulfide anti-oxidant buffer to 10 mL lead actetate trapping solution which was in turn acidified and sparged as described. Recovery of hydrogen sulfide in 20 mL sulfide anti-oxidant buffer was estimated from electrode potential of the solution. Standards representative of complete recovery were treated in an identical manner except the appropriate amounts of hydrogen were added directly to the sulfide anti-oxidant buffer (20 mL final volume) after sparging of a blank lead acetate solution. Values are the mean of at least seven determinations.

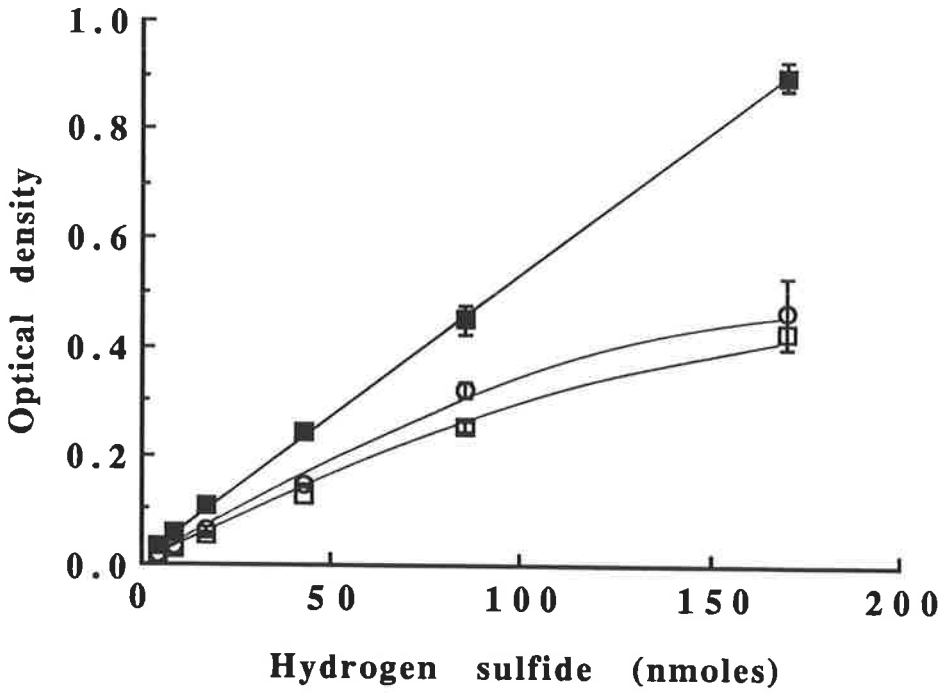


Figure 3.3 The intensity of colour development during the detection of hydrogen sulfide by various quantitative methods.

Hydrogen sulfide was reacted with *N,N*-dimethyl-*p*-phenylene diamine (O) and *N,N*-diethyl-*p*-phenylene diamine (□) by the method of Stratford (1983), or *N,N*-diethyl-*p*-phenylene diamine by the method of Acree *et al.* (1971) (■). Optical densities were determined at 667, 672 and 672 nm respectively. Values are the mean of at least four determinations.

Tris(hydroxymethyl)aminomethane (TRIS) or phosphate buffers since these were more readily acidified to allow efficient colourimetric hydrogen sulfide quantitation. Again, Stratford's (1983) adaptation of the method of Gustafsson (1960) for determining hydrogen sulfide in the sulfite reductase assay, proved inferior to that of Acree and coworkers (1971), modified to utilise the diethyl amine reagent. For example, for the detection of 91 nmoles of hydrogen sulfide in 4 mL of 0.20 M potassium phosphate buffer (pH 7.3), an optical density (672 nm) of only 0.475 ± 0.007 was obtained compared with one of 0.688 ± 0.008 when determined by the latter procedure.

Sodium sulfite concentrations of 0.3 and 1 mM are commonly used in the enzyme assay reaction mixture (Prabhakararao and Nicholas, 1969; Yoshimoto and Sato, 1968; Stratford and Rose, 1985). Colourimetric hydrogen sulfide detection was inhibited at sulfite concentrations approaching 0.3 mM. Consequently, a concentration of 0.1 mM was routinely employed since this provided sufficient precursor for sulfite reductase, which if all was converted to hydrogen sulfide, would exceed the upper limit of the quantifying method.

Nonetheless, no significant sulfite reductase activity was detected in extracts of late-log phase cultures of *Saccharomyces cerevisiae* strain 77 prepared and assayed in buffers of the above phosphate and sulfite concentrations. Despite numerous reports to the contrary (De Vito and Dreyfuss, 1964; Prabhakararoa and Nicholas, 1969, 1970; Stratford and Rose, 1985) it was concluded that this was a result of enzyme inactivation which occurred at the low ionic strength of these phosphate buffers (Naiki, 1965; Yoshimoto and Sato, 1970). Therefore, extraction and reaction mixture buffer concentrations were increased, but only to 0.25 M to limit interference with detection of hydrogen sulfide (Table 3.3). Increases in the addition rate of the acidic working-strength amine reagent at either buffer concentration were of limited value. Although an NADPH-linked sulfite reductase activity could be readily demonstrated in 0.25 M potassium phosphate buffer (pH 7.3), it remained quite labile even with storage of extracts on ice.

Table 3.3 The influence of phosphate buffer concentration and reagent addition rate on colour development during[a] colourimetric quantitation of hydrogen sulfide.

Reagent volume (μL)	Buffer concentration (M)		
	0	0.25	0.30
350	-	85 ± 2	63 ± 2
300	100 ± 4	91 ± 4	61 ± 4
250	-	82 ± 2	40 ± 14
200	-	80 ± 2	19 ± 19

Hydrogen sulfide (9.1 nmoles) was added to 4 mL of water (0 M control) or potassium phosphate buffer (pH 7.3), reacted as described with working-strength amine reagent, and the extinction of samples determined at 672 nm.

[a] Values are expressed as a percentage of the control ± S.E.M and are the mean of at least three determinations.

b. Efficacy of inhibitors of proteolytic activity

Yoshimoto and Sato (1968a) recommended the inclusion of 1 mM (ethylenediaminetetraacetic acid) EDTA in all buffers, though no reason was given. Because of its lower affinity for magnesium ions, a requirement of sulfite reductase, ethyleneglycol-bis-[β-aminoethyl ether] *N,N,N′,N′*-tetraacetic acid (EGTA) rather than EDTA was assessed for its ability to stabilise the NADPH-linked sulfite reductase activity of cell-free extracts of *Saccharomyces cerevisiae*. Preparation and assay of the cell-free extract in 0.25 M phosphate buffers devoid of EGTA yielded low levels of enzyme activity as measured by the formation of hydrogen sulfide. However, inclusion of EGTA to 1 mM in the reaction mixture buffer increased the observed hydrogen sulfide formation by over ten-fold after the introduction of the cell-free extract. A further increase in NADPH-linked sulfite reductase activity was observed where EGTA was added to the 0.25 M phosphate buffer in which cells were disrupted, giving a total increase of the order of 12-fold over extracts prepared and assayed in unsupplemented buffers.

The specific action of EGTA remains unclear. It will be apparent that EGTA is most effective in protecting the NADPH-linked sulfite reductase activity of cell-free extracts when included to 1 mM in the reaction mixture buffer during the 1 hour assay period at 30°C. There are two possible explanations for this effect of EGTA. The chelating agent is inhibiting an interaction between sulfite reductase and extract components, e.g. proteases, which otherwise results in losses of enzyme activity, particularly during extended incubation at the higher assay temperature. Alternatively, EGTA may be acting to minimise an inhibitory/degradative interaction between components of the cell-free extract and those of the reaction mixture used to quantify enzyme activity. To examine the first possibility, cell-free extracts were pre-incubated in the presence or absence of 1 mM EGTA at 30°C for up to 90 minutes and subsequently assayed in the presence of EGTA for residual enzyme activity. As shown in Figure 3.4,

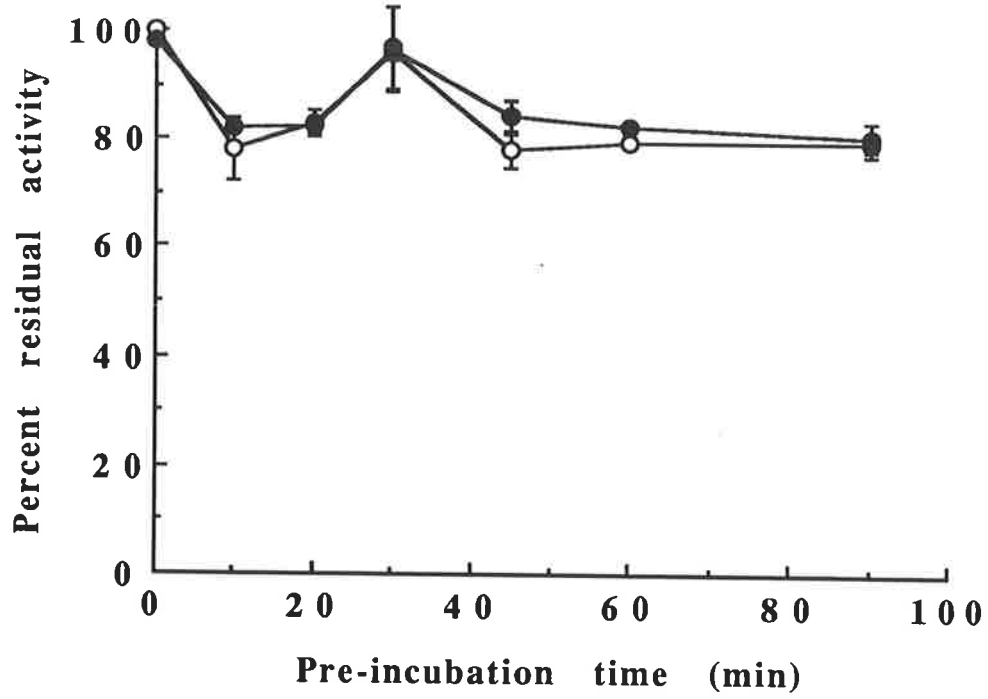


Figure 3.4 Effect of preincubation of cell-free extracts of *Saccharomyces cerevisiae* in the presence or absence of EGTA on the detected NADPH-linked sulfite reductase activity.

Cell-free extracts of 15 h cultures of strain 77 which had become nitrogen-depleted 45 min earlier were prepared as described in 0.25 M potassium phosphate buffer (pH 7.3) with (●) or without (○) the inclusion of EGTA to 1 mM and incubated at 30°C for the prescribed time. Extracts were then assayed for residual NADPH-linked sulfite reductase activity in the described EGTA-containing (1 mM) reaction mixture. Values are the mean of three determinations and are expressed as a percentage of the non-preincubated EGTA free control ± S.E.M.

although total enzyme activity varies, exclusion of EGTA from cell-free extracts during preincubation at 30°C was of little significance. Conversely, exclusion of EGTA from the NADPH-linked sulfite reductase activity assay for a portion of the assay incubation period, markedly reduced enzyme activity (Figure 3.5). Furthermore, loss of enzyme activity was irreversible as shown by the observation that incubation of the 60 minute sample (Figure 3.5; residual activity: 58 ± 0.5%) for a further 60 minute after the addition of EGTA to 1 mM stabilised remaining enzyme activity (residual activity: 62 ± 3.5%) but failed to restore full activity.

It appeared possible that EGTA was optimising NADPH-linked sulfite reductase activity during the assay procedure by preventing inhibition/degradation of glucose-6-phosphate dehydrogenase, the NADPH regenerating enzyme of the sulfite reductase assay. This notion was examined by monitoring the reduction of NADP to NADPH over time at 340 nm. Figure 3.6 clearly demonstrates that NADPH formation is not reduced in the absence of EGTA, in fact the opposite appears to be true.

Despite the use of EGTA or the protease inhibitor, phenylmethylsulfonyl flouride (PMSF), losses of enzyme activity continued to be observed particularly during extended storage of extracts on ice (Table 3.4). NADPH-linked sulfite reductase activity of cell-free extracts proved particularly labile during dialysis of samples. While there appeared to be some merit in the use of PMSF in addition to EGTA during extended storage of extracts at 0°C, this was not the case where extracts were subjected to dialysis at the same temperature. Since dialysis was the only situation under which extracts were incubated for periods of several hours before being assayed, further use of PMSF was abandoned. A cocktail of the serine-protease inhibitors PMSF, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*α-p-tosyl-L-lysine chloromethyl ketone (TLCK) each at concentrations of 100 mM in *N*,*N*-dimethylformamide (DMF) together with trypsin

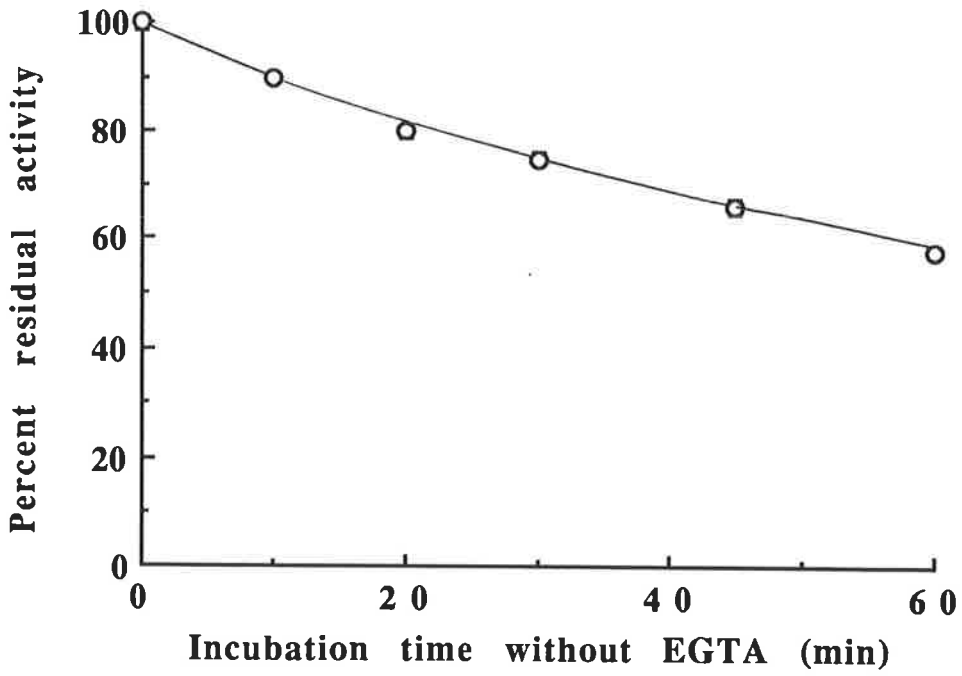


Figure 3.5 Effect on detected NADPH-linked sulfite reductase activity of cell-free extracts of *Saccharomyces cerevisiae* of conducting initial portion of enzyme assay in the absence of EGTA.

Cell-free extracts of 15 h cultures of strain 77 which had become nitrogen-depleted 45 min earlier were prepared as described in 0.25 M potassium phosphate buffer (pH 7.3) devoid of EGTA. Assays of cell-free extracts for NADPH-linked sulfite reductase activity were initiated in the described reaction mixture devoid of EGTA and incubated at 30°C. After the prescribed time, EGTA was added to enzyme assays to 1 mM and incubation of samples continued for the remainder of the 1 h incubation period.

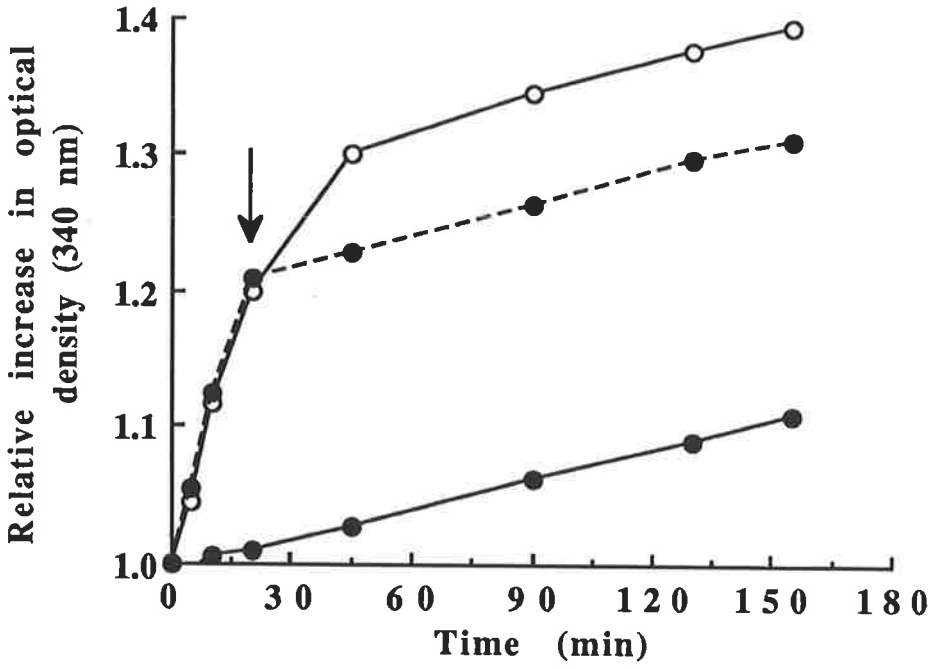


Figure 3.6 Effect of EGTA on the nett formation of NADPH during the assay of NADPH-linked sulfite reductase activity of cell-free extracts of *Saccharomyces cerevisiae* strain 77.

Cell-free extracts of 15 h cultures which had become nitrogen-depleted 45 min earlier were prepared as described in 0.25 M potassium phosphate buffer (pH 7.3) devoid of EGTA. Assays of cell-free extracts for NADPH-linked sulfite reductase activity were conducted in the absence (O) or presence of EGTA at 1 mM present either throughout the assay period (●, solid line) or from the indicated time for addition of EGTA (●, broken curve).

Table 3.4 Effect of protease inhibitors on the stability of the NADPH-linked sulfite reductase activity of cell-free extracts of *Saccharomyces cerevisiae*[a]

Inhibitor[b]	Duration of incubation prior assay (h)	Relative hydrogen sulfide formation[c]
- (control)	1.5	1.00 ± 0.07
EGTA	1.5	1.28 ± 0.02
PMSF	1.5	1.15 ± 0.06
EGTA and PMSF	1.5	1.19 ± 0.03
-	6	0.43 ± 0.01
EGTA	6	0.50 ± 0.01
PMSF	6	0.74 ± 0.00
EGTA and PMSF	6	0.71 ± 0.01
-	6 (dialysis)	0.10 ± 0.00
EGTA	6 (dialysis)	0.25 ± 0.00
PMSF	6 (dialysis)	0.23 ± 0.01
EGTA and PMSF	6 (dialysis)	0.22 ± 0.02

All extracts were assayed for NADPH-linked sulfite reductase activity in reaction mixture prepared as described in 0.25 M potassium phosphate buffer (pH 7.3) containing 1 mM EGTA.

[a] Extracts prepared from 15 h cultures of strain 77 which had become nitrogen-depleted 45 min earlier.

[b] Inhibitor was added to 0.25 M potassium phosphate buffer (pH 7.3) in which washed cells were resuspended and disrupted. EGTA was added to 1 mM; PMSF was added to 0.1 mM.

[c] Values expressed as a proportion of the control ± S.E.M.

inhibitor at 2 $mg.L^{-1}$ diluted 100-fold in cell-free extract, similarly failed to protect enzyme activity.

c. Cold-sensitivity of NADPH-linked sulfite reductase activity

The NADPH-linked sulfite reductase activity of cell-free extracts was confirmed to be cold-sensitive by comparing losses of enzyme activity from extracts incubated at 0°C and 25°C (Figure 3.7). Losses were reduced through the inclusion of glycerol in the extract and assay buffer. Since the complete disruption of cells resuspended in 30% glycerol was greatly delayed, a glycerol content of 20% was routinely used in all buffers for the preparation and assay of cell-free extracts.

d. Optimal storage conditions for whole cells

It was of interest to measure the NADPH-linked sulfite reductase activity of *Saccharomyces cerevisiae* cultures throughout fermentation. As it was not always convenient nor desirable to assay enzyme activity of culture samples immediately, it was therefore necessary to identify the optimal storage conditions for whole cells. A cell free-extract was prepared from a culture of *Saccharomyces cerevisiae* strain 77 and maintained at 0°C for 24 hours before being assayed for NADPH-linked sulfite reductase activity. This sample acted as a control and was arbitrarily taken to represent 100% residual activity. By comparison, cells which had been stored for 24 hours at 0°C in the original culture medium before being processed and assayed, retained only 63 ± 3.0% of control activity. Only 14 ± 0.1% residual activity was detected when the above cells were stored at -20°C. A maximal retention of enzyme activity of 85 ± 9.6% of the control was observed when cells were resuspended in 0.25 M potassium phosphate buffer (pH 7.3) containing 20% glycerol before freezing at -20°C for 24 hours. In general cells were stored for no longer than 12 hours before being processed and assayed.

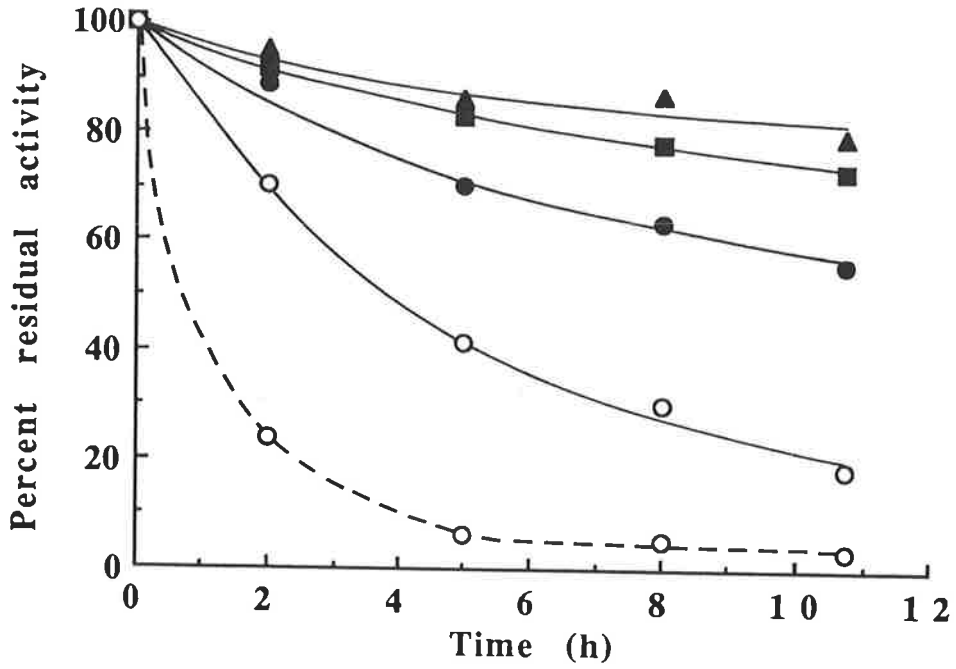


Figure 3.7 Influence of temperature and exposure to various concentrations of glycerol on the retention of NADPH-linked sulfite reductase activity by cell-free extracts of *Saccharomyces cerevisiae*.

Extracts of 15 h cultures of strain 77 which had become nitrogen-depleted 45 min earlier were prepared in 0.25 M potassium phosphate buffer (pH 7.3) containing no glycerol (○), 10 % glycerol (●), 20 % glycerol (■) or 30% glycerol (▲), and were incubated at 0°C (solid curve) or 25°C (dotted curve) for the indicated time before being assayed.

3.3 DISCUSSION

The active removal of hydrogen sulfide from fermentation media by sparging and its concentration in a trapping solution prior to quantitation overcomes several shortcomings of earlier approaches to the study of hydrogen sulfide production. Foremost, total excreted hydrogen sulfide can be collected for quantitation. Unlike determinations of residual hydrogen sulfide in fermentation media, this volatile compound does not escape quantitation by entrainment in evolved carbon dioxide or reaction with medium components, the likelihood of the latter being increased with residence time of hydrogen sulfide in the medium. Furthermore, trapped hydrogen sulfide is stabilised allowing its determination at leisure. Quantitation is carried out independently of potential interference from the fermentation medium and can be performed by several methods. The fact that temporal information can be derived through the frequent replacement of hydrogen sulfide traps is of importance to metabolic studies. Finally, absence of a need for direct sampling of the medium allows anaerobic integrity of the fermentation to be maintained.

A potentiometric and a colourimetric approach to the measurement of hydrogen sulfide has been described. Although the sulfide-specific ion-selective electrode has previously demonstrated its suitability for quantifying hydrogen sulfide directly in fermentation media (MacRostie, 1968; Schütz and Kunkee, 1977; Kovac *et al.*, 1987), the novel protocol described here has several advantages. Apart from those already listed above, hydrogen sulfide can be measured under ideal conditions in a sulfide anti-oxidant buffer, thus optimising electrode performance and simplifying its use. In addition, instrument flexibility is increased by sulfide trapping. This allows the measurement of samples from a range of fermentation media without the suggested need to prepare standard solutions in buffers reflecting the composition of the sample matrix (MacRostie, 1968).

Ideally, hydrogen sulfide which is sparged from fermentations would be collected directly into sulfide anti-oxidant buffer (SAOB). Acidification of SAOB by evolved carbon dioxide erodes the trapping efficiency of this buffer thus necessitating the use of an intermediate trapping solution, namely lead acetate. Efficient trapping and complete recoveries of hydrogen sulfide from this solution have been confirmed. Modifications to a method reported by Acree and coworkers (1971) resulted in a colourimetric method of significantly improved sensitivity and reproducibility suited for the routine determination of hydrogen sulfide in large numbers of samples.

By addressing the inadequacies of earlier protocols (Yoshimoto and Sato, 1968a, 1968b, 1970; Prabhakararoa and Nicholas, 1969, 1970; Stratford and Rose, 1985), a procedure for the determination of NADPH-linked sulfite reductase activity has been developed which can be applied to the *Saccharomyces cerevisiae* yeast strains used in the present study. During method development, at least three modes for the loss of enzyme activity were observed. These demonstrated a dependence on ionic strength, metal ions, and storage temperature. Apparent differences in the susceptibility of enzyme preparations to these factors implicates the possibility of a strain dependence of the phenomena. This suggestion could be verified by a study of the strains used by other researchers.

The irreversible nature of the metal-ion dependent losses of NADPH-linked sulfite reductase enzyme activity indicated that EGTA protects this activity rather than merely optimises it. Losses of enzyme activity do not appear to be a consequence of the deterioration of the NADPH regenerating system of the sulfite reductase assay. Although there exist several intracellular proteolytic enzymes in *Saccharomyces cerevisiae* yeast which require metal ion co-factors (Achstetter and Wolf, 1985) and could be expected to be active in cell-free extracts of this organism, these did not appear to be operating in the absence of the reaction mixture. This inactivational mechanism does not appear to be due to a serine-protease.

It is proposed that while being a requirement of sulfite reductase, magnesium ions present in the assay reaction mixture might also permit the functioning of proteases in the cell-free extract, especially in the absence of EGTA. Confirmation of at least gross proteolytic degradation of sulfite-reductase may be obtained by examination of partially purified preparations of the enzyme which have been incubated with various combinations of EGTA and magnesium ions at 30°C and visualised following separation by polyacrylamide gel electrophoresis.

Interestingly, cold-sensitivity of the NADPH-linked sulfite reductase activity of cell-free extracts of *Saccharomyces cerevisiae* appears to have been described by only one other group (Cherest *et al.*, 1971), despite several independent laboratories having studied this enzyme. This study therefore is the first which has undertaken and succeeded in efforts to ameliorate the instability of this enzymic activity against cold-storage.

It is concluded that the method described in this chapter for the collection and potentiometric or colourimetric quantitation of hydrogen sulfide is suitable for determining the production of hydrogen sulfide in fermentation media. Similarly, extensive modifications of existing methods have produced an improved procedure for the detection of NADPH-linked sulfite reductase activity in yeast extracts.

Chapter 4

Hydrogen sulfide production by *Saccharomyces* yeasts

4.1 INTRODUCTION

Before the biochemical basis for hydrogen sulfide formation had begun to be established, the selection and use of 'low hydrogen sulfide-producing' yeast strains was one of the more effective means of controlling hydrogen sulfide production during the oenological fermentation. Until recent decades, selection was based on empirical observations made in the winery. More contemporary studies have involved screening yeast strains for hydrogen sulfide production on hydrogen sulfide-indicator media under aerobic conditions, or else in simple, defined, liquid media or chemically undefined grape juices (Nickerson, 1953; Rankine, 1963; Zambonelli *et al.*, 1984; Romano *et al.*, 1985; Abad and Gómez, 1987; Thornton and Bunker, 1989). These studies are of limited value in assessing how yeast perform under oenological conditions since the composition of these defined media often bears little resemblance to grape juice and individual undefined grapes juices may produce spurious results. This question was specifically addressed in this study by using a chemically-defined synthetic grape juice medium (SGJM) in either liquid or solid form (1.11 M glucose, 200 $g.L^{-1}$) in addition to the widely used simple, defined BiGGy agar (56 mM glucose, 10 $g.L^{-1}$) and yeast carbon base medium (0.7 M glucose, 125 $g.L^{-1}$).

Studies conducted in SGJM may also be expanded to incorporate a more detailed examination of the factors contributing to hydrogen sulfide production. Demonstration of a statistically significant link between the formation of hydrogen sulfide and a deficiency of assimilable nitrogen during the fermentation of randomly selected grape juices, was only possible by virtue of the

number of samples examined (n = 104; Vos and Gray, 1979). Subsequent experiments involving ammonium supplementation strengthened the suggested interaction between nitrogen and sulfur metabolism. However, little evidence exists to support the proposed proteolytic/cysteine degradative route for hydrogen sulfide formation (Nelson and Young, 1986; Rosi *et al.*, 1987).

Model media investigations would greatly facilitate efforts to identify the mechanism of importance for the production of hydrogen sulfide under oenological conditions. A study of this type has, under laboratory conditions, demonstrated the regulation of hydrogen sulfide formation by ammonium ions, disputed the involvement of proteolysis and implicated sulfite as an important precursor for hydrogen sulfide production (Stratford and Rose, 1985). A comparable investigation conducted under model-oenological conditions is detailed in this chapter.

4.2 RESULTS

4.2.1 Yeast strain screening for hydrogen sulfide production

a. Bismuth-containing indicator media

Nineteen strains of *Saccharomyces cerevisiae* yeast were screened for their propensity to produce hydrogen sulfide on BiGGy and synthetic grape juice agar (SGJA) indicator media. The latter contained either sulfate as sole-sulfur source or both sulfate and sulfite when prepared with bismuth citrate or bismuth sulfite, respectively. The importance of aerobic incubation was examined.

