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REVIEW ARTICLE

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Malo-ethanolic fermentation in *Saccharomyces* and *Schizosaccharomyces*

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Abstract Yeast species are divided into the K(+) or K(−) groups, based on their ability or inability to metabolise tricarboxylic acid (TCA) cycle intermediates as sole carbon or energy source. The K(−) group of yeasts includes strains of *Saccharomyces*, *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii*, which is capable of utilising TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources. Although grouped together, these yeasts have significant differences in their abilities to degrade malic acid. Typically, strains of *Saccharomyces* are regarded as inefficient metabolisers of extracellular malic acid, whereas strains of *Sch. pombe* and *Z. bailii* can effectively degrade high concentrations of malic acid. The ability of a yeast strain to degrade extracellular malic acid is dependent on both the efficient transport of the dicarboxylic acid and the efficacy of the intracellular malic enzyme. The malic enzyme converts malic acid into pyruvic acid, which is further metabolised to ethanol and carbon dioxide under fermentative conditions via the so-called malo-ethanolic (ME) pathway. This review focuses on the enzymes involved in the ME pathway in *Sch. pombe* and *Saccharomyces* species, with specific emphasis on the malate transporter and the intracellular malic enzyme.

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Introduction

Several yeast species are recognised for their ability to metabolise extracellular L-malic acid and fall into either the K(−) or K(+) yeast groups, depending on their ability to utilise L-malic acid and other tricarboxylic acid (TCA) cycle intermediates as sole carbon or energy source (Barnett and Kornberg 1960; Barnett et al. 1990; Goto et al. 1978; Rodriquez and Thornton 1990; Saayman et al. 2000; Whiting 1976). The K(+) group includes *Candida sphaerica* (Côrte-Real et al. 1989), *C. utilis* (Cássio and Leão 1993), *Hansenula anomala* (Côrte-Real and Leão 1990), *Pichia anomala* (Amador et al. 1996) and *Kluyveromyces marxianus* (Queiros et al. 1998), which have the ability to utilise TCA cycle intermediates as sole energy and carbon sources, with no requirement for other assimilable sugars. The K(−) group can utilise TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources (Barnett and Kornberg 1960). Strains of *Saccharomyces* sensu stricto (*Sac. cerevisiae*, *Sac. paradoxus*, *Sac. pastorianus*, *Sac. uvarum*, *Sac. bayanus*), *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii* are all classified as K(−) yeasts. Although grouped together, the yeasts in this category have diverse aptitudes to metabolise L-malic acid. Typically, strains of *Saccharomyces* are regarded as inefficient metabolisers of extracellular L-malic acid; and the synthesis of L-malic acid in some strains of *Saccharomyces* has even been reported (Fatichenti et al. 1984; Pines et al. 1996,1997; Ramon-Portugal et al. 1999; Schwartz and Radler 1988). In contrast, strains of *Sch. pombe* and *Z. bailii* can degrade high concentrations of L-malic acid (Baranowski and Radler 1984; Kuczynski and Radler 1982; Osothsilp 1987; Osothsilp and Subden 1986b; Rodriquez and Thornton 1989; Taillandier and Strehaiano 1991; Taillandier et al. 1988).

Genetic and biochemical characterisation of the L-malic acid-utilising pathways in several K(−) and

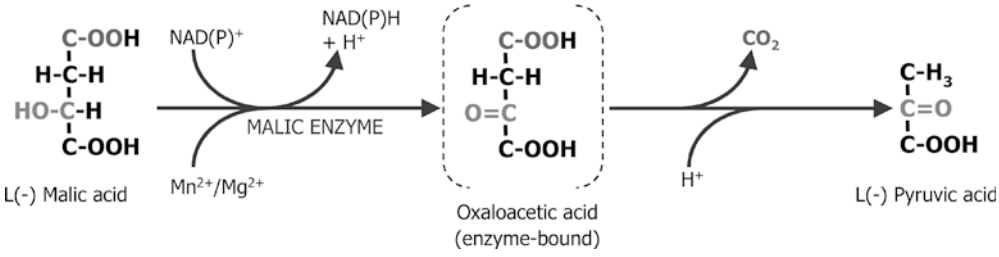


Fig. 1 The NAD(P)-dependent malic enzyme catalyses the oxidation of L-malic acid to oxaloacetic acid, followed by decarboxylation to pyruvic acid

