The Biochemistry of Malic Acid Metabolism by Wine Yeasts - A Review

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L-Malic acid is an essential intermediate of cell metabolism and the D,L-racemic mixture is used as an acidulant in a variety of foods and beverages. In the wine industry, it plays an important role during grape must fermentation, contributing to the "fixed acidity" that is important. The latter is important in defining the quality of wine. Genetic and biochemical characterisation of the L-malate utilising pathways in several yeast species has indicated that the physiological role and regulation of L-malate metabolism differ significantly between the K(-) and K(+) yeasts. A variety of factors influence the ability of a yeast species to effectively degrade L-malate, including the conditions associated with wine fermentation and the yeast's intrinsic ability to transport and effectively metabolise L-malate inside the cell. This paper reviews the ability **of different yeast species associated with grapes and wine to degrade extracellular L-malate, and the underlying mechanisms in the differential utilisation of L-malate by different yeast species.**

INTRODUCTION

The production of good quality wines often requires[1] the proper adjustment of wine acidity in relation to the other wine components to create a well-balanced bottled product. The different chemical and biological factors that contribute to the presence, role and degradation of L-malate during wine fermentation have been discussed in detail by Volschenk *et al.* (2006). The traditional method to deacidify wine involves the conversion of L-malic acid to L-lactic acid and CO2 during malolactic fermentation by strains of *Oenococcus oeni.* The complexities associated with traditional malolactic fermentation in wine, however, necessitate alternative approaches to reduce wine acidity. One of the options is biological deacidification with yeast strains that are able to degrade excess malic acid present in the grape must. However, not all yeasts that are able to degrade extracellular malic acid are able to survive the conditions associated with grapes, must and wine, and some may even be considered responsible for undesirable characteristics in wine. The metabolic patterns in yeasts also differ significantly, with varying levels of sensitivity to the levels of oxygen and glucose. For example, the degradation of extracellular L-malate in some yeasts is subject to strong substrate induction and carbon repression, whereas other genera require the presence of glucose or a similar carbon source for the degradation of L-malate.

The degradation of L-malate has been studied in detail in only a few yeast species, including *S. cerevisiae* (Boles *et al,* 1998; Volschenk *et al.,* 2003), *S. pombe* (Osothsilp, 1987; Subden *et al,* 1998; Viljoen *et al.,* 1994; 1998; 1999), *Candida utilis* (Cássio and Leão, 1993, Saayman *et al,* 2000; 2006), with limited investigations done in *Hansenula anomala (Pichia anomala)* (Côrte-Real and Leão, 1990; Amador *et al,* 1996), *Pichia stipitis* (Thornton and Rodriques, 1996) and *Kluyveromyces marxianus* (Queiros *etal,* 1998), Z *bailii* (Kuczynski and Radler, 1982) and *Schizosaccharomyces pombe van malidevorans* (Rodriques and Thornton, 1990). Yeast species that are recognised for their ability to metabolise extracellular L-malate fall into either the K(-) or K(+) yeast groups, depending on their ability to utilise L-malate and other tricarboxylic acid (TCA) cycle intermediates as sole carbon or energy source (reviewed in Volschenk *et al,* 2003). The K(+) group includes *Candida sphaerica, C. utilis, H. anomala, P. stipitis* and *K. marxianus,* which all have the ability to utilise TCA cycle intermediates as sole carbon sources.

The K(-) group of yeasts comprises those yeasts capable of utilising TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources. According to this definition, *S. cerevisiae, S. pombe, S. pombe var. malidevorans* and Z *bailii* are all classified as K(-) yeasts (Volschenk *et al,* 2003). Although grouped together, the K(-) yeasts display significant differences in their ability to degrade L-malate. The yeast 5. *cerevisiae* is regarded as a poor metaboliser of extracellular malate, which has been attributed to the lack of a mediated transport system for the acid (Salmon, 1987). Strains of *S. pombe* and Z *bailii* can degrade high concentrations of L-malate, but only if glucose or another assimilable carbon source is present (Baranowski and Radler, 1984; Rodriquez and Thornton, 1989).

Genetic and biochemical characterisation of the L-malate utilising pathways in several yeast species indicates that the physiological role and regulation of L-malate metabolism differs significantly between the K(-) and K(+) yeasts. The factors involved could include the substrate affinity of the malic enzyme, the rate of transport of L-malate into the cell, and other stimulatory or inhibitory influences exerted by glucose, fructose or malic acid (Gao and Fleet, 1995). In general, L-malate metabolism in K(-) yeasts is characterised by the absence of glucose repression or substrate induction. In contrast, the regulation of L-malate metabolism in K(+) yeasts typically exhibits strong glucose repression together with substrate induction (Côrte-Real and Leão, 1990; Amador *etal,* 1996; Cássio and Leão, 1993; Queiros *etal,* 1998).

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Yeasts that dominate on grapes include species of *Rhodotorula, Cryptococcus, Candida, Hanseniaspora* (anamorph *Kloeckera)* and *Mitchnikowia* (Fleet, 2003; Jolly *et al.,* 2006). Damaged grapes have shown an occurrence of *Hanseniaspora, Candida* and *Metchnikowia* species, together with species of *Saccharomyces* and *Zygosaccharomyces.* However, *Saccharomyces cerevisiae,* the principle wine yeast, is not prevalent on grapes, which may be ascribed to the surface chemistry of the grape, tolerance to natural stresses and chemical inhibitors, and interactions with other bacterial and fungal species (Fleet, 2003). During grape juice fermentation, species of *Hanseniaspora (Kloeckera), Candida* and *Mitchnikowia* initiate the fermentation, with *Pichia, Issatchenkia* and *Kluyveromyces* species often present as well. Growth of these species declines by mid-fermentation when *Saccharomyces cerevisiae* becomes dominant, mostly due to their ethanol sensitivity (limited to 5-7%). *Schizosaccharomyces pombe, Zygosaccharomyces bailii* and *Zygosaccharomycesfermentati* are well known for their tolerance of high ethanol concentrations (>10%), but are often out-competed by other wine yeasts due to their slow growth.

A variety of yeasts, bacteria and filamentous fungi contribute to the microbial ecology of grapes and wine and to the chemical composition of wine, with yeasts having the dominant influence due to their role in alcoholic fermentation. However, our understanding of the different factors involved in the degradation of L-malate by yeasts associated with grapes and wine is limited and requires further investigation. The following discussion will first provide an overview of general L-malate metabolism in yeast cells, followed by a discussion of the degradation of L-malate in *S. cerevisiae, S. pombe* and *C. utilis,* with specific reference to the transport of L-malate and the structure, function and regulation of the malic enzyme in different yeast species.

