Glycerol Production by the Yeast *Saccharomyces cerevisiae* and its Relevance to Wine: A Review

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Submitted for publication: February 1998
Accepted for publication: July 1998
Key words: Glycerol, yeast, wine, genetic manipulation, environmental factors

Glycerol is a sugar alcohol produced as a by-product of the ethanol fermentation process by *Saccharomyces cerevisiae.* In wines, levels between 1 and 15 g/*l* are frequently encountered and the higher levels are thought to contribute to the smoothness and viscosity of wine. Glycerol and ethanol levels are inversely related, which may add an additional favourable attribute to wine. The metabolic pathways involved in glycerol synthesis, accumulation and utilisation by yeast are now better understood since a number of the genes involved in glycerol metabolism have been cloned, sequenced and their functions established. These fundamental studies now permit the glycerol levels produced by yeast to be raised by either the specific control of the culture conditions or by the manipulation of the genetic and molecular properties of the yeast. In some instances, the level of glycerol produced under laboratory conditions has been significantly raised. However, a number of undesirable by-products also accumulate during the fermentation and an improved understanding of the glycerol metabolic flux is required before wines with a consistently elevated glycerol concentration can be produced.

Glycerol is an economically important alcohol with a slightly sweet taste and with applications in the food, beverage, pharmaceutical and chemical industries. This nontoxic triol is soluble in water and other polar solvents, but insoluble in non-polar organic solvents. In beverages such as wine and beer, glycerol is thought to impart certain sensory qualities. Although most commercial glycerol is produced by chemical synthesis, there are instances when biological synthesis by yeast is significant. The well-known wine, brewing and bakers yeast *Saccharomyces cerevisiae* is the most important glycerol-producing yeast. In this organism glycerol plays important roles in physiological processes such as combating osmotic stress, managing cytosolic phosphate levels and maintaining the NAD^+/NADH redox balance (see Blomberg & Adler, 1992; Prior & Hohmann, 1997; Hohmann, 1997 for reviews).

The manipulation of *S. cerevisiae* to produce either higher of lower glycerol levels could be advantageous to the food and beverage industries (Pretorius & Van der Westhuizen, 1991; Rankine & Bridson, 1971). This can be achieved by either manipulating the genetic properties of the yeast or by controlling the culture conditions. In the former instance, classical genetic and newer molecular techniques have been used, whereas in the latter instance, the nutritional and other environmental factors such as temperature, nitrogen source and osmotic stress can regulate glycerol production by yeast.

In this review, the significance of glycerol in beverages, especially wine, and the intrinsic and extrinsic factors influencing glycerol production by yeast will be examined in detail.

Significance of Glycerol:

Glycerol in wine and other beverages: Glycerol is an important constituent of wine formed as a by-product during the fermentation process, and is the most abundant constituent except for carbon dioxide and ethanol. In early studies Pasteur found that up to 3,6% of sugar in wine was fermented to glycerol (Prescott & Dunn, 1949). Most extensive studies have shown that, depending upon the yeast strain, medium and process conditions, 4 to 10% of the carbon source is converted to glycerol (Radler & Schütz, 1982) and that the levels are generally to be found in the range of 7 to 10% of that of ethanol (Ciani & Ferraro, 1996). Although the concentration of glycerol in grape must is low, the level may be much higher in wines produced from mould-infected grape musts where glycerol is produced in the grape before fermentation by yeast (Ravji, Rodriguez & Thornton, 1988). The variety and degree of ripeness of the grapes used to prepare must can also affect the glycerol level in the wine (Ough, Fong & Amerine, 1972; Radler & Schütz, 1982; Rankine & Bridson, 1971).

The amount of glycerol in wine has only been reported in a limited number of surveys despite its relative significance as a fermentation product. Typical levels found in wine vary from 1 to 15 g/*l* with average values approximately 7 g/*l* (Mattick & Rice, 1970a; Rankine & Bridson, 1971). Glycerol concentrations have been found to be higher in red wines from New York

Acknowledgements: The authors sincerely thank the Foundation for Research Development (Core and THRIP programmes), Winetech and the Flemish/South Africa Cooperative Agreement for financial assistance. The awarding of an FRD bursary to K.T. Scanes is gratefully acknowledged.

State (Mattick & Rice, 1970b), from California (Ough, Fong & Amerine, 1972) and from Australia (Rankine & Bridson, 1971) compared to white wines. An investigation of 11 Australian Pale Dry sherries yielded a mean glycerol level of 3,4 g/*l*, whereas a mean value of 6,7 g/*l* was found in 16 samples of Tawny port (Rankine & Bridson, 1971). Notable in all these surveys is the considerable variation in glycerol levels in the wine products and the lack of any investigation of the relationship between wine quality and glycerol levels. No reports on the glycerol levels in South African wines have been published to our knowledge.