K(+) yeast species, including *Sch. pombe*, *C. utilis*, *K. marxianus*, *Z. bailii* and *Sac. cerevisiae*, indicated that the physiological role and regulation of L-malic acid metabolism differs significantly between the K(−) and K(+) yeasts. In general, L-malic acid metabolism in K(−) yeasts is characterised by the absence of glucose repression or substrate induction (Osothsilp and Subden 1986b; Rodriquez and Thornton 1989). In contrast, the regulation of L-malic acid metabolism in K(+) yeasts typically exhibits strong glucose (or catabolite) repression and rapid substrate induction, which leads to a diauxic shift in growth pattern, where glucose is exhausted before L-malic acid is utilised as a carbon source (Amador et al. 1996; Cássio and Leâo 1993; Côrte-Real and Leâo 1990; Côrte-Real et al. 1989; Queiros et al. 1998).

In general, the ability to metabolise extracellular L-malic acid depends on an efficient uptake system for L-malic acid (i.e. active import via a malate transporter) and a L-malic acid-converting enzyme (i.e. fumarase, malolactic enzyme, malate dehydrogenase or a malic enzyme). This discussion focuses on the uptake of L-malic acid and the intracellular degradation thereof via malic enzymes found in *Sch. pombe* and *Saccharomyces* strains. This fundamental knowledge is of particular importance in industrial processes where the degradation or biosynthesis of L-malic acid is required. For example, L-malic acid is one of the dominant organic acids in wine and the degradation of excess L-malic acid in grape must is of major importance in the production of quality wines that require a judicious balance between the sugar, acid and flavour/aroma components. However, strains of *Saccharomyces* routinely used for wine fermentation in general do not degrade L-malic acid effectively during alcoholic fermentation. Furthermore, the degree of L-malic acid degradation varies from strain to strain and, although the underlying mechanisms of this phenomenon provided the focus of recent studies (Redzepovic et al. 2003), they are not yet well understood.

Malic enzymes

Since the first description of a malic enzyme in pigeon liver more than 50 years ago (Ochoa et al. 1947), ''malic enzyme'' activities have been identified in several organisms, including prokaryotes (*Bacillus subtilis*, *B. stearothermophilus*, *Clostridium thermocellum*, *Corynebacterium glutamicum*, *Escherichia coli*, *Pseudomonas putida*, *Rhizobium meliloti*, *Sulfolobus solfataricus*; Driscoll and Finan 1996; Gourdon et al. 2000; Kobayashi et al. 1989), parasitic flagellates (*Tritrichomonas foetus*; Vaňáčová et al. 2001), yeasts, fungi (*Aspergillus nidulans*, *Mortierella alpina*, *Mucor circinelloides*, *Neocallimastix frontalis*; Song et al. 2001; Van der Giezen et al. 1998; Wynn et al. 1999), plants (Drincovich et al. 2001; Edwards and Andreo 1992; Edwards et al. 1998; Lance and Rustin 1984; Laporte et al. 2002), birds (Goodridge 1968a, 1968b; Goodridge and Ball 1966, 1967), mammals (Coleman and Kuzava 1991) and humans (Bukato et al. 1995; Kochan et al. 1995; Loeber et al. 1994; Xu et al. 1999; Yang et al. 2000). The malic enzyme catalyses the oxidative decarboxylation of L-malic acid to pyruvic acid and CO_2, linked to the reduction of the pyridine nucleotides, NAD^+ or $NADP^+$ (Fig. 1). During fermentative sugar metabolism in yeast, pyruvic acid, 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-malic acid is thus in effect converted to ethanol, this pathway is referred to as the ''malo-ethanolic (ME) fermentation pathway''.