CENTRAL ROLE OF MALIC ACID IN YEAST METABOLISM

L-Malate plays a pivotal role in the metabolism of C_3 and C_4 compounds in different subcellular compartments in the yeast cell. Depending on the cellular requirements, malate can be oxidised, dehydrated or decarboxylated (see Fig. 1). The oxidation of L-malate provides oxaloacetate for the turnover of the TCA cycle or for gluconeogenesis via phosphoenolpyruvate (PEP), while the decarboxylation of L-malate provides pyruvate for amino acid and other biosynthetic pathways. Synthesis of L-malate via malate synthase in the glyoxalate cycle provides a mechanism for the combination of two C_2 molecules (glyoxalate and acetyl-CoA) to form a C_4 molecule (Fraenkel, 1982).

The TCA cycle functions primarily in mitochondria where it allows for the complete degradation of pyruvate produced during glycolysis (see Fig. 1). The TCA cycle is only functional under aerobic conditions and is required for oxidative growth on pyruvate, lactate, acetate and ethanol (Boulton *et al.,* 1996). The cycle allows for the metabolic flow of carbon between various metabolic pathways and is a major source of NADH for the production of ATP via oxidative phosphorylation. Some of the enzymes of the TCA cycle are also present in the cytosol and peroxisome where they catalyse similar reactions. Under anaerobic conditions

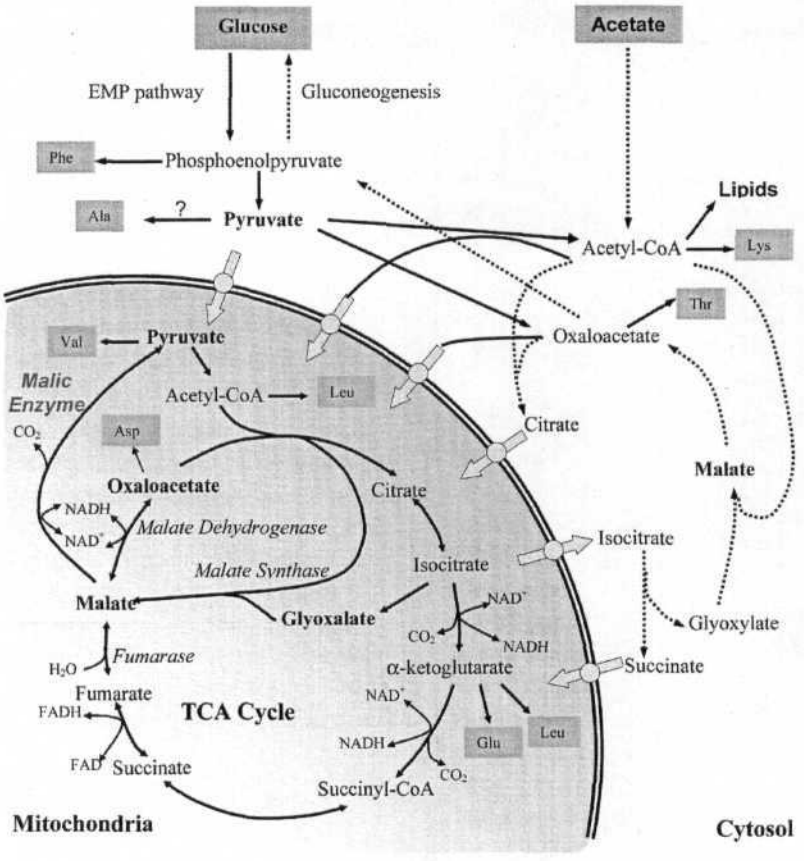


FIGURE 1
L-Malate plays a pivotal role in yeast metabolism. Dashed arrows represent reactions that do not operate during growth on glucose.

and in the presence of high concentrations of glucose, cells of *S. cerevisiae* do not have functional mitochondria (Fraenkel, 1982), but cytosolic enzymes similar to those in the TCA cycle produce the necessary biosynthetic intermediates.

The enzymatic reactions of the TCA cycle include the hydration of fumarate to L-malate via fumarase and the oxidation of L-malate to oxaloacetate via malate dehydrogenase (see Fig. 1). The reversible hydration of fumarate to L-malate is catalysed by the mitochondrial fumarase (encoded by *FUM1* gene in *S. cerevisiae,* Zubay *et al,* 1993), followed by oxidation to oxaloacetate by malate dehydrogenase *(S. cerevisiae MDH1),* with NAD^+ serving as the electron acceptor. The equilibrium of this reaction is in the direction of malate oxidation, favoured by the continued consumption of oxaloacetate in the TCA cycle. Both fumarase and malate dehydrogenase are regulated by the substrate concentration and the requirement for either the reductive or oxidative arm of the TCA cycle (Boulton *et al.,* 1996). NADH is produced by various enzymes of the TCA cycle during the oxidative arm of the TCA cycle. If the regeneration of NAD^+ from NADH via the conversion of acetaldehyde to ethanol under fermentable conditions is restricted, the reductive arm of the TCA cycle can be used to regenerate NAD^+, with the production of L-malate and succinate.

In *S. cerevisiae,* the production of L-malate most likely occurs via the reduction of oxaloacetate, which is synthetised by a biotin-dependent pyruvate carboxylase. The production of L-malate by yeast is greatly influenced by culture conditions: it is favoured by high sugar concentrations (20-30%), pH-values of about pH 5 and limiting levels of nitrogen (100-250 mg N/L), as well as the required presence of CO2 (Radler, 1993). Although part of the malate found in wine might have been synthesised by yeasts, the usually high level of amino acids and low pH of wine may inhibit the formation of malate.

The glyoxalate cycle in yeast peroxisomes is primarily associated with the complete degradation of fatty acids via (3-oxidation. However, it also plays an important role in the synthesis of C_4 compounds from C2 carbon substrates by employing some of the TCA cycle enzymes (Fraenkel, 1982). In *S. cerevisiae,* peroxisomal malate synthesis from glyoxalate is catalysed by malate synthase (MLS) as part of the glyoxalate pathway (Van Roermund *et al.,* 1995). This reaction combines the two C_2 molecules glyoxalate and acetyl-CoA into the C_4 molecule L-malate, which allows for the synthesis of TCA cycle intermediates in the peroxisome when the mitochondria are non-functional under anaerobic or high glucose conditions.