Glycerol does not contribute to the aroma of the wine due to its non-volatile nature but does contribute to the smoothness (Eustace & Thornton, 1987). The threshold taste level of glycerol is observed at 5,2 g/*l* in wine (Noble & Bursick, 1984) and between 3,8 and 4,4 g/*l* in water (Berg *et al.*, 1955), whereas a change in the viscosity is only perceived at a glycerol level of 25 g/*l*. Hinreimer *et al.* (1955) noted that this threshold taste level increased with acidity and ethanol concentration. Furthermore, glycerol apparently enhances the flavour components present in shochu, a Japanese fermented beverage made from barley, rice and sweet potatoes (Omori *et al.*, 1995). Therefore, these observations point to glycerol contributing only indirectly to wine qualities and indicate that the overproduction of glycerol by wine strains of *S. cerevisiae* could improve the sensory qualities of the wine (Pretorius & Van der Westhuizen, 1991).

Glycerol concentrations in other fermented beverages such as beer and shochu are similar or less than those observed in wine, although scant attention has apparently been paid to these beverages (Panchal & Stewart, 1980; Omori *et al.*, 1995). Klopper *et al.* (1986) reported glycerol levels of 1,3-1,7 g/*l* in Pilsener-type beer and levels of 1,5-2,9 g/*l* in top-fermented beers. Since the formation of glycerol is increased during osmotic stress, this compound will be very important during high-gravity fermentations (very strong worts) and as the brewing industry attempts to introduce higher-gravity brewing fermentation, the significance of glycerol in beer will increase (G.G. Stewart, personal communication).

Industrical production of glycerol by fermentation: Until the nineteenth century glycerol was produced by the saponification of fats and oils in making soaps, but after Pasteur (1860) noted that glycerol was a significant by-product of the yeast ethanol fermentation process, a way was opened to develop an industrial process. The consistently low glycerol yields resulted in a concerted effort to manipulate the process in order to improve yields. The two most successful strategies were the soluble sulphite and the alkaline processes. In the former instance, the addition of sodium sulphite to the fermentation broth formed a complex with acetaldehyde and steered yeast metabolism away from ethanol production towards glycerol (Neuberg & Reinfurth, 1918). Fermentation at high pH also improved the glycerol yield significantly (Connstein & Lüdecke, 1919). Commercially, both processes have fallen into disuse and are unable to compete with the more efficient chemical synthesis of glycerol (Vijaikshore & Karanth, 1986).

Glycerol metabolism in yeasts:

Glycerol synthesis: Glycerol is synthesised in the cytosol of the yeast. Glucose is phosphorylated as it enters the cell and it is converted through the normal glycolytic steps to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate in equimolar amounts (Fig. 1). Most of the dihydroxyacetone phosphate, however, is converted to glyceraldehyde-3-phosphate by the interconverting enzyme triose phosphate isomerase. This enzyme has a greater affinity for dihydroxyacetone phosphate than glyceraldehyde-3-phosphate. Ethanol is formed from glyceraldehyde-3-phosphate via pyruvate and in this process NADH is reduced to form NAD^+. Dihydroxyacetone phosphate is converted to glycerol in a two-step reaction involving an NADH-dependent glycerol-3-phosphate dehydrogenase (Albertyn, Van Tonder & Prior, 1992) and a phosphatase. This latter enzyme was thought to be non-specific (Tsuboi & Hudson, 1956) until recently, when *GPP1* and *GPP2* genes encoding specific glycerol-3-phosphatases were discovered (Norbeck *et al.*, 1996).

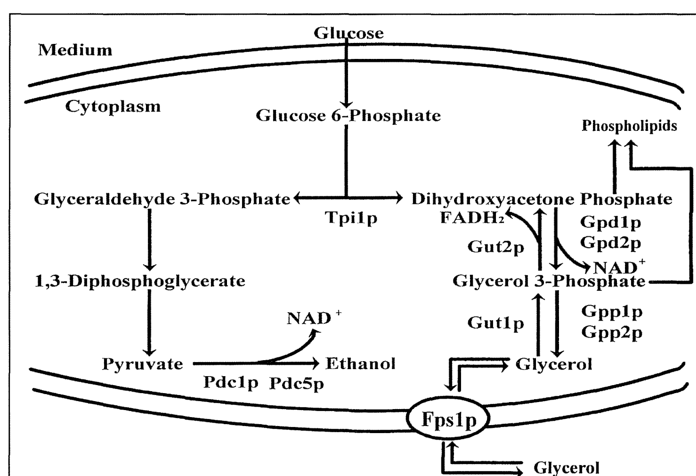

FIGURE 1

Metabolic reactions of importance in the glycerol metabolism of *Saccharomyces cerevisiae*. Glycolysis and the reduction of glycolytic intermediate dihydroxyacetone phosphate to glycerol-3-phosphate and the subsequent oxidation of NADH to NAD^+ leads to the formation of glycerol.