Yeast strains were classified as either high, medium, low or trace producers of hydrogen sulfide based on the intensity of the brown colouring of colonies on indicator media. Reference strains representing the full range of reaction intensities observed for the strains examined here were in descending order, 73, 77, 81 and 6523 (Figure 4.1). Similarly, strains 73, 72, 108 and 6523 acted as reference strains for growth on synthetic grape juice agar (SGJA) containing a bismuth salt as the hydrogen sulfide indicator (Figure 4.2). Screening and classification of yeasts accordingly identified strains 59, 72, 73 and 92F as predominantly high hydrogen sulfide producers whereas strains 6523, 6842, 7070 and 10728 produced trace amounts of hydrogen sulfide (Table 4.1). The extent of hydrogen sulfide production by individual strains during aerobic growth on BiGGy agar was largely unaltered during anaerobic growth. However, strains which produced higher amounts of hydrogen sulfide during aerobic growth on SGJA tended to yield colonies of similar or lighter colour when grown under anaerobic conditions on the same medium. The degree of growth was similar in both cases. Hydrogen sulfide production by yeasts inoculated onto SGJA was little affected by the inclusion of sulfite in addition to sulfate in the medium.

b. Hydrogen sulfide production in nitrogen-sufficient SGJM

Eight *Saccharomyces cerevisiae* strains were examined for their capacity to produce hydrogen sulfide in nitrogen-sufficient liquid media under conditions approximating those seen in oenology (Table 4.2). Except for strain 59 which appeared to be a constitutive producer, all strains produced only trace amounts of hydrogen sulfide under these conditions compared with the range in the extent of hydrogen sulfide production observed on solid media.

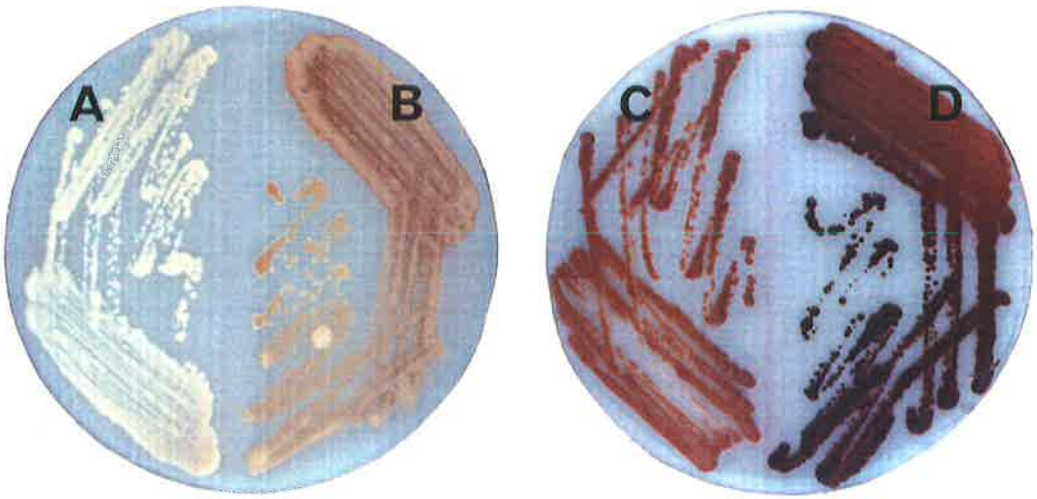


Figure 4.1 Relative reaction intensities of *Saccharomyces cerevisiae* yeast strains during growth on BiGGy indicator agar containing 12 mM bismuth sulfite.

Yeast strains: A, 6523; B, 81; C, 77; D, 73.

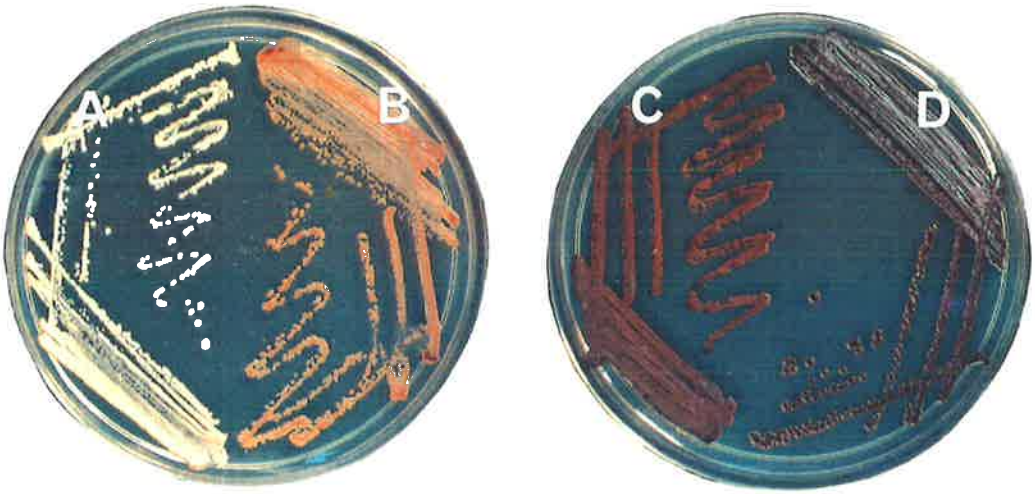


Figure 4.2 Relative reaction intensities of *Saccharomyces cerevisiae* yeast strains during growth on synthetic grape juice agar (SGJA) indicator media containing 12 mM bismuth sulfite.

Yeast strain: A, 6523; B, 108; C, 77; D, 73.

Table 4.1 Hydrogen sulfide production by *Saccharomyces cerevisiae* yeast strains grown on bismuth-containing indicator media under aerobic or anaerobic conditions.

	BiGGy Agar (Bismuth sulfite)		SGJA[a] (Bismuth sulfite)		SGJA[a] (Bismuth citrate)	
Strain	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
59	+++	+++	+++	+	+++	+
64	++	++	+++	+	++	+
65	+	+	+/-	+/-	+	+/-
70	++	++	+/-	+/-	+	+
72	+++	+++	++	++	++	++
73	+++	+++	+++	++	+++	++
77	++	++	+/-	+	++	++
78	+	+	++	++	+++	+
81	+	+	+/-	+/-	+	++
92F	+++	+++	++	+	+++	+/-
94	+	++	++	+	++	++
95	+	+	++	+	+	++
108	+/-	++	+	+	+	++
109	++	+++	++	+	+++	++
115	++	++	+	+	+	++
6523	+/-	+/-	+/-	+/-	+/-	+/-
6842	+/-	+	+	+	+	+
7070	+/-	+/-	+/-	+/-	+	+/-
10278	+/-	+/-	+/-	+/-	+/-	+/-

Strains are classified as high (+++), medium (++), low (+) and trace (+/-) hydrogen sulfide producers upon comparison with reference strains as described in the text.

[a] Synthetic grape juice agar.

Table 4.2 Comparison of strains of *Saccharomyces cerevisiae* for production of hydrogen sulfide under fermentation conditions[a].

Yeast strain	Attained biomass (g dry weight.L^{-1})	Hydrogen sulfide produced	
		µmol.L^{-1}	nmol.g dry weight^{-1}
59	8.9	8.98 ± 3.29	1012 ± 371
65	14.5	0.65 ± 0.23	44 ± 16
70	9.1	1.09 ± 0.38	120 ± 42
72	10.2	0.88 ± 0.18	87 ± 17
73	12.7	0.62 ± 0.18	49 ± 13
77	11.7	0.62 ± 0.06	54 ± 4
81	10.2	0.47 ± 0.09	47 ± 7
92F	10.7	0.53 ± 0.18	51 ± 16

[a] Fermentations were conducted at 25°C in synthetic grape juice medium containing sulfate (5mM) as sole sulfur source and mixed amino acids as nitrogen source.

4.2.2 Hydrogen sulfide production by yeast during fermentation

a. Initial observations in grape juice fermentations

Hydrogen sulfide was produced by *Saccharomyces cerevisiae* strain 77 in two distinct peaks of activity during the fermentation of a Sultana grape juice at 15°C (Figure 4.3). The first peak of hydrogen sulfide evolution developed mid-fermentation following the depletion of assimilable nitrogen from the must. The second peak occurred toward the end of the fermentation, commencing at a residual glucose concentration of approximately 0.1 M. The maintenance of assimilable nitrogen concentrations in the grape juice suppressed the 'mid-fermentation' production of hydrogen sulfide (Figure 4.4). The presence of assimilable nitrogen only partially suppressed the peak of hydrogen sulfide producing activity which developed following complete catabolism of glucose.

The remainder of this chapter will be devoted to findings relating to the nitrogen-responsive formation of hydrogen sulfide. A limited investigation of the causes for the formation of the 'end-of-fermentation' peak of hydrogen sulfide was undertaken and is discussed in detail in Chapter 6.

b. Fermentations in defined media

The formation of hydrogen sulfide by three yeast strains was examined at time points immediately prior to and following the depletion of ammonium ions. Air-sparged fermentations were conducted at 30°C in yeast carbon-base medium. *Saccharomyces cerevisiae* strain 81 produced only trace amounts of hydrogen sulfide over the course of the fermentation (Figure 4.5). However, strains 77 (Figure 4.6) and 72 (Figure 4.7) produced hydrogen sulfide in greater amounts, the maximal rates of production developing shortly after the depletion of

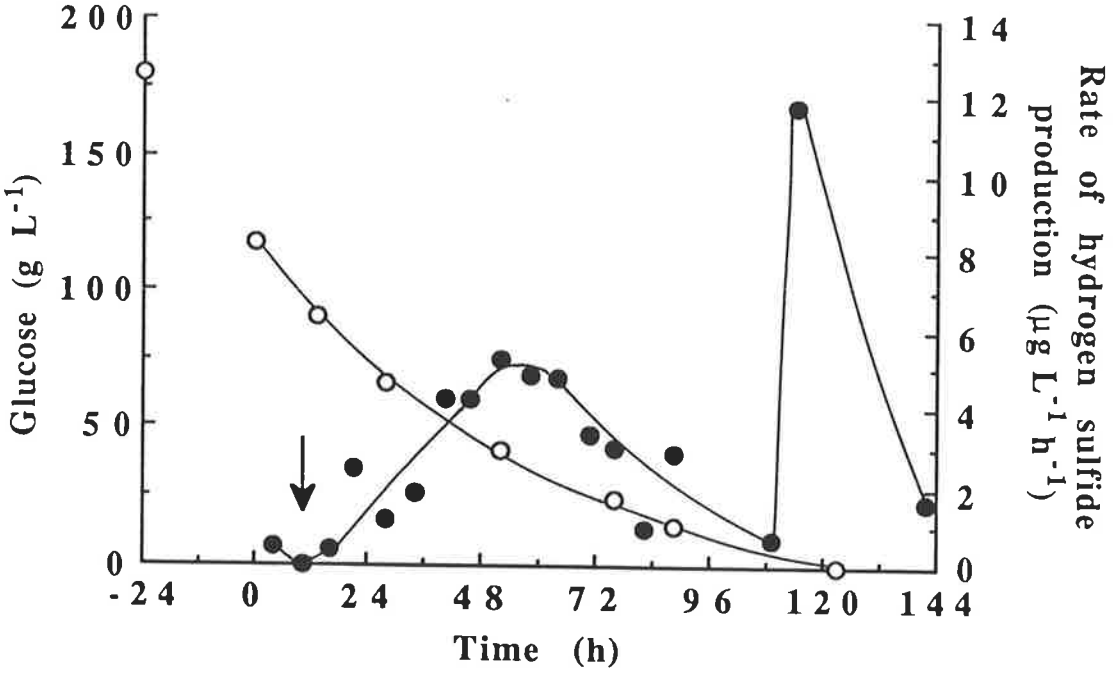


Figure 4.3 Hydrogen sulfide production (●) and glucose concentration (○) during the fermentation of a Sultana grape juice by *Saccharomyces cerevisiae* strain 77 at 15°C.

Grape juice was obtained from a commercial winery approximately 24 h after inoculation.The juice (200 mL) was dispensed into anaerobic fermentation flasks (250 mL) and monitored for hydrogen sulfide production. The point of ammonium ion depletion is indicated with an arrow.

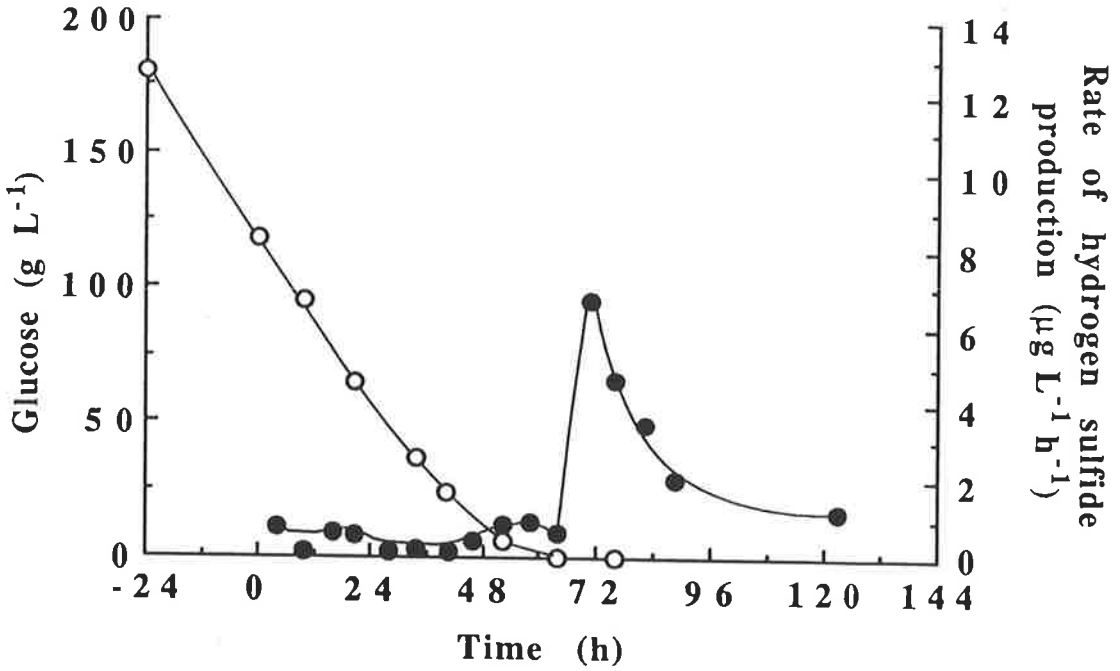


Figure 4.4 Hydrogen sulfide production (●) and glucose concentration (○) during the fermentation of a Sultana grape juice by *Saccharomyces cerevisiae* strain 77 at 15°C.

Grape juice was obtained from a commercial winery approximately 24 h after inoculation. The juice (200 mL) was dispensed into anaerobic fermentation flasks (250 mL) and monitored for hydrogen sulfide production. Assimilable nitrogen concentrations were monitored and maintained throughout the fermentation through the addition of ammonium ions.

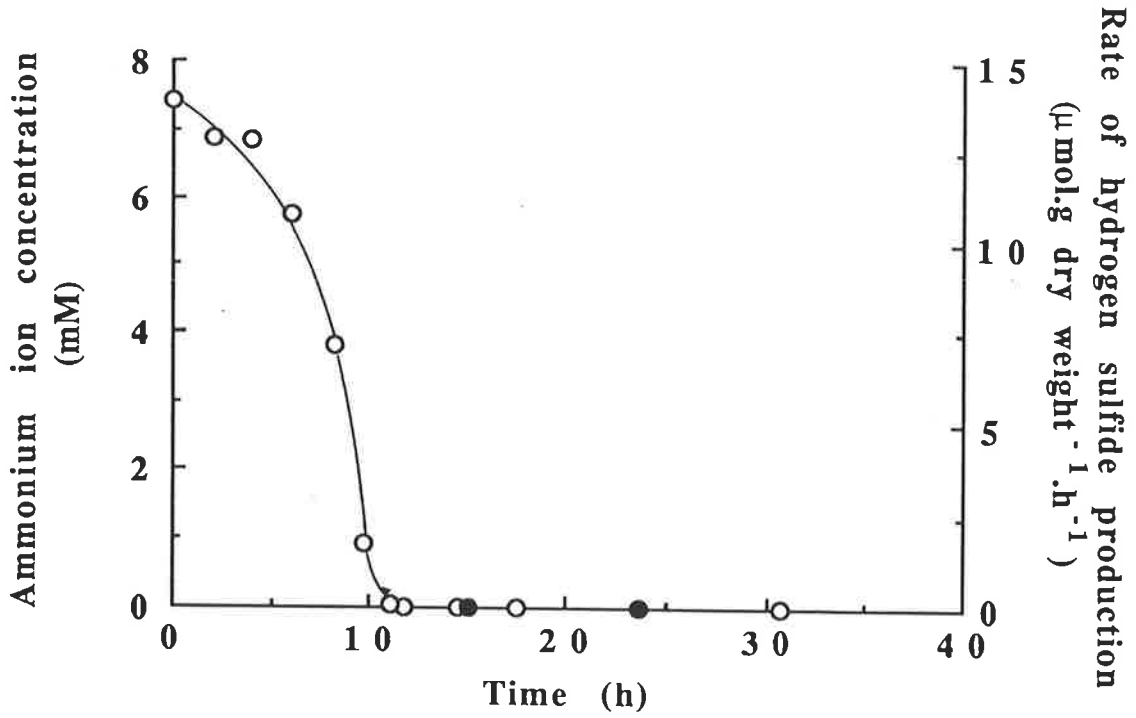


Figure 4.5 Ammonium ion concentration (O) and hydrogen sulfide production (●) by *Saccharomyces cerevisiae* strain 81 grown at 25°C in yeast carbon-base medium containing sodium metabisulfite (263 μM). Hydrogen sulfide was sparged from the fermentation with a stream (1 $L.L^{-1}.min^{-1}$) of air.

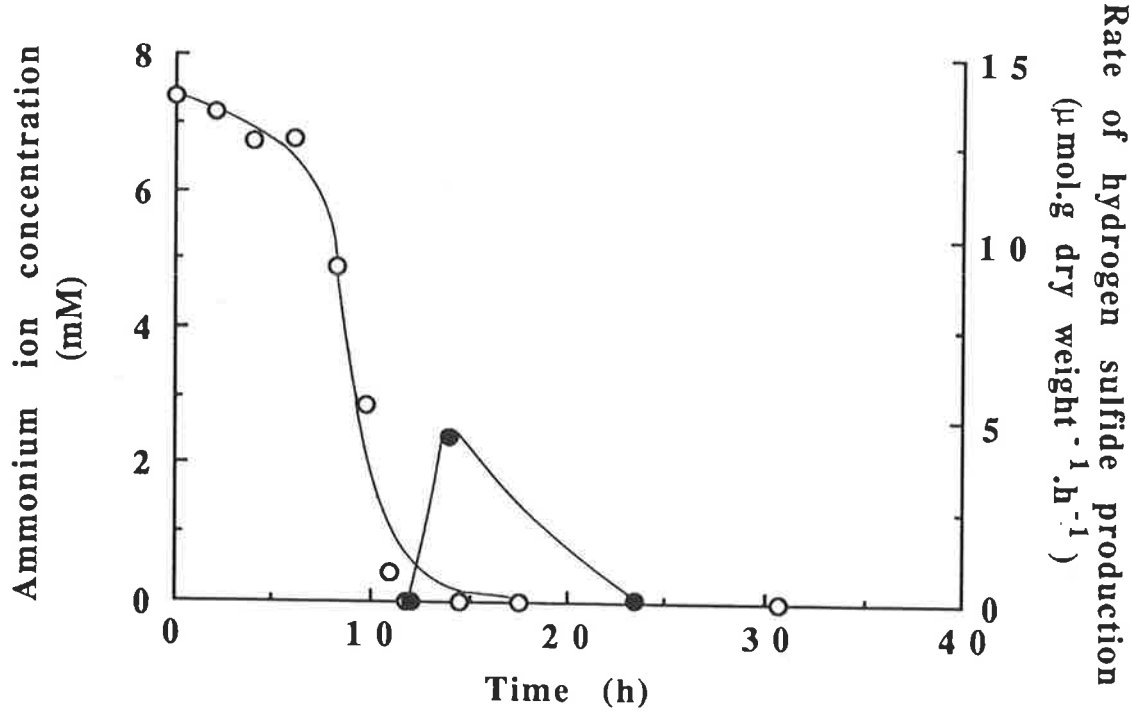


Figure 4.6 Ammonium ion concentration (O) and hydrogen sulfide production (●) by *Saccharomyces cerevisiae* strain 77 grown at 25°C in yeast carbon-base medium containing sodium metabisulfite (263 μM). Hydrogen sulfide was sparged from the fermentation with a stream (1 $L.L^{-1}.min^{-1}$) of air.

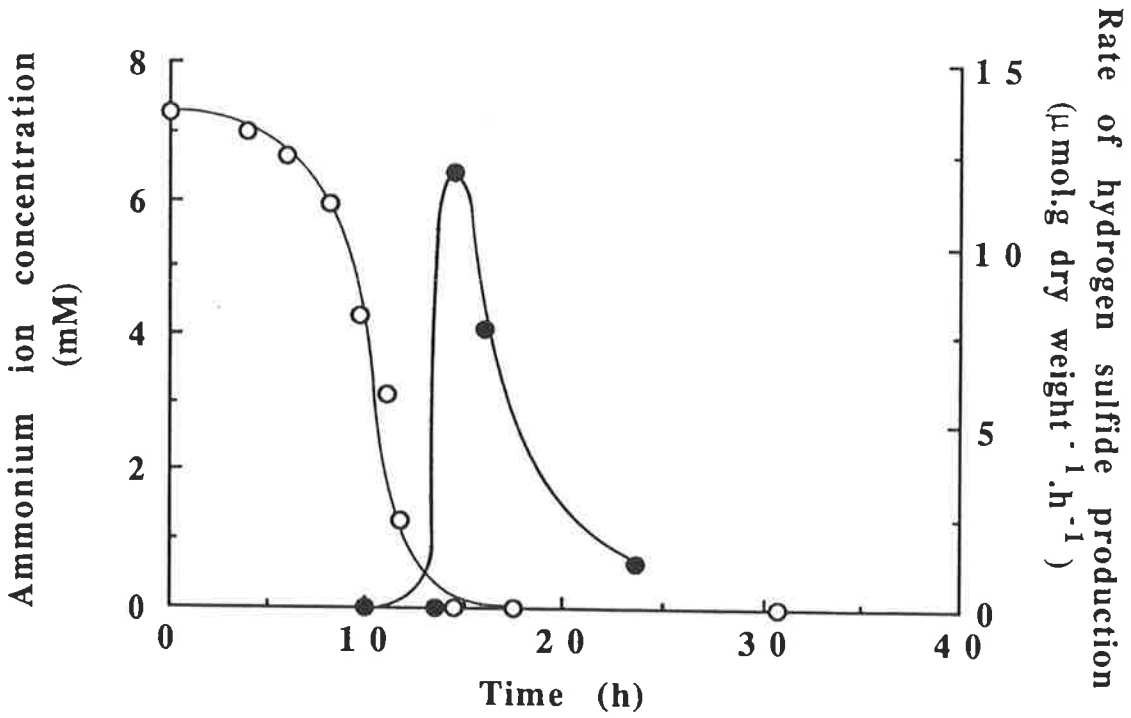


Figure 4.7 Ammonium ion concentration (O) and hydrogen sulfide production (●) by *Saccharomyces cerevisiae* strain 72 grown at 25°C in yeast carbon base medium containing sodium metabisulfite (263 μM). Hydrogen sulfide was sparged from the fermentation with a stream (1 $L.L^{-1}.min^{-1}$) of air.

ammonium ions from the medium. The amounts of hydrogen sulfide formed by strains 81, 77 and 72 over 30 hours were, respectively, trace, 34.2 and 74.5 μmol. L^{-1}. These results agree well with the relative reaction intensities of these strains on indicator agar media.

Sparging of fermentations with oxygen-free nitrogen rather than air reduced the final biomass from 1.65 $g.L^{-1}$ to 0.95 $g.L^{-1}$ for strain 72. A concomitant reduction in the maximum rate of hydrogen sulfide production was observed under these conditions (Figure 4.8). In accord with observations in earlier grape juice fermentations, hydrogen sulfide production was reduced in the presence of supplements of ammonium ions (Figure 4.9).

Differences were evident between strains of *Saccharomyces cerevisiae* with regard to maximal rates and extent of hydrogen sulfide production during growth in synthetic grape juice medium containing limited nitrogen (Table 4.3). The kinetics of hydrogen sulfide production and ammonium ion utilisation from synthetic grape juice media mirrored those seen during growth in yeast carbon-base medium. Specifically, within 30-60 minutes of the depletion of ammonium ions from the medium, a maximal rate of hydrogen sulfide production developed which was, in turn, observed to decline progressively over several hours.

Hydrogen sulfide production by *Saccharomyces cerevisiae* strain 77 demonstrated a growth phase dependence, such that maximum rates and extent of production were observed where depletion of assimilable nitrogen occurred during the exponential phase of growth (Figure 4.10). The NADPH-linked sulfite reductase activity of a culture of strain 77 was similarly observed to peak during the active growth phase (Figure 4.11). Conversely, when induced by a depletion of nitrogen during the stationary phase of the culture, hydrogen sulfide formation

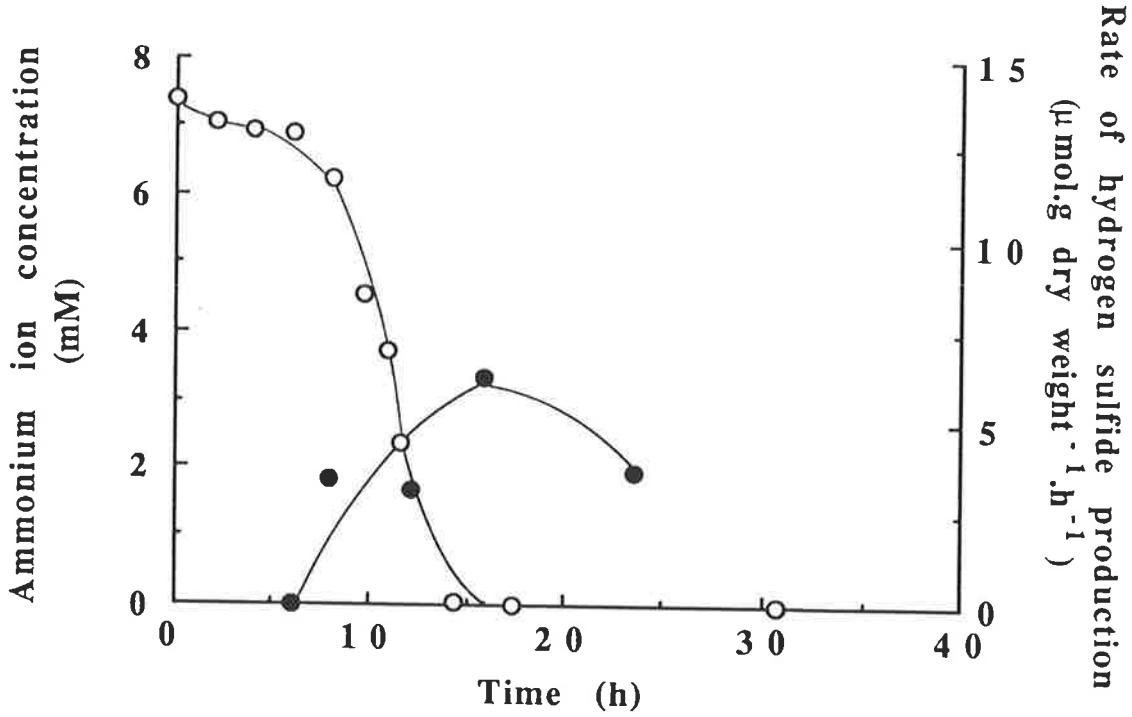


Figure 4.8 Ammonium ion concentration (O) and hydrogen sulfide production (●) by *Saccharomyces cerevisiae* strain 72 grown at 25°C in yeast carbon-base medium containing sodium metabisulfite (263 μM). Hydrogen sulfide was sparged from the fermentation with a stream (1 $L.L^{-1}.min^{-1}$) of high-purity nitrogen.

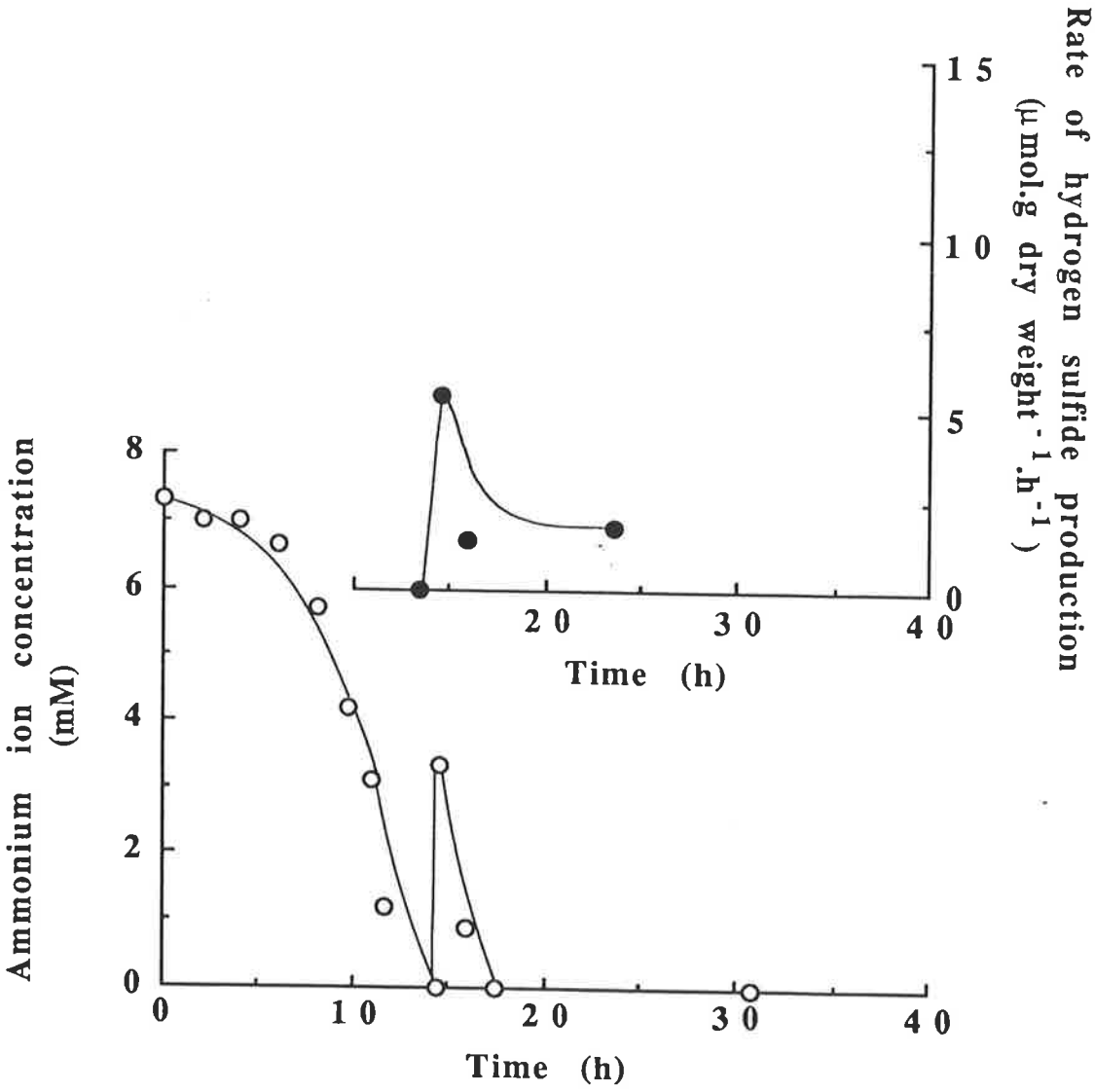


Figure 4.9 Ammonium ion concentration (O) and hydrogen sulfide production (●) by *Saccharomyces cerevisiae* strain 72 grown at 25°C in yeast carbon base medium containing sodium metabisulfite (263 μM). Hydrogen sulfide was sparged from the fermentation with a stream (1 $L.L^{-1}.min^{-1}$) of air. Ammonium ions were added at 14.5 hours to a concentration of 3.33 mM.