Malic enzymes (EC 1.1.1.38–40; Outlaw and Springer 1983) in general exhibit a high degree of amino acid homology (Viljoen et al. 1994; Xu et al. 1999; Yang et al. 2000), but differ in their intracellular localisation (cytosolic, mitochondrial or hydrogenosomal), substrate affinity and specificity (L-malic acid and/or oxaloacetic acid), 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). 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 (Driscoll and Finan 1996; Song et al. 2001).

The end-products of the malic enzyme reaction, i.e. pyruvic acid, CO_2 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, or (2) pathways where the NADP-dependent enzymes play a role in reductive biosynthesis processes. 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 pyruvic acid. For example, the human NAD-dependent malic enzyme is pivotal in energy production via glutamine in rapidly growing tissues, such as the spleen, thymus, mucosal cells of small intestine and tumour cells (Bagetto 1992; McKeehan 1982; Sauer et al. 1980). In contrast, the reverse reaction of the NADP-dependent malic enzyme (which involves the carboxylation of pyruvic acid to malic acid) plays an important housekeeping role in the anapleurotic reactions of the TCA cycle of both prokaryotes and eukaryotes (Sauer et al. 1999; Wedding 1989).

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 (Coleman and Kuzava 1991; Goodridge 1968a, 1968b; Goodridge and Ball 1966, 1967; Gourdon et al. 2000; Leveille et al. 1968; Nunes et al. 1996; Tanaka et al. 1983; Wynn et al. 1999; Xu et al. 1999). When ATP supplies are abundant, acetyl-coenzyme A can be diverted into fatty acids as an energy reserve. However, mitochondrial acetyl-coenzyme A must be converted to citric acid via the tricarboxylate transport system (Fig. 2) to participate in fatty acid synthesis in the cytosol. Citrate synthase fuses acetyl-coenzyme A with oxaloacetic acid to produce citric acid that is transported from the mitochondria to the cytosol. Once in the cytosol, citric acid is converted back to oxaloacetic acid via the energy-dependent citrate lyase. The oxaloacetic acid is then reduced to L-malic acid via malate dehydrogenase; and L-malic acid can be oxidised to pyruvic acid via the malic enzyme, with the production of NADPH that can feed into the fatty acid biosynthesis pathway. Pyruvic acid can also be re-imported back into the mitochondria. Similarly, L-malic acid can be transported back into the mitochondria and used to produce NADH via the mitochondrial malate dehydrogenase.

The role of NADP-malic enzyme in lipid biosynthesis in filamentous fungi was studied in depth and strong evidence was obtained that malic enzyme activity is a key factor in ensuring maximal lipid accumulation (Wynn and Ratledge 1997, 2000). The direct relation between malic enzyme activity and lipid accumulation was until recently still speculative, since maximum lipid accumulation in fungi was not necessarily linked to maximum NADP-malic enzyme activity (Song et al. 2001; Wynn et al. 1999). However, the finding of multiple isoforms of NADP-malic enzyme in some fungi and the evolution of specific isoforms under specific growth conditions of high lipogenesis clarified the critical role of NADP-malic enzymes in lipid biosynthesis (Savitha et al. 1997; Song et al. 2001; Zink 1972). 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,