The two most important functions of glycerol synthesis in yeast are related to redox balancing and the hyperosmotic stress response. Osmotic stress is one of the most common types of stress imposed on yeasts, making it necessary for the cell to develop mechanisms to survive under these conditions. One of the consequences of hyperosmotic stress is the rapid diffusion of water from the cell into the surrounding medium. To prevent this many yeasts accumulate mainly glycerol but other polyols such as arabitol, mannitol, meso-erythritol (Brown, 1976) and xylitol (Spencer & Spencer, 1978) are also produced. These solutes, known as compatible solutes, serve to equilibrate the intra- and extracellular environment which is essential for growth under osmotically stressful conditions (Brown, 1978; Larsson, 1986).

Since NADH is oxidised to form NAD^+ during the production of glycerol (Fig. 1), the maintenance of the NAD^+/NADH redox balance under anaerobic conditions is essential (Nordström, 1966; Van Dijken & Scheffers, 1986; Ansell *et al.*, 1997). Lowry and Zitomer (1984) noted that the aerobic production of glycerol occurs as long as glucose is present resulting in respirofermentative growth. This indicates that the cell requires glycerol production to maintain the redox balance as respiration is repressed by glucose and by oxygen limitation. Interestingly glycerol production is found to be higher in a minimal medium than in a complex medium as *de novo* synthesis of amino acids from glucose and ammonia leads to an excess of NADH which is re-oxidised by the formation of glycerol (Albers *et al.*, 1996). The inability of mutants unable to produce glycerol to grow under anaerobic conditions and their intracellular accumulation of surplus NADH supports the essential function of glycerol synthesis in the maintenance of the redox balance (Ansell *et al.*, 1997).

Glycerol may also have roles in oxidative stress resistance, in regenerating cytosolic inorganic phosphate and in nitrogen metabolism, but these functions require greater clarification (Prior & Hohmann, 1997). When glycerol-3-phosphate is dephosphorylated to form glycerol, a phosphate ion is released and in this way glycerol production may also regulate the cytosolic phosphate levels (Thevelein & Hohmann, 1995). As glucose is taken into the cell in the unphosphorylated form, this phosphate must be released at a later stage or else a phosphate inbalance will develop, as is found in trehalose (*tps 1* Δ) mutants (Thevelein & Hohmann, 1995). Overproduction of the *GPD1* gene rescues this phenotype, indicating that glycerol production is important in the recycling of the inorganic phosphate. Furthermore, when *S. cerevisiae* cells are under conditions of nitrogen limitation, glycerol synthesis is increased in order that excess cytosolic ATP can be consumed (Larsson *et al.*, 1998). It is also thought that glycerol production could act as a protectant against cytosolic oxygen radicals (Chaturverdi, Bartiss & Wong, 1997). The mechanism of this protection still has to be investigated.

GPD1 encodes the cytosolic glycerol-3-phosphate dehydrogenase and is located on chromosome IV of *S. cerevisiae* (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b). The predicted size of this gene product is 391 amino acids and the molecular mass is 42,8 kDa. Gpd1p was found to share a strong homology with NAD-dependent glycerol-3-phosphate dehydrogenases isolated from other eukaryotic organisms. A significant difference between the Gpd1p and its counterparts from other eukaryotes is the presence of an amino-terminal extension of about thirty amino acids in length. This extension contains characteristic features of mitochondrial signal peptides (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b). The importance of Gpd1p in glycerol synthesis was confirmed by the deletion of the *GPD1* gene, resulting in lowered glycerol production, and the strain exhibited an osmosensitive phenotype (Albertyn *et al.*, 1994b).

Eriksson *et al.* (1995) cloned a second gene encoding a NAD-dependent glycerol-3-phosphate dehydrogenase and called it *GPD2*. This *GPD1* homologue, which is found on chromosome VII, encodes a polypeptide 385 amino acids in length and has an estimated molecular mass of 42,3 kDa. As was found for *GPD1*, *GPD2* exhibited a high degree of homology with glycerol-3-phosphate dehydrogenase genes from other organisms. This polypeptide, like Gpd1p, contains an amino-terminal extension, but the Gpd2p extension differs remarkably from that of Gpd1p. The function of these extensions is unclear at this stage. The pI of Gpd2p (6,8) differs markedly from the pI of Gpdlp (5,3).