Table 4.3 Hydrogen sulfide production by strains of *Saccharomyces cerevisiae* following nitrogen depletion.

Yeast strain	Hours to ammonium depletion[a]	Glucose catabolised (mmoles)	Hydrogen sulfide production	
			Maximum rate (μmol.g dw^{-1}.h^{-1})[b]	Total (μmoles)[c]
65	16.0 ± 1.0	218 ± 13	0.54 ± 0.10	20.7 ± 2.1[1]
72	14.8 ± 0.3	265 ± 32	2.72 ± 1.06	46.3 ± 4.0[2]
77	16.6 ± 0.5	222 ± 18	1.09 ± 0.46	24.8 ± 4.0[12]
81	13.3 ± 0.2	285 ± 24	1.45 ± 0.36	10.8 ± 2.6[34]
6523	14.4 ± 0.1	201 ± 11	0.86 ± 0.30	5.9 ± 0.1[3]
10728	14.3 ± 0.3	215 ± 5	0.47 ± 0.06	3.6 ± 0.4[4]

Saccharomyces cerevisiae yeasts were grown at 25°C in synthetic grape juice medium containing ammonium ions (8.3 mM) and sulfite (263 μM as sodium metabisulfite). Values are the mean of at least four determinations ± S.E.M.

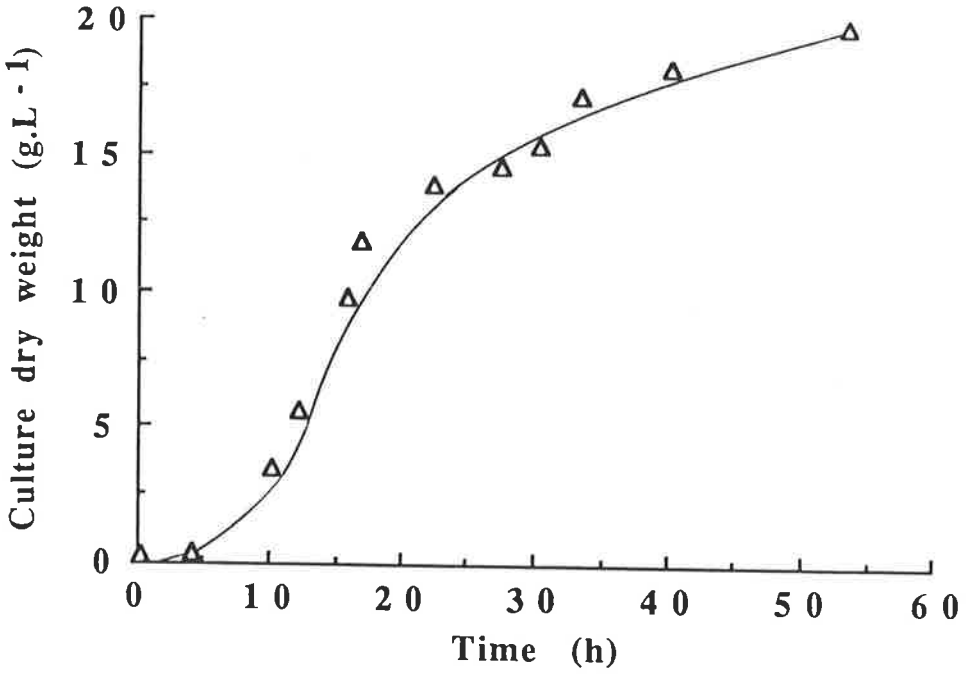
[a] Determined to the nearest 15 min.

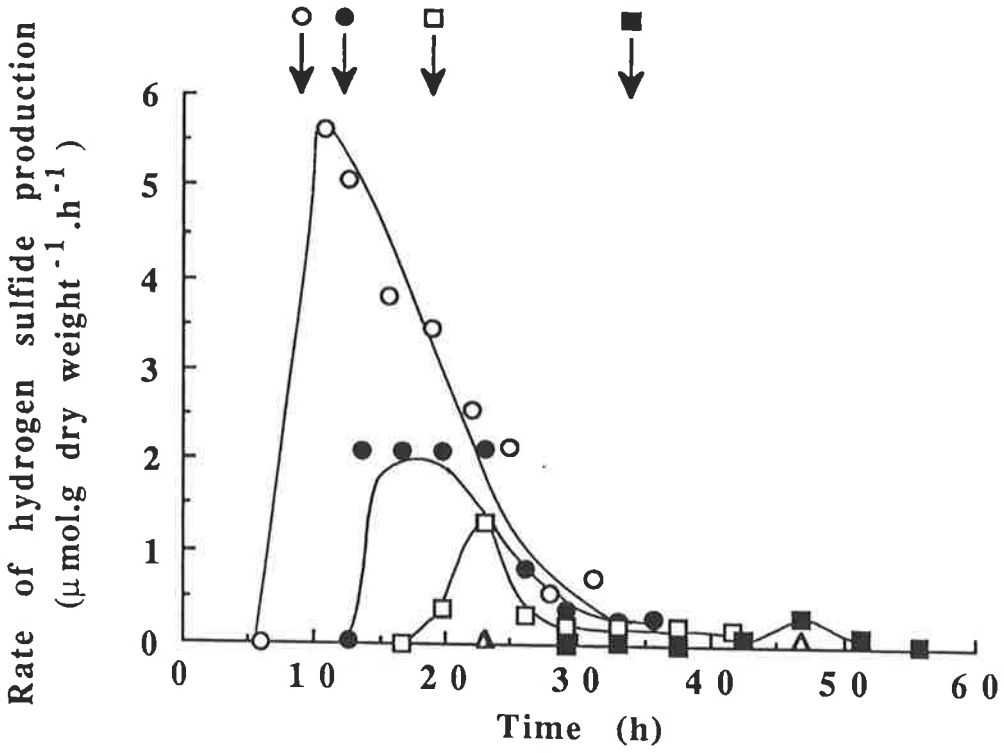
[b] μmoles.g dry weight^{-1}.h^{-1}.

[c] Total hydrogen sulfide produced over 19.5 h after the depletion of ammonium ions from the medium. All values quoted, except those with the same superscript, are significantly different ($p \leq 0.05$).

Figure 4.10 Effect on hydrogen sulfide production of ammonium ion depletion at progressively later stages of the growth of *Saccharomyces cerevisiae* strain 77.

Cultures were grown at 25°C in synthetic grape juice medium which contained initial ammonium ion concentrations of 3.3 (○), 8.3 (●), 16.6 (□), 24.9 (■) and 33.3 (△) mM. Sodium metabisulfite was added to 132 mM at 1 hour prior to the depletion of ammonium ions (labelled arrows) and hydrogen sulfide production was monitored for a further 24 hours.


Culture dry weight (g.L-1)
20
15
10
5
0
0
10
20
30
40
50
60
Time (h)


Rate of hydrogen sulfide production
(μmol.g dry weight-1.h-1)
6
5
4
3
2
1
0
0
10
20
30
40
50
60
Time (h)

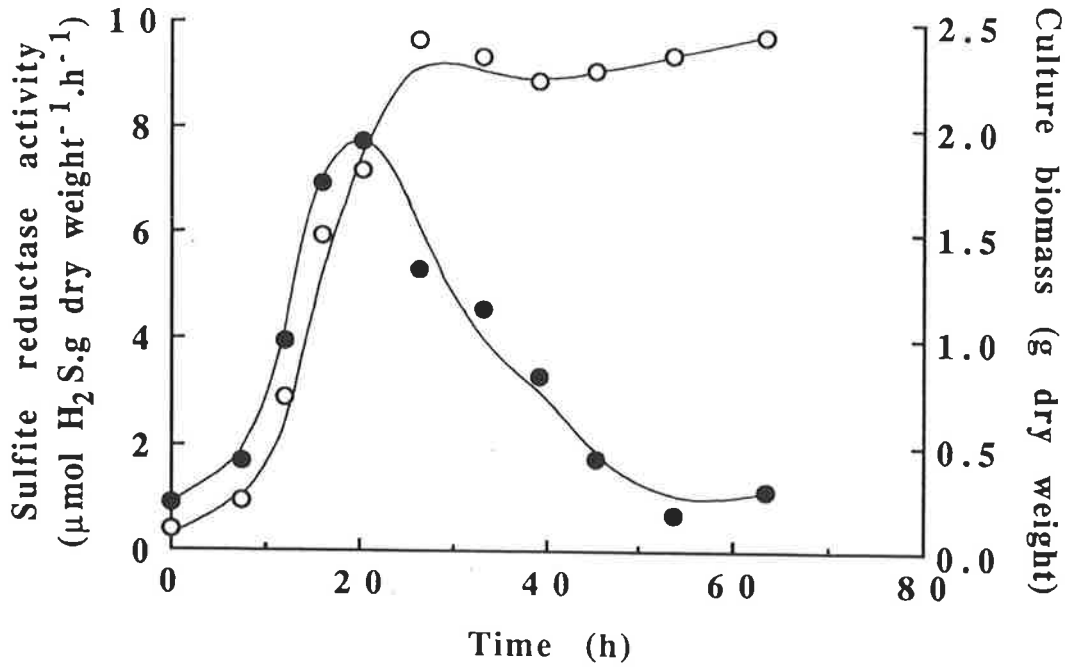


Figure 4.11 Sulfite reductase activity during fermentation by *Saccharomyces cerevisiae* strain 77. Fermentation was conducted at 25°C in synthetic grape juice medium containing ammonium ions (33.3 mM) and sulfite (263 µM as sodium metabisulfite). Biomass formation (O) and NADPH-linked sulfite reductase activity (●) were determined at regular intervals throughout fermentation.

was short-lived and occurred at a reduced rate. This reponse was reflected by the corresponding level of sulfite reductase activity of the culture at this time.

c. Suppression of hydrogen sulfide production by assimilable nitrogen compounds

A more detailed examination of the regulation of hydrogen sulfide production by assimilable nitrogen was carried out for *Saccharomyces cerevisiae* strains 72 (Figure 4.12) and 77 (Figure 4.13) grown in SGJM under initially anaerobic conditions. In both cases, low initial rates of hydrogen sulfide production were increased several-fold upon the depletion of ammonium ions from the fermentation medium. Hydrogen sulfide production was subsequently suppressed through ammonium ion supplementation and sustained at a comparatively low rate until the added ammonium ions were depleted. Hydrogen sulfide production resumed thereafter.

Of 19 amino acids examined individually or in combination, the majority demonstrated an ability similiar to ammonium ions in suppressing ~~suppress~~ hydrogen sulfide production by *Saccharomyces cerevisiae* strain 77 (Figure 4.14). In the case of strain 72, a smaller number of amino acids proved effective in suppressing hydrogen sulfide production. For both strains 72 and 77, supplementation of fermentations with cysteine, both individually and in combination with methionine, consistently lead to increased rates of hydrogen sulfide formation compared with the unsupplemented control. While this increase was of the order of ten-fold for strain 77, strain 72 exhibited an increase of 25- to 35-fold.

When fermentations were initiated in media containing a limited sole-source of assimilable nitrogen, the amounts of hydrogen sulfide produced by *Saccharomyces cerevisiae* strain 77 differed depending on the nature of the

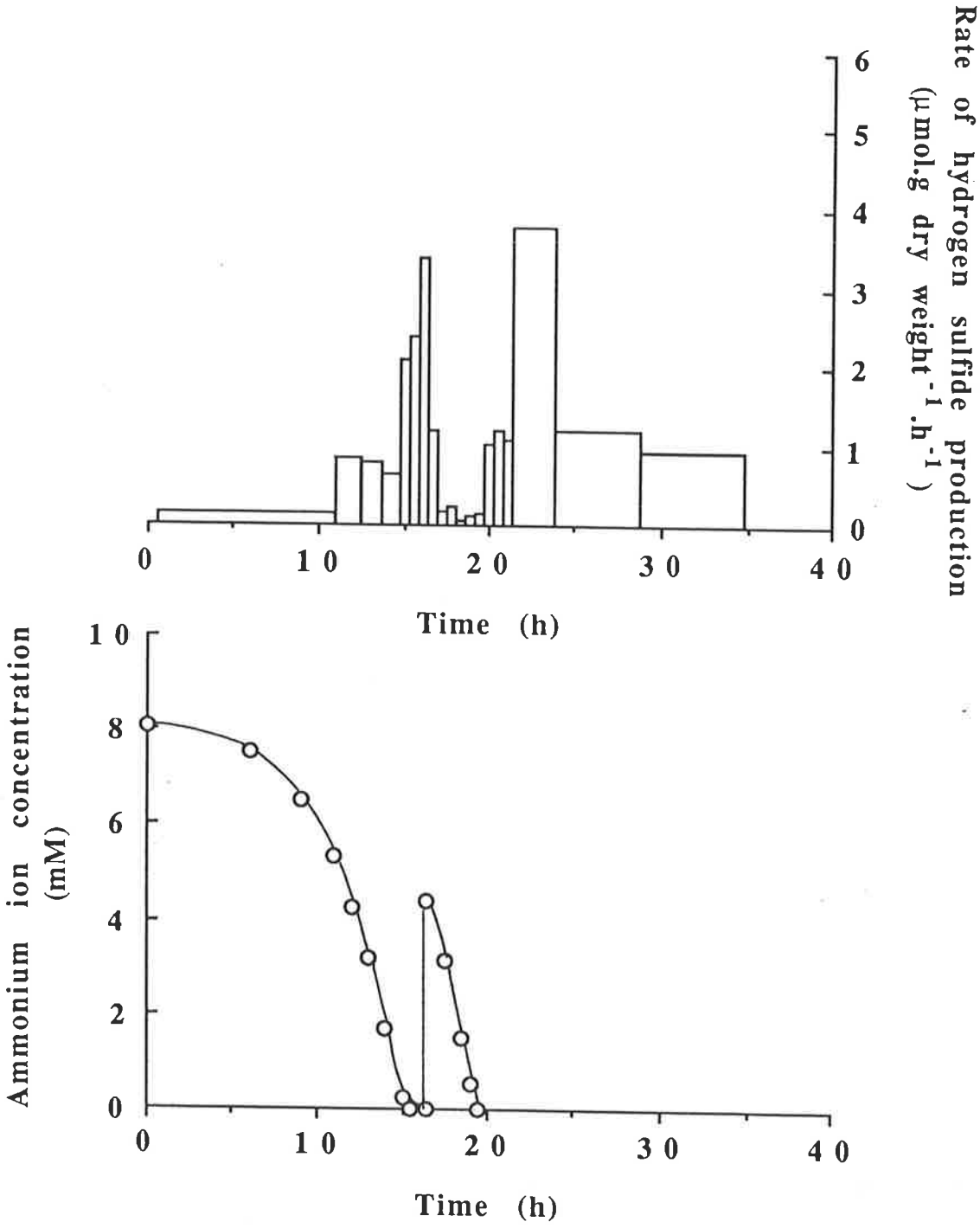


Figure 4.12 Ammonium ion concentration (O) and hydrogen sulfide production (histogram) by *Saccharomyces cerevisiae* strain 72 at 25°C in air-saturated synthetic grape juice medium containing sodium metabisulfite (263 μM). Ammonium ions were added at 16.5 hours to a concentration of 4.2 mM.

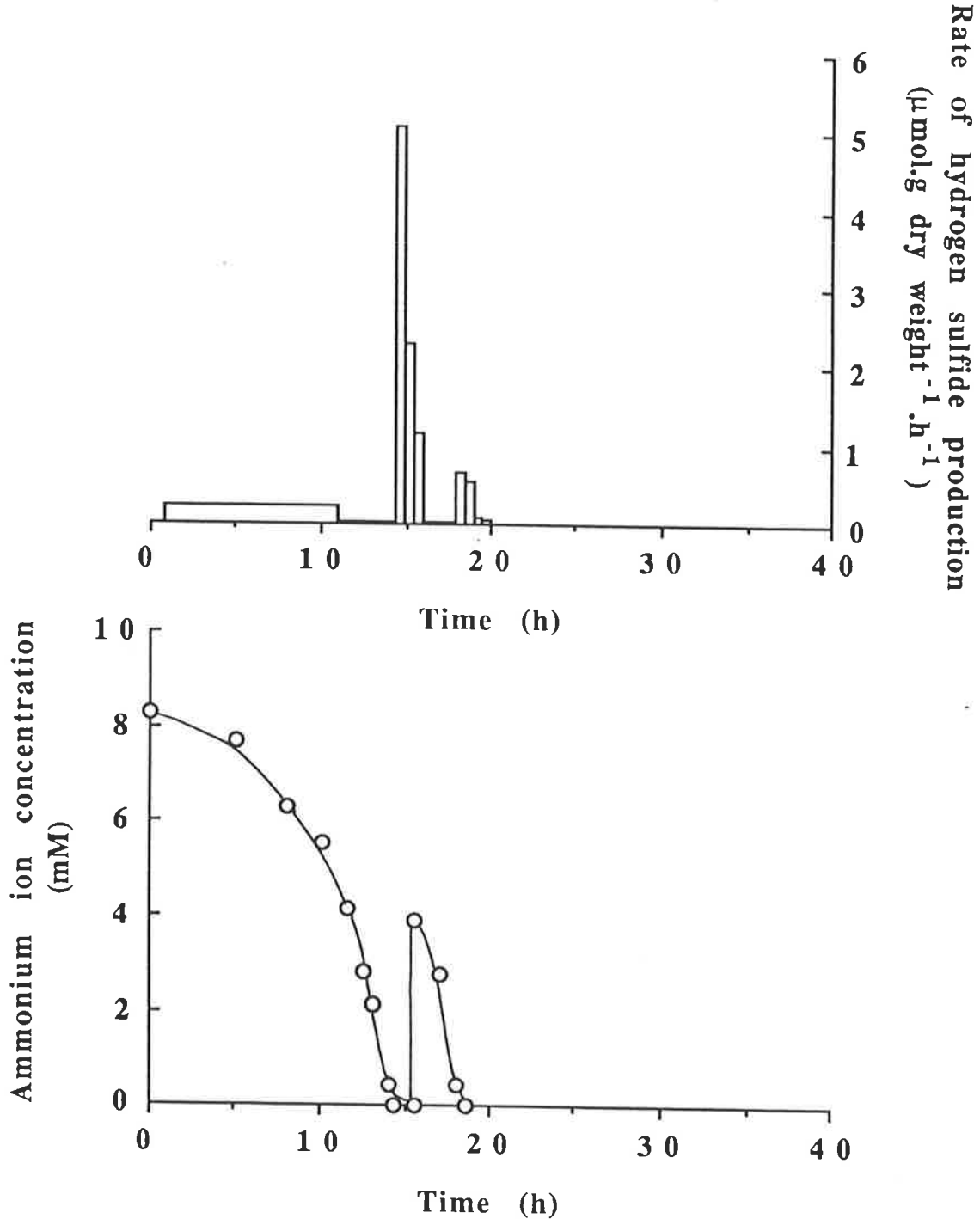


Figure 4.13 Ammonium ion concentration (O) and hydrogen sulfide production (histogram) by *Saccharomyces cerevisiae* strain 77 at 25°C in air-saturated synthetic grape juice medium containing sodium metabisulfite (263 μM). Ammonium ions were added at 15.5 hours to a concentration of 3.9 mM.

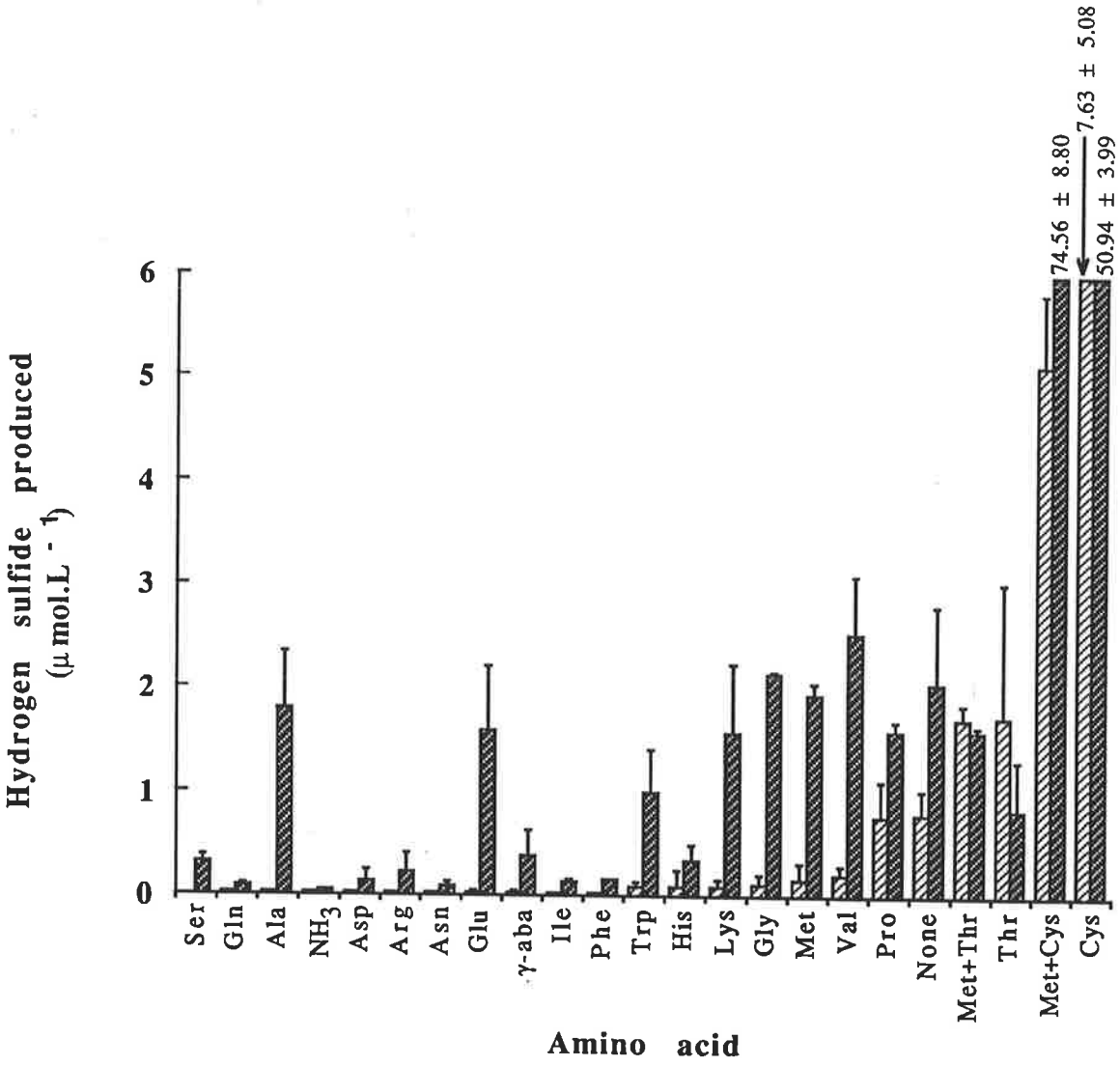


Figure 4.14 Suppression of hydrogen sulfide production in *Saccharomyces cerevisiae* strains 77 and 72 by amino acids and ammonium ions .

Fermentations were initiated in synthetic grape juice medium (4.5 L) containing sodium metabisulfite (50 $mg.L^{-1}$) and ammonium ions (150 $mg.L^{-1}$) at 25°C. One hour prior to ammonium ion depletion, aliquots (180 mL) were anaerobically transferred into fermentation flasks (250 mL), supplemented with amino acids or ammonium ions to a total equivalent of 200 mg $N.L^{-1}$ and monitored for hydrogen sulfide production over the following 6 hours. All samples, except the unsupplemented control, were confirmed to contain residual assimilable nitrogen. Values are the mean of at least two determinations ± S.E.M.

nitrogen source (Table 4.4). Larger quantities of hydrogen sulfide were formed following growth on nitrogen sources which supported higher growth rates (shorter doubling times).

d. Inorganic sulfur precursor compounds

The formation of hydrogen sulfide from sulfate under nitrogen sufficient conditions has already been shown to be low for seven of the eight *Saccharomyces cerevisiae* strains examined (refer to Table 4.2). The high rates of hydrogen sulfide production described above for *Saccharomyces cerevisiae* strains occurred only in response to a depletion of assimilable nitrogen from synthetic grape juice medium containing both sulfate and sulfite. This was also shown for strain 77 (Table 4.5). However, unlike hydrogen sulfide production from sulfate and sulfite-containing media, the stage of the fermentation at which assimilable nitrogen became depleted was of no significance to hydrogen sulfide production from sulfate by this strain.

Kinetic studies using *Saccharomyces cerevisiae* strain 77 demonstrated that hydrogen sulfide production induced by a deficiency of assimilable nitrogen was confined to a relatively small, short lived peak of activity during growth in media containing sulfate as sole sulfur source at either of two sulfate concentrations (Figure 4.15). The ability of the former culture to produce hydrogen sulfide in large amounts, despite the cessation of formation from sulfate, was demonstrated upon supplementation of the medium with sulfite. Two possible roles of sulfite in this excessive formation of hydrogen sulfide are (a) as principal precursor or (b) as inducer of formation from sulfate. By repeating the previous experiment utilising ^{35}S-sulfate, the sulfur precursor of the first peak of hydrogen sulfide-producing activity was shown to be sulfate, whereas the excessive formation of hydrogen sulfide which followed sulfite addition occurred

Table 4.4 Influence of nitrogen source on hydrogen sulfide production, glucose catabolism and culture doubling time by *Saccharomyces cerevisiae* strain 77.

Nitrogen source	Glucose catabolised (mmoles)	Doubling Time (min)	Hydrogen sulfide production	
			Total (μmoles)	Maximum rate (μmol.gdw^{-1}.h^{-1})[a]
NH_4^+	341	324	16.40	2.04
Arg	326	348	11.03	1.35
Phe	348	408	3.49	1.38
Tyr	207	450	0.94	1.37
Arg, Phe, Tyr	296	384	7.72	0.99

Fermentations were conducted at 25°C in synthetic grape juice medium containing 526 mM sulfite as sodium metabisulfite. Assimilable nitrogen was provided to total nitrogen concentration of 7.5 mM by a single nitrogen compound or an equal ratio of three amino acids. Cultures were monitored for a total of 49.5 hours which was 36 hours beyond nitrogen depletion in the ammonium grown culture.

[a] μmol.g dry weight^{-1}.h^{-1}.

Table 4.5 Hydrogen sulfide production by *Saccharomyces cerevisiae* strain 77 during the fermentation of synthetic grape juice media containing various sulfur sources.

Sulfur sources					
Sulfate			Sulfate and sulfite		
Initial ammonium content (mM)	Hours to ammonium depletion[a]	Hydrogen sulfide produced ($\mu mol.L^{-1}$)[b]	Initial ammonium content (mM)	Hours to ammonium depletion[a]	Hydrogen sulfide produced ($\mu mol.L^{-1}$)[b]
44.4	-	0.16	33.3	-	0.88
33.3	-	0.17	24.9	34	9.24
22.2	24	0.25	16.6	19	26.12
11.1	13.5	0.15	8.3	12.5	47.98
4.4	8	0.10	3.3	9	55.60

Fermentations were initiated in synthetic grape juice medium containing 5 mM sulfate with or without 263 µM sulfite (as sodium metabisulfite) and incubated at 25°C.

[a] Determined to the nearest 30 min.

[b] Total hydrogen sulfide produced over 24 h after the depletion of ammonium ions from the medium.

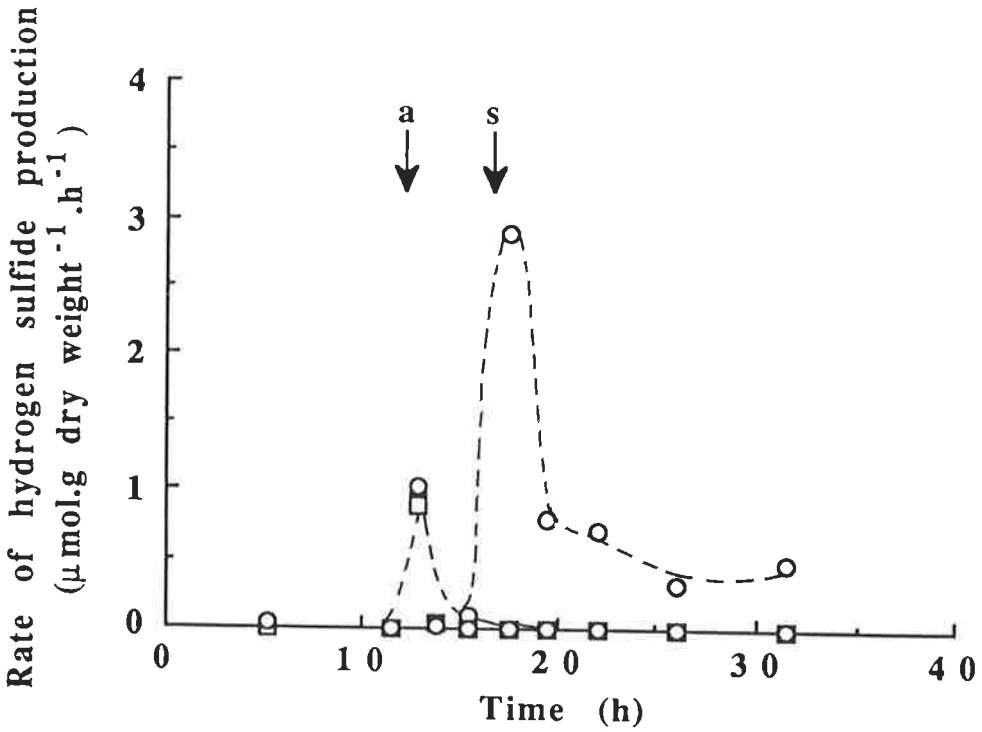


Figure 4.15 Hydrogen sulfide production by *Saccharomyces cerevisiae* strain 77 grown in media containing various sulfur sources.

Fermentations were conducted at 25°C in synthetic grape juice medium containing ammonium ions (8.3 mM) and 5 mM (O) or 50 mM (□) sulfate. Hydrogen sulfide production was determined from fermentations of media free of sulfite (solid curves) or supplemented to 263 μM sulfite (as sodium metabisulfite; O, broken curve) at the time indicated (s) following the point of ammonium ion depletion (a).

essentially without utilisation of sulfate (Figure 4.16). Accumulation of ^{35}S-sulfate into yeast biomass immediately prior to the depletion of ammonium ions occurred at a rate of 42.5 µmol.g dry weight^{-1}.h^{-1}.

The effect of sulfite on hydrogen sulfide formation from sulfate was determined by initiating fermentations in media containing both sulfate and sulfite. Compared with control fermentations in media containing only sulfate, hydrogen sulfide production occurred in larger amounts and at a greater rate upon disappearance of nitrogen from a sulfate and sulfite containing medium. The contribution from sulfate was, however, variable such that for *Saccharomyces cerevisiae* strain 77 (Figure 4.17), the rate of hydrogen sulfide production from ^{35}S-sulfate was reduced in the presence of sulfite, whereas this parameter remained unchanged for *Saccharomyces cerevisiae* strain 72 (Figure 4.18).