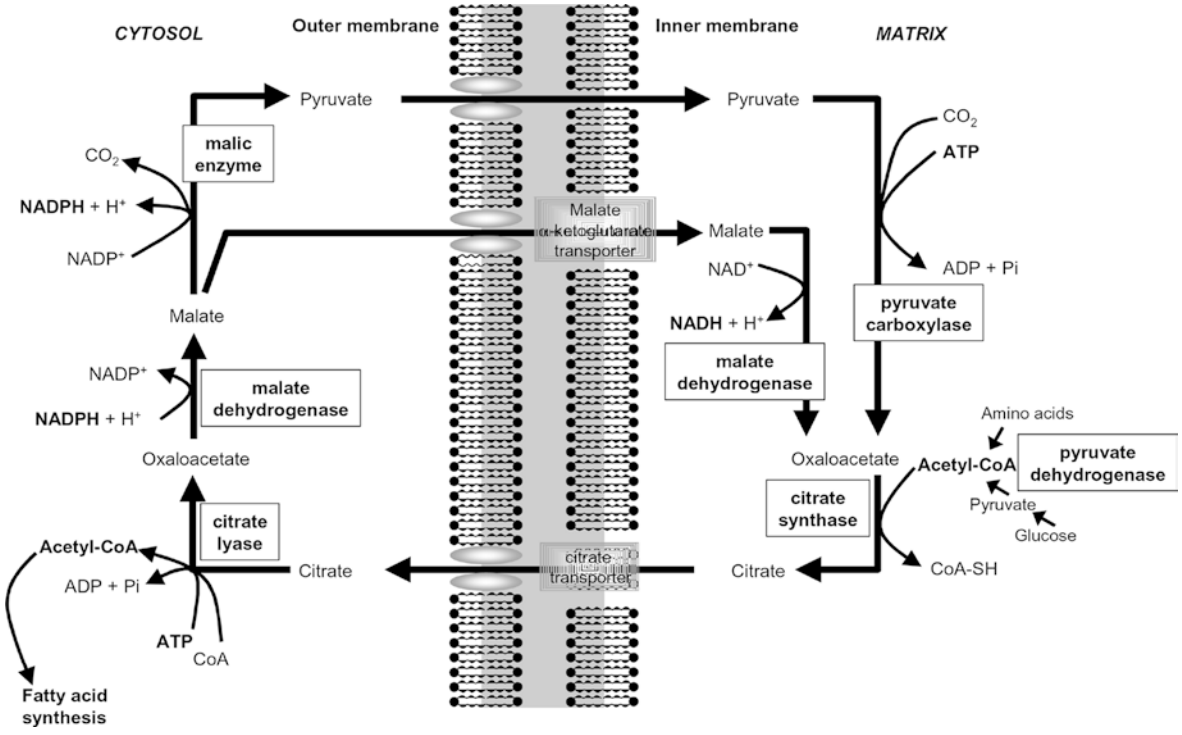


Fig. 2 The role of the malic enzyme in lipid biosynthesis and desaturation through the provision of cytosolic NADPH in mouse and human. The tricarboxylate transport system is responsible for the export of acetyl-coenzyme A from the mitochondrial matrix into the cytosol, where the fatty acid biosynthetic pathway is situated (adapted from Biocarta, http://www.biocarta.com/)

whilst others have other cellular functions (Song et al. 2001).

Malic acid transport in yeast

Two classes of malate transporters have been described for yeast and fungi, i.e. those that are repressed by glucose and those that are not. In the K(+) yeasts *K. lactis*, *C. utilis*, *H. anomala* and *C. sphaerica*, the malate transport system was found to be substrate-inducible and subject to glucose repression (Camarasa et al. 2001; Cássio and Leão 1993; Côrte-Real and Leão 1990; Côrte-Real et al. 1989). Saayman et al. (2000) showed that *C. utilis* effectively degraded extracellular fumarate and L-malate, but glucose or other assimilable carbon sources repressed the transport and degradation of these dicarboxylic acids. The transport of the two dicarboxylic acids was shown to be strongly inducible by either fumarate or L-malate, while kinetic studies suggest that the same protein transports both dicarboxylic acids.

In the K(−) yeasts *Z. bailii* and *Sch. pombe*, L-malic acid transport was found to be active in the presence of glucose and not induced by the substrate (Baranowski and Radler 1984; Osothsilp and Subden 1986b). In support of the *Sch. pombe* requirement for fermentable carbon sources for L-malic acid utilisation, it was postulated that sugar metabolism provides the required energy by inducing the proton motive force for active transport of L-malic acid (Camarasa et al. 2001; Magyar and Panyik 1989; Osothsilp and Subden 1986a, 1986b; Taillandier and Strehaiano 1991). However, strains of *Saccharomyces* lack the machinery for the active transport of L-malic acid and rely on rate-limiting simple diffusion for the uptake of extracellular L-malic acid (Delcourt et al. 1995).