A comparison of the amino acid sequence of *GPD1* and *GPD2* showed a 69% identity between the two peptides. The peptides were found to be remarkably homologous, with only 68 amino acid substitutions of which the majority were conservative (Eriksson *et al.*, 1995). The promoter regions of *GPD1* and *GPD2* have a very low degree of homology and this points to different regulatory mechanisms. Indeed it was found that the expression of *GPD1* is usually increased by hyperosmotic stress, whereas *GPD2* expression is increased by anaerobic conditions (Ansell *et al.*, 1997). Whereas the deletion of *GPD2* results in no obvious phenotype under aerobic conditions (Eriksson *et al.*, 1995), under anaerobic conditions the deletion mutant is growth-defective, pointing to an involvement of *GPD2* in redox control (Ansell *et al.*, 1997). Strains containing the double mutations, *gpdl gpd2* that fail to produce detectable glycerol, are highly sensitive to high osmolarity and do not grow anaerobically.

The two glycerol-3-phosphatases of *S. cerevisiae* have been purified and the genes (*GPP1* and *GPP2*) encoding these proteins have been identified (Norbeck *et al.*, 1996). The two proteins have 95% amino acid identity and have respective molecular masses of 30,4 and 27,8 kDa. The intracellular concentration of glycerol-3-phosphatase (the Gpp2p but not the Gpplp isoform) increases in cells subjected to hyperosmotic stress.

The level of glycerol in *S. cerevisiae* and the expression of *GPD1* and *GPP2* are partially controlled by the HOG (**H**igh **O**smolarity **G**lycerol) signal transduction pathway when cells are placed under hyperosmotic stress (Albertyn *et al.*, 1994b; Norbeck *et al.*, 1996). The HOG pathway is a mitogen-activated pathway (MAP) kinase cascade (a simplified version is shown in Fig. 2) that is related to a number of other similar pathways such as the yeast pheronome response pathway (Herskowitz, 1995). Changes in osmolarity are detected by two distinct putative transmembrane osmosensors (Slnlp and Sholp). Under conditions of low osmolarity Slnlp is active, causing the auto-phosphorylation of a histidine residue. This phosphate group is transferred to an aspartate residue in the receiver domains of Sln1p and the signal transducers Ypd1p as well as Ssk1p. Once the Ssk1p is phosphorylated, it is inactivated resulting in the absence of HOG-pathway stimulation (Maeda, Wurgler-Murphy & Saito, 1994). Under conditions of high osmolarity Slnlp is inactive allowing Ssklp to activate the protein kinases Ssk2p and Ssk22p. Ssk2p and Ssk22p are thought to be a redundant MAP kinase kinase kinase (MAPKKK). These in turn activate the MAP kinase kinase (MAPKK) Pbs2p of the HOG signal transduction pathway through a Ser-Thr phosphorylation and the MAP kinase (MAPK) Hoglp is then activated by a Thr-Tyr phosphorylation (Maeda, Takekawa & Saito, 1995). The second osmosensor Sho1p activiates Pbs2p via Ste11p at NaCl concentrations of 200 mM and higher (Brewster *et al.*, 1993; Maeda, Wurgler-Murphy & Saito, 1994; 1995; Posas & Saito, 1997).

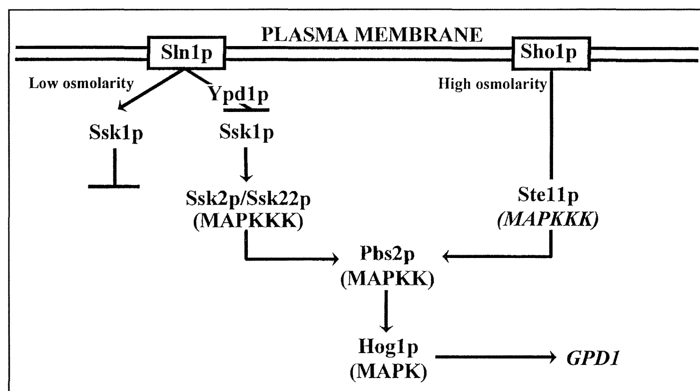

FIGURE 2

A schematic representation on the HOG pathway (Albertyn, 1996). A signal is passed on via two distinct putative osmosensors. This signal then goes through a MAP kinase system until it eventually reaches a number of targets, one of which is *GPD1*, thus regulating glycerol synthesis.

There has been much speculation as to whether "cross-talk" occurs between the different MAP-kinase pathways (Davenport *et al.*, 1995), but recently Posas & Saito (1997) have put forward a hypothesis that this uncontrolled interference between pathways is eliminated by the formation of enzyme complexes. They noted that although Ste11p plays a role in both the HOG pathway and the mating pathway, there is little "cross-talk" as Pbs2p acts as a scaffold protein in the HOG pathway, while Ste5p plays this role in the case of the mating pathway.