4.3 DISCUSSION

4.3.1 Comparison of hydrogen sulfide production on indicator media and under fermentation conditions

Although bismuth-containing indicator media have been extenstively used for screening the hydrogen sulfide production trait in yeasts, no verification for relevance to oenological coditions appears to have been made. The performance of *Saccharomyces cerevisiae* yeasts on nutritionally complete BiGGY indicator media correlated well with their broad classification as non-, low- or high-hydrogen sulfide producers as listed in Table 2.1. Generally, only minor changes were evident in the pattern of relative reaction intensities seen between strains during growth on a synthetic grape juice agar (SGJA) which more closely modelled the composition of grape juice. Similarly, incubation of cultures under

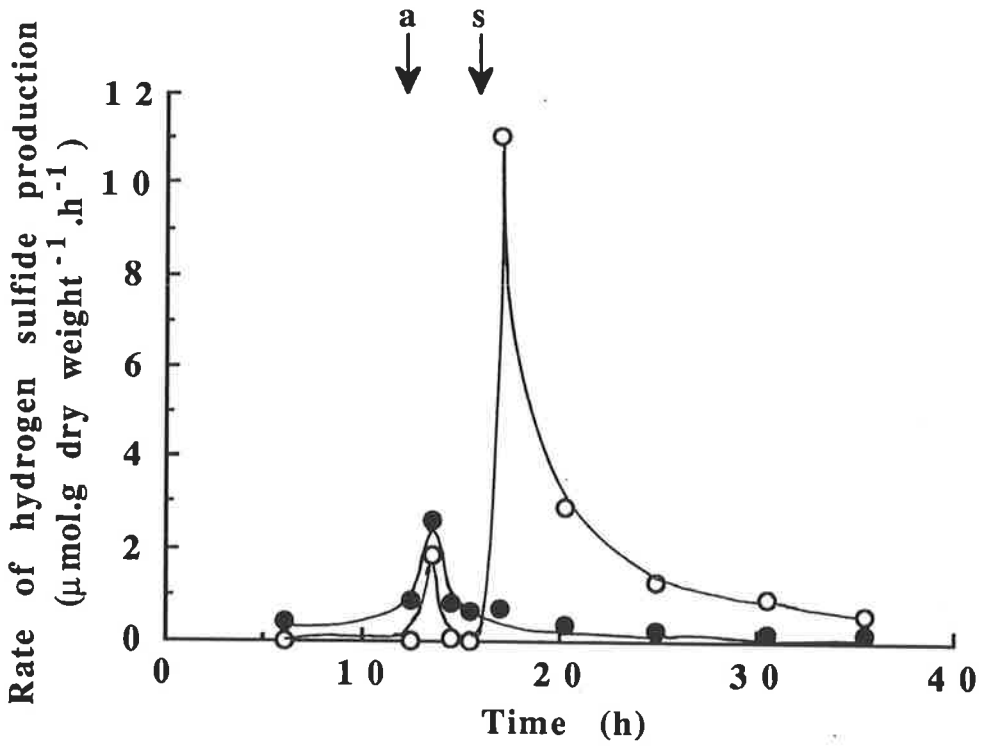
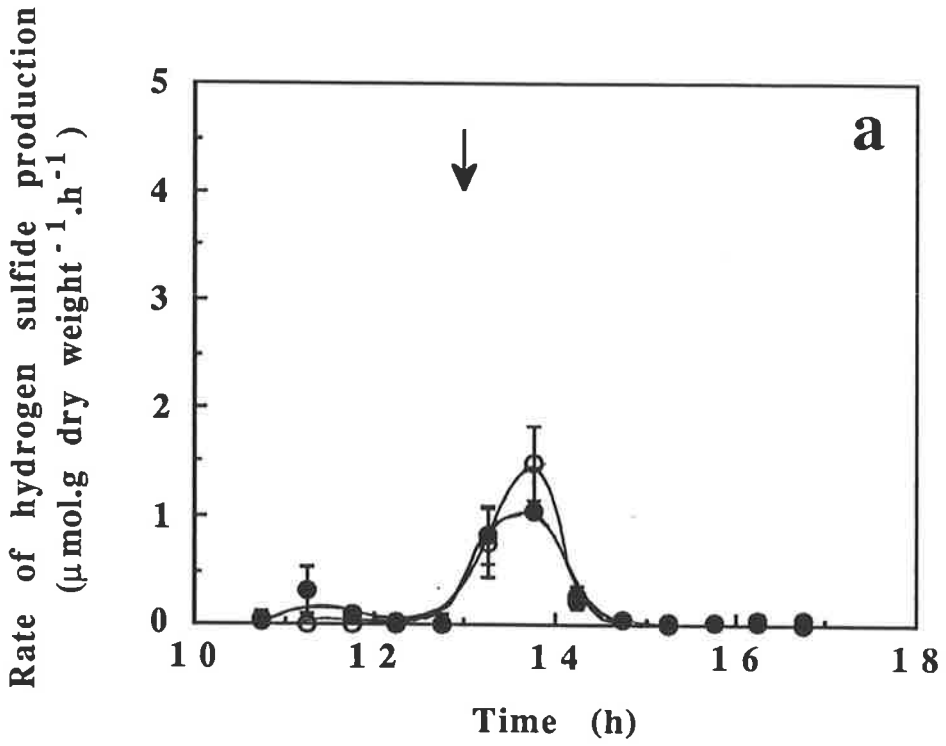


Figure 4.16 Total hydrogen sulfide production (O) and production from ^{35}S sulfate (●) by *Saccharomyces cerevisiae* strain 77.

Fermentations were conducted at 25°C in synthetic grape juice medium containing ammonium ions (8.3 mM) and ^{35}S-sulfate (5 mM, 4 µCi.mmol^{-1} specific activity). Sulfite was added to 260 µM (as sodium metabisulfite) at the time indicted (s) following the point of ammonium ion depletion (a).

Figure 4.17 Total hydrogen sulfide production (O) and production from ^{35}S-sulfate (●) by *Saccharomyces cerevisiae* strain 77.

Fermentations were conducted at 25°C in synthetic grape juice medium containing ammonium ions (8.3 mM), ^{35}S-sulfate (5mM, 5 mM, 4 $\mu Ci.mmol^{-1}$ specific activity) alone (a) or together with sulfite (260 μM as sodium metabisulfite) (b). The point of ammonium ion depletion is indicated by an arrow.


a
Rate of hydrogen sulfide production
(μmol.g dry weight⁻¹.h⁻¹)
0
1
2
3
4
5
10
12
14
16
18
Time (h)

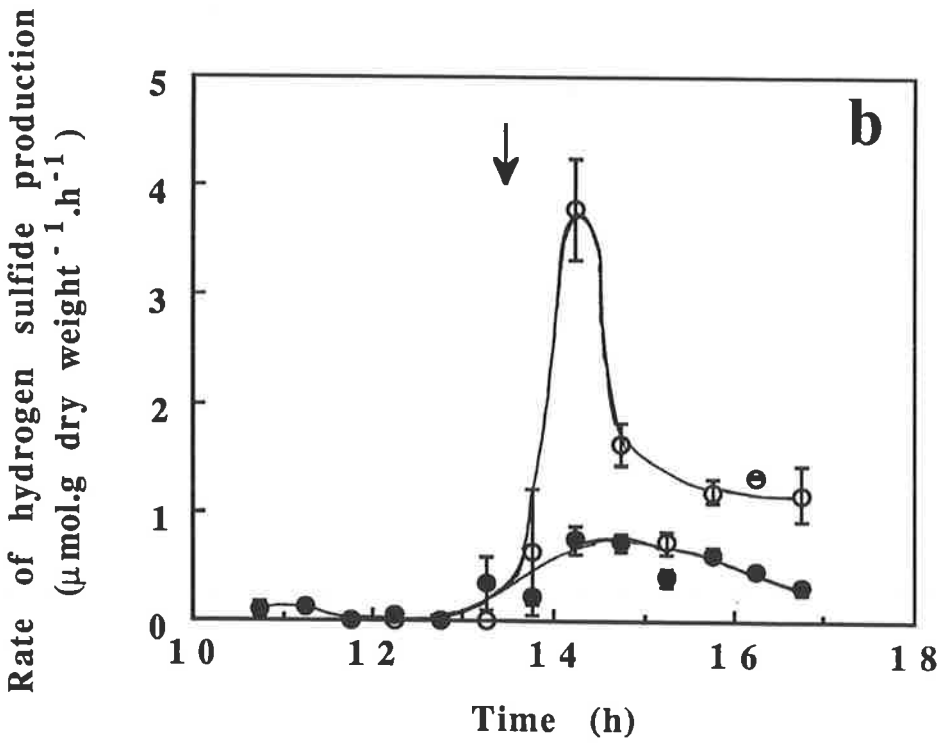
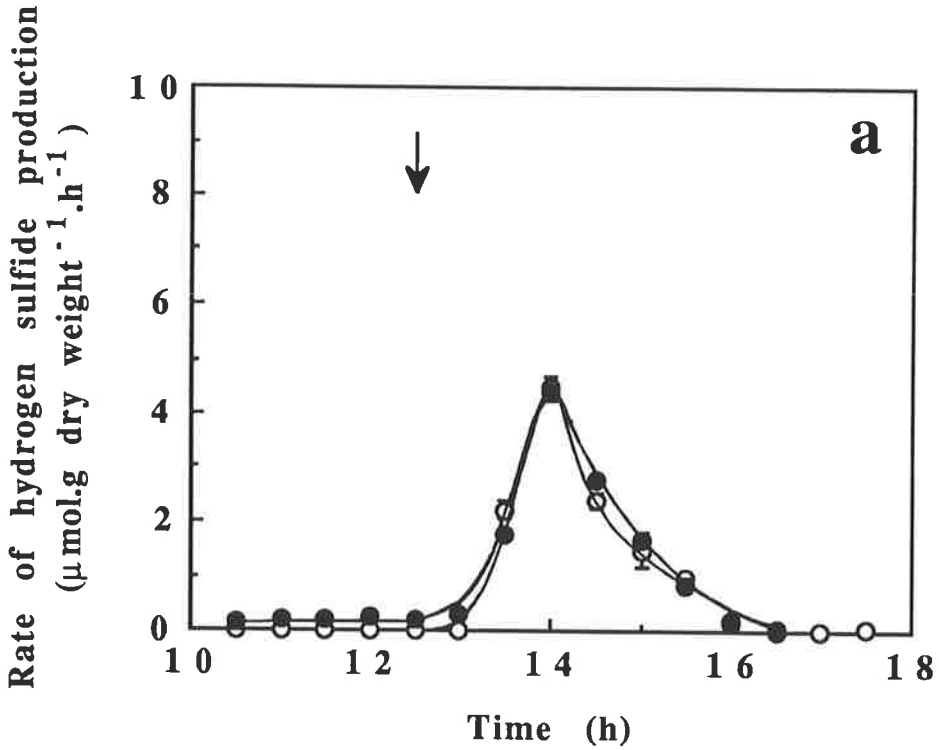
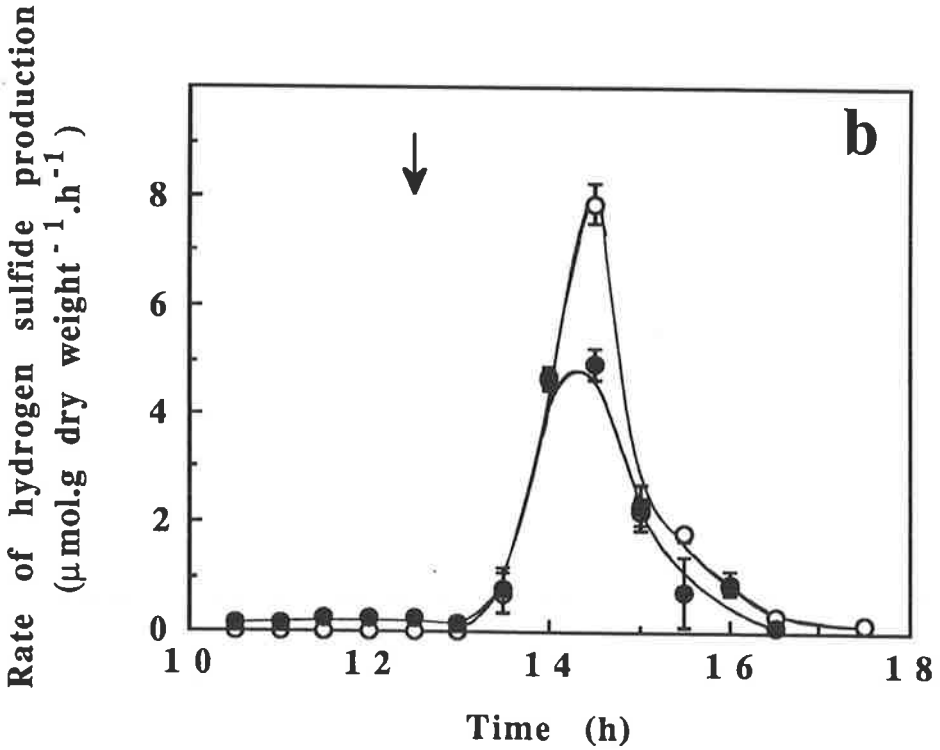

b
Rate of hydrogen sulfide production
(μmol.g dry weight⁻¹.h⁻¹)
0
1
2
3
4
5
10
12
14
16
18
Time (h)

Figure 4.18 Total hydrogen sulfide production (O) and production from ^{35}S-sulfate (●) by *Saccharomyces cerevisiae* strain 72.

Fermentations were conducted at 25°C in synthetic grape juice medium containing ammonium ions (8.3 mM), ^{35}S-sulfate (5 mM, 4 $\mu Ci.mmol^{-1}$ specific activity) alone (a) or together with sulfite (260 μM as sodium metabisulfite) (b). The point of ammonium ion depletion is indicated by an arrow.


a
Rate of hydrogen sulfide production
(μmol.g dry weight⁻¹.h⁻¹)
10
8
6
4
2
0
10
12
14
16
18
Time (h)


b
Rate of hydrogen sulfide production
(μmol.g dry weight⁻¹.h⁻¹)
8
6
4
2
0
10
12
14
16
18
Time (h)

anaerobic conditions had little effect on hydrogen sulfide production. Contrary to subsequent observations in nitrogen-limited liquid fermentations, sulfite in SGJA did not increase hydrogen sulfide formation.

Laboratory studies (Eschenbruch, 1974a; Vos and Gray, 1979) and empirical observations in the wine industry indicate that individual yeast strains differ in the amounts of hydrogen sulfide they produce between fermentations and/or batches of grape juices. Thus must composition is a likely candidate cause for the variability in hydrogen sulfide production observed. In this context it is interesting to note the lack of response by individual yeast strains to variations in the nutritional composition seen across the bismuth-containing indicator media. The uniformity of hydrogen sulfide formation for an individual yeast strain, despite the different test conditions, used lends support to the notion that what is being observed is the full, deregulated expression of this characteristic of yeast strains, the maximum level of which is being genetically determined (Zambonelli, 1964; Takahashi *et al.*, 1980). Similarly the 'non hydrogen sulfide-producing' strains 6523, 6842, 7070, 10728, have undetectable sulfite reductase activity (Zambonelli *et al.*, 1984).

By comparison with indicator-media studies, seven of eight yeast strains cultivated under optimised fermentation conditions in nutritionally complete synthetic grape juice medium (SGJM) produced only trace amounts of hydrogen sulfide. Hence these could no longer be distinguished according to their characteristic production of this volatile. Only when cultures were stressed by the depletion of assimilable nitrogen from either SGJM or yeast carbon-base medium, did the amounts of hydrogen sulfide produced by yeast strains reflect the relative reaction intensities seen on bismuth-containing indicator media. Trace formation of hydrogen sulfide in nutritionally complete SGJM confirms that this attribute formed a selection criteria for the initial isolation of these strains of wine yeasts.

Conversely, strain 59, is a possible wild yeast isolate (not used commercially for wine production) and therefore has not been subject to this selection process. The degree of hydrogen sulfide formation by this strain is indicative of the potential detriment that non-wine yeasts may inflict on the wine fermentation. It is nonetheless interesting to note that yeast strains empirically classed as 'low-' or 'non-hydrogen sulfide producers' are capable of high rates of production upon metabolic stress. Since these strains enjoy widespread use in the wine industry, it would appear that fermentations are generally conducted in grape juices of sufficient natural or supplemented nitrogen content. It should be noted that the use of assimilable nitrogen supplements is permitted by law in almost all wine-producing countries.

Given the similarity between the reactions of yeast on indicator media and in SGJM or yeast carbon-base media upon nitrogen depletion, it is suggested that the two represent an analogous response of sulfur metabolism to metabolic stress. High rates of hydrogen sulfide formation on indicator media are unlikely to result from a localised depletion of assimilable nitrogen since growth of yeast on SGJA containing reduced amounts of assimilable nitrogen limited growth but did not alter hydrogen sulfide production (data not shown). Instead the reactions of yeast on BiGGy or SGJA may be explained in two ways. First the complexing of cellular hydrogen sulfide by accumulated bismuth prevents sulfur amino acid biosynthesis. This results in derepression of enzymes of the sulfate reduction sequence in an attempt to meet metabolic requirements for hydrogen sulfide. Second, yeast cells are stimulated to produce hydrogen sulfide at full capacity as a protective mechanism by which heavy metal ions are detoxified (Ono *et al.*, 1991).

It is concluded that hydrogen sulfide formation on bismuth-containing indicator media is a reflection of the potential for hydrogen sulfide production observed in response to a depletion of assimilable nitrogen during fermentation

under model oenological conditions. Furthermore, as reported by Zambonelli and colleagues (1984) and as will be discussed below, this potential appears to be indicative of the maximal sulfite reductase activity of these yeast strains. Therefore, this rapid screening methodology will be useful to the winemaker in determining the risk of hydrogen sulfide formation associated with the use of a given yeast strain, particularly during the fermentation of a grape juice of a composition conducive to this outcome.

4.3.2 Metabolic basis for hydrogen sulfide formation by *Saccharomyces cerevisiae* following assimilable nitrogen starvation

Monitoring of industrial fermentations completed under controlled laboratory conditions highlighted the existence of two peaks of hydrogen sulfide producing activity. The sensitivity of the 'mid-fermentation peak' to ammonium ions largely accounts for the widespread use of supplements of assimilable nitrogen to grape juices prior to inoculation with yeast and during fermentation upon the detection of hydrogen sulfide. The often observed, but apparently not previously reported, second peak of activity is a source of frustration for the winemaker as it is not readily suppressed through the addition of assimilable nitrogen. Furthermore, it often develops at a stage of fermentation when the evolution of carbon dioxide is not sufficiently vigorous to strip the volatile from the wine. This latter phase of hydrogen sulfide production is the subject of Chapter 6.

Results from temporal supplementation studies conducted under model oenological conditions in defined media resemble the data of Stratford and Rose (1985) obtained under semi-defined laboratory conditions. Regulation of sulfur metabolism, specifically, that leading to hydrogen sulfide formation, is clearly demonstrated for assimilable nitrogen compounds by the following observations.

Small amounts of hydrogen sulfide were formed in the presence of assimilable nitrogen. Following the depletion of this nutrient from the medium, the rate of hydrogen sulfide production increased dramatically, often by several orders of magnitude, peaking within 30 to 60 minutes. This activity could be rapidly suppressed by ammonium and, to varying degrees, amino acid supplementation of the medium. High rates of hydrogen sulfide formation were restored upon depletion of the ammonium supplements. Response times appear to be considerably longer in industry since the efficiency of entrainment is not as great as that observed for the sparged fermentation system used in this study.

It is noteworthy that amino acids which support high growth rates (refer Table 1.7) or those that are able to act as precursors for *O*-acetylserine or *O*-acetylhomoserine synthesis, were generally the most potent suppressants of hydrogen sulfide production. The relative ease with which these compounds enter the cytosolic nitrogen pool and/or supply the synthesis of the acetylated precursors of cysteine and methionine, hence expediting the incorporation of hydrogen sulfide into these amino acids, may account for this. The possibility of the synthesis of a nitrogenous inhibitor of sulfite reductase activity being facilitated in the same manner needs to be also considered.

Cysteine supplemented cultures produced hydrogen sulfide in greater quantities than unsupplemented cultures. This observation is attributed to the exploitation of this amino acid principally as a nitrogen source and hence its catabolism to ammonium, pyruvate and hydrogen sulfide directly (Tokuyama, *et al.*, 1973). Given that cysteine is involved in the regulation of the sulfate reduction sequence (SRS) either itself or via an interconversion with methionine, it is suggested that the observed hydrogen sulfide is entirely of amino acid origin. Threonine, on the other hand inhibits homoserine dehydrogenase which catalyses the biosynthesis of homoserine, a common

precursor of threonine, isoleucine and methionine (White, 1978). Thus despite the availability of a reasonable nitrogen source, formation of methionine and its derivatives is reduced. Elevated rates of hydrogen sulfide production presumably become apparent due to a derepression of the sulfate reduction sequence and/or a reduced incorporation of hydrogen sulfide into sulfur amino acid biosynthesis.

As predicted by reaction on bismuth-containing media, the extent of hydrogen sulfide production exhibited strain variation such that strain 72 gave rise to a larger, sustained production of hydrogen sulfide upon nitrogen depletion than did strain 77. The observed maxima for hydrogen sulfide formation of 55.6 $\mu mol.L^{-1}$ over 24 hours by strain 77 grown in SGJM and 74.5 $\mu mol.L^{-1}$ over 30 hours by strain 72 grown in yeast carbon-base medium compares favourably with that of 28.8 $\mu mol.L^{-1}$ for strain WE 14 (73) (Vos and Gray, 1979) and 16.0 $\mu mol.L^{-1}$ (Monk, 1986) reported for grape juice fermentations. Stratford (1983) quoted a value of 265 $\mu mol.L^{-1}$ for an aerobic, defined-medium fermentation conducted under laboratory conditions.

Several models have been proposed for the action of assimilable nitrogen and/or consequences of its absence. Vos and Gray (1979) suggested that hydrogen sulfide was formed through the enzymic degradation of cysteine which had been liberated from grape juice proteins by a nitrogen deficiency-induced yeast proteolytic activity. Based on the mean of the values quoted in Chapter 1 for the protein content of grape juice, and assuming equal representation of all amino acids in grape proteins, complete catabolism of protein-derived cysteine could potentially yield hydrogen sulfide at a rate of *ca.* 670 $\mu mol.L^{-1}$. Given the rapidity of the hydrogen sulfide response to nitrogen depletion, yeast would need to elaborate a proteolytic activity capable of degrading grape proteins to at least transportable penta-peptides or smaller (Becker *et al.*, 1973) within approximately 30 minutes. An activity of this potency has not been demonstrated to date (Nelson

and Young, 1986; Rosi *et al.*, 1987; Lagace and Bisson, 1990). At any rate, such an activity could be expected to simultaneously liberate numerous amino acids which have been demonstrated to be as effective as ammonium ions in suppressing hydrogen sulfide production. A final piece of evidence negating this hypothesis is the fact that nitrogen depleted cultures in protein-free SGJM or yeast carbon-base media (Stratford and Rose, 1985) are able to form hydrogen sulfide in amounts of the order of, or greater than, those seen during grape juice fermentations.

A second candidate mechanism for the metabolic formation of hydrogen sulfide involves the dissimilatory metabolism of cellular pools of sulfur-containing nitrogen compounds such as proteins, peptides and amino acids. The induction of intracellular proteolytic and degradative enzymes as a result of nitrogen limitation has been described (reviewed by Cooper, 1982a; Achstetter and Wolf, 1985). However, for reasons discussed above pertaining to the relative speeds of enzyme induction and hydrogen sulfide production, the generation of hydrogen sulfide through the catabolism of anything more complex than free amino acids, is unlikely to be the main route by which this sulfur compound becomes readily detectable in nitrogen-limited fermentations.

The role of free sulfur-amino acids remains in doubt. Watson (1976) reported that ammonium grown *Saccharomyces cerevisiae* yeast strain Y48 contained no detectable free cysteine and only 1.6 µmol free methionine. Even if strain 77 possessed a similar cellular methionine content, this organic precursor of hydrogen sulfide production would potentially account for only 23% of the maximum hydrogen sulfide produced by this strain. The contributions to cellular organic sulfur by glutathione and *S*-adenosylmethionine are smaller still (Maw, 1965; Lawrence and Cole, 1968). Determination of the exact degree of involvement of these compounds in hydrogen sulfide production upon assimilable nitrogen

depletion could be achieved through the use of a radioactive sulfur source. Organisms initially cultivated on such a compound would be transferred to a medium containing unlabelled sulfur, deprived of assimilable nitrogen and the ratio of unlabelled hydrogen sulfide to labelled hydrogen sulfide of cellular origin determined.

The proposal of alternative mechanisms for hydrogen sulfide production requires the identification of an appropriate sulfur-containing precursor compound(s). This task is somewhat simplified since sulfate and sulfite are the only sulfur compounds present in either yeast carbon-base medium or SGJM in amounts sufficient to supply the observed hydrogen sulfide production. Radiolabelling experiments confirmed the reduction of sulfate and sulfite to hydrogen sulfide in media containing these compounds.

In the yeast *Saccharomyces cerevisiae*, reduction of sulfate and sulfite to hydrogen sulfide occurs via the sulfate reduction sequence as a prelude to the biosynthesis of the sulfur-containing amino acids. It has been proposed that during nitrogen starvation, a concomitant cellular depletion of sulfur amino acids and their metabolic derivatives results in a derepression of synthesis of the enzymes of the sulfate reduction sequence (refer to Figure 1.3; de Vitta and Dreyfuss, 1964; Cherest *et al.*, 1969, 1971, 1973) and in turn an increased formation of hydrogen sulfide (Stratford and Rose, 1985).

Again, the rapid onset of hydrogen sulfide formation tends to reduce this mechanism to one of importance in the long term and not the 30 to 60 minute time frame observed here. Further the availability of precursors for such synthesis could be expected to be restricted. Instead, the switch to detectable hydrogen sulfide production is likely to be due to a continuing production of the thiol ion despite its reduced sequestration to sulfur amino acid biosynthesis caused by a

nitrogen deficiency-induced shortage of the appropriate nitrogenous precursors (Stratford and Rose, 1985).

The key enzyme in this scheme for hydrogen sulfide formation is sulfite reductase. Based on observations made using ^{35}S-sulfate and estimates of the contribution of sulfur to yeast total dry weight (Maw, 1965), sulfur accumulation rates and hence sulfite reductase activities of at least 42.5 and 22.5 µmol.g dry weight^{-1}.h^{-1}, respectively, are possible. Consequently, the maximum observed rates of hydrogen sulfide production of 2.8 µmol.g dry weight^{-1}.h^{-1} from sulfate alone or 11.1 µmol.g dry weight^{-1}.h^{-1} from sulfate together with sulfite, are clearly achievable through the action of the sulfite reductase enzyme. The fact that hydrogen sulfide is not produced upon nitrogen depletion at the same rate at which sulfur can be accumulated by the cell, indicates the existence of a regulatory mechanism(s) or metabolic block(s) limiting the reduction of these precursors.

Several additional pieces of evidence point to the sulfite reductase enzyme as the principal mediator of hydrogen sulfide production upon nitrogen starvation. As an essential step in the biosynthesis of sulfur amino acids, sulfite reductase activity can be expected to reflect the growth/metabolism-related demand for these amino acids. Therefore, the potential for hydrogen sulfide production upon nitrogen depletion should be determined by the growth/metabolic rate of the culture immediately prior to depletion. In support of this, maximum rates and extents of hydrogen sulfide production were apparent when disappearance of nitrogen from the fermentation medium occurred during the exponential, rather than stationary, phase of yeast growth. Taking the specific rate of nitrogen utilisation as a measure of growth rates or metabolic activity, positive correlations were seen between this parameter and both total hydrogen sulfide production ($r = 0.961$) and the mean rate of hydrogen sulfide

production over 12 hours after nitrogen depletion (r = 0.832). Further, the manipulation of growth rates through the use of single amino acid nitrogen sources of differing efficiencies (see Table 1.7), produced changes in total hydrogen sulfide production which were inversely correlated with culture doubling times (r = -0.994).

The most convincing evidence was obtained from data relating to the profile of sulfite reductase activity through fermentation. A relationship is evident between estimates of sulfite reductase activity and maximum rates of hydrogen sulfide production occurring upon nitrogen depletion at various stages of fermentation. Analogous observations were made for strain TC8 by Stratford and Rose (1985) under laboratory conditions. Therefore the pattern of sulfite reductase activity throughout fermentation reflects the expected growth-related demand for the sulfur amino acids. Thus, observed rates of hydrogen sulfide production upon nitrogen-depletion are an indication of the preceding rate of sulfur accumulation. In turn, maximum rates of hydrogen sulfide formation for a given strain are likely to be determined by the specific activity of the sulfite reductase enzyme which is a genetic characteristic of the strain (Zambonelli *et al.*, 1984).

The presence of sulfite in addition to sulfate in the fermentation medium consistently increased the rate and extent of hydrogen sulfide production upon the disappearance of assimilable nitrogen from the medium. The observed additional formation of hydrogen sulfide did not occur from ^{35}S-sulfate and was concluded to be derived from the added sulfite, the only alternate sulfur source present in the medium. Under these conditions, strain 77 demonstrated a preferential utilisation of sulfite, raising the possibility that sulfate accumulation and reduction in this strain are especially sensitive to sulfite inhibition

(McCready and Din, 1974; Breton and Surdin-Kerjan, 1977; Yoshimoto and Sato, 1968a; Dott and Trüper, 1976).

Interestingly, when sulfite was added to cultures after the onset and subsequent diminution of hydrogen sulfide production from sulfate, the ensuing production was supplied essentially at the complete exclusion of sulfate. Thus, while the capacity for excessive hydrogen sulfide production is retained by yeast cells for several hours after the depletion of ammonium ions from the medium, the ability for the utilisation of sulfate for this production is less enduring. Analogous observations made from sulfite supplementation experiments lead Stratford and Rose (1985) to propose that when presented with both sources of sulfur, hydrogen sulfide production utilised only sulfite as a precursor. However, in light of the data presented here for fermentations initiated with both sulfur sources, the conclusion that sulfate exclusion is absolute may be erroneous.

The fact that hydrogen sulfide production *per se* can be increased by sulfite additions to beyond the levels seen from sulfate alone, demonstrates that accumulation and/or reduction of sulfate to sulfite are the rate limiting steps for the formation of hydrogen sulfide from sulfate. Given the input of energy required for the formation of sulfite from exogenous sulfate, that is, energisation of active transport and the expenditure of two molecules of ATP for sulfate activation, tight regulation of this pathway would seem imperative. Since hydrogen sulfide is readily lost from the yeast cell, regulatory mechanisms involving feedback inhibition by hydrogen sulfide and, hence its metabolic precursors, do not satisfactorily explain the control and rapid cessation of hydrogen sulfide production from sulfate.

In an effort to explain the relatively small and short-lived nature of hydrogen sulfide formation from sulfate, it is proposed that this precursor either

fails to enter the yeast cell or to be reduced to hydrogen sulfide. Given the longevity of the ability to reduce sulfite of exogenous origin and, as will be discussed later, the half-life of sulfite reductase of several hours in nitrogen depleted cells, a failure of sulfate accumulation seems to be the most plausible mechanism for this phenomenon.