The peroxisomal malate dehydrogenase (MDH3) catalyses the reduction of oxaloacetate to malate with the concomitant oxidation of NADH to NAD^+ (Van Roermund *et al,* 1995). It was suggested that the peroxisomal membrane is impermeable to NADH *in vivo,* therefore the NADH produced during P-oxidation of fatty acids has to be deoxidised inside the peroxisome. The malate dehydrogenase therefore serves as part of a recycling mechanism where it catalyses the oxidation of NADH to provide NAD^+ for (3-oxidation. Since the peroxisomes are also believed to be impermeable to oxaloacetate, the conversion of oxaloacetate to L-malate allows for the export of reducing equivalents from the peroxisome. The malate dehydrogenases in the cytosol (MDH2) or mitochondrion (MDH1) can then oxidise the L-malate back to oxaloacetate for further metabolism.

During fermentative sugar metabolism in yeast, pyruvate, an important branching point in carbohydrate metabolism, is further decarboxylated to acetaldehyde by pyruvate decarboxylase and subsequently reduced to ethanol by alcohol dehydrogenase. Since L-malate is thus in effect converted to ethanol, this pathway is referred to as the malo-ethanolic fermentation pathway. Malo-ethanolic fermentation (MEF) is carried out mostly by yeast species such as 5. *pombe* and strains of 5. *cerevisiae* that convert malate into pyruvate by means of an intracellular malic enzyme (Volschenk *et al,* 2003).

DEGRADATION OF L-MALATE IN DIFFERENT YEAST SPECIES

A number of yeast species found in wine, *e.g. S. cerevisiae, S. pombe, S. pombe van malidevorans* and Z *bailii,* can utilise TCA cycle intermediates while growing on glucose (Thornton and Rodriques, 1996). Strains of 5. *pombe* and Z *bailii* can degrade high concentrations of L-malate, but only if glucose or another assimilable carbon source is present (Baranowski and Radler, 1984; Rodriquez and Thornton, 1989). In contrast, *H. anomala, C. sphaerica, P. stipitis* and *Pachysolen tannophilus* can utilise malic acid as sole carbon source, but this ability is repressed in the presence of sugar. Strains of *S. pombe van malidevorans* are also effective in malic acid utilisation (Gao and Fleet, 1995), with low efficiencies observed for *S. cerevisiae, C. stellata, Candida colliculosa* and *K. apiculata.* Most strains of 5. *cerevisiae* can only utilise L-malate in the presence of one or more fermentable carbon sources, but L-malate degradation is weak compared to *S. pombe,* which seems to be evolutionarily optimised for L-malate degradation.

The ability of a yeast strain to degrade extracellular L-malate is dependent on the efficient transport of the dicarboxylic acid, as well as the efficiency of the intracellular malic enzyme (Ansanay *et al,* 1996; Volschenk *et al,* 1997a; 1997b). In addition, factors such as the availability of glucose and oxygen also influence the efficacy of malic acid degradation. For example, under semi-anaerobic conditions, utilisation of L-malate was reduced at high and low levels of glucose in *S. pombe var. malidevorans* and Z *bailii* (Rodriques and Thornton, 1990). In contrast, *P. stipitis* and *P. tannophilus* utilised malate rapidly in the absence of glucose, with reduced utilisation in the presence of glucose, suggesting that malate utilisation is under catabolite repression.

S. pombe

Cells of 5. *pombe* display an extreme tolerance for high L-malate concentrations, as levels of up to 29 g L-malate/L can be degraded without any negative effect on cell growth, sugar metabolism or ethanol-producing abilities (Temperli *et al,* 1965). Taillandier and Strehaiano (1991) showed that L-malate is not integrated into biomass after its degradation by *S. pombe,* but is completely metabolised to ethanol and CO2 during anaerobiosis, and to CO2 under aerobiosis (Mayer and Temperli, 1963). As a K(-) yeast, *S. pombe* degrades L-malate only in the presence of glucose or another assimilable carbon source under both aerobic and anaerobic conditions (De Queiros and Pareilleux, 1990; Magyar and Panyik, 1989), suggesting that the metabolism of malate requires energy, probably for the transport of L-malate into the cell (Taillandier and Strehaiano, 1991).

Three enzymes are involved in L-malate degradation in *S. pombe,* namely the malate transporter, malic enzyme and a mito-

chondrial malate dehydrogenase (see Fig. 2). The malate transporter (mae1p) uses an H^+-symport system for the active transport of L-malate (Grobler *et al.,* 1995), while the NAD-dependent malic enzyme (mae2p) catalyses the oxidative decarboxylation of L-malate to pyruvate and CO_2 (Viljoen *et al.,* 1994). The *S. pombe* malate transporter is unable to transport fumarate, although fumarate inhibits the uptake of L-malate (Saayman *et al,* 2000). The cytosolic NAD-dependent malic enzyme (EC 1.1.1.38) has a high substrate affinity (K_m = 3.2) (Maconi *et al.,* 1984) and sequence analysis of mae2p revealed a strong phylogenetic link with other malic and malolactic enzymes (Viljoen *et al.,* 1994; Groisillier and Lonvaud-Funel, 1999).

The mitochondrial malate dehydrogenase (EC 1.1.1.37) oxidises L-malate to oxaloacetate in the TCA cycle and is responsible for 10% of the degradation of malate under aerobic conditions, with the remaining L-malate being directly converted to pyruvate and CO_2 via the malic enzyme. Therefore, both the malic enzyme and malate dehydrogenase are required for malate utilisation in *S. pombe* during aerobiosis. Under fermentative (non-aerated) conditions, when functional mitochondria are restricted, the cytosolic malic enzyme is exclusively involved in the degradation of intracellular L-malate (Osothsilp, 1987).