ME pathway in *Sch. pombe*

As a K(−) yeast, *Sch. pombe* utilises L-malic acid (or other TCA cycle intermediates) only in the presence of glucose or other assimilable carbon sources (De Queiros and Pareilleux 1990; Fuck and Radler 1972; Magyar and Panyik 1989; Osothsilp 1987; Osothsilp and Subden 1986b; Rankine 1966; Taillandier et al. 1988). *Sch. pombe* displays an extreme tolerance for high L-malic acid concentrations, as levels of up to 29.0 g L-malic acid/l can be degraded without any negative effect on cell growth. Temperli et al. (1965) also found that this highly active metabolism of L-malic acid had no effect on the yeast's sugar metabolism or ethanol-producing abilities. Our understanding of why *Sch. pombe* degrades L-malic acid in this manner and the specific metabolic role of ME fermentation in this yeast was partially gained through primary biochemical characterisation of the L-malic acid transport and enzymatic conversion to pyruvic acid by *Sch. pombe* cells. Detailed molecular analysis of the mechanisms involved in regulating L-malic acid degradation in *Sch. pombe* further contributed to our understanding of the physiological role of the ME pathway in yeast.

L-Malic acid metabolism in *Sch. pombe* involves three enzymes, i.e. the malate permease, the cytosolic malic enzyme (EC 1.1.1.38) and a mitochondrial malate dehydrogenase enzyme (EC 1.1.1.37; Osothsilp and Subden 1986a). Under fermentative (non-aerated) conditions when functional mitochondria are restricted, the cytosolic malic enzyme of *Sch. pombe* is exclusively involved in the degradation of intracellular L-malic acid. However, during aerobiosis (respiration), when mitochondria are functional, both the malic enzyme and malate dehydrogenase play a role in the metabolism of L-malic acid. The malate dehydrogenase contributes to approximately 10% of the L-malic acid degradation during aerobiosis, while the remaining L-malic acid is directly converted to pyruvic acid and CO_2 via the malic enzyme (Osothsilp 1987; Osothsilp and Subden 1986a; Subden et al. 1998).

Transport of L-malic acid via *Sch. pombe* malate permease (mae1p)

Genetic analysis of L-malic acid metabolism in *Sch. pombe* originated in the 1980s, when mutants of *Sch. pombe* defective in L-malic acid metabolism (*mau*$^-$ mutants) were generated and characterised (Osothsilp and Subden 1986a). Based on classic genetic analysis, the mutants were found to group into three complementation groups, namely malate permease, malic enzyme and malate dehydrogenase mutants. The malate transporter (*mae1*) and malic enzyme (*mae2*) genes involved in L-malic acid metabolism were subsequently cloned from a genomic library of *Sch. pombe* (Grobler et al. 1995; Subden et al. 1998; Viljoen et al. 1994).

The structural gene of the malate permease (*mae1*), localised on chromosome I of *Sch. pombe*, encodes an open reading frame of 1,314 bp that translates into a putative protein of 438 amino acids with a calculated molecular mass of approximately 49 kDa (Grobler et al. 1995). On the transcription level, preliminary expression studies of the *Sch. pombe* malate permease (*mae1*) gene confirmed that it is constitutively expressed and is not subject to catabolite repression (Grobler et al. 1995; Osothsilp 1987).

A hydropathy profile of the predicted amino acid sequence revealed a protein with ten membrane-spanning or associated domains and hydrophilic N- and C-termini. The predicted secondary structure of the protein is similar to models proposed for other integral membrane proteins from both prokaryotes and eukaryotes. Conserved motifs found in other transport proteins were also identified, e.g. a leucine zipper motif, a C-terminal PEST motif and several N-linked glycosylation and protein kinase C phosphorylation sites (Grobler et al. 1995; Rogers et al. 1986). The biological role of these protein motifs in *Sch. pombe* has not yet

been established and requires more in-depth physiological studies. In addition, the putative mae1p protein did not contain a N-terminal membrane-targeting signal, but the existence of an internal membrane signal motif was suggested (Grobler et al. 1995).