High rates of transport-protein turnover may account for the loss of sulfate transport capabilities by a mechanism somewhat reminiscent of that proposed to account for the rapid decay of glucose transport rates in nitrogen-starved cells (Lagunas *et al.*, 1982; Salmon, 1989). Monitoring the accumulation of sulfate by cultures in which protein synthesis has been arrested through cycloheximide addition, would clarify this issue. Considering that in the natural environment yeasts are rarely exposed to high concentrations of sulfite, the evolution of highly labile sulfate transport proteins would have proven to be an extremely efficient means of preventing the futile commitment of energy to hydrogen sulfide formation in the absence of a suitable nitrogen source.

In conclusion, hydrogen sulfide production on solid indicator media shares some similarity to the response of yeast cells to nitrogen starvation under oenological conditions. As such surveys utilising these methodologies are of value to the winemaker.

While the evolution of hydrogen sulfide under conditions modelling the oenological fermentation appears to be a constitutive attribute of some yeast strains, the key cause of this phenomenon is proposed to be a cellular limitation of assimilable nitrogen which is suggested to arise out of a depletion of assimilable nitrogen from the medium. The principal metabolic mechanism involved is the continued reduction of organic sulfur compounds via the existing enzymic machinery of the cell despite the absence of the reactive nitrogen-

precursors of the sulfur amino acids. The enzyme sulfite reductase is central to this process. The propensity of strains to produce excessive hydrogen sulfide is suggested to be related to their intrinsic levels of sulfite reductase activity. The availability of the energetically preferred sulfite moiety permits maximal rates and durations of hydrogen sulfide production to develop.

During the grape juice fermentation, two phases of hydrogen sulfide production are evident, only that occurring during the active growth phase being responsive to assimilable nitrogen supplements. Supplementation of the medium with assimilable nitrogen is only effective in controlling the formation of hydrogen sulfide during the active growth phase.

Chapter 5

Sulfite reductase activity in *Saccharomyces cerevisiae*

5.1 INTRODUCTION

The important conclusions from Chapter 4 are that; (a) nitrogen depletion triggers excessive hydrogen sulfide production under conducive conditions, and (b) that sulfite is the important source of sulfur for this production of hydrogen sulfide. It has been suggested that these results are consistent with the formation of hydrogen sulfide by sulfite reductase in excessive amounts relative to the availability of the hydrogen sulfide-sequestering molecules, *O*-acetlyserine and *O*-acetlyhomoserine. This mechanism, depends on sulfite reductase being maintained despite a nitrogen deficiency or on any other mechanism which could inhibit its activity.

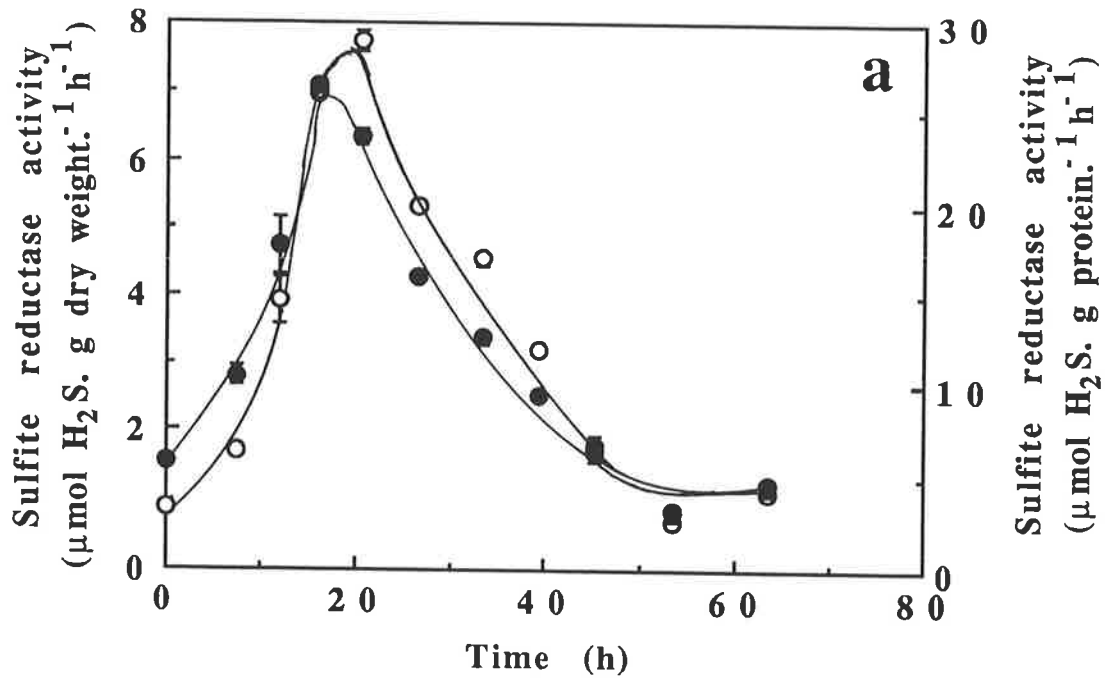
To date, the majority of reports in the literature have dealt only with the biochemical properties of sulfite reductase. Few have examined its behaviour during the course of fermentation let alone under conditions of metabolic stress, particularly that induced by a deficiency of assimilable nitrogen. The aims of this chapter are to determine the specific action of assimilable nitrogen in the regulation and suppression of the sulfite reductase enzyme and its fate during nitrogen deficiency.

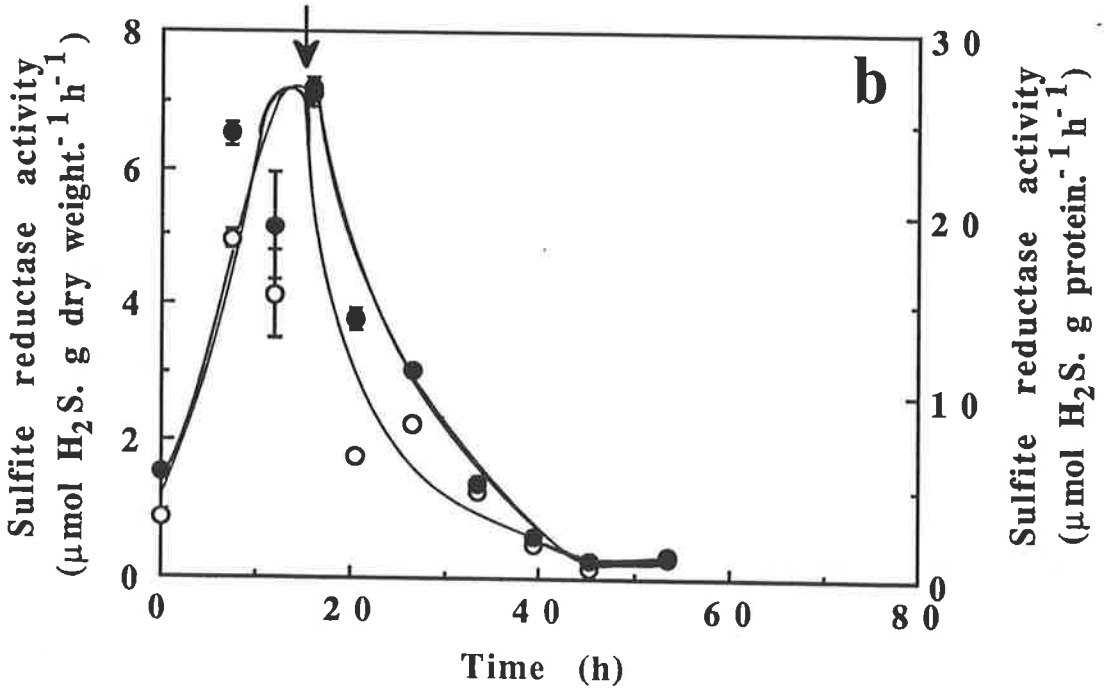
5.2 RESULTS

Parallel fermentations in nitrogen sufficient or nitrogen deficient media were used to study the effect of assimilable nitrogen on the NADPH-linked sulfite reductase activity of *Saccharomyces cerevisiae* strain 77. As introduced in the previous chapter (Figure 4.11), a rapid rise in activity was observed at the commencement of exponential growth in both cultures (Figure 5.1). In the

Figure 5.1 Development of NADPH-linked sulfite reductase activity during the growth of *Saccharomyces cerevisiae* strain 77 under nitrogen excess (a) or limiting (b) conditions.

Fermentations were conducted at 25°C in air-saturated synthetic grape juice medium containing ammonium ions (8.3 mM) and sodium metabisulfite (263 μM). Ammonium ions were either maintained throughout fermentation through supplementation or allowed to become depleted at the time indicated with an arrow. The duplicate cultures were sampled at regular intervals and the level of NADPH-linked sulfite reductase activity, protein content and biomass determined as described. Values are reported in units against culture dry weight (O) or protein content (●).


a
Sulfite reductase activity (μmol H_2S. g dry weight.$^{-1}$ h^{-1})
Sulfite reductase activity (μmol H_2S. g protein.$^{-1}$ h^{-1})
Time (h)
0
2 0
4 0
6 0
8 0
2
4
6
8
1 0
2 0
3 0


b
Sulfite reductase activity (μmol H_2S. g dry weight.$^{-1}$ h^{-1})
Sulfite reductase activity (μmol H_2S. g protein.$^{-1}$ h^{-1})
Time (h)
0
2 0
4 0
6 0
8 0
2
4
6
8
1 0
2 0
3 0

continual presence of assimilable nitrogen this activity peaked shortly before the stationary phase of growth whereafter it declined with progress of the fermentation. At no point during the course of this fermentation was hydrogen sulfide evolved in detectable amounts from the medium.

By comparison, the evolution of large amounts of hydrogen sulfide were detected through the blackening of lead acetate-impregnated indicator columns following the depletion of assimilable nitrogen from the medium. The existing high NADPH-linked sulfite reductase activity of the culture in turn decayed at an initially rapid rate to values of approximately 50% or less of that seen at the peak of activity. Subsequently, sulfite reductase activity decreased at a rate similar to that seen at comparable stages of fermentation by cultures grown in the presence of assimilable nitrogen.

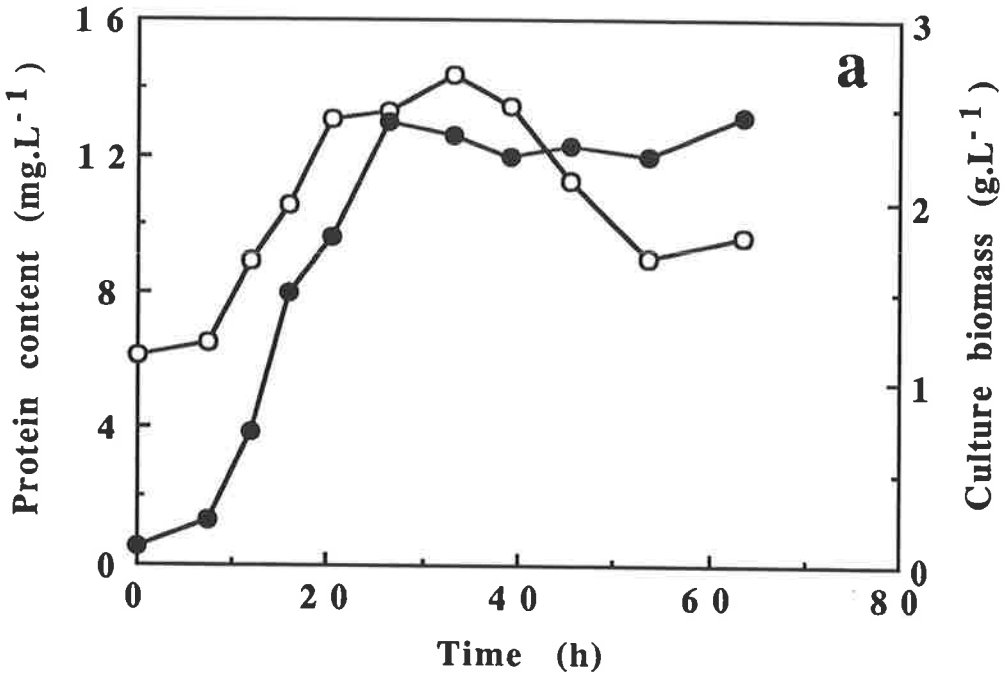
A difference was evident between culture biomass and cellular protein content with respect to their relative rate of change during fermentation (Figure 5.2). The latter was observed to be particularly variable following the exhaustion of nitrogen from the medium.

In the short term, the NADPH-linked sulfite reductase activity of *Saccharomyces cerevisiae* strain 77 was independent of the nitrogen status of the culture (Figure 5.3). While there was close agreement between the maximum rates of hydrogen sulfide production from the culture and those detected during estimation of sulfite reductase activity, the latter remained stable during periods of complete ammonium suppression of hydrogen sulfide production. Only during extended incubation of cultures in the absence of assimilable nitrogen did the levels of sulfite reductase activity begin to decline together with the rates of hydrogen sulfide production by the culture.

Cultivation of organisms in synthetic grape juice medium containing methionine as sole nitrogen source, proved an effective means of reducing the

Figure 5.2 Development of biomass (●) and cellular protein (○) during the growth of *Saccharomyces cerevisiae* strain 77 under nitrogen excess (a) or limiting (b) conditions.

Fermentations conditions as described for Figure 5.1.


a
Protein content (mg.L-1)
Culture biomass (g.L-1)
Time (h)
0
4
8
12
16
0
1
2
3
0
20
40
60
80

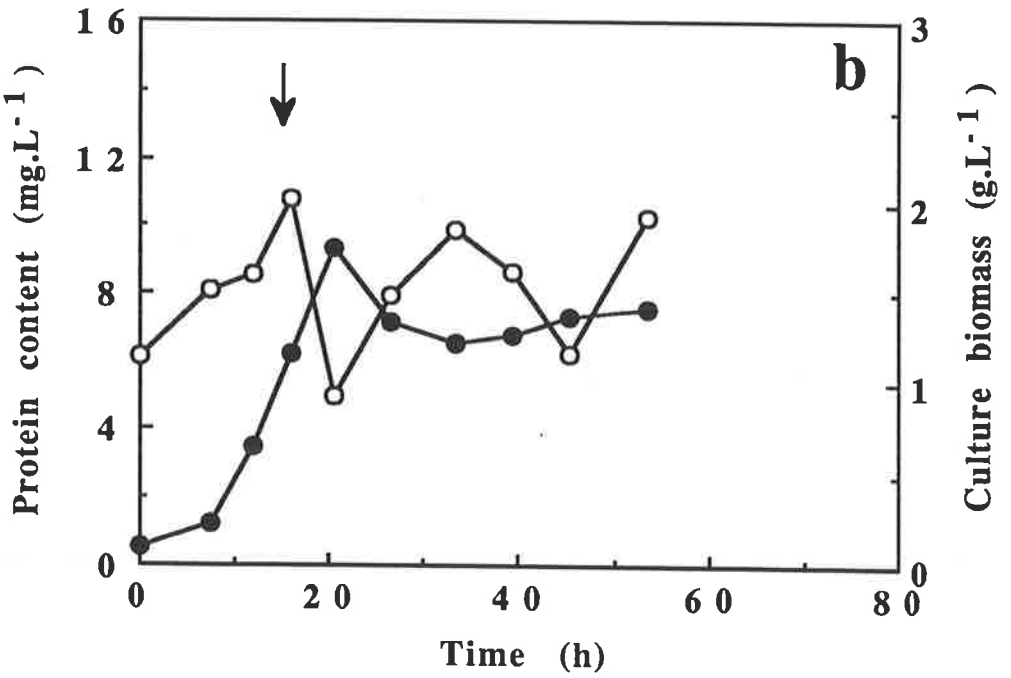
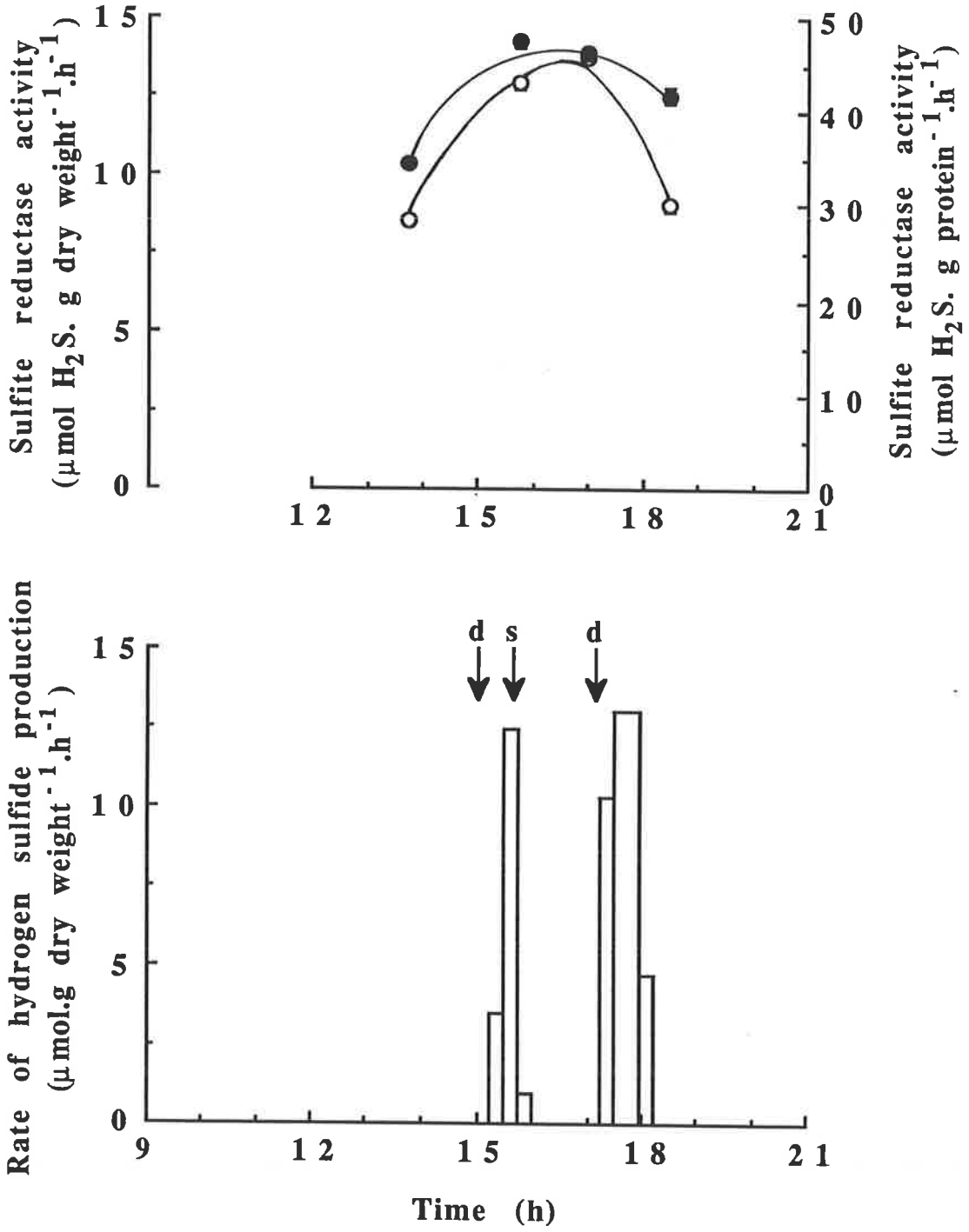

b
Protein content (mg.L-1)
Culture biomass (g.L-1)
Time (h)
0
4
8
12
16
0
1
2
3
0
20
40
60
80

Figure 5.3 Hydrogen sulfide production and NADPH-linked sulfite reductase activity during the growth of *Saccharomyces cerevisiae* strain 77.

Fermentations were conducted at 25°C in air-saturated synthetic grape juice medium containing ammonium ions (8.3 mM) and sodium metabisulfite (263 μM). The points of ammonium ion depletion are indicated with an arrow (d). Supplementation of the medium to 4.2 mM ammonium ions occurred at 15.75 hours as indicated with an arrow (s). Values for NADPH-linked sulfite reductase activity are reported in units against culture dry weight (O) or protein content (●).


Sulfite reductase activity
(μmol H_2S. g dry weight^{-1}.h^{-1})
15
10
5
0
50
40
30
20
10
0
12
15
18
21
Sulfite reductase activity
(μmol H_2S. g protein^{-1}.h^{-1})
Rate of hydrogen sulfide production
(μmol.g dry weight^{-1}.h^{-1})
d
s
d
9
Time (h)

detectable NADPH-linked sulfite reductase activity of these cultures compared with those grown on ammonium (Figure 5.4). Although a peak of enzyme activity was still evident during the exponential phase of growth of the former culture, it was only of the order of 30% of that seen in ammonium grown cells.

Methionine also appeared to have a direct effect on the NADPH-linked sulfite reductase activity of cell-free extracts. When extracts from cultures of strain 77 which had been starved of nitrogen for 30 minutes were assayed in the presence of methionine at 1 and 5 mM, detectable enzyme activity was reduced to 89% ± 1 and 73% ± 1, respectively, of the unsupplemented controls. Ammonium supplementation to 1 mM produced a relative activity of 101% ± 1.

Based on results reported in Figure 5.1, the half-life of the NADPH-linked sulfite reductase activity of nitrogen depleted cultures was of the order of five hours. A more detailed examination of cultures of strain 77 during the period immediately following the depletion of assimilable nitrogen from the medium or the addition of cycloheximide, produced comparable estimates of enzyme half-life of 4.25 and 5.75 hours, respectively (Figure 5.5).

5.3 DISCUSSION

At the onset of this investigation, some importance was given to the selection of the most appropriate units in which to express detected NADPH-linked sulfite reductase activity. Since it was highly desirable to compare this activity to rates of hydrogen sulfide production from fermentations, preference was given to units incorporating a measure of culture biomass, namely, µmoles hydrogen sulfide formed per gram dry weight per hour.

The more conventional expression of sulfite reductase activity against protein content of cell-free extracts, is considered inappropriate during nitrogen limitation studies since, as confirmed here, protein contents exhibited greater

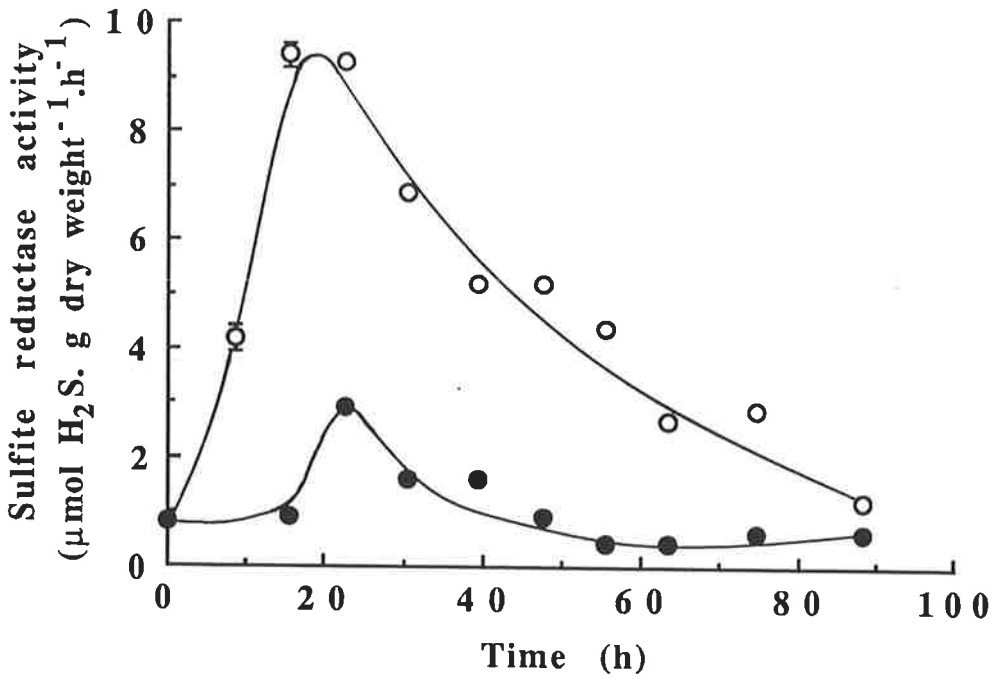


Figure 5.4 Development of NADPH-linked sulfite reductase activity during the growth of *Saccharomyces cerevisiae* strain 77 on ammonium (O) or methionine (●) as sole nitrogen sources

Fermentations were conducted at 25°C in air-saturated synthetic grape juice medium containing ammonium ions or methionine (8.3 mM) and sodium metabisulfite (263 μM). Nitrogen sources were maintained in excess through supplementation. Cultures were sampled at regular intervals and the level of NADPH-linked sulfite reductase activity and biomass determined as described.

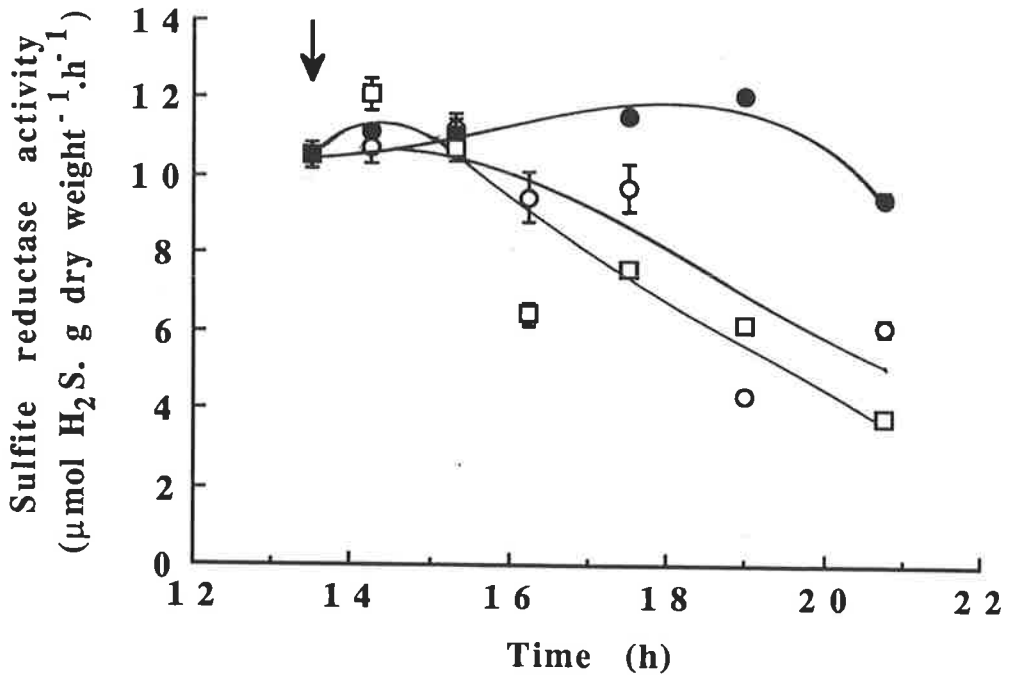


Figure 5.5 Decay of the NADPH-linked sulfite reductase activity of *Saccharomyces cerevisiae* strain 77.

Fermentations were conducted at 25°C in air-saturated synthetic grape juice medium containing ammonium ions (8.3 mM) and sodium metabisulfite (263 μM). Immediately prior the depletion of ammonium from the medium (indicated with an arrow) cultures were either unsupplemented (○) or else supplemented with ammonium ions (11.1 mM) alone (●) or together with cycloheximide (35.5 mM) (□). Samples were then taken immediately and at subsequent intervals for the determination of the residual level of NADPH-linked sulfite reductase activity and biomass determined.

variability than culture biomass under these conditions. In particular, for determinations made shortly after nitrogen depletion, expression of units of enzyme activity against protein content produces artificially high values due to the decline of cellular 'proteinacious' material which occurs at this point. Accordingly, the dry weight-based expression of sulfite reductase activity reported for Figure 5.1 is considered to be more meaningful.

The data presented in this chapter address several of the key questions surrounding the action of sulfite reductase on the production of hydrogen sulfide during nitrogen starvation. In the short-term, sulfite reductase activity is relatively stable and apparently independent of the nitrogen status of the culture. Therefore, it can be concluded that assimilable nitrogen does not suppress hydrogen sulfide production by diminishing the sulfite-reducing capacity of the yeast cell. Conversely, elevated rates of hydrogen sulfide formation during nitrogen starvation cannot be attributed to a corresponding *de novo* synthesis of sulfite reductase. The likelihood of there existing a general cellular deficiency of nitrogenous precursors under these conditions is the most probable explanation for the absence of such synthesis. Thus, it is suggested that the production of hydrogen sulfide observed under nitrogen-depleted conditions is merely a manifestation of the existing enzymic capacity of the cell.

The fact that no detectable hydrogen sulfide was observed emanating from nitrogen sufficient/supplemented fermentations despite the presence of high levels of NADPH-linked sulfite reductase activity can be accounted for in two possible ways. Either, the existing enzyme activity is controlled *in vivo* by a post-translational inhibitory mechanism, or alternatively, enzyme activity is precisely matched to the growth-related demand for hydrogen sulfide required for sulfur-amino acid biosynthesis. Evidence exists in favour of both explanations.

Strengthening the first proposal are the observations from inhibition studies wherein direct methionine supplementation of cell-free extracts was

shown to decrease the inherent NADPH-linked sulfite reductase activity. Previous studies have examined only the benzyl-viologen dependent sulfite reductase activity for its sensitivity to methionine inhibition and have found no effect (Dott and Trüper, 1976). Thus, the inhibitory effect of this amino acid on the NADPH-linked activity of sulfite reductase of cell-free extracts of *Saccharomyces cerevisiae* is being described here for the first time.

Additional evidence implicating the existence of an inhibitor of sulfite reductase, presumably nitrogenous in nature, is the finding that enzyme activity of cell-free extracts derived from nitrogen-sufficient cultures, could be optimised by *ca.* 20% by dialysis, while that of nitrogen starved cells could not (data not shown). Further work is required to confirm this mode of enzyme regulation. Nevertheless, such a mechanism of feedback inhibition seems a logical and effective means of controlling the short term activity of the sulfite reductase enzyme.

The bulk of available data supports the proposal that sulfite reductase activity is matched to metabolic requirements for hydrogen sulfide. Key observations leading to his conclusion are; (a) the peak of sulfite reductase activity observed here under model oenological conditions and elsewhere under laboratory conditions (Dott and Trüper, 1978; Stratford and Rose, 1985) develops when the demand for hydrogen sulfide for cysteine and methionine biosynthesis would be expected to be highest, during exponential growth, (b) the existence of a repressive mechanism controlling sulfite reductase biosynthesis indicates that enzyme will be produced only in amounts dictated by cellular hydrogen sulfide requirements (Cherest *et al.*, 1971; Dott and Trüper, 1978), (c) as discussed in Chapter 4, rates and extent of hydrogen sulfide formation were strongly correlated with various measures of culture growth rates, thus, the finding that rates of hydrogen sulfide formation by the NADPH-linked sulfite reductase activity of cell-free extracts closely match the maximal rates of hydrogen sulfide evolution induced by a deficiency of assimilable nitrogen, is highly significant,

(d) even after extended dialysis of the enzyme extracts, the rates of hydrogen sulfide formation observed during assays of sulfite reductase activity did not exceed the theoretical or measured rates of sulfur accumulation also referred to in Chapter 4, and (e) sulfite reductase activity is greatly reduced when a direct source of organic sulfur is provided.

Since current data favour the latter proposal, it therefore appears that the principal role of assimilable nitrogen in the suppression of hydrogen sulfide production during nitrogen starvation is through the provision of biosynthetic precursors of sulfur amino acids into which hydrogen sulfide is sequestered. Thus, where assimilable nitrogen is not provided, the formation of hydrogen sulfide continues in an uncontrolled manner as long as reducing power and a suitable sulfur source are available. Decay of hydrogen sulfide formation is primarily determined by the half-life of the existing enzyme.