The other important gene for glycerol synthesis (*GPD2*) is induced under anaerobic conditions by a pathway which is independent of the HOG pathway. Under aerobic conditions *GPD2* is induced when bisulphite is added to the medium. From these results Ansell *et al.* (1997) concluded that *GPD2* expression is controlled by a novel, oxygen-independent, signalling pathway which appears to regulate metabolism under conditions when oxygen is limited.

Glycerol assimilation: Glycerol can be used as a source of carbon by *cerevisiae* in the absence of glucose (Sprague & Cronan, 1977). The utilisation of glycerol is coupled to respiration via a glycerol kinase converting glycerol to glycerol-3-phosphate, which is oxidised to dihydroxyacetone phosphate by a flavin-dependent and membrane-bound mitochondrial glycerol-3-phosphate dehydrogenase (Fig. 1) (Gancedo, Gancedo & Sols, 1968; Ronnow & Kielland-Brandt, 1993). The mitochondrial glycerol-3-phosphate dehydrogenase also participates, in conjunction with the cytoplasmic NAD^+-linked glycerol-3-phosphate dehydrogenase, in the aerobic glycerol phosphate shuttle system (Haddock & Jones, 1977). The role of this shuttle system is not clear at this stage, but it has been proposed to oxidise cytosolic NADH by conversion of dihydroxyacetone phosphate to glycerol-3-phosphate via the cytosolic glycerol-3-phosphate dehydrogenase followed by the re-oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate via the flavin-dependent mitochondrial glycerol-3-phosphate dehydrogenase. The gene encoding the mitochondrial glycerol-3-phosphate dehydrogenase (*GUT2*) is strongly repressed in the presence of glucose (Ronnow & Kielland-Brandt, 1993; Sprague & Cronan, 1997), but the shuttle is used extensively by cells grown on non-fermentable reduced substrates such as ethanol (Larsson *et al.*, 1997).

The glycerol kinase is encoded by the gene *GUT1* (Pavlik *et al.*, 1993), while the glycerol-3-phosphate dehydrogenase is encoded by *GUT2* (Ronnow & Kielland-Brandt, 1993). Mutants defective in either *GUT1* or *GUT2* are unable to grow on glycerol (Pavlik *et al.*, 1993; Ronnow & Kielland-Brandt, 1993). *GUT1*, located on chromosome VIII, encodes a protein of 709 amino acids that has 40,8% identity with the *Escherichia coli* glycerol kinase, and 42,1% identity with the *Bacillus subtilis* enzyme. *GUT2* encodes a polypeptide that has a predicted molecular mass of 68,8 kDa and is 615 amino acids in length. This gene, which was mapped to chromosome IX of *S. cerevisiae*, showed respectively 27%, 26% and 32% identity with the anaerobic glycerol-3-phosphate dehydrogenase from *E. coli*, with the aerobic glycerol-3-phosphate dehydrogenase from *E. coli* and with the *B. subtilis* glycerol-3-phosphate dehydrogenase (Ronnow & Kielland-Brandt, 1993).

The glycerol metabolic pathways also play an important role as a source of the precursors necessary for the synthesis of membrane non-ether phospholipids and the maintenance of the cell membrane (Fig. 1) (Pieringer, 1989; Racenis *et al.*, 1992). Dihydroxyacetone phosphate is a precursor for the synthesis of the membrane ether phospholipids (Racenis *et al.*, 1992).

Glycerol permeability: Although the utilisation of sugars requires the existence of transport systems, uncharged compounds with only two or three carbon atoms can penetrate *S. cerevisiae* by simple diffusion. The poor diffusion rate to glycerol, however, raised the question as to whether the entrance of glycerol into the cell by simple diffusion would be sufficient to account for the observed growth rate (Gancedo, Gancedo & Sols, 1968). Furthermore, under osmotic stress conditions, the nonosmotolerant *S. cerevisiae* was originally thought to maintain the intracellular glycerol content by redirecting its carbohydrate metabolism towards glycerol thereby compensating for the glycerol lost to the external environment (Edgley & Brown, 1983). By contrast other osmotolerant yeasts such as *Zygosaccharomyces rouxii* (Van Zyl, Kilian & Prior, 1990), *Debaryomyces hansenii* (Lucas, Da Costa & Van Uden, 1990) and *Pichia sorbitophila* (Lages & Lucas, 1995) have active transport mechanisms to maintaint the intracellular glycerol content under osmotic stress. Recently a gene *FPS1*, which encodes a channel protein belonging to the MIP family, was shown to act as a yeast glycerol transport facilitator controlling both glycerol influx and efflux (Luyten *et al.*, 1995; Sutherland *et al.*, 1997). The MIP family has more than 80 members found in organisms such as plants, animals, fungi and bacteria (Park & Saier, 1996). Some of the members of this family function as aquaporins regulating water flux across the membrane, while others act as glycerol facilitators. Under hyperosmotic stress the facilitator restricts the intracellular glycerol loss to the medium, whereas under osmostress-free conditions, intracellular glycerol release occurs rapidly (Luyten *et al.*, 1995). A similar channel has been found in *E. coli* (Heller, Lin & Wilson,

1980), but one important difference between this facilitator and the *S. cerevisiae* version is the absence of long C- and N-terminal extensions in the *E. coli* facilitator (Van Aelst *et al.*, 1991; Andre, 1995). These extensions appear to play a role in the regulation of the opening and closing of the pore in *cerevisiae* (Sutherland, 1996).