S. cerevisiae

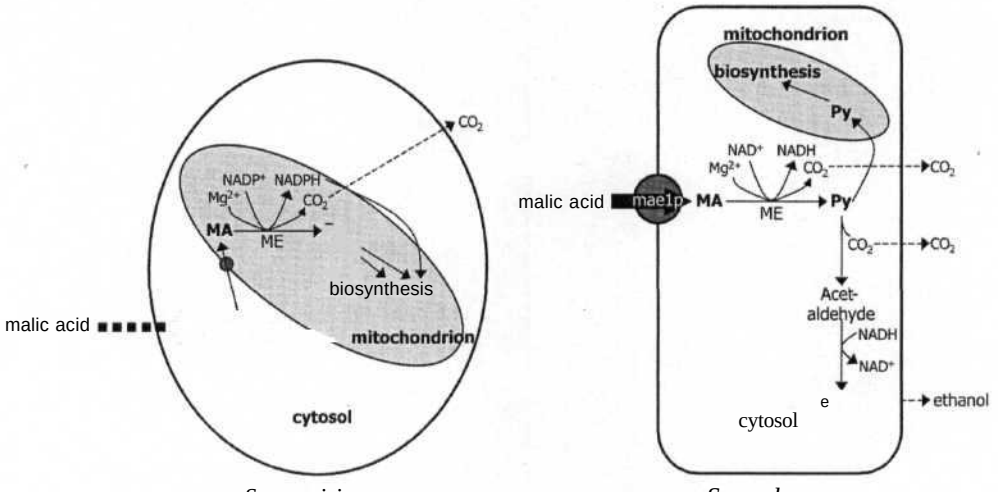
Although *S. cerevisiae* and *S. pombe* are both classified as K(-) yeasts, L-malate degradation is significantly weaker in *S. cerevisiae* for the following reasons: (1) *S. cerevisiae* lacks an active transport system for L-malate and extracellular L-malate enters the cells by means of simple diffusion; (2) the malic enzyme of *S. cerevisiae* has a significantly lower substrate affinity for L-malate (K_m = 50 mM) than that of *S. pombe* (K_m = 3.2 mM) (Temperli *et al.,* 1965; Fuck *et al.,* 1973); and (3) the *S. cerevisiae* malic enzyme is mitochondrial, whereas the *S. pombe* malic enzyme is cytosolic (see Fig. 2). The *MAE1* gene, identified as the structural gene for the *S. cerevisiae* malic enzyme, encodes a putative protein of 669 amino acids with 47% homology to the *S. pombe* malic enzyme (Boles *et al.,* 1998). The mitochondrial location of the *S. cerevisiae* malic enzyme suggests that this enzyme is essentially subjected to the regulatory effect of fermentative glucose metabolism, such as mitochondrial deterioration (Volschenk *et al.,* 2003).

The *S. cerevisiae* malate dehydrogenases are encoded by three genes, of which one gene product is located in the cytosol (Minard and McAlister-Henn, 1991) and the other two are located in the mitochondrion and peroxisome, respectively (Steffan and McAlister-Henn, 1992). The cytosolic enzyme, MDH2, has an estimated molecular mass of 42 kDa and is the target of glucose-induced proteolytic degradation. The apparent K_m of MDH2 for L-malate is 11.8 mM, which suits its function in the conversion of L-malate to oxaloacetate and is in sharp contrast to the K_m of 0.28 mM for MDH1, the mitochondrial isoenzyme.

C. utilis

Cells of *C. utilis* effectively degrade extracellular L-malate and fumarate, but glucose or other assimilable carbon sources repress the transport and degradation of these dicarboxylic acids (Saayman *et al.,* 2000). The transport of both dicarboxylic acids was strongly inducible by either fumarate or L-malate, while kinetic studies suggest that the two dicarboxylic acids are transported by the same protein.

Cloning and molecular analysis of the *C. utilis* malic enzyme gene, *CME1,* revealed a high degree of homology with the malic enzyme genes of *S. pombe* and *S. cerevisiae* (Saayman *et al.,* 2006). The *C. utilis* malic enzyme uses either NAD^+ or $NADP^+$ for the decarboxylation of oxaloacetate, but only NADP+ for the decarboxylation of L-malate. Based on its substrate specificity and cofactor requirements, the *C. utilis* malic enzyme can therefore be classified as L-Malate: NAD^+ oxidoreductase (oxaloacetate decarboxylating; EC 1.1.1.38). DNA sequence analysis revealed the absence of a mitochondrial targeting signal, suggesting that the *C. utilis* malic enzyme is cytosolic, similar to that of *S. pombe.* However, the expression of the two enzymes is subject to quite different regulatory signals; the *S. pombe* malic enzyme is induced under conditions that may result in redox imbalances, while the *C. utilis* malic enzyme is subject to catabolite repression (discussed in more detail in a following section).



S. cerevisiae *S.pombe*

FIGURE 2

The main differences in L-malic acid degradation between *S. cerevisiae* and *S. pombe* involve the transport of malic acid, the substrate affinity of the malic enzyme and the compartmentalisation of the malic enzymes in these two yeast species. MA, malic acid; Py, pyruvate; ME, malic enzyme; and mae1, malate transporter (Volschenk *et al.,* 2003).

DICARBOXYLIC ACID TRANSPORT IN YEAST

Two classes of dicarboxylic acid transporters have been described for yeast, *i.e.* those that are repressed by glucose and those that are not glucose-sensitive. In the K(+) yeasts *C. sphaerica* (Côrte-Real *et al,* 1989), *C. utilis* (Cássio and Leão, 1993) and *H. anomala* (Côrte-Real and Leão, 1990), transport of L-malate and other dicarboxylic acids across the plasma membrane is substrate-inducible and subject to glucose repression. These yeast species are able to use L-malate as sole carbon and energy source. In contrast, L-malate transport in the K(-) yeasts *S. pombe* and Z *bailii* occur only in the presence of glucose or another assimilable carbon source, with no substrate induction observed (Baranowski and Radler, 1984; Osothsilp and Subden, 1986b).

5. *pombe*

The dicarboxylic acid carrier of *S. pombe* was shown to be a proton dicarboxylate symport system that allows mediated transport and accumulation as a function of ApH (Sousa *et al,* 1992), with simple diffusion of the undissociated acid in the presence of high malate concentrations and low pH values. The *S. pombe* proton-malate symport is constitutive and remains active in the presence of high glucose concentrations (Sousa *et al,* 1992), but the transport of L-malate is inhibited by ethanol or acetate. In support of 5. *pombe's* requirement for fermentable carbon sources for L-malate utilisation, it was postulated that sugar metabolism provides the required energy by inducing the proton motive force for active transport of L-malate (Camarase *et al.,* 2001; Taillandier and Strehaiano, 1991).

Competitive inhibition of the initial uptake rates of labelled L-malate and succinic acid by D-malate, fumarate, oxaloacetate, a-ketoglutarate, maleate and malonate suggested a common 'dicarboxylate transport system' (Sousa *et ah,* 1992). However, Grobler *et al.* (1995) and Saayman *et al.* (2000) observed that although α-ketoglutarate and fumarate acted as competitive inhibitors of L-malate transport, neither substrate was transported by *S. pombe* cells. Molecular analysis of the 49 kDa *S. pombe* malate permease (maelp) revealed a protein with ten membrane-spanning or associated domains, and a similar structure to models proposed for integral membrane proteins from both prokaryotes and eukaryotes (Grobler *et al.,* 1995).