The similarity seen in the half-lives of sulfite reductase prepared from cycloheximide supplemented and nitrogen starved cultures promotes the possibility that the losses of activity in the two occur via the same mechanism. In the former, a complete inhibition of protein synthesis would prevent the maintenance of cellular levels of the sulfite reductase enzyme. The arrest of protein synthesis in response to nitrogen deficiency could produce the same outcome. The marginally delayed/slower rate of decay could be attributed to the turnover of other cellular nitrogen components.

The long half-life of the sulfite reductase enzyme suggests it is of interest to consider the efficiency of repression of enzyme synthesis by methionine. In accord with Dott and Trüper (1978) who reported enzyme repression of the order of 80-85%, sulfite reductase activity was reduced to approximately 30% during growth on methionine as sole nitrogen source. By contrast, Cherest and coworker (1971) attained a 25-fold reduction in enzyme activity. The proposal of the existence of an inhibitor of sulfite reductase is

consistent with the observed absence of hydrogen sulfide formation despite the incompleteness of repression of this enzyme during growth solely on methionine.

To conclude, the NADPH-linked sulfite reductase activity of *Saccharomyces cerevisiae* strain 77 appears sufficient to account for the rates of hydrogen sulfide production observed upon the disappearance of nitrogen from the fermentation medium. The evidence presented here supports the contention that in the long term regulation of sulfite reduction is primarily achieved through a repression of enzyme synthesis by methionine and/or its derivatives. Although some evidence points to the involvement of an inhibitor, possibly methionine, of the sulfite reductase enzyme, the activity of existing enzyme appears to be essentially uncontrolled, dissipating only through enzyme degradation with a half-life of *ca.* five hours. Control of hydrogen sulfide evolution in nutritionally complete media seems to be primarily accomplished by a complete channelling of hydrogen sulfide into the biosynthesis of the organic sulfur compounds, methionine and cysteine.

Chapter 6

'End-of-fermentation' hydrogen sulfide production by *Saccharomyces cerevisiae* wine yeast

6.1 INTRODUCTION

The observations made in laboratory-monitored, industrial grape juice fermentations (Chapter 4) highlighted the existence of two distinct phases of hydrogen sulfide production. The first occurred most commonly during the active growth phase of the culture and developed in response to a deficiency of assimilable nitrogen but was essentially suppressed by additions of the same. The second peak of activity developed toward the end of the fermentation and was poorly suppressed by supplements of assimilable nitrogen. An investigation was undertaken to identify the causes of this second peak of hydrogen sulfide production, from which some initial observations are detailed here.

6.2 RESULTS

Key observations pertaining to the 'end-of-fermentation' formation of hydrogen sulfide during the industrial fermentation of Sultana grape juice are described below. Production occurred despite the presence of assimilable nitrogen (ammonium ions) in the must. The onset of production occurred between the point at which the must fell below 2 to 3° Bé (0.2 to 0.3 M glucose equivalent) and as late as 1 to 2 days after the complete catabolism of must sugar. The addition of further ammonium (as diammonium hydrogen phosphate) failed to alleviate the problem.

Preliminary observations were obtained for samples of fermenting Sultana grape juice which had been inoculated with strain 77 at the winery, maintained at a readily detectable assimilable nitrogen content with supplements

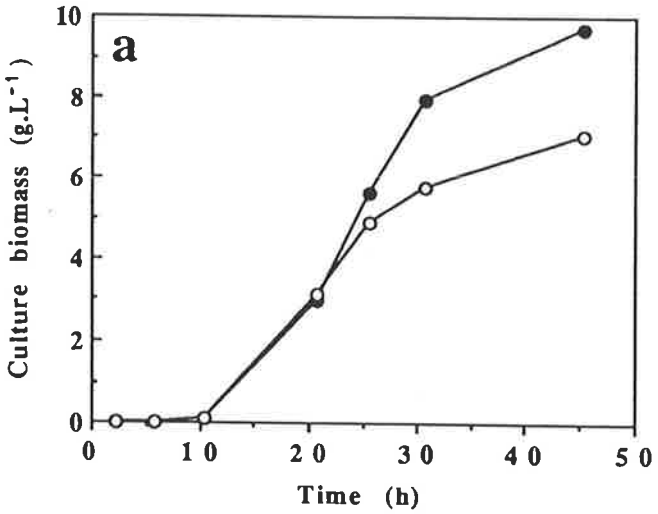
of ammonium ions, dispensed into fermentation flasks and monitored for total hydrogen sulfide production. Control flasks which had received no supplements other than ammonium ions produced hydrogen sulfide in the amounts similar to those already described (refer to Figure 4.4). In flasks which had received limited amounts of oxygen (50 mL air:200 mL medium), hydrogen sulfide production was reduced by a mean of 91% for four independent determinations. This was in agreement with empirical observations from the winery. The possible role of oxygen in the suppression of hydrogen sulfide production was examined by measuring the accumulation of ^{14}C-arginine and the ^{14}C- labelled ammonium analogue (Roon *et al.*, 1975a), methylamine, by cultures which had been grown under various degrees of aeration or supplementation with the oxygen-substitutes, ergosterol and oleic acid (Tween 80).

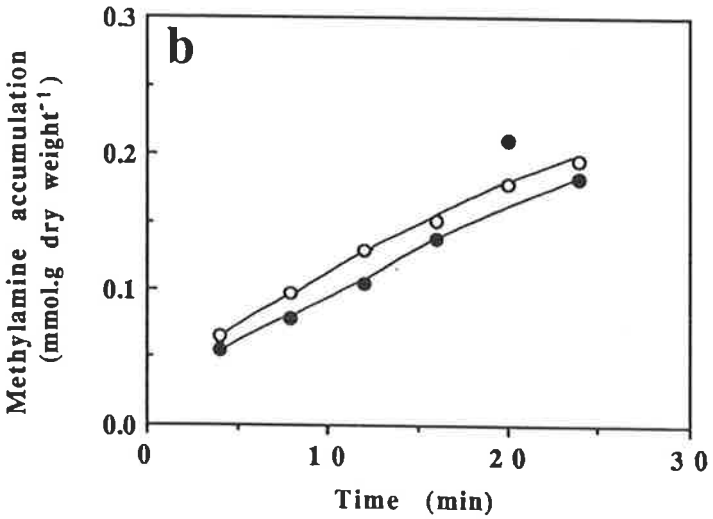
The addition of ergosterol and oleic acid (Tween 80) to the medium at the time of inoculation resulted in the formation of a greater biomass than the anaerobically grown, unsupplemented controls (Figure 6.1). When cells from these cultures were assayed after 45 hours incubation they failed to differ markedly in their ability to accumulate methylamine or arginine. Greater differences were apparent between samples of partially aerated and anaerobic cultures especially when taken close to the point at which the growth curves of these cultures diverged (Figure 6.2). Thus at 14 hours, the partially aerated culture demonstrated an ability to accumulate methylamine at a mean rate of approximately 0.25 mmol.g dry weight^{-1}.min^{-1}, whereas the anaerobic culture displayed only a baseline level of activity (Figure 6.3). These differences were reduced for samples of the cultures which were taken at times progressively later after the point of divergence.

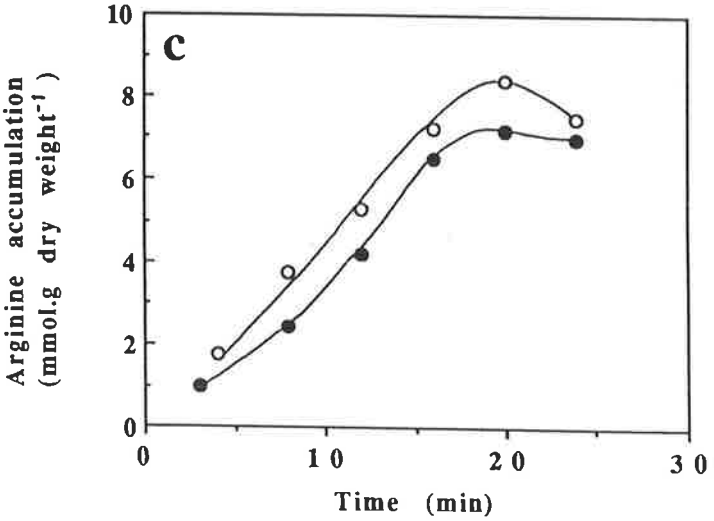
The effect of aeration on arginine accumulation was not as apparent. Cells collected after 14 hours incubation from the initially aerated culture exhibited a greater ability to accumulate arginine than the anaerobic control. As

Figure 6.1 Effect of oxygen substitutes on growth and methylamine and arginine accumulation by *Saccharomyces cerevisiae* strain 77.

Following inoculation of nitrogen-sparged synthetic grape juice medium to $1x10^6$ cells.mL^{-1}, fermentations were conducted at 25°C under a headspace of nitrogen. The medium was unsupplemented (O) or contained ergosterol (10 $mg.L^{-1}$) and Tween 80 (0.5 $mL.L^{-1}$) (●). The relative development of culture biomass was monitored (a) to the point where samples of the cultures were taken for the determination of ^{14}C-methylamine (b) and ^{14}C-arginine (c) accumulation.


a
Culture biomass (g.L-1)
10
8
6
4
2
0
0
10
20
30
40
50
Time (h)


b
Methylamine accumulation (mmol.g dry weight-1)
0.3
0.2
0.1
0.0
0
10
20
30
Time (min)


c
Arginine accumulation (mmol.g dry weight-1)
10
8
6
4
2
0
0
10
20
30
Time (min)

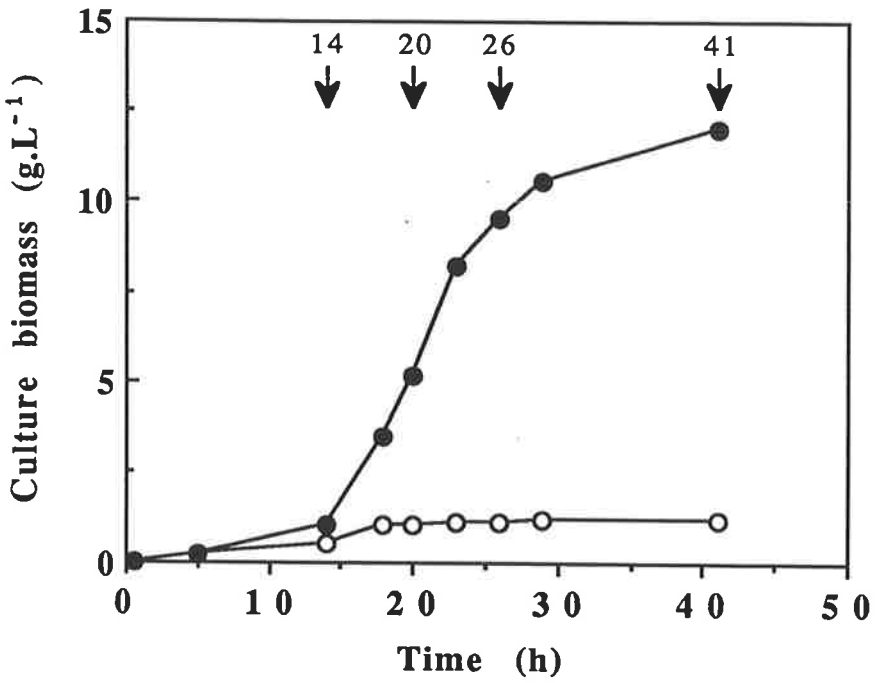
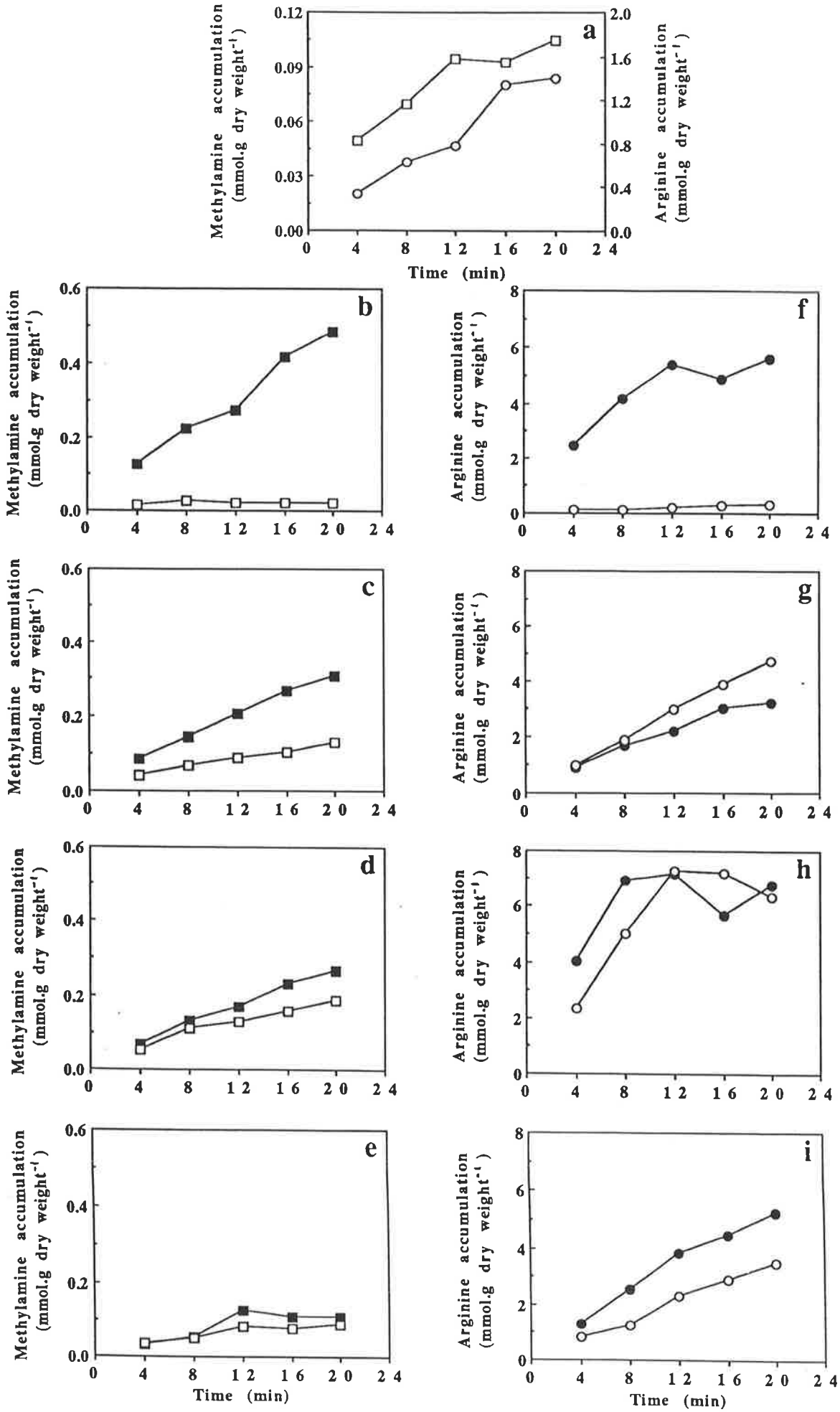


Figure 6.2 Effect of initial access to oxygen on the growth of *Saccharomyces cerevisiae* strain 77.

Following inoculation of nitrogen-sparged synthetic grape juice medium to $1x10^6$ cells.mL^{-1}, fermentations were conducted at 25°C under a headspace of nitrogen. Fermentation flasks were unsupplemented (O) or dosed with 50 mL of sterile air (●). Cells were harvested at the time points indicated by arrows and assayed for their ability to accumulate nitrogen compounds (refer to Figure 6.3).

Figure 6.3 Effect of initial access to oxygen on methylamine and arginine accumulation by *Saccharomyces cerevisiae* strain 77.

The accumulation of nitrogen compounds by cells which had been allowed initial access to oxygen (air) (●,■) was compared to that of anaerobic cultures (○,□). The ability of cells to accumulate ^{14}C-methylamine (■,□) was determined at time points 14 (b), 20 (c), 26 (d) and 41 (e) hours after inoculation. Similarly, accumulation of ^{14}C-arginine (●,○) was determined at time points 14 (f), 20 (g), 26 (h) and 41 (i) hours after inoculation. Fermentations were conducted as described for Figure 6.2.


a
b
c
d
e
f
g
h
i
Methylamine accumulation (mmol.g dry weight^{-1})
Arginine accumulation (mmol.g dry weight^{-1})
Time (min)

for methylamine accumulation, these differences were far less evident for subsequent samples.

6.3 DISCUSSION

Being poorly suppressed by assimilable nitrogen, hydrogen sulfide produced late in fermentation might be suggested to arise via an alternate mechanism to that discussed in previous chapters. However, given that oxygen appeared to ameliorate this phase of hydrogen sulfide production, it was proposed that a reduced ability to accumulate assimilable nitrogen may be involved. In this case, a resulting cellular depletion of nitrogenous precursors would invoke the same mechanism of hydrogen sulfide formation.

Several groups have reported an increased ability of cells to accumulate free amino nitrogen or specific amino acids upon being allowed access to oxygen or through enrichment with aerobically-synthesised sterols or unsaturated fatty acid residues (Haukeli and Lie, 1976; Keenan and Rose, 1979; Calderbank *et al.*, 1984, 1985). The introduction of oxygen or oxygen-substitutes to a yeast culture will generally stimulate growth (Haukeli and Lie, 1976; Aries and Kirsop, 1977; David and Kirsop, 1973), presumably by satisfying a growth-limiting deficiency of these metabolites. Growth will continue until either a second factor(s) restricts growth or the supplements are completely assimilated and the membrane content of the growth-limiting lipids again decreases through cell division.

In this study, supplementation of fermentations with oxygen-substitutes generated additional biomass. However, within the time-frame of the experiment, growth was again seen to be limited. Assuming the added lipids permitted the observed formation of an additional *ca.* 3 $g.L^{-1}$ biomass, then the corresponding ergosterol content would be of the order of 0.3%, well below that seen under optimal conditions of *ca.* 2% and approaching the suggested growth-limiting content of 0.02% (Strydom *et al.*, 1982). Accordingly, it is proposed that the failure

of the supplemented culture to differ from the control with regard to the capacity to accumulate either methylamine or arginine, is attributable to the them being sampled too long after supplementation. Thus any differences between the cultures were lost, as attested to by the fact that the growth of both cultures was being subject to limitation.

By comparison, differences were evident in relative rates of assimilable nitrogen accumulation when cultures were sampled shortly after the divergence of the growth curves and before the growth of the partially aerated culture began to be restricted. The accumulation of methylamine was particularly sensitive to decay as the fermentation progressed. This may be interpreted to indicate a strong dependence of this transporter on a high membrane content of aerobically-synthesised lipids. It is noteworthy that the rate to which methylamine accumulation declined was in good agreement to that observed in Figure 6.1 wherein cultures were sampled 45 hours after supplementation with oxygen-substitutes. Similar agreement was seen between the rates of arginine accumulation.

As for methylamine, differences in arginine accumulation between aerated and anaerobic cultures were most evident for samples taken near the point of supplementation. Arginine accumulation appeared to be more stable than that of methylamine with the progress of fermentation.

The importance of the latter point is emphasised by the fact that in the last stages of the fermentation of grape juice, typically low in assimilable nitrogen (refer to Table 1.3), the most predominant, if not only, form of this nutrient remaining is ammonium as added by the winemaker. Thus, it could be expected that the loss of the ability to effectively accumulate ammonium at a rate sufficient to meet metabolic requirements will result in a depletion of the cellular nitrogen pool and in turn the induction of hydrogen sulfide formation. In this regard, the supplementation of fermentations with selected amino acids rather than

ammonium ions may be more effective in suppressing end-of-fermentation hydrogen sulfide production. There is a need to determine the relative activity of nitrogen transport systems late in fermentation so as to identify the most efficient form of nitrogen supplementation.

It should be noted that the experimental design used in this study does not take into account the influence of ethanol on the transport of assimilable nitrogen compounds. Given the inhibitory influence of this species on nitrogen transport (Thomas and Rose, 1979) and the ability of unsaturated fatty acid residues to offer some protection against this, it is highly likely that in the fermentation environment, the accumulation of ammonium and amino acids by lipid-depleted cells will be markedly reduced.

Finally, the data presented in this chapter do not exclude other possible explanations for this phase of hydrogen sulfide formation. It will be recalled that the precursors of the sulfur-amino acids into which hydrogen sulfide is ordinarily sequestered, also possess a carbon component. Hence the depletion of these precursors due to a reduced accumulation of carbon sources may induce hydrogen sulfide formation in the same manner as nitrogen deficiency. The sensitivity of glucose transporters to nitrogen deficiencies has been described (Busturia and Lagunas, 1986; Salmon, 1989) and hence a nett effect of the combined decline in nitrogen and carbon accumulation may be the depletion of the nitrogen-containing carbon precursors of sulfur amino acids. Alternatively, such a deficiency may arise out of the effects of ethanol on the glucose transport system of yeast (Leão and van Uden, 1985) or else, purely out of a depletion of carbon upon completion of fermentation.

In conclusion, a previously unreported phase of hydrogen sulfide production has been described. Although this phase does not respond as readily to assimilable nitrogen supplements as that developing due to a nitrogen starvation during the active growth phase, the metabolic basis may be the same. It is

proposed that as a result of the depletion of aerobically-synthesised membrane lipid components, nitrogen transport, especially that mediating the uptake of methylamine and presumably ammonium, is unable to meet the cellular metabolic requirements for nitrogen. Consequently, a deficiency of the precursors of sulfur-amino acids brings about the reduced sequestration of hydrogen sulfide into methionine and cysteine biosynthesis. The results of nitrogen accumulation studies are consistent with this proposal. The likelihood of ethanol exacerbating this scenario has been suggested but needs to be confirmed.

A possible role for a carbon deficiency in the depletion of the precursors of methionine and cysteine has been discussed, though further experimental work is required to clarify this issue.

Chapter 7

Assimilable nitrogen requirements of *Saccharomyces cerevisiae* wine yeasts

7.1 INTRODUCTION

The alcoholic beverage industries are acutely aware of the intrinsic importance of assimilable nitrogen to many aspects of yeast metabolism. Attempts to quantify the requirements of yeasts for assimilable nitrogen during fermentation have largely been prompted by the association of several fermentation problems with imbalances, and in particular deficiencies, in the supply of assimilable nitrogen compounds.

Most commonly, research aimed at alleviating such deficiencies has targeted the vineyard where efforts have been made to effect gross increases in assimilable nitrogen content of grapes. Given the increased microbial instability that accompanies wines with high residual assimilable nitrogen contents, this approach together with the use of nitrogen supplements may not always be appropriate. A more desirable alternative may be to identify, alter, through the manipulation of fermentation conditions, and exploit differences between yeast strains with regard to their requirements for assimilable nitrogen. In this way, existing grape juice nitrogen contents could be more efficiently used resulting in a partial or complete reduction in the use of supplements; an important consideration for a wine industry which today is striving to minimise the use of additives while producing a wine without faults and of optimal quality.

To date, no studies have been carried out to determine the quantitative and qualitative nitrogen requirements of individual strains of *Saccharomyces cerevisiae* wine yeasts under defined conditions. Extensive efforts to establish ranges and minimum levels of assimilable nitrogen required for a complete or

satisfactory rate of fermentation have overlooked the contribution of individual yeast strains. By and large, research in this area has been preoccupied with the influence of the physical and chemical fermentation parameters while yeast strains appear to have been regarded as equal.

The potential to attribute strain-dependent aspects of hydrogen sulfide production to a strain-dependent trait of assimilable nitrogen utilisation is considerable in light of the pivotal role of nitrogen in the regulation of sulfate/sulfite reduction. Outlined in this chapter is an initial characterisation of the assimilable nitrogen requirements of several strains of *Saccharomyces cerevisiae* wine yeasts, together with an identification of influential fermentation factors. The relative fermentation efficiency of a number of yeast strains is examined under nitrogen-limited conditions.

7.2 RESULTS

7.2.1 Extent and order of amino acid and ammonium accumulation

The extent of amino acid utilisation from synthetic grape juice medium containing all amino acids in excess of yeast requirements is summarised in Table 7.1. Significant differences were evident in the degree to which individual yeast strains exploited available assimilable nitrogen during the catabolism of 1.11 M glucose from the air-saturated medium under an anaerobic headspace. For four strains, which fermented an additional 25% glucose, the mean amino acid utilisation of 405 mg $N.L^{-1}$ was increased by approximately 5% to 427 mg $N.L^{-1}$.

When presented graphically, the above data obtained for eight yeast strains grown in media containing 1.11 M glucose exhibit some noteworthy features (Figure 7.1). Irrespective of yeast strain, arginine, serine, glutamate, threonine, aspartate and lysine generally supplied the bulk of yeast nitrogen

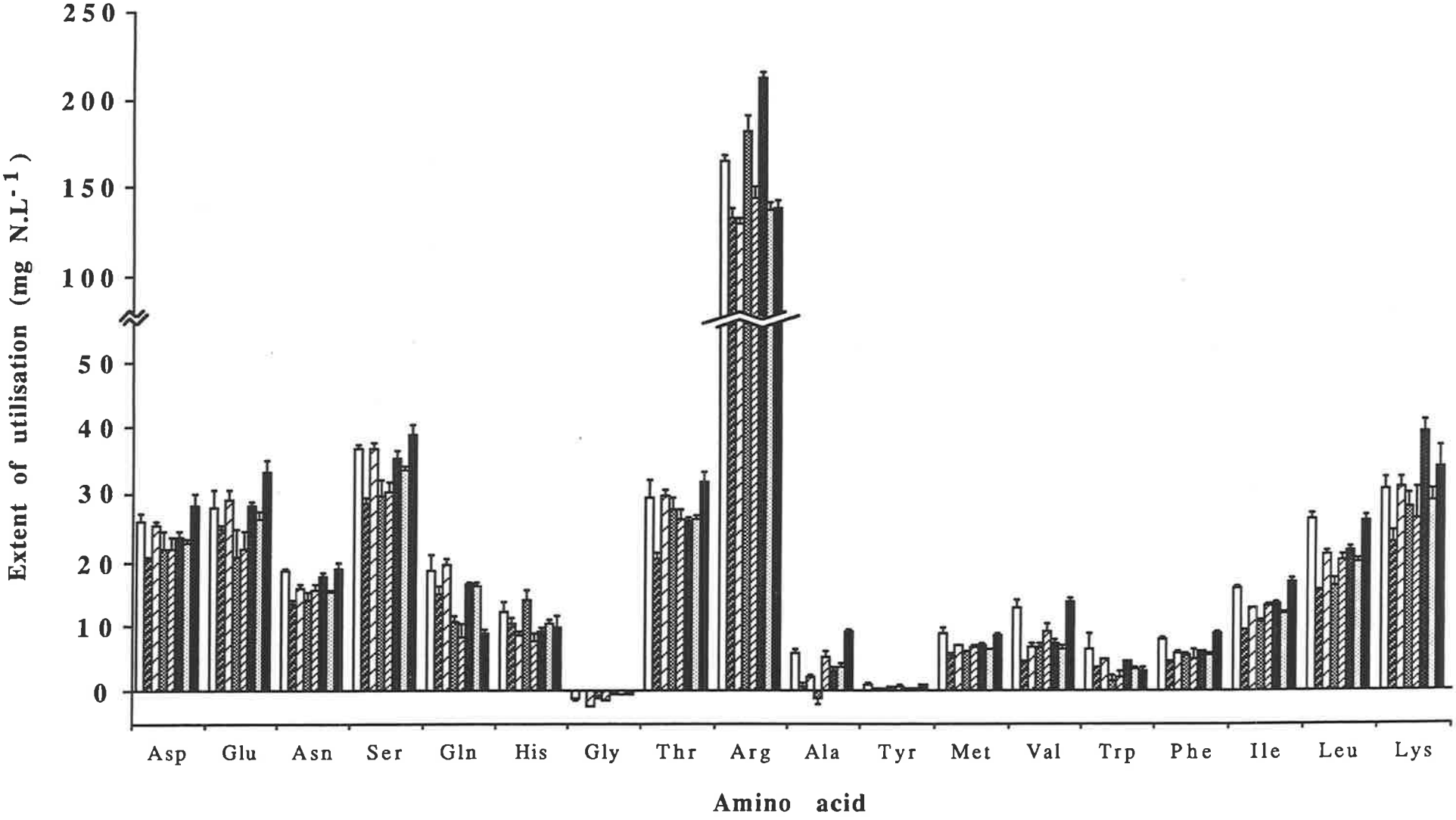
Table 7.1 Amino acid utilisation by *Saccharomyces cerevisiae* yeast strains during growth under a nitrogen headspace at two glucose concentrations.

Fermentations were conducted at 25°C in air-saturated synthetic grape juice medium under a headspace of nitrogen (medium:headspace ratio, 1:1). The medium contained 1.11 M or 1.39 M glucose and excess amino acids (20 and 25 mL amino acid stock per litre, respectively). Samples were taken at regular intervals and residual amino acids determined. Fermentations were concluded once the residual glucose content fell below 11 mM. Values quoted are the means of four determinations ± S.E.M. Totals with the same letter entered below are significantly different ($p \leq 0.05$).