Recent physiological evidence points to the existence of an active glycerol transport mechanism in *S. cerevisiae* (Sutherland *et al.*, 1997). This proton symport is apparently repressed in the presence of glucose but derepressed when grown on glycerol and ethanol.

Environmental factors influencing the production of glycerol: The wide variation in levels of glycerol produced by strains in different media points to an influence of environmental factors on the metabolism in yeast (Radler & Schütz, 1982).

Temperature: Increasing fermentation temperature results in greater glycerol production in must. Rankine & Bridson (1971) found that the concentration of glycerol formed during the fermentation of Riesling grape must by 14 wine yeast strains increased by 1 g/*l* when the temperature was changed from 15° to 25°C and suggested that the pathway to glycerol becomes more active at the higher temperature. The optimum temperature for maximum glycerol production by commercial wine yeast strains of *S. cerevisiae* varies between 22°C (Gardner, Rodrigue & Champagne, 1993) and 32°C (Ough, Fong & Amerine, 1972) and appears to be influenced by the grape variety used to prepare the must (Ough, Fong & Amerine, 1972). Furthermore, the lower glycerol levels in white wines compared to red wines can possibly be explained by the lower fermentation temperatures used to produce white wine (Ough, Fong & Amerine, 1972). The cultivation conditions, however, can also impact on the optimum fermentation temperature. When various wine yeast strains fermented Beaujolais grape juice under static conditions, the highest glycerol levels were found at 20°C, whereas under agitated conditions these strains produced the highest level of glycerol at 25°C (Gardner, Rodrigue & Champagne, 1993).

pH and acidity: Studies conducted at the beginning of this century on industrial glycerol production by *S. cerevisiae* indicated that higher glycerol yields were obtained under alkaline conditions (Connstein & Lüdecke, 1919; Eoff, Linder & Beyer, 1919). Freeman & Donald (1957) reported the optimum pH range for sulphite-directed production of glycerol to lie between 6,7 and 7,0. Increasing the pH of Riesling grape must from 3,3 to 3,8 resulted in 14 wine yeast strains yielding only slightly greater glycerol levels, suggesting that little can be done to increase glycerol production by manipulation of the pH generally required for wine fermentations (Rankine & Bridson, 1971). Fermentation of must with a low pH is desirable as this limits the growth of spoilage micro-organisms and enhances flavour development (Jackson, 1994).

Sugar concentration and osmotic stress: Typically must from grape cultivars such as *Vitis vinifera* has a sugar concentration of 200 g/*l* or more at maturity consisting of equal proportions of glucose and fructose (Jackson, 1994). This sugar concentration is equivalent to a water acitivity (a_w) of 0,975 to 0,98 (unpublished data). Whereas the minimum growth a_w (glucose) of *S. cerevisiae* is 0,9 (Van Eck, Prior & Brandt, 1993), the a_w found in grape must does slightly inhibit the growth rate of *S. cerevisiae* but can also lead to other physiological responses. The most notable of these responses is the increased synthesis and accumulation of glycerol in order to compensate for greater osmotic stress imposed by the sugar concentration of must. In related studies on *S. cerevisiae,* the glycerol yield in continuous culture was three to four fold greater at 0,971 a_w than at 0,994 a_w (Kenyon, Prior & Van Vuuren, 1986) and at least a two-fold increase in extracellular glycerol concentration was observed when the a_w was reduced from 0,998 to 0,98 (Albertyn, Hohmann & Prior, 1994a). Panchal & Stewart (1980) found that when a brewing yeast *Saccharomyces uvarum (carlsbergensis)* was grown on minimal media containing 10% sucrose and non-metabolisable sorbitol (to adjust a_w to 0,98), glycerol production increased by 50%, whereas the ethanol produced decreased by approximately 30%. This enhanced glycerol production occurred concomitantly with increased levels of the cytosolic NADH-dependent glycerol-3-phosphate dehydrogenase (Andre, Hemming & Adler, 1991; Albertyn, Hohmann & Prior, 1994a).