Proton flux studies with *Sch. pombe* during L-malic acid transport strongly suggest that the malate permease operates as a proton-dicarboxylate symporter and that the proton motive force (ΔpH) is the driving force behind L-malic acid uptake (Camarasa et al. 2001; Osothsilp and Subden 1986b; Sousa et al. 1992). According to the pK_a parameters for L-malic acid dissociation ($pK_{a1} = 3.41$, $pK_{a2} = 5.1$), the negatively charged mono-anionic form of L-malic acid is transported by carrier-mediated active transport at pH 3.5. At pH values lower than 3.4, mainly the undissociated form of the acid enters cells of *Sch. pombe* by simple diffusion (Baranowski and Radler 1984; Camarasa et al. 2001; Osothsilp and Subden 1986b; Rodriquez and Thornton 1990; Sousa et al. 1992, 1995). Taillandier et al. (1988) first demonstrated that the uptake of L-malic acid in *Sch. pombe* displays saturation kinetics data typical of carrier-mediated active transport. The energy requirement of active L-malic acid transport was demonstrated by the inhibitory effect of energy metabolism inhibitors on L-malic acid transport, such as oxidative phosphorylation uncouplers and electron transport inhibitors (Osothsilp and Subden 1986b). Recently, active transport by mae1p was confirmed when the *mae1* gene was expressed in *Sac. cerevisiae* under the regulation of the 3-phosphoglycerate kinase (*PGK1*) promoter (Camarasa et al. 2001; Volschenk et al. 1997b).

Cells of *Sch. pombe* have the ability to metabolise mainly two TCA cycle intermediates: both L-malic acid and oxalacetic acid undergo vigorous oxidative decarboxylation, while the other TCA cycle intermediates are poorly metabolised (Krebs 1952). Mayer and Temperli (1963) ascribed this phenomenon to the presence of a "permeability barrier" for these slowly metabolised TCA cycle intermediates in *Sch. pombe*. Competitive inhibition studies on the initial transport rate of C^{14}-labelled L-malic acid in the presence of other TCA cycle intermediates or dicarboxylic acids, such as succinic, fumaric, oxalacetic, α-ketoglutaric, maleic and malonic acid, indicated that the malate permease of *Sch. pombe* might act as a general transporter for all these acids, but not for lactic, pyruvic and citric acid (Sousa et al. 1992). More in-depth investigations into the transport mechanism of the *Sch. pombe* malate transporter revealed that the malate permease is able to act as a selective general dicarboxylic acid transporter. Although the preferred substrate of the *Sch. pombe* malate transporter is L-malic acid, other dicarboxylic acids (such as succinic, malonic, α-ketoglutaric acid) are also weakly transported by this protein (Camarasa et al. 2001; Grobler et al. 1995). However, fumaric acid is not actively transported by mae1p, but competes with L-malic acid during transport, probably by binding to the active site of the protein and thereby blocking L-malic acid transport (Saayman et al. 2000). This could be ascribed to the structural relatedness of malic and fumaric acid; and it may also apply to other TCA cycle intermediates and dicarboxylic acids that may compete for the transporter.