Amino Acid	Amino acid utilisation (mg $N.L^{-1}$)											
	1.11 M initial glucose								1.39 M initial glucose			
Strain	**59**	**65**	**70**	**72**	**73**	**77**	**81**	**92**	**59**	**72**	**73**	**92**
Asp	25.8 ± 1.1	20.1 ± 0.3	25.2 ± 0.6	21.5 ± 2.8	21.5 ± 1.8	23.3 ± 0.9	22.4 ± 0.5	28.2 ± 1.7	17.7 ± 0.9	21.8 ± 0.4	18.6 ± 0.9	22.3 ± 0.2
Glu	27.9 ± 2.6	24.5 ± 0.7	29.1 ± 1.4	20.3 ± 4.2	21.5 ± 2.7	28.1 ± 0.7	25.9 ± 1.3	33.0 ± 2.0	18.0 ± 1.3	22.0 ± 1.5	20.2 ± 1.1	25.6 ± 0.2
Asn	18.3 ± 0.3	13.3 ± 0.5	15.6 ± 0.7	13.8 ± 1.2	15.3 ± 0.9	17.5 ± 0.6	14.9 ± 0.4	18.6 ± 0.8	17.4 ± 0.6	18.4 ± 0.0	18.2 ± 0.4	17.2 ± 1.2
Ser	36.6 ± 0.7	28.4 ± 0.8	36.7 ± 0.9	29.6 ± 2.3	30.2 ± 1.5	35.3 ± 1.1	33.5 ± 0.6	38.9 ± 1.4	30.9 ± 0.9	34.5 ± 0.3	32.6 ± 0.9	35.7 ± 0.2
Gln	18.3 ± 2.3	14.8 ± 1.2	19.2 ± 1.0	10.4 ± 0.9	8.2 ± 1.9	16.0 ± 0.3	16.0 ± 0.6	8.6 ± 0.8	18.5 ± 1.0	19.5 ± 0.2	19.9 ± 1.2	21.1 ± 0.3
His	11.9 ± 1.4	10.1 ± 0.9	8.5 ± 0.6	13.9 ± 1.3	7.3 ± 1.3	9.0 ± 0.5	10.2 ± 0.6	9.6 ± 1.8	15.1 ± 0.8	15.0 ± 0.7	11.5 ± 0.3	10.8 ± 0.2
Gly	-1.2 ± 0.4	-0.1 ± 0.0	-2.3 ± 0.1	-0.8 ± 0.5	-1.5 ± 0.0	-0.4 ± 0.1	-0.6 ± 0.1	-0.7 ± 0.1	1.7 ± 0.2	1.1 ± 0.4	1.9 ± 0.1	2.8 ± 0.2
Thr	29.3 ± 2.6	20.1 ± 0.8	29.5 ± 1.0	27.5 ± 1.6	25.9 ± 1.6	25.6 ± 0.8	26.1 ± 0.6	31.8 ± 1.2	24.8 ± 0.6	30.6 ± 0.1	25.9 ± 0.7	27.9 ± 0.1
Arg	164.7 ± 3.3	132.3 ± 5.1	128.4 ± 3.8	181.8 ± 8.1	143.2 ± 7.3	211.5 ± 3.6	136.5 ± 4.1	137.6 ± 4.2	170.6 ± 2.8	199.0 ± 1.3	161.9 ± 2.8	137.4 ± 3.3
Ala	5.7 ± 0.5	0.7 ± 0.5	2.0 ± 0.5	-1.2 ± 0.9	5.1 ± 0.9	2.9 ± 0.7	3.6 ± 0.5	8.8 ± 0.5	-2.4 ± 0.7	-0.3 ± 0.2	5.3 ± 0.5	4.9 ± 0.6
Tyr	1.0 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.7 ± 0.0	0.6 ± 0.3	0.2 ± 0.1	0.3 ± 0.1	0.9 ± 0.1	0.7 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.0
Met	8.7 ± 0.8	5.4 ± 0.3	6.7 ± 0.2	6.0 ± 0.1	6.7 ± 0.2	6.9 ± 0.2	6.2 ± 0.2	8.5 ± 0.3	8.5 ± 0.2	7.5 ± 0.1	8.2 ± 0.2	8.7 ± 0.1
Val	12.5 ± 1.2	4.3 ± 0.3	6.5 ± 6.5	6.4 ± 0.8	9.0 ± 1.2	7.3 ± 0.6	6.2 ± 0.6	13.4 ± 0.5	10.8 ± 0.4	9.0 ± 0.5	10.3 ± 0.4	12.0 ± 0.4
Trp	6.4 ± 2.2	3.5 ± 0.2	4.6 ± 0.1	1.6 ± 0.9	2.2 ± 0.9	4.5 ± 0.1	3.4 ± 0.2	2.8 ± 0.6	7.6 ± 0.1	6.1 ± 0.1	8.1 ± 0.3	7.7 ± 0.3
Phe	7.6 ± 0.4	4.2 ± 0.2	5.7 ± 0.4	5.4 ± 0.3	4.7 ± 1.6	5.8 ± 0.3	5.4 ± 0.3	8.6 ± 0.5	8.0 ± 0.2	6.6 ± 0.3	6.9 ± 0.1	7.9 ± 0.1
Ile	15.5 ± 0.5	9.2 ± 0.1	12.4 ± 0.3	10.5 ± 0.3	12.9 ± 0.3	13.1 ± 0.3	11.6 ± 0.3	16.5 ± 0.6	11.9 ± 0.4	9.6 ± 0.2	12.0 ± 0.2	13.0 ± 0.3
Leu	26.1 ± 0.7	15.1 ± 0.2	20.5 ± 0.6	15.9 ± 1.2	19.7 ± 0.8	21.1 ± 0.6	19.4 ± 0.5	25.6 ± 1.1	22.6 ± 0.8	17.6 ± 0.6	21.0 ± 0.3	22.6 ± 0.7
Lys	30.5 ± 1.6	22.4 ± 1.8	30.7 ± 1.6	27.8 ± 2.2	25.9 ± 4.7	39.3 ± 1.7	28.6 ± 1.9	33.7 ± 3.4	37.6 ± 0.8	35.6 ± 0.4	34.0 ± 1.4	36.4 ± 0.8
TOTAL	446 ± 12	328 ± 12	379 ± 11	391 ± 22	358 ± 12	467 ± 10	369 ± 12	424 ± 11	420 ± 11	454 ± 5	417 ± 8	415 ± 2
	bhpvw	abcdef	ghi		ikvxyz	agjklmno	jpqrstu	clqx	dmry	s	entz	fouw

Figure 7.1 Amino acid utilisation by *Saccharomyces cerevisiae* yeast strains during growth under a nitrogen headspace.

Strains 59 (□), 65 (▨), 70 (◪), 72 (▩), 73 (◪), 77 (■), 81 (▩) and 92F (■) were grown in synthetic grape juice medium containing 1.11 M glucose under the fermentation conditions described for Table 7.1. Values quoted are the mean of four determinations ± S.E.M.


250
200
150
100
50
40
30
20
10
0
Extent of utilisation (mg N.L-1)
Asp
Glu
Asn
Ser
Gln
His
Gly
Thr
Arg
Ala
Tyr
Met
Val
Trp
Phe
Ile
Leu
Lys
Amino acid

requirements, with arginine alone contributing between 32.5 and 46.5% of the total nitrogen utilised. Typically, glycine was excreted during fermentation.

Apart from a dependence on yeast strain and initial glucose content of the medium, preliminary investigations revealed assimilable nitrogen requirements to be increased by allowing the culture initial access to oxygen (Table 7.2). Mean nitrogen utilisation was increased by 68% from 398 mg $N.L^{-1}$ to 670 mg $N.L^{-1}$ for five strains examined under the two degrees of aeration. Times required to attain the complete catabolism of glucose (1.11 M) were markedly reduced for fermentations initiated with a headspace of air (Table 7.3). Increasing initial glucose content by 25% produced a 12% higher mean assimilable nitrogen utilisation of 814 mg $N.L^{-1}$. In all cases, arginine remained the single greatest contributor to yeast nitrogen requirements. Supplementation of air-headspace fermentations to 155 mM ammonium reduced amino acid removal but increased nett assimilable nitrogen utilisation by *ca.* 20% in the case of strain 72.

Temporal studies revealed that the accumulation of amino acids from the medium by strains 77 and 72 occurred in an ordered manner (Figures 7.2 and 7.3). The inclusion of ammonium in the medium brought about the delayed and/or reduced accumulation of several amino acid species, glutamate, arginine, glycine, valine and phenylalanine being especially sensitive. Based on the time-lag before onset of accumulation and the extent of accumulation or the time taken for the initial concentration to be reduced by 50%, amino acids and ammonium were classified into three accumulation groups (Table 7.4). The mean kinetics of removal for the three accumulation groups are shown for strains 72 and 77 (Figure 7.4). The greatest influence of ammonium supplementation was effected on the accumulation kinetics of the group B and C amino acids.

Table 7.2 Amino acid utilisation by *Saccharomyces cerevisiae* yeast strains during growth at two glucose concentrations with initial access to oxygen.

Fermentations were conducted at 25°C in air-saturated synthetic grape juice medium under a headspace of air (medium:headspace ratio, 1:1). The medium contained 1.11 M or 1.39 M glucose and excess amino acids (40 and 50 mL amino acid stock per litre, respectively). Samples were taken at regular intervals and residual amino acids determined. Fermentations were concluded once the residual glucose content fell below 11 mM.

[a] Values extrapolated from incomplete fermentation.

Amino Acid	Amino acid utilisation (mg $N.L^{-1}$)									
	1.11 M initial glucose						1.39 M initial glucose			
Strain	**59**	**65[a]**	**72**	**73**	**77**	**108**	**59**	**72**	**73**	**77**
Asp	40.4	28.5	44.7	34.8	33.2	32.4	50.1	42.0	40.4	40.4
Glu	51.4	28.7	53.0	40.1	34.0	35.4	58.7	40.3	38.7	36.1
Asn	29.2	18.6	24.3	25.1	23.2	25.1	36.7	30.2	30.4	33.1
Ser	51.5	38.5	58.8	48.7	47.3	47.9	72.2	63.3	52.0	62.9
Gln	30.8	18.2	31.8	26.1	26.1	22.0	32.3	28.5	29.1	37.5
His	20.4	11.7	20.4	16.8	17.7	20.1	27.8	20.2	14.3	21.0
Gly	-2.0	0.1	-1.8	-1.4	-0.7	0.4	-1.5	-0.9	-6.3	0.2
Thr	37.3	28.9	41.1	33.9	33.7	32.4	48.3	40.5	42.9	42.4
Arg	287.0	169.0	315.0	276.0	302.0	193.0	350.0	312.0	331.0	348.0
Ala	9.7	0.1	1.2	8.8	5.3	9.9	9.3	10.2	-3.3	8.8
Tyr	1.1	0.4	1.5	0.8	0.5	1.4	1.6	0.9	-0.5	1.9
Met	12.1	9.1	11.7	11.0	10.9	11.4	15.4	13.3	12.7	13.6
Val	22.0	9.1	20.5	16.6	14.3	19.6	25.9	18.4	18.9	17.9
Trp	5.1	2.9	3.6	3.6	3.9	2.9	5.8	3.4	5.8	5.8
Phe	13.4	7.0	12.9	9.4	9.0	12.5	16.0	10.5	11.8	10.6
Ile	22.4	22.4	21.8	18.9	17.6	20.0	27.2	22.3	21.4	21.1
Leu	40.8	23.8	38.9	33.1	32.2	37.3	52.3	39.9	41.8	40.4
Lys	85.1	38.4	80.5	66.1	78.8	61.0	90.5	68.4	65.5	84.9
TOTAL	758	455	780	668	689	585	918	763	746	827

Table 7.3 Influence of initial access to oxygen on fermentation duration.

Yeast strain	Total fermentation time (h)	
	Nitrogen headspace	Air headspace
59	89.0	55.5
72	91.0	38.5
73	75.5	40.0
77	74.0	44.0

Fermentations were conducted in synthetic grape juice medium containing 1.11 M glucose under the conditions described for Tables 7.1 and 7.2.

Figure 7.2 Evolution of amino acids and ammonium ions during fermentation of synthetic grape juice medium by *Saccharomyces cerevisiae* strain 72.

Fermentations were conducted at 25°C in synthetic grape juice medium containing 1.11 M glucose and limited nitrogen (12.5 mL amino acid stock per litre) alone (a-c) or together with ammonium ions (155 mM; d-f). Fermentations were concluded once the residual glucose content fell below 11 mM. Values quoted are the mean of four determinations.

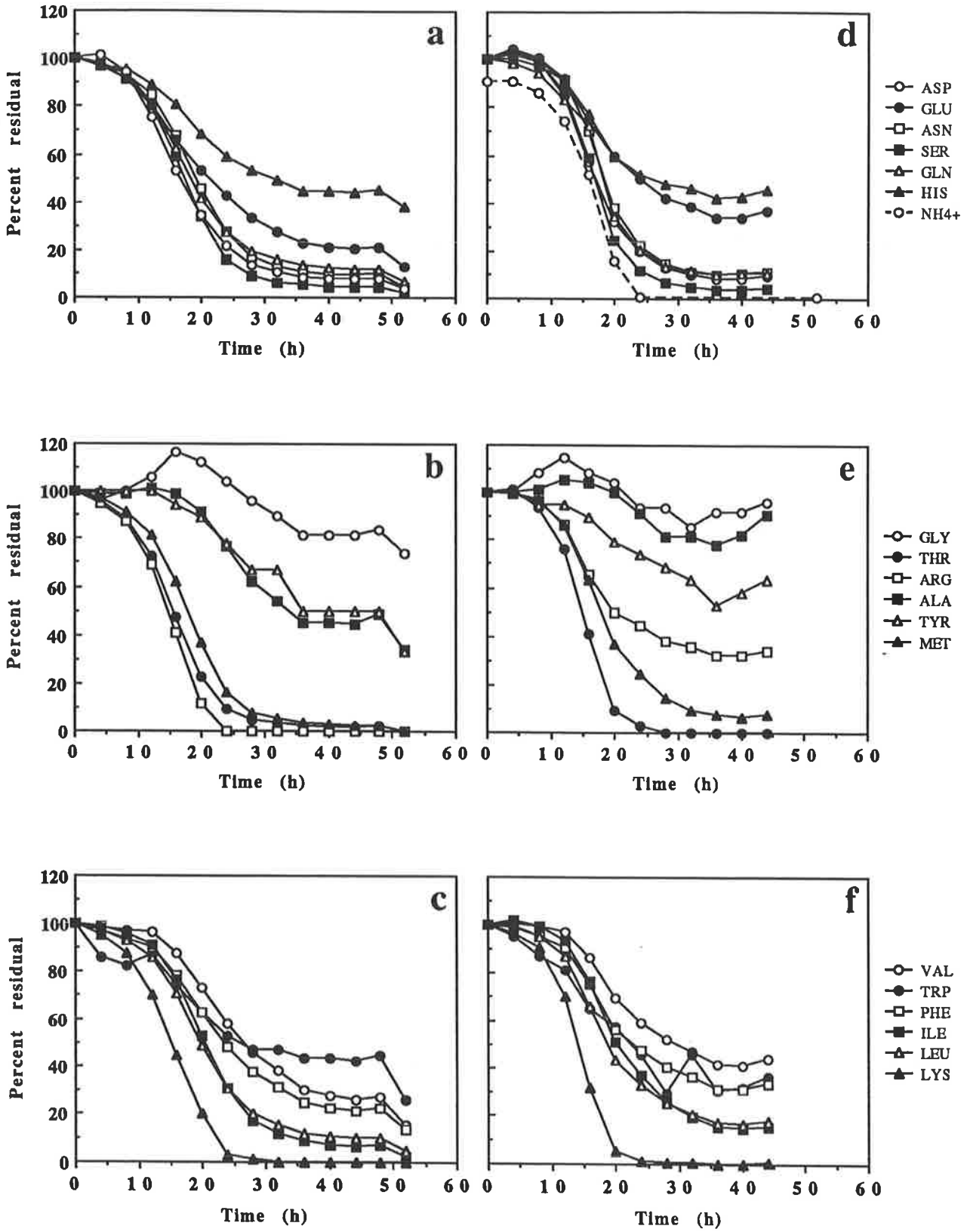

a
b
c
d
e
f
Percent residual
Time (h)
0
10
20
30
40
50
60
80
100
120
ASP
GLU
ASN
SER
GLN
HIS
NH4+
GLY
THR
ARG
ALA
TYR
MET
VAL
TRP
PHE
ILE
LEU
LYS

Figure 7.3 Evolution of amino acids and ammonium ions during fermentation of synthetic grape juice medium by *Saccharomyces cerevisiae* strain 77.

Fermentations were conducted at 25°C in synthetic grape juice medium containing 1.11 M glucose and limited nitrogen (12.5 mL amino acid stock per litre) alone (a-c) or together with ammonium ions (155 mM; d-f). Fermentations were concluded once the residual glucose content fell below 11 mM. Values quoted are the mean of four determinations.

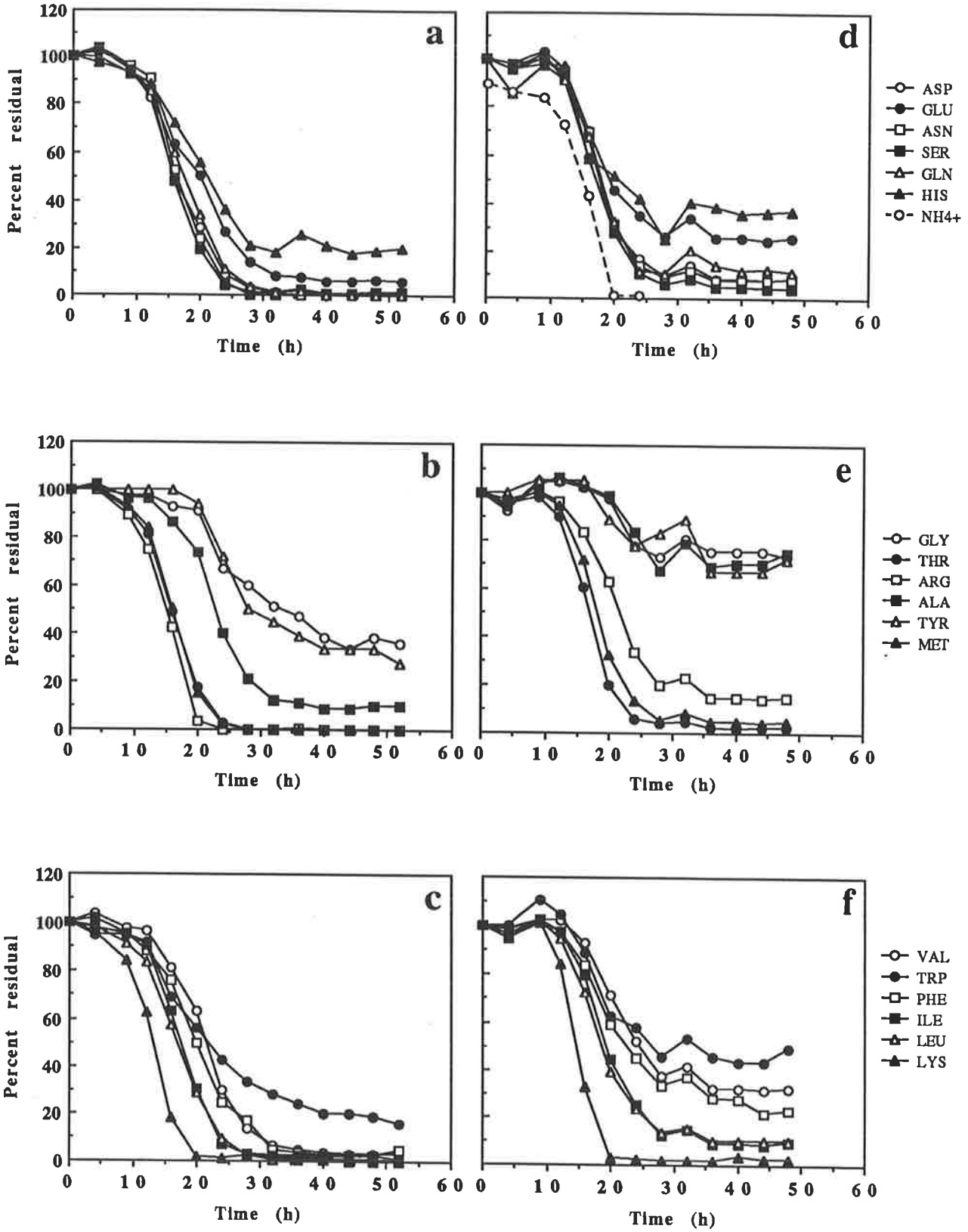

a
b
c
d
e
f
Percent residual
Time (h)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
ASP
GLU
ASN
SER
GLN
HIS
NH4+
GLY
THR
ARG
ALA
TYR
MET
VAL
TRP
PHE
ILE
LEU
LYS

Table 7.4 Classification of amino acids and ammonium ion according to the time-course of removal from synthetic grape juice medium by *Saccharomyces cerevisiae* strains 72 and 77.

Group A	Group B	Group C
Ammonium	Alanine	Glycine
Arginine	Glutamate	Tryptophan
Asparagine	Histidine	Tyrosine
Aspartate	Phenylalanine	
Glutamate	Valine	
Isoleucine		
Leucine		
Lysine		
Methionine		
Serine		
Threonine		

Fermentations conditions were as described for Figures 7.2 and 7.3.

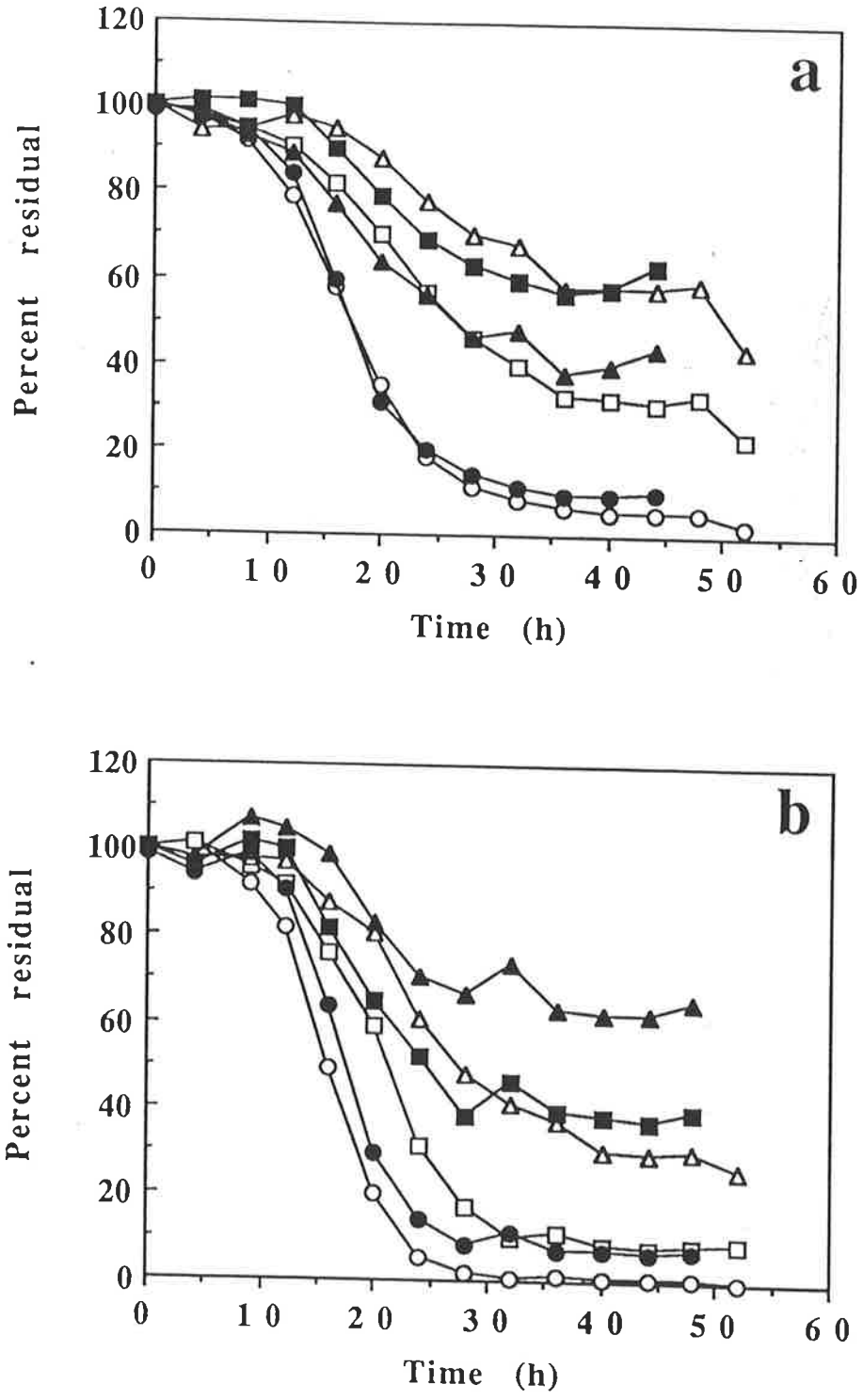


Figure 7.4 Mean kinetics of accumulation for Group A (○,●), Group B (□,■) and Group C (△,▲) nitrogen compounds during growth in the presence (solid symbols) or absence (open symbols) of ammonium ions (155 mM) by *Saccharomyces cerevisiae* strains 72 (a) and 77 (b).
Experimental conditions are as described for Figures 7.2 and 7.2.

7.2.2 Responses to nitrogen-limitation

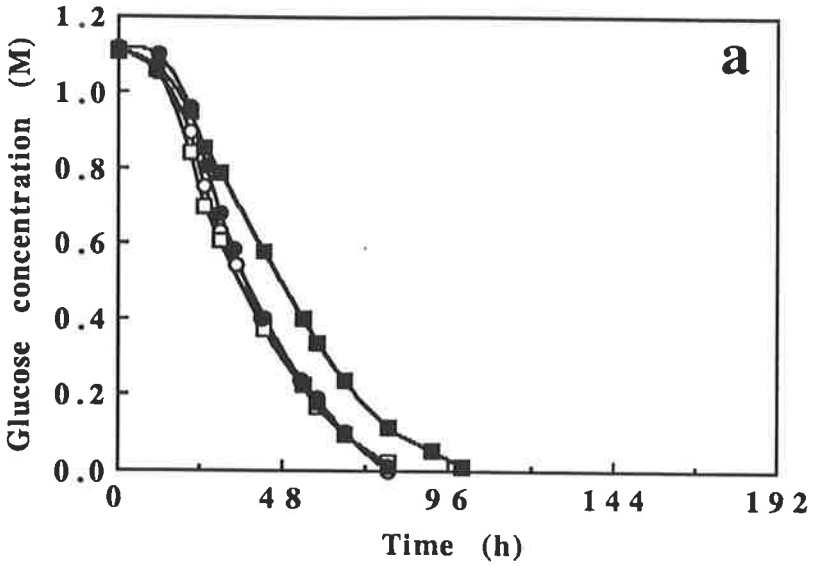
Four yeast strains were compared for their glucose catabolic rates during growth in synthetic grape juice media containing nitrogen (as ammonium ions) at 390 mg $N.L^{-1}$ and 78 mg $N.L^{-1}$. The maximum glucose catabolic rates during growth in the high-nitrogen media were essentially the same for three of the four yeast strains, ranging between 22 to 27 $mM.h^{-1}$, whereas the fourth stain, 81, lagged, with a rate of 17 $mM.h^{-1}$ (Figure 7.5). Conversely, under nitrogen-limited conditions, greatly reduced catabolic rates of 4.0, 6.6, 3.8 and 4.8 $mM.h^{-1}$ were evident for strains 65, 72, 77 and 81, respectively. The rates and extent of glucose catabolism under nitrogen-limited conditions did not correlate with the observed demand for amino acids as detailed in Table 7.1.

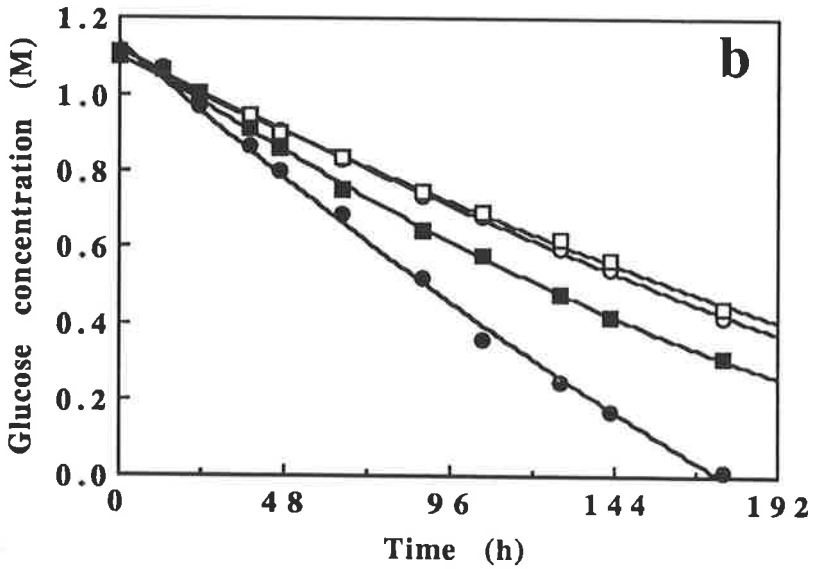
Differences in the initial ammonium content of the fermentation medium were reflected in the maximum biomass of the four strains, being *ca.* 6 g dry $weight.L^{-1}$ (data not shown) at 390 mg $N.L^{-1}$ and *ca.* 4 g dry $weight.L^{-1}$ at 78 mg $N.L^{-1}$ (Figure 7.5). The observed variations in fermentation rates under nitrogen-limited conditions were not attributable to differences in biomass production.

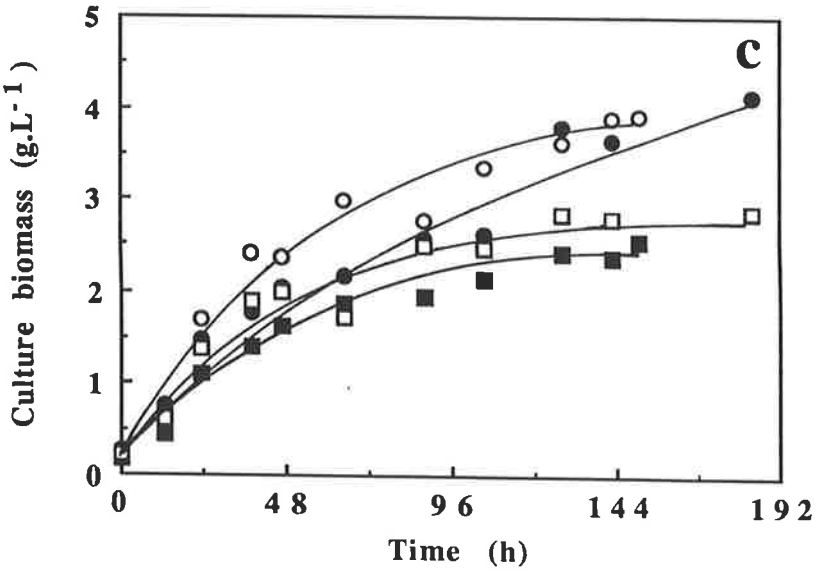
A range of rates and extents of glucose catabolism analogous with that observed between yeast strains could be produced for a single yeast strain by initiation of fermentation with differing amounts of assimilable nitrogen (Figure 7.6). Maximal fermentation rates were evident in media with higher initial ammonium concentrations. Fermentation rates were seen to decrease shortly after the depletion of ammonium from the medium. Since media containing an initial ammonium ion content of 33.3 mM provided assimilable nitrogen in excess of yeast requirements, a total nitrogen utilisation of 30.1 mM (420 mg $N.L^{-1}$) could be determined for the catabolism of 1.11 M glucose.

Figure 7.5 Glucose catabolism (a, b) and growth (c) of *Saccharomyces cerevisiae* strains during growth in limited-nitrogen media.