S. cerevisiae

Strains of the K(-) yeast 5. *cerevisiae* lack the machinery for the active transport of L-malate and rely on rate-limiting simple diffusion for the uptake of extracellular L-malate (Delcourt *et al.,* 1995). However, *S. cerevisiae* is able to utilise short-chain monocarboxylic acids, such as lactate and pyruvate, as sole carbon and energy sources under aerobic conditions. This is done by means of a proton symport process via a monocarboxylate permease (lactate/pyruvate permease, Jenlp), which is repressed by glucose and induced by non-fermentable substrates such as ethanol and lactate (Bojunga and Entian, 1999; Casal *et al.,* 1999).

C. utilis

Cells of *C. utilis* can utilise short-chain carboxylic acids as sole source of carbon and energy (Barnett *et al.,* 1990; Côrte-Real and Leão, 1990). When cells of *C. utilis* are grown in media with lactate, L-malate, citrate or other metabolisable carboxylic acids, they are able to transport mono-, di- and tri-carboxylic acids across the plasma membrane by two mediated transport systems: a proton symport specific for mono-, di- and tri-carboxylates and a facilitated transport system able to accept the undissociated form of the acids (as well as some amino acids). Both transport systems are inducible and subject to glucose repression (Cássio and Leão, 1991, 1993; Leão and Van Uden, 1986).

Two distinct transport modes were reported for L-malate and succinate in *C. utilis:* a low-affinity system (K_m of 1.5 mM for L-malate and K_m of 1.8 mM for succinate) and a high-affinity system (K_m of 4 mM for L-malate and K_m of 0.3 mM for succinate). All the other dicarboxylic acids, as well as lactate, pyruvate and citrate, use the low-affinity transport system. Amino acids such as glycine and glutamate competitively inhibited the uptake of succinate at high concentration levels, suggesting that they also use the low-affinity system (Cássio and Leão, 1993). A common carrier protein was suggested for L-malate, fumarate, oxaloacetate and α-ketoglutarate, since they are all competitive inhibitors of the high-affinity succinate transport system. The high-affinity L-malate transport system in *C. utilis* (K_m = 0.11 mM at pH 3.5, Saayman *et al.,* 2000) can be described as a proton-dicarboxylate symport while the low-affinity component is not dependent on transmembrane proton-motive forces. This is consistent with the hypothesis that the undissociated acid is transported by facilitated diffusion.

A significant difference in the transport of fumarate and L-malate has been observed for *C. utilis* and *S. pombe;* cells of *S. pombe* were unable to transport fumarate, although fumarate acted as a competitive inhibitor, whereas cells of *C. utilis* were able to actively transport both L-malate and fumarate (Saayman *et al.,* 2000). The uptake of both dicarboxylic acids by *C. utilis* was induced by either of the substrates and kinetic data suggested that fumarate and L-malate were transported by the same carrier protein in *C. utilis.* Transport of either fumarate or L-malate by *C. utilis* was repressed in the presence of glucose and was also insignificant in the presence of other carbon sources such as raffinose and glycerol/ethanol (Saayman *et al.,* 2000), confirming that the dicarboxylic acids are only transported in the presence of either of the inducers and when no alternative carbon source is available. In contrast, the *S. pombe* proton-malate symport system is constitutive, and can only metabolise L-malate in the presence of glucose or another assimilable carbon source (Sousa *et al.,* 1992).

YEAST MALIC ENZYMES: STRUCTURE, FUNCTION AND REGULATION

Malic enzymes catalyse a reversible oxidative decarboxylation of L-malate to yield pyruvate and CO_2, with the concomitant reduction of $NAD(P)^+$ to NAD(P)H (see Fig. 3). In addition to the $NAD(P)^+$ dinucleotide, malic enzymes require divalent cations (Mn^{2+} or Mg^{2+}) as cofactors. The three isozymes, EC 1.1.1.38-40 generally exhibit a high degree of amino acid homology (Viljoen *et al.,* 1994; Xu *et al.,* 1999), but differ in their intracellular localisation (cytosolic, mitochondrial or hydrogenosomal), substrate affinity and specificity (L-malate and/or oxaloacetate), co-factor specificity [either NAD^+ (EC 1.1.1.38, EC 1.1.1.39) or $NADP^+$ (EC 1.1.1.39, EC 1.1.1.40)] and the degree to which the decarboxylation reaction is reversible (Voegele *et al.,* 1999).

Malic enzymes are widely distributed in nature and have been reported in the yeasts *S. pombe* (Viljoen *et al,* 1994), *Rhodotorula glutinis* (Fernández *et al,* 1967), Z. *bailii* (Kuczynski and Radler, 1982), *S. cerevisiae* (Boles *et al,* 1998) and *C. utilis* (Saayman *et*

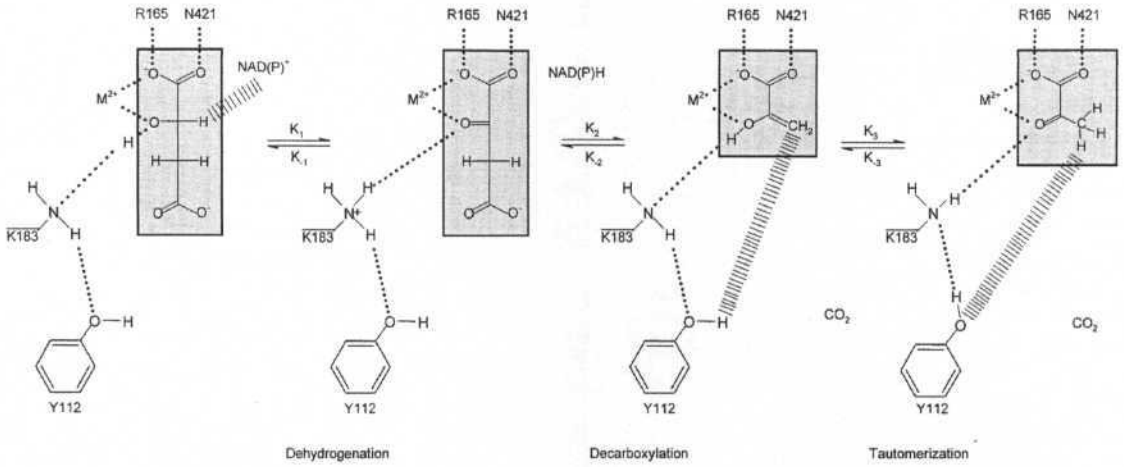


FIGURE 3

Active site of malic enzymes showing a possible catalytic mechanism for malic enzymes (Chang and Tong, 2003). Residue numbers refer to those of the human mitochondrial-NAD-dependent malic enzyme. The malate molecule (shaded box) is shown with the interactions with the metal ion (M^{2+}), nicotinamide cofactor ($NAD(P)^+$) and the Lys183 side chain (K183). The hydride transfers occurs between the C2 atom of malate and the C4 atom of $NAD(P)^+$, and the proton transfer between Tyr112 (Y112) and the C3 atom of the substrate.