Nitrogen: The nitrogen source in the culture medium of anaerobically-grown *S. cerevisiae* has a significant impact on the glycerol yield (Albers *et al.*, 1996). The yield in ammonium-grown cultures was more than double that of yeast grown in cultures with a mixture of amino acids as nitrogen source (Albers *et al.*, 1996; Omori *et al.*, 1995). This observation was related to the maintenance of the redox balance. Ammonium-grown cultures require *de novo* synthesis of amino acids, giving rise to excess NADH which must be re-oxidised by glycerol synthesis. The relative concentrations of ammonia and amino acids can vary during the ripening of grapes (Jackson, 1994), which could have an influence on the glycerol levels in wines fermented from musts in various stages of ripening.

Sulphur dioxide: Sulphur dioxide has been known for some time to increase the production of glycerol by yeast and the addition of sulphite ions to the medium is a strategy that was exploited in the commercial production of glycerol during the first half of this century (Prescott & Dunn, 1949). Sulphur dioxide is also a normal constituent of wine as a result of yeast metabolism and is frequently added to must to inhibit wild yeast and spoilage bacteria (Jackson, 1994). Sulphur dioxide in high concentrations is toxic to yeasts, but wine yeast strains are generally less sensitive than other yeasts strains. The effect of sulphur dioxide on glycerol production is apparently strain dependent. The addition of 100 mg/*l* sulphur dioxide to Riesling must resulted in an average 8% increase in glycerol production by 14 yeast strains (Rankine & Bridson, 1971). The increase was as high as 20% in wine produced by some strains whereas in wine produced by other strains little or no increase was observed. In further studies, the addition of up to 200 mg/*l* sulphur dioxide had a negligible effect on glycerol production by a commercial wine yeast strain (Ough, Fong & Amerine, 1972) and the impact of sulphur dioxide on glycerol production was only marked at concentrations greater than 200 mg/*l* (Gardner, Rodrigue & Champagne, 1993). In the light of the modern trend to limit the use of sulphur dioxide in wines, it is

unlikely that glycerol levels can be raised by this method in commercial wine production.

Other factors affecting glycerol production: S. cerevisiae cultures are found to produce greater glycerol levels when agitated or aerated (Radler & Schütz, 1982; Gardner, Rodrigue & Champagne, 1993). The increased glycerol production is difficult to explain since NADH should be oxidised by respiration, thereby reducing the need to reduce dihydroxyacetone phosphate to glycerol-3-phosphate. High cell populations in must (up to 10^8/m*l*) have been found to give between 10 and 20% greater glycerol levels than must inoculated with a 100-fold lower inoculum level irrespective of when the cultures were inoculated (Radler & Schütz, 1982).

Many minerals are found in grapes and are added to must during the fermentation process as contaminants from equipment (Jackson, 1994). Little attention has been given to the influence of minerals on glycerol production until recently. Ansell (1997) showed that glycerol levels produced by a laboratory strain of *S. cerevisiae* were more than double when cultured under iron-limited versus non-limited conditions (0,6 g/*l* $FeSO_4$).

Manipulation of glycerol levels by genetic and molecular techniques: The possiblity of improving the quality of wines lacking body and fullness by increasing the glycerol levels has led to a limited number of attempts to manipulate wine yeasts using classical genetic and molecular techniques. These techniques are not described here in detail and the reader is referred to reviews by Pretorius & Van der Westhuizen (1991) and Barre *et al.* (1993) for more information.

Manipulation by genetic techniques: The considerable variation in glycerol levels found in various yeast strains cultivated under standard culture conditions indicates that this genetic diversity could be used to breed yeasts with elevated glycerol levels (Rankine & Bridson, 1971; Radler & Schütz, 1982). Eustace & Thornton (1987) undertook a breeding programme by investigating the glycerol levels of 11 commercial homothallic wine yeasts and 3 heterothallic haploid yeast strains. Selected strains were hybridised by spore-cell matings and the zygotes and spores were selected for futher hybridisation with the original parent in order to maintain genetic stability based on their glycerol levels. Compared to the 3,0-6,6 g/*l* glycerol levels found in the original breeding stock, three generations of hybridisation resulted in yeast strains which produced 10-11 g/*l* glycerol. Furthermore a decrease in the ethanol yield of the high-producing strains was noted and this also led to a higher glycerol/ethanol ratio compared with the original strains. Two promising strains were tested for their ability to produce wine in a commercial-scale fermentation trial. The wine was reported to be of acceptable quality, with glycerol and ethanol concentrations similar to those found in laboratory experiments.

In an investigation of the glycerol levels produced by *S. cerevisiae* strains isolated from spontaneous fermentations, Prior *et al.* (1998) found that some strains produced elevated levels. Using techniques similar to those of Eustace & Thornton (1987), hybrid strains were obtained after the third hybridisation with glycerol levels as high as 18 g/*l*. Interestingly, a 2:2 segregation for glycerol production of four ascospores from asci was observed indicating that glycerol production is under genetic control. The high glycerol levels produced by two spores were accompanied by elevated production of acetic acid and acetaldehyde. This indicates that the higher production of glycerol results in a redox imbalance that is only relieved by the oxidation of acetaldehyde to acetic acid. Butanediol and acetoin production was also higher in spores that produce the highest glycerol levels.