Strains 65 (○), 72 (●), 77 (□) and 81 (■) were grown at 25°C in air-saturated synthetic grape juice medium containing 1.11 M glucose and an initial ammonium concentration of 390 mg $N.L^{-1}$ (a) or 78 (b,c) mg $N.L^{-1}$.


a
Glucose concentration (M)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
48
96
144
192
Time (h)


b
Glucose concentration (M)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
48
96
144
192
Time (h)


c
Culture biomass (g.L-1)
5
4
3
2
1
0
0
48
96
144
192
Time (h)

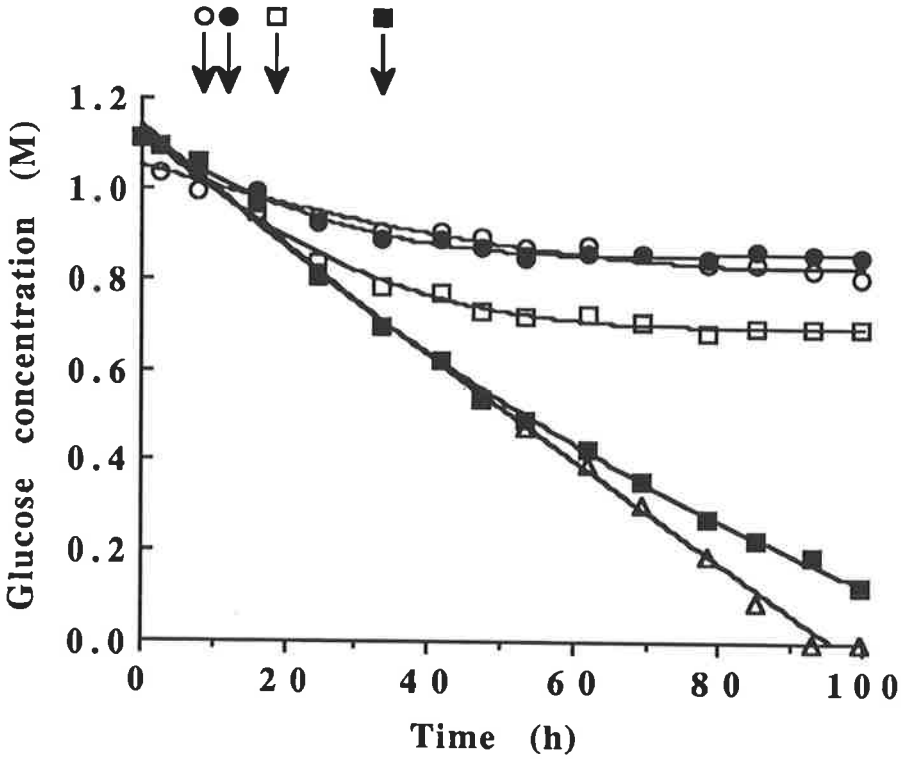


Figure 7.6 Influence of initial assimilable nitrogen concentration on glucose catabolism by *Saccharomyces cerevisiae* strain 77.

Cultures were grown at 25°C in synthetic grape juice medium which initially contained ammonium ion concentrations of 47 (○), 117 (●), 233 (□), 349 (■) and 466 (△) mg $N.L^{-1}$. The point of ammonium ion depletion is indicated (labelled arrows).

7.3 DISCUSSION

A temporal study of amino acid accumulation was initially undertaken with the aim of identifying amino acids which may be important to the suppression of hydrogen sulfide formation. The observation that most amino acids are as effective as ammonium in suppressing hydrogen sulfide production (refer to Chapter 4) suggests that individual amino acids have no special importance beyond their role as nitrogen sources. Jones and coworkers (1969) confirmed this through radiolabelling experiments which emphasised the high degree of transamination reactions which occur upon absorption of amino acids. Obvious exceptions to this proposal are the amino acids such as methionine, cysteine and threonine which are directly involved in the biosynthesis or regulation of the sulfur amino acids (de Robichon-Szulmajster and Corrivaux, 1963; Cherest *et al.*, 1971; Wainwright, 1971; Cherest *et al.*, 1973). Invariably these amino acids do not occur in significant concentrations in grapejuices to be to be of concern in commercialfermentations (Refer to Table 1.3).

The observed order of amino acid accumulation could, therefore, be expected to be a reflection of the ease with which these species are transported and degraded to supply the nitrogen requirements of the cell, that is, their relative efficiency as nitrogen sources. Strengthening this proposal is the existence of a highly significant correlation ($r = 0.69$; $p \leq 0.01$) between culture doubling times during growth on a single amino acid nitrogen source (refer to Table 1.7) and the times reported in the present study for the corresponding amino acid to attain 50% depletion (Table 7.5). Thus, in general, the more efficient sources of nitrogen are preferentially accumulated earlier in fermentation.

As an example, assuming that all of the nitrogen atoms in the arginine molecule can be utilised by yeast, arginine represents an efficient nitrogen source from a transport point of view (Seaston *et al.*, 1973). This fact may account for the observation that arginine is absorbed both early in fermentation and in

Table 7.5 Kinetics of absorption of nitrogen compounds during fermentation by *Saccharomyces cerevisiae* strain 77.

Nitrogen compound	Hours to 50% depletion		Relative position		Group
Alanine	29	(>50)	11	(13)	B
Ammonium	-	(16)	-	(2)	A
Arginine	15	(21)	2	(7)	A
Asparagine	18	(18)	5	(4)	A
Aspartate	17	(17)	4	(3)	A
Glutamate	21	(22)	7	(8)	B
Glutamine	17	(19)	4	(5)	A
Glycine	>42	(>50)	13	(13)	C
Histidine	27	(24)	10	(10)	B
Ileucine	19	(20)	6	(6)	A
Leucine	19	(19)	6	(5)	A
Lysine	14	(15)	1	(1)	A
Methionine	17	(18)	6	(4)	A
Phenylalanine	22	(23)	8	(9)	B
Serine	17	(17)	4	(3)	A
Threonine	16	(16)	3	(2)	A
Tryptophan	24	(25)	9	(11)	C
Tyrosine	32	(>50)	12	(13)	C
Valine	24	(27)	9	(12)	B

Fermentations conditions were as described for Figure 7.3.

large amounts such that it represents the primary source of nitrogen irrespective of yeast strain or fermentation conditions used.

By comparison, many of the amino acids of Group C, whose accumulation is largely mediated by the general amino acid permease, contain fewer nitrogen atoms and are symported with two protons (Eddy, 1978, 1982). The presence and activity of the various amino acid transport systems has been put forward as a physiological basis for the observed order of amino acid accumulation (Rose and Keenan, 1981; Bisson, 1991). The kinetics of accumulation will therefore be determined by the nett result of the interaction of factors such as permease substrate affinities (K_m), maximum transport rates (V_{max}) (refer to Table 1.6) as well as the degree and timing of transporter expression, as affected by nitrogen catabolite repression (see review by Wiame *et al.*, 1985), trans- and feedback inhibition (Cooper, 1982b) and sensitivity to ethanol toxicity.

It is interesting to note that despite there being differences in medium composition, yeast strains and fermentation conditions, the order of amino acid removal from synthetic grape juice medium (Table 7.5) shared many similarities to those sequences quoted in or derived from earlier reports from both brewing and oenological researchers (refer to Table 1.4). This tends to suggest that the observed absorption kinetics can not be attributed to the specific conditions used here, but rather, are a trait common to several yeast strains. The failure of brewing researchers to recognise strain variations in the pattern of amino acid utilisation may be due to the relative uniformity of wort compared with the high variability of grape juices. Recently, Bisson (1991) described a pattern of amino acid consumption from grape juice wherein the onset of accumulation of all amino acids from grape juice occurred in the early stages of fermentation. Only ammonium, arginine, alanine and γ-amino butyric acid were still being accumulated in the latter stages of fermentation, though this appears to be primarily a consequence of the high initial concentration of these species in the must.

One of the more important points to arise out of this study of the kinetics of amino acid accumulation, is the fact that ammonium supplementation of the medium delayed and reduced the accumulation of arginine. In light of the involvement of the arginine catabolite, urea, in ethyl carbamate formation (Ough, 1976; Ough *et al.*, 1988; Montiero *et al.*, 1989), ammonium supplementation has the potential to influence the evolution of this potential carcinogen. Henschke and Ough (1991) demonstrated that for arginine rich musts with high total assimilable nitrogen contents, arginine catabolism leads to urea excretion by susceptible strains, the re-metabolism of which did not occur unless total assimilable nitrogen content became low. The inhibition of urea transport by good nitrogen sources may explain the delayed uptake of urea (Cooper and Sumrada, 1975).

It may be concluded from the work of Henschke and Ough (1991) that the supplementation of fermentations with progressively greater amounts of ammonium could be expected to increasingly delay the arginine catabolism/urea secretion and re-metabolism phenomenon. Thus, for lower ammonium:arginine ratios, arginine catabolism is delayed such that urea re-metabolism is not effected before the completion of fermentation leaving high residual urea concentrations. Conversely, for higher ammonium:arginine ratios (requiring degrees of supplementation only permissible in the U.S.A.) arginine accumulation and catabolism is so delayed that very little urea is formed. Clearly additional work is required before the complex interactions which are taking place are understood sufficiently to allow the manipulation of conditions to minimise the formation of urea and hence, potentially, ethyl carbamate.

Another area of possible future research interest is the modification of wine aroma profile by influencing the metabolism of precursors of aroma-active compounds. Amino acid examples of such precursors include alanine, serine, valine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan and γ-amino butyric acid (reviewed by Rapp and Versini, 1991). Alterations in the metabolism

of these amino acids may be possible by influencing the rate and extent of amino acid accumulation through appropriate degrees and timing of ammonium or amino acid supplementation of the fermentation.

This study appears to be the first systematic comparison of *Saccharomyces cerevisiae* wine yeast strains for their individual amino acid-nitrogen demand. The results show that yeast strains are not uniform in this characteristic, but instead, possess significant differences. In the past, researchers have been primarily concerned with the effect of initial glucose concentration on nitrogen requirements. The influence of yeast strain was not considered and is no doubt an explanation for some of the differences seen between the estimates of nitrogen demand listed in Table 1.5.

Dittrich and Sponholz (1975) are one of a few groups to provide information relating to amino acid utilisation by yeast from a medium (grape must) containing all amino acids in excess. A demand of 174 mg $N.L^{-1}$ could be calculated from their data. Although low in comparison with the values determined here, Ough's group (1991) have demonstrated that this may be partly attributable to the lower fermentation temperature (20°C) used by Dittrich and Sponholz (1975). The work of Ough and coworkers (1991) similarly permits the determination of extents of assimilable nitrogen utilisation under nitrogen-excess conditions. Calculation of mean values for the four Californian wine yeasts studied, Epernay, Flor, Prise de Mouse and Montrachet, gave nitrogen consumptions of 392, 402, 449 and 473 mg $N.L^{-1}$, respectively. The values reported for Montrachet are likely to be an underestimate given that four of the eleven musts used in this study did not contain an excess of all amino acids. Nonetheless, these determinations are in good agreement with the findings detailed here.

The existence of such differences in nitrogen demand has great potential in the food and alcoholic beverage industries as a alternative approach to dealing with fermentation problems stemming from a deficiency of assimilable nitrogen.

Given that here, the eight strains examined under anaerobic conditions differed in their nitrogen demands by as much as 140 mg $N.L^{-1}$, the winemaker is presented with considerable scope for ensuring the unhindered fermentation of grape must of low assimilable nitrogen content possibly without the need for nitrogen supplements. Since the present study is not an exhaustive survey, it is highly likely that the described range of nitrogen demands will be broadened as more strains are included.

A comparison of relative assimilable nitrogen utilisation by individual yeast strains and the corresponding extent or maximum rates of hydrogen sulfide production seen upon nitrogen depletion of the medium (refer to Table 4.3) showed there to be no correlation ($r = 0.22$). Therefore the hypothesis that the propensity of a yeast strain to produce hydrogen sulfide is related to its relative demand for assimilable nitrogen, must be rejected. Instead it is proposed that nitrogen demand merely determines how soon, in a limited-nitrogen fermentation, a yeast strain experiences nitrogen starvation and in turn is induced to produce hydrogen sulfide, the rate and extent of production being dictated by another factor, namely, the characteristic level of sulfite reductase activity. Thus while not influencing specific aspects of hydrogen sulfide production, nitrogen demand remains an important characteristic of yeast which warrants further definition in light of the its function in determining the development of hydrogen sulfide as such.

Of great interest are factors which increase the ratio of nitrogen utilised:glucose catabolised. Ammonium ion supplementation and initial aeration of the culture proved to be two such factors. The current report of oxygen related increases of 68% in nitrogen consumption confirms the work of Ingledew and Kunkee (1985) who demonstrated increases of between 33 and 190%. While initial access to oxygen dramatically reduces fermentation time, this advantage may be outweighed by the associated increase in yeast biomass and the greater consumption of nitrogen for the fermentation of the same quantity of glucose.

The latter will have serious ramifications for the fermentation of a must of limited nitrogen content.

At this stage it is difficult to separate the cause and effect relationship of the concomitant increases in nitrogen utilisation and yeast growth seen with ferment aeration. However, it appears that oxygen acts by stimulating the oxygen-dependent synthesis of growth-limiting sterols and unsaturated fatty acids (Strydom *et al.*, 1982; Ingledew and Kunkee, 1985; Larue and Lafon-Lafourcade, 1989; Mauricio *et al.*, 1991). Consequently, by aiding in the maintenance of membrane integrity and an optimisation of membrane-bound transport systems, nitrogen carriers included (reviewed by Henschke and Rose, 1991), cellular metabolic process are sustained for longer to bring about increased yeast growth and thus, increased nitrogen demand. Controlling the aeration of fermentations is therefore an important step by which the winemaker can ensure that optimal use of the existing nitrogen content of must is made by the inoculated yeast.

Supplementation of the medium with ammonium (as diammonium hydrogen phosphate) also increased yeast nitrogen demand. Although the extent of accumulation of numerous amino acids was decreased, the complete utilisation of the added ammonium produced a nett increase in nitrogen consumption compared with an unsupplemented fermentation. As has been discussed above, the Group B and Group C amino acids were especially prone to delayed/reduced accumulation brought about by ammonium supplementation, a fact which appears related to the lower relative efficiencies of these groups of nitrogen sources. Thus it is suggested that by preferentially utilising ammonium, yeast growth is stimulated which produces an overall increase utilisation of assimilable nitrogen.

The full significance of these findings remains unclear. Some caution may be advisable when supplementing fermentations with large amounts of

ammonium particularly in the early stages of fermentation when a greater proportion of nitrogen is likely to be diverted to biomass formation (Agenbach, 1977; Bely *et al.*, 1990). Although the biomass/metabolic activity of the culture is sufficient to complete fermentation without further nitrogen supplements, if further additions of nitrogen are not made or are restricted by legal limits, nitrogen depletion will induce the formation of hydrogen sulfide. Based on the findings in Chapters 4 and 5, the amounts of hydrogen sulfide formed by these cultures will be high given the large biomass and high metabolic activity of the supplemented culture.

The increase in nitrogen utilisation which accompanies the fermentation of larger quantities of sugar has been frequently observed (refer to Table 1.5; Bely *et al.*, 1990; Ough *et al.*, 1991). The mean increase of 5% (22 mg $N.L^{-1}$) reported here for the anaerobic fermentation of an additional 25% (0.28 M) glucose in an air-saturated medium, compares with the 30 mg $N.L^{-1}$ increase described by Agenbach (1977) for a similar variation in sugar concentration. The extra consumption of nitrogen may reflect the greater need for maintenance of carbon catabolic machinery as the result of extended fermentation. Therefore, selection of a suitable strain for the fermentation of a grape must of known nitrogen content should take into account grape must sugar content (Ingledew and Kunkee, 1985).

Estimates of nitrogen requirements of yeast have in the past been determined according to two criteria. Maximal or total values are based on the actual or the predicted extent of assimilable nitrogen utilisation which enables maximal growth and/or sugar catabolic rates to be achieved (Agenbach, 1977; Vos *et al.*, 1979; Ingledew and Kunkee, 1985). Alternatively, these values are determined under conditions of nitrogen excess when nitrogen utilisation is not restricted by nitrogen availability but rather some other growth-limiting factor, such as membrane lipid content. Minimum nitrogen requirements on the other hand, perhaps the more useful measurement, are indications of the levels of

assimilable nitrogen below which the completion of fermentation or the maintenance of a satisfactory rate of fermentation, is not achieved.

Minimum nitrogen requirements fall between 70 and 190 mg $N.L^{-1}$ for the fermentation of media with sugar concentrations in the range of 0.62 to 1.29 M as glucose (refer to Table 1.5). More recently, the figure has been put at 140 mg $N.L^{-1}$, based on both grape must and defined media fermentations (Bely *et al.*, 1990, 1991). It is not surprising, therefore, that the fermentation of synthetic grape juice medium of moderate nitrogen content (390 mg $N.L^{-1}$) revealed little differences between yeast strains, all of which completed fermentation in much the same time and at a satisfactory rate. To a certain extent, these observations are a reflection of the fact that these wine yeast strains have been selected for this ability. By comparison, under nitrogen-limited conditions (78 mg $N.L^{-1}$), there emerged differences in the ability of these strains to complete fermentation.

In agreement with Agenbach (1977), differences in fermentation rates seen between the higher and lower initial nitrogen contents were directly proportional ($r = 0.96$; $p \leq 0.01$) to the difference in initial nitrogen content. Despite being reduced by about five-fold, the fermentation rate seen in the lower nitrogen-containing media would not be regarded as being 'sluggish' or 'stuck' as judged by general winemaking criteria. These descriptors are in fact rarely defined in objective terms, Houtman and du Plessis (1986) being one of the few groups to have done so. Accordingly, their threshold rate for a slow fermentation is 2.2 $mM.h^{-1}$ while that for a sluggish fermentation is 1 $mM.h^{-1}$.

By definition a 'stuck' fermentation is one in which sugar catabolism has ceased. Thus although often referred to as such, stuck fermentations are not commonly described in the literature. Salmon (1989) showed that upon nitrogen depletion, fermentation proceeded at an ever decreasing rate but he did not report truly stuck fermentations. In fact, Bely and coworkers (1990) only demonstrated a complete cessation of fermentation when initiated with a nitrogen content of

below 37 mg $N.L^{-1}$; one quarter the minimum requirement for satisfactory fermentation. In this study, the initial nitrogen content at which a stuck fermentation developed was in the vicinity of less than 60 mg $N.L^{-1}$.

In accord with Salmon (1989), the initiation of fermentations with progressively decreasing amounts of assimilable nitrogen resulted in a corresponding decline of fermentation rate upon nitrogen depletion. While fermentation continued after the disappearance of assimilable nitrogen from the medium for initial nitrogen concentrations of greater than 60 mg $N.L^{-1}$, it is important not to lose sight of the fact that the formation of hydrogen sulfide was also induced at this point (refer to Figures 7.6 and 4.10).

This report seems to be the first to describe the existence of differences between yeast strains with regard to their relative rates of glucose catabolism upon the depletion of assimilable nitrogen. The belief that strains with a low demand for nitrogen under nitrogen-excess would have a greater ability to complete fermentation under nitrogen-limited conditions was not substantiated since fermentation rates after nitrogen depletion were not constant across the strains examined. Furthermore, the fermentation rates that were observed at this point did not show a simple inverse relationship to the extent to which nitrogen was diverted to biomass formation by these strain. The direct quantitation of cellular protein content or assimilated nitrogen, rather than culture dry weight is likely to be more appropriate in clarifying this last point.

Thus while yeast nitrogen demands under nitrogen-excess may be indicative of how early in the fermentation of a nitrogen-limited must this nutrient will become depleted, resulting in hydrogen sulfide formation, the ability to complete fermentation once this has occurred does not appear to be determined simply by the biomass which has developed at this point, but rather by the ability of strains to retain a fermentative capacity. The latter is most probably being linked to the half-lives of the glucose transporters (Busturia and

Lagunas, 1986; Salmon, 1989), the catabolic enzymes themselves or the efficiency of nitrogen cycling.

In summary, several aspects of the metabolism of and requirement for assimilable nitrogen compounds by yeast have been characterised in terms of their importance to yeast growth, fermentation and hydrogen sulfide production. Yeast strains differ in the quantities of assimilable nitrogen, as amino acids and ammonium, which they utilise under conditions of nitrogen-excess. Given that the use of supplements of assimilable nitrogen during wine production is most likely to be further restricted by legislation, a viable alternative method for effecting the complete fermentation of nitrogen deficient juices and/or ensuring low residuals of nitrogen is the use of low nitrogen-requiring yeast strains. Control of fermentation aeration and the judicious use of ammonium supplements will further facilitate the optimal use of existing grape juice nitrogen content.

Demands for nitrogen also showed no correlation with the propensity of these strains to produce hydrogen sulfide, but rather appear important in determining the immediacy of onset of hydrogen sulfide production in nitrogen-limited fermentations. Nitrogen demands were unrelated to the longevity of the fermentative capacity of yeasts upon nitrogen depletion or the biomass formation during the same. This study has highlighted that while minimum nitrogen requirements for the completion of fermentation are frequently below the nitrogen content of grape must, additional quantities of nitrogen are required to ensure a suppression of hydrogen sulfide production.

Finally, a pattern of amino acid and ammonium accumulation has been described. This pattern was similar for the two strains studied and shared many parallels with previous reports. The ability of ammonium ions to delay the accumulation of specific amino acids has potential applications in controlling the formation of urea and altering the formation of compounds which contribute to the aroma profile of the wine.

Chapter 8

General conclusions

The research outlined in this thesis has been aimed at developing an understanding of the factors that influence hydrogen sulfide production during wine fermentation. Previous studies have provided considerable information on the biochemistry of hydrogen sulfide production but it has been difficult to relate this work to oenological conditions. In the light of the present study, it is possible to construct a model of the relationship between the production of hydrogen sulfide and the nitrogen demand of yeasts. This relationship appears crucial to the excessive production of hydrogen sulfide.

The model predicts that under conditions of nitrogen excess, hydrogen sulfide is condensed with nitrogenous precursor compounds to yield the corresponding sulfur amino acids (Figure 8.1a). When nitrogen becomes depleted, the nitrogenous precursors are in short supply. Consequently, only a limited amount of hydrogen sulfide is sequestered into sulfur amino acid biosynthesis (Figure 8.1b). The excess diffuses from the cell where it becomes a problem for the winemaker.

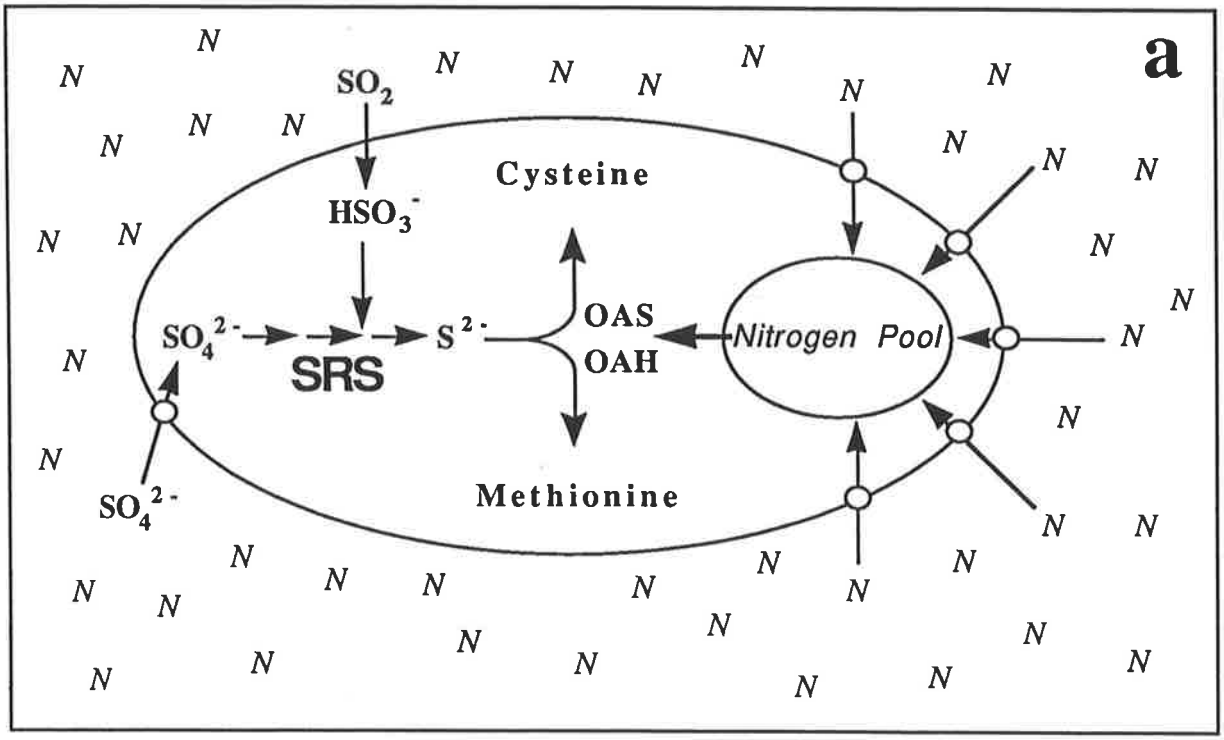
Both sulfate and sulfite can be reduced to hydrogen sulfide, though sulfite is energetically preferred. Furthermore, unlike sulfate, there are no mechanisms controlling the supply of exogenous sulfite to sulfite reductase, the key enzyme involved. As a result, during nitrogen-starvation, hydrogen sulfide production from sulfite can greatly exceed that seen from sulfate.

There is little evidence to suggest that the activity of sulfite reductase is regulated once the enzyme has been synthesised. In light of the stability of

sulfite reductase, repression of enzyme biosynthesis is largely a long-term mechanism for the control of hydrogen sulfide production. Thus, the complete assimilation of hydrogen sulfide is proposed to be the principal short-term mechanism by which this volatile is prevented from diffusing into the growth medium. Hence the importance of assimilable nitrogen to the suppression of excessive hydrogen sulfide production during fermentation stems from its role in the provision of nitrogenous precursors of sulfur amino acids.

The winemaker can, therefore, control hydrogen sulfide production by ensuring a ready supply of assimilable nitrogen. At present nitrogen additions are routinely made at inoculation and then upon detection of hydrogen sulfide. However, high residual nitrogen contents may lead to increased microbial instability of the wine. This study suggests that a less random approach is possible. Specifically, the winemaker will now be able to predict whether additions of assimilable nitrogen will be necessary at all. If needed, additions can be made to precisely match yeast nitrogen-requirements for the completion of the fermentation. Early indications suggest that additions should be made later in fermentation so as to minimise the stimulation of yeast growth.

Most importantly, this study highlights the existence of the additional possibility of matching yeast nitrogen-demand to the nitrogen content of individual musts. A broad range of yeast requirements for nitrogen has been identified and some fermentation parameters influencing nitrogen demand have been delineated. Accordingly, the selective use of yeast strains and fermentation conditions has great potential for the optimal utilisation of existing must assimilable nitrogen. This approach promises the achievement of complete and hydrogen sulfide-free fermentation while avoiding the use of nitrogen supplements and their associated detrimental features.


a
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Cysteine
SO4 2-
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OAS
OAH
Nitrogen Pool
SO4 2-
Methionine

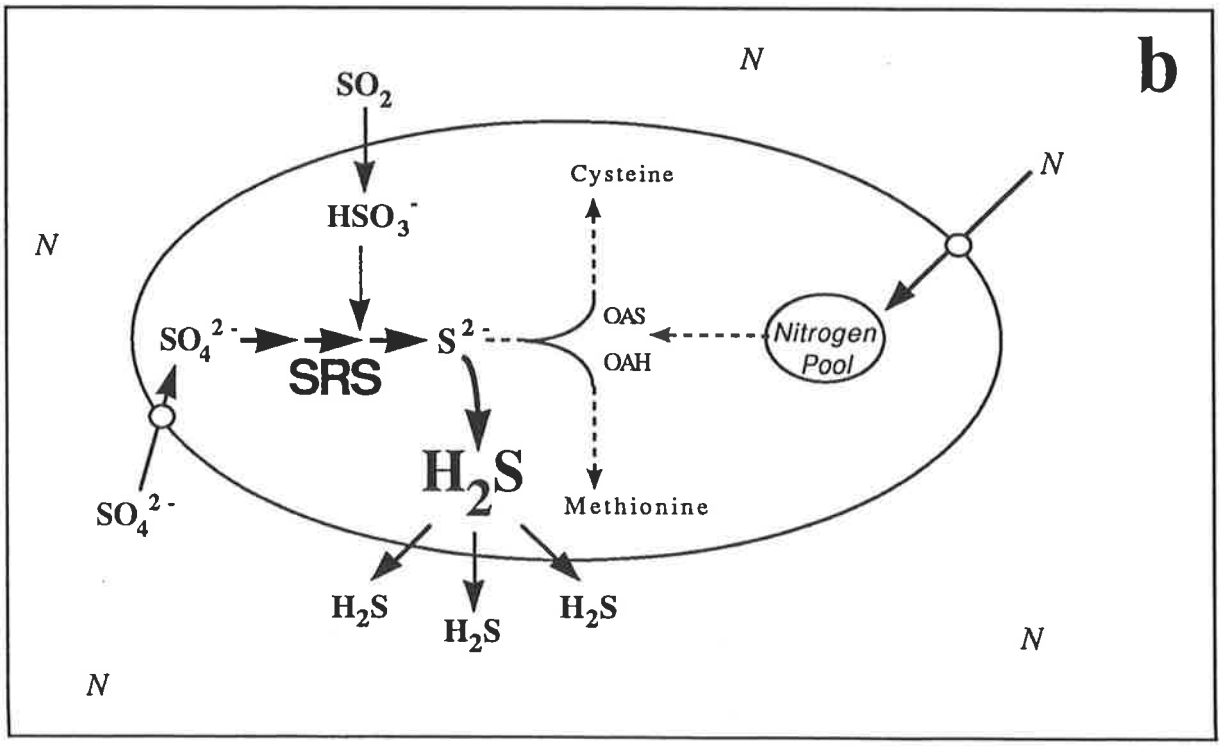
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Figure 8.1

The foremost requirements in fulfilling this goal are the accurate quantitation of grape must assimilable nitrogen contents, yeast quantitative and qualitative nitrogen requirements and the identification of all factors affecting these parameters. Meeting these criteria is facilitated by this study. A model for the type of data that needs to be collected about the yeast strains and the experimental methodologies for acquiring them, are outlined here.

It will be clear that determination of assimilable nitrogen content of grape musts requires quantitation of individual amino acids and ammonium, and possibly small peptides. This sort of analysis is beyond the routine analytical capabilities of a typical winery. Therefore, a prime future objective should be the development of a rapid assay for assimilable nitrogen. Only when this is accomplished will it be possible to make informed decisions about the sufficiency of must nitrogen content.

To make full use of existing strain variations in nitrogen demand, the present survey of yeast strains needs to be expanded. In addition, it would be desirable to identify strains which have novel qualitative nitrogen demands. The potential to reduce urea excretion through reduced utilisation of arginine has already been demonstrated by molecular biological techniques (Suizi *et al.*, 1990). The existence of a spontaneous mutant with the same attributes has not been reported. Similarly, the identification of a mutant strain which has an ability to utilise proline, despite the presence of other nitrogen sources, would be highly desirable given the predominance of proline in musts. In all cases, an important consideration will be the influence of efficiency of nitrogen utilisation or selective amino acid utilisation on the other fermentation characteristics.

Molecular biological techniques have great potential to accelerate the acquisition of a selection of yeast strains with a range of qualitative and

quantitative nitrogen requirements which are tailored to almost all must nitrogen contents. Similarly, while the identification of low hydrogen sulfide producing strains could be accomplished by traditional screening techniques, a molecular genetic approach would aid the process. Low hydrogen sulfide producing strains could be constructed through site directed mutagenesis or the reduction or modification of sulfite reductase promotor activity. If low hydrogen sulfide production and/or nitrogen efficiency are able to be localised to single or a small region of the genome, then it may be possible to insert these desirable attributes into any yeast strain which lacks them but possess other traits such as the generation of desirable organoleptic properties. However, as there are ethical problems associated with the use of genetically engineered organisms, it is essential that the more accepted screening approaches be fully exploited.

It remains for the wine industry to fully appreciate the importance of intracellular assimilable nitrogen for the effective control of hydrogen sulfide production. The reliance of winemakers on nitrogen supplements to prevent stuck fermentations and hydrogen sulfide production will become more difficult to justify with the move towards the reduced use of chemicals in food and beverage manufacture. Therefore, it is imperative that the winemaker pay close attention to must nitrogen content and provide greater input into the activities of viticulturalists. In this way fermentation problems arising out of a deficiency of nitrogen may be essentially abolished.

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