al., 2006). The *S. cerevisiae, C. utilis* and *S. pombe* enzymes are bifunctional (can react with both malate and oxaloacetate), whereas the Z. *bailii* enzyme can only decarboxylate malate, suggesting that a different enzyme effects the decarboxylation of oxaloacetate. Whereas the *S. pombe* malic enzyme can only use NAD^+ (EC 1.1.1.38) as cofactor (Temperli *et al,* 1965), the *S. cerevisiae* malic enzyme uses both NAD^+ and $NADP^+$ as electron acceptor, with NAD^+ being favoured (Kuczynski and Radler, 1982). The *C. utilis* malic enzyme uses either NAD^+ or NADP for the decarboxylation of oxaloacetate, but only NADP+ for the decarboxylation of L-malate (Saayman *et al.,* 2006).

Yeast malic enzymes also differ with respect to their substrate affinities and metal requirements. The *S. pombe* malic enzyme has a very high substrate affinity (K_m = 3.2 mM) (Temperli *et al.,* 1965) as opposed to that of *S. cerevisiae* (K_m = 50 mM) (Osothsilp, 1987). The cytosolic malic enzymes from *C. utilis* and *S. pombe* require the divalent cations Mn^{2+} or Mg^{2+} for activity, in contrast to the mitochondrial *S. cerevisiae* malic enzyme that prefers Mn^{2+} (Osothsilp and Subden, 1986a; Osothsilp, 1987; Saayman *et al,* 2006).

Malic enzyme structure

The malic enzymes isolated and purified thus far show a subunit composition of two to ten identical subunits, with the malic enzyme from *S. pombe* being a homodimer (Osothsilp, 1987). These malic enzymes have a similar overall tertiary structure, with small local differences, which have important structural implications on their catalytic and regulatory mechanisms. Eight highly conserved regions, regions A-H, were identified in malic enzymes from various prokaryotic and eukaryotic organisms (Viljoen *et al.,* 1994). These regions represent clusters of highly conserved residues separated by spacer regions with less homology, but conserved in length. The amino acid sequences of the *C. utilis* (CMElp), *S. cerevisiae* (MAElp) and *S. pombe* (mae2p) malic enzymes showed various levels of homology: 37% between CMElp and mae2p, 68% between CMElp and MAElp, and 47% between MAElp and mae2p (Saayman *et al,* 2006). Phylogenetic analyses of the evolutionary relationship between CMElp and 44 known malic enzymes revealed that the *C. utilis* malic enzyme clusters together with *S. pombe* and *S. cerevisiae,* with a closer relationship with *S. cerevisiae.* The results suggested that sequence homologies among malic enzymes from various organisms are primarily determined by phylogenetic relationships between the organisms, rather than being the result of functional constraints related to catalytic properties and intracellular localisation.

The active site residues of malic enzymes can be roughly divided into four categories: (1) divalent cation-binding residues; (2) substrate-binding residues; (3) $NAD(P)^+$ cofactor binding residues; and (4) catalytic residues. The divalent cation is bound deep in the active site cleft and is octahedrally coordinated by six oxygens, one each from three side-chain carboxylate groups, two from the substrate or inhibitor and one from a water molecule (see Fig. 3). The metal ion serves as a bridge between L-malate to properly position the substrate at the active site center and to help polarise the C2 hydroxyl group during the initial stage (Chou *et al.,* 1995). The metal ion acts as a Lewis acid in the subsequent decarboxylation of oxaloacetate and plays a vital role in chelating the negatively charged enolate-pyruvate intermediate (Chang *et al.,* 2002).

Malate is bound in the active site such that the C2 hydroxyl is essentially in the same plane as the Cl carboxylate group (see Fig. 3) (Chang and Tong, 2003). The C2 hydroxyl and one of the Cl carboxylate oxygen atoms are ligands to the divalent cation. Malate is also involved in a large network of hydrogen-bonding and ionic interactions with the enzyme. The bound conformation of the oxaloacetate molecule is consistent with its role as an analogue of the enol-pyruvate transition-state intermediate as well as the pyruvate product (Tao *et al,* 2003). The active site also contains several hydrophobic residues and the majority of them do not have direct interactions with the substrate, but instead help to shield the active site region from the solvent in the closed form (Chang and Tong, 2003).

Catalysis by malic enzymes generally proceeds in three steps: dehydrogenation of malate to produce oxaloacetate *(k\),* decarboxylation of oxaloacetate to produce enolpyruvate (k_2), and tautomerisation of enolpyruvate to produce pyruvate (k_3) (see Fig. 3)

(Cleland, 1999). The divalent cation at the optimal position helps catalyse all the steps of the reaction, which explains its requirement for catalysis by malic enzymes. For the oxidative decarboxylation of malate, a general base is needed to extract the proton from the C2 hydroxyl group to initiate the dehydrogenation reaction *(k\).* For the tautomerisation reaction (k3), a general acid is needed to protonate the enolpyruvate intermediate at the C3 position, and a general base is needed to extract the proton from the C2 hydroxyl of this intermediate. In the complex with malate, the proton on the C2 atom is pointed toward the C4 atom of the nicotinamide ring of NAD^+, with a hydride transfer distance of about 2 Å (Chang and Tong, 2003). This explains the stereospecificity of malic enzymes for L-malate, as D-malate cannot adopt the same binding mode (Xu *et al,* 1999).

Physiological role of malic enzymes

Based on the divergent regulation of malic enzymes in different organisms, tissues and cellular compartments, and the evolutionary preservation of malic enzymes throughout a wide spectrum of organisms in nature, it is believed that malic enzymes are responsible for various essential physiological functions in living organisms (Driscol and Finan, 1996; Song *et al,* 2001). The end products of the malic enzyme reaction, *i.e.* pyruvate, CO2 and NAD(P)H, feed into numerous biological pathways that can be broadly defined as: (1) pathways where NAD^+-dependent malic enzymes are involved in oxidative metabolic processes that yield ATP via the electron transport system, or (2) pathways where the NADP dependent enzymes play a role in reductive biosynthesis processes (see Fig. 2). In line with this broad metabolic view, the NAD^+-dependent malic enzyme isoforms usually play an important role in cellular ATP biosynthesis via the production of NADH and pyruvate. Under fermentative conditions, the pyruvate generated by the cytosolic NAD-dependent malic enzyme is further metabolised to ethanol, probably to ensure that the redox balance is maintained.