The observation that higher glycerol yields are obtained in media containing inorganic nitrogen than in media with amino nitrogen led Omori *et al.* (1995) to devise a screening technique to select for mutant *S. cerevisiae* strains resistant to amino acid analogues. They isolated a number of mutants resistant to 5,5,5-trifluoroleucine and *p*-fluoro-DL-phenylalanine that produced up to 50% more glycerol than the parent strain and also lower ethanol levels. Interestingly, these mutants had lower alcohol dehyrogenase levels compared to the parent strain, indicating that a decrease in alcohol dehydrogenase activity can in some mutants direct the metabolic flux away from ethanol towards glycerol.

Manipulation by molecular techniques: The elevated levels of glycerol found in some strains of *S. cerevisiae* might be related to the activity of glycerol-3-phosphate dehydrogenase. While Eustace & Thornton (1987) could find no relationship between glycerol-3-phosphate dehydrogenase activity and the glycerol levels of their wine yeast hybrids, the reverse was found by Radler & Schütz (1982) and Michnick *et al.* (1997). This led the latter authors to propose that glycerol-3-phosphate dehydrogenase may be the rate-limiting step in glycerol production by yeast and that the overexpression of this enzyme could increase glycerol synthesis. When they inserted an expression vector carrying the *GPD1* gene under the control of the *ADH1* promoter into a haploid *S. cerevisiae* wine strain, they observed that the glycerol production increased from 4,3 g/*l* (in the control strain) to 14 g/*l* with a simultaneous decrease in ethanol production. In synthetic must containing 200 g/*l* glucose, a glycerol level of 28 g/*l* (4 times more than the control) was produced accompanied with a reduction in ethanol but higher levels of pyruvic acid, acetaldehyde, acetic acid, acetoin, succinic acid and 2,3 butanediol. While the higher glycerol levels were welcomed, concern was expressed about the need to reduce the acetaldehyde and acetic acid levels before such strains could be used in wine-making.

When *GPD2*, the isogene of *GPD1* whose gene product is apparently involved in redox control, was overexpressed in a laboratory strain of *S. cerevisiae,* a two-fold increase in glycerol was noted but also with a concomitant increase in acetic acid (De Barros Lopes *et al.,* 1996). This laboratory strain failed to ferment grape juice and hence the enological properties of the strain could not be thoroughly investigated. When a wine yeast strain was transformed with *GPD2* under the control of an *ADH1* yeast promoter and overexpressed, glycerol levels as high as 20 g/*l* were obtained in grape must.

Ciriacy & Breitenbach (1979) reported that the deletion of the gene encoding triose phosphate isomerase (*TPI1*) in yeast leads to an accumulation of dihydroxyacetone phosphate. This prompted Compagno. Boschi & Ranzi (1996) to examine what effect this

would have on the production of glycerol. They found that when yeast *tpi1*Δ mutants were cultivated on glucose, the main product of the fermentation was glycerol instead of ethanol, and glycerol levels as high as 17 g/*l* were produced from 40 g/*l* glucose. Unfortunately it was noted that the level of acetate produced increased from 0,5 g/*l* to 1,8g/*l*. Furthermore, increasing levels of glucose as substrate inhibited growth and only respirative growth was possible. Nevoigt & Stahl (1996) found that glycerol could be overproduced when the gene encoding the pyruvate decarboxylase regulatory subunit (*PDC2*) was deleted in *S. cerevisiae*. This increase in glycerol from 0,64 to 2,9 g/*l* was accompanied by a decrease in the ethanol production from 7,9 to 5,6 g/*l* when cultivated on 18 g/*l* glucose.

CONCLUSIONS

The recent progress made in the investigation of the physiology and molecular biology of glycerol metabolism in *S. cerevesiae* has opened a number of possibilities for specific manipulation of glycerol levels produced during ethanol fermentation. However, our understanding of the process is still incomplete. For example, we know little about the mechanisms that yeast use to control the metabolic flux of glycerol and whether certain rate-limiting steps in glycerol production exist. The rate-limiting step is a concept which has proved useful when studying metabolic regulation. Unfortunately it appears that this concept is an over-simplification, as a single rate-limiting reaction occurs infrequently (Groen *et al.*, 1982; Westerhoff, 1995). A more thorough flux-control analysis of glycerol metabolism is required based on a sound understanding of the relevant properties of the regulatory enzymes at the molecular level (Nimmo & Cohen, 1987). This strategy should permit selection of desired properties in wine yeasts manipulated using either acceptable genetic techniques or more specific molecular techniques.

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