NADP-dependent malic enzyme isoforms found in bacteria, yeast, fungi, birds and mammals play a role in primarily biosynthetic reactions, especially lipid biosynthesis and desaturation, through the provision of NADPH (Gourdon *et al,* 2000; Tanaka *et al.,* 1983; Xu *et al.,* 1999). The current accepted hypothesis suggests that several isoforms of NADP-malic enzyme exist in fungi through the action of post-translational modifications (either partial proteolytic cleavage, phosphorylation or dephosphorylation) and that specific isoforms of the NADP-malic enzyme are directly associated with lipid accumulation, whilst others have other cellular functions (Song *et al.,* 2001).

In 5. *cerevisiae,* NADPH is generated in only a few reactions: (1) via the two dehydrogenases of the pentose-phosphate (PP) pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), (2) via the $NADP^+$-dependent isocitrate dehydrogenase, (3) via the $NADP^+$-dependent acetaldehyde dehydrogenase, and (4) via the NADP-dependant malic enzyme. The *S. cerevisiae* mitochondrial NAD(P)-dependent malic enzyme, MAElp, is located in the center of the metabolic network of *S. cerevisiae,* converting malate (an intermediate of the TCA cycle) into pyruvate (a key metabolite for yeast in the split between respiration and fermentation), with the production of one NADPH molecule (Boles *et al,* 1998). In contrast, the NAD^+-dependent malic enzyme from *S. pombe* seems to play a role in the provision of cytosolic NADH under fermentative conditions (Groenewald and Viljoen-Bloom, 2001). The high substrate affinity and cytosolic location of the *S. pombe* malic enzyme enables the yeast to effectively degrade malate to ethanol during alcoholic fermentation (Sousa *et al.,* 1995; Taillandier *et al.,* 1995). A similar function is plausible for the *C. utilis* NAD-dependent malic enzyme, although it is subject to different regulatory mechanisms (Saayman et a/., 2006).

Due to the respiro-fermentative metabolism of *S. cerevisiae,* carbon flow is steered away from biosynthesis towards ethanol production under both anaerobic and aerobic conditions (Fiechter *et al,* 1981; Pronk *et al.,* 1996). However, even under fermentative conditions, some degree of biosynthetic activity is essential for the yeast cell's survival. Biosynthesis results in a net consumption of NADPH and a net production of NADH and, since alcoholic fermentation is a redox-neutral process, ethanol formation does not account for the re-oxidation of assimilatory NADH. This redox dilemma is solved by *S. cerevisiae* and other yeasts by reducing glucose to glycerol, with the associated re-oxidation of NADH (Larson *et al,* 1998; Van Dijken and Scheffers, 1986).

Regulation of yeast malic enzymes

Various mechanisms have been described for regulating the level of malic enzyme activities. Regulation of the levels of mRNA via transcriptional and post-transcriptional regulation is normally found in higher eukaryotic cells, whereas malic enzymes in fungi and bacteria are usually regulated through competition and activation by other dicarboxylic acids.

S. pombe mae2p

Analysis of the transcriptional regulation of the 5. *pombe* malic enzyme gene indicated that two m-acting elements in the *mae2* promoter, UAS1 and UAS2, are required for basal expression, while three negative-acting elements (URSs) are involved in general derepression of *mae2* (Viljoen *et al,* 1999). Transcription of *mae2* in 5. *pombe* was induced after growth on high glucose or salt concentrations or under non-aerated conditions. This response was linked to the involvement of both the cAMP-dependent (Pkal) and general stress-activated (Styl) signal transduction pathways (Groenewald and Viljoen-Bloom, 2001). It is possible that there are two levels of regulation for the *mae2* gene in response to glucose. The first level involves a mild carbon regulated induction in response to high glucose concentrations *(e.g.* 8% glucose), and the second a stronger induction in response to osmotic stress conditions *(e.g.* 30% glucose). Both these conditions can result in redox imbalances, which are rectified by increasing the production of glycerol, with the corresponding oxidation of NADH to NAD^+ (Bakker *et al,* 2001). The additional NAD^+ must be reduced to NADH, potentially via the malic enzyme, to maintain the NAD^+/NADH redox balance within the cell.

S. cerevisiae MAElp.

Expression of the *S. cerevisiae MAE1* gene was found to be relatively low, but constitutive during continuous cultivation on different carbon sources, *i.e.* glucose, ethanol and acetate (Boles *et al,* 1998). A clear induction of *MAE1* expression was observed during anaerobic growth of *S. cerevisiae* on glucose in continuous culture, with a 3-fold increase in transcription and a 4-fold increase in the enzyme activity in cell extracts.

The degradation of extracellular L-malate during alcoholic fermentation varied greatly between different *Saccharomyces* species: *S. paradoxus* degraded 28% to 38% L-malate, whereas *S. cerevisiae* and *S. bayanus* degraded only 17% and 8% of the L-malate during alcoholic fermentation (Redzepovic *et al.,* 2003). Transcriptional analyses showed that expression of the malic enzyme genes from *S. paradoxus* and *S. cerevisiae* increased towards the end of fermentation, once glucose was depleted, whereas the level of transcription in the non-degrading stain, *S. bayanus,* decreased towards the end of fermentation. Only *S. paradoxus* showed an increased degradation of L-malate in response to the increase in malic enzyme expression, suggesting that it was able to utilise the L-malate as a secondary carbon source. Given the different promoter sequences observed for the three *Saccharomyces* strains (Redzepovic *et al.,* 2003), it is plausible that different transcription regulatory mechanisms exist in *S. paradoxus,* which could explain this yeast's higher aptitude to degrade L-malate. These results also implicated the native malic enzyme gene as one of the pivotal role players involved in the differential ability of *Saccharomyces* species to degrade L-malate (Volschenk *et al,* 2003).

C. utilis CMElp

Saayman *et al.* (2006) demonstrated a 10-fold increase in transcription of *CME1* when grown in media containing 0.5% malate as sole carbon source, relative to growth in media containing 2% glucose. Malic enzyme activity was induced more than 35-fold (relative to growth in 2% glucose) in cells grown in 0.5% L-malate as sole carbon source, while carbon sources that can provide pyruvate for the TCA cycle repressed the malic enzyme activity. This confirmed that the glucose repression/substrate induction observed for malate degradation and malate transport in *C. utilis* also applies to the *C. utilis* malic enzyme, suggesting concerted regulatory mechanisms that govern the degradation of extracellular malate in this organism.

The strong carbon sensitivity displayed for both the malate transporter and malic enzyme can be linked to the ability of C. *utilis* to utilise intermediates of the TCA cycle as the only source of carbon and energy. The transport and degradation of L-malate will be delayed when better carbon sources (e.g. glucose) are available (Saayman *et al.,* 2006). When no better assimilable carbon source is available, the cell will adjust its metabolism to utilise L-malate for the provision of pyruvate for biogenesis as well as energy production via the TCA cycle. An interesting observation was that growth in 2% glucose under non-aerated conditions (exclusion of oxygen) increased the enzyme activity 2-fold. Under aerobic growth conditions the yeast tends to channel most of its pyruvate into the TCA cycle, resulting in an adequate supply of intracellular TCA cycle intermediates. Under non-aerated conditions the mitochondrial TCA enzymes may be repressed (McCammon *et al.,* 2003), which leaves the cytosolic malic enzyme with the task of replenishing TCA intermediates for other cellular requirements. Since the *C. utilis* malic enzyme is predicted to be cytosolic (Saayman *et al.,* 2006), induction of the malic enzyme activity will therefore contribute to replenishing of the TCA cycle intermediates.

Genetically modified malate-degrading *S. cerevisiae* strains

Biological deacidification of wine with malolactic fermentation has been the method of choice for most winemakers as it is perceived to be a natural or spontaneous process (reviewed in Volschenk *et al.,* 2006). However, due to inherent problems associated with malolactic fermentation, alternative biological methods for the deacidification of wine have been investigated, including the heterologous co-expression of the *S. pombe* malate transporter *(mael)* and malic enzyme *(mae2)* genes, as well as *mael* together with the *Oenococcus oeni* malolactic gene *(mleA)* gene. Deacidification of wine with a malo-degrading yeast strain will eliminate the use of bacterial starter cultures for malolactic fermentation, which in turn will reduce the risk of the production of hazardous compounds such as biogenic amines and ethyl carbamate. Furthermore, use of the malo-degrading yeast is expected to eliminate delays in cellar operations due to the simultaneous completion of alcoholic fermentation and deacidification, which in turn will minimise the risk of spoilage by oxidation and the proliferation of spoilage microorganisms.

The genes encoding the *S. pombe* malate permease *{mael)* and *O. oeni* malolactic *(mleA)* genes were cloned and successfully expressed in laboratory strains of *S. cerevisiae* (Volschenk *et al.,* 1997a; 1997b). Recently, a genetically engineered commercial wine yeast, ML01, was constructed through stable integration of the *mael* and *mleA* genes into the genome of *S. cerevisiae* S92, a Prise de Mousse strain (Husnik *et al.,* 2006). The recombinant malolactic strain retained all the qualities and characteristics of S92, as well as the ability to complete the malolactic fermentation during the initial stages of alcoholic fermentation. The strain received GRAS (Generally Regarded as Save) status from the FDA and Canadian regulatory authorities and has been commercialised in Moldova and the USA.

The general inefficient degradation of extracellular malate by strains of *S. cerevisiae* is ascribed to the absence of an active transport system for the uptake of malate, as well as low substrate affinity of the intracellular malic enzyme (Ansanay *et al.,* 1996; Volschenk *et al.,* 1997a; 1997b). The uptake and intracellular degradation of L-malate by an industrial *Saccharomyces bayanus* EC1118 strain was greatly improved with the integration of the *S. pombe mael* and *mae2* genes, encoding the malate transporter and malic enzyme, respectively (Volschenk *et al.,* 2004). This introduced a strong malo-ethanolic phenotype that resulted in complete degradation of extracellular L-malic acid degradation within 1, 1.5, 2 and 5 days in Cabernet Sauvignon, Ruby Cabernet, Colombard and Chardonnay grape musts, respectively, without negatively affecting the fermentation ability of the yeast or wine flavour perception.

The *C. utilis* malic enzyme gene *(CME1)* was also subcloned and introduced into a *S. cerevisiae* laboratory strain together with the *S. pombe* malate transporter gene *(mael)* (Saayman *et al.,* 2006). The recombinant malic enzyme was active in *S. cerevisiae* and the degradation ability of the recombinant strain compares well with results obtained for the co-expression of the *S. pombe mael* and *mae2* genes in *S. cerevisiae.*

CONCLUSIONS

L-Malic and tartaric acid are the most prominent organic acids in wine and play a crucial role in winemaking processes and wine quality, including the organoleptic quality and the physical, biochemical and microbial stability of wine. The production of premium wines depends on the oenologist's skill to adjust wine acid-

ity to obtain the optimum balance with other wine components, to produce wine with optimum colour and flavour. Various yeast species are associated with grapes, grape must and fermenting wine, but only a few are able to degrade L-malic acid, and even fewer are suitable for the fermentation process. In general, strains of *Saccharomyces* degrade only 3% to45% L-malic acid present in grape must during alcoholic fermentation, with relatively minor modifications in total acidity during vinification. This varying degree of L-malic acid degradation in *Saccharomyces* strains can in part be ascribed to different levels of malic enzyme expression.

Fundamental knowledge about the nature and regulation of malo-ethanolic pathways in different yeasts is therefore imperative in our understanding of the physiological role of L-malate and its degradation in yeast. This may result in better strategies for the deacidification of grape must and wine through the selection of appropriate wine yeast strains, manipulating the fermentation conditions or even the introduction of a heterologous malo-alcoholic phenotype into wine yeast strains. The commercial availability of a malo-ethanolic wine yeast will be especially beneficial to the production of fruity-floral wines, such as Gewurtzraminer and Riesling, as well as the deacidification of high-acid wines in the cool-climate viticultural regions of the world. Furthermore, it will eliminate the use of bacterial starter cultures for malolactic fermentation, which in turn will reduce the risk of the production of hazardous compounds such as biogenic amines and ethyl carbamate. Furthermore, use of the malo-ethanolic yeast is expected to eliminate delays in cellar operations due to the simultaneous completion of alcoholic fermentation and deacidification, which in turn will minimise the risk of spoilage by oxidation and the proliferation of spoilage microorganisms. Another potential benefit of the malo-ethanolic wine yeast is in the production of distilled beverages for distilling purposes since higher levels of ethanol are produced during fermentation with the malo-ethanolic yeast.

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