Sch. pombe malic enzyme (mae2p)

The structural gene of the *Sch. pombe* malic enzyme, *mae2*, was cloned and characterised as an open reading frame of 1,695 bp located on chromosome III of *Sch. pombe* (Viljoen et al. 1994). DNA sequence analysis of the *Sch. pombe* malic enzyme gene did not indicate the presence of a mitochondrion-targeting signal, suggesting that the malic enzyme functions in the cytosol of *Sch. pombe* cells. A high degree of amino acid similarity was observed between the putative *Sch. pombe* mae2p protein and malic enzymes from various prokaryotic and eukaryotic organisms (Viljoen et al. 1994). A high degree of similarity was also observed between the eukaryotic malic enzyme and the malolactic enzyme of lactic acid bacteria. The phylogenetic tree obtained with amino acid sequences of malolactic enzymes and different malic enzymes showed that these two types of enzymes might have a common ancestor. In fact, the malic enzymes of *E. coli*, *Sch. pombe* and *Sac. cerevisiae* showed a closer phylogenetic link with the malolactic enzymes of lactic acid bacteria than with malic enzymes from other organisms (Groisillier and Lonvaud-Funel 1999).

The biologically functional form of most eukaryotic malic enzymes is a homotetrameric protein composed of four identical sub-units (Lee and Chang 1990; Mitsch et al. 1998). In *Sch. pombe*, however, the malic enzyme was predicted to be a dimer consisting of two identical subunits with a molecular mass of 60 kDa (Temperli et al. 1965). The enzyme has an optimal pH range of 3.5–4.0, with a high substrate affinity for L-malic acid ($K_m = 3.2$ mM; Temperli et al. 1965). The malic enzyme catalyses the direct 1,4-decarboxylation of L-malic acid to pyruvic acid and CO_2 (Fig. 1). The enzyme in *Sch. pombe* is bifunctional, reacting with either L-malic acid or oxalacetic acid and requiring NAD^+ and the divalent cations Mn^{2+} or Mg^{2+} for activity (Osothsilp 1987; Osothsilp and Subden 1986a). The metal ion serves as a bridge between L-malic acid to properly position the L-malic acid at the active centre and to help polarise the C-2 hydroxyl group during the initial stage (Chou et al. 1995). The metal ion acts as a Lewis acid in the subsequent decarboxylation of oxaloacetic acid and plays a vital role in chelating the negatively charged enolate–pyruvate intermediate (Chang et al. 2002).

Molecular analysis of the *Sch. pombe* malic enzyme gene and its deduced amino acid sequence revealed eight highly conserved regions, A–H, present in various prokaryotic and eukaryotic malic enzymes (Viljoen et al. 1994, 1998). These regions represent clusters of highly conserved residues separated by spacer regions with less homology, but conserved in length. Four of the conserved regions were implicated in the binding of

NAD(P)H, L-malic acid or divalent cations. Although the physiological importance of the other conserved regions is yet unknown, their importance should not be disregarded. A single point mutation in the *Sch. pombe* malic enzyme gene at nucleotide 1331 (G-to-A) changed amino acid 444 from a glycine into an aspartate residue in the conserved region H and completely abolished the malic enzyme activity (Viljoen et al. 1998).

An eminent attribute of the conversion of L-malic acid to ethanol in *Sch. pombe* is the stoichiometric nature of this conversion, i.e. one mole of L-malic acid is converted into one mole of ethanol and CO_2, with no apparent link between L-malic acid utilisation and cell growth or biomass production under fermentative conditions (Magyar and Panyik 1989; Mayer and Temperli 1963; Taillandier and Strehaiano 1991; Taillandier et al. 1988, 1995). The transcriptional regulation of the *mae2* gene of *Sch. pombe* under different conditions was therefore investigated to shed some new light on the function of malic enzyme in this yeast. In accordance with the general characteristics of L-malic acid metabolism in K(−) yeasts, the expression of the *mae2* gene was not induced by the substrate, malic acid. Expression studies revealed an increase in transcription of the *mae2* gene under high glucose (8%) and anaerobic (fermentative) conditions (Groenewald and Viljoen-Bloom 2001; Viljoen et al. 1999). Subsequent deletion and mutational analysis of the *mae2* gene promoter identified the presence of several *cis*-acting regulatory elements, including upstream activator sequences and repressor sequences that play a role in the regulation of the *mae2* gene in *Sch. pombe*. The specific role of these *cis*-acting regulatory elements in the regulation of the *mae2* gene expression could be linked to the cAMP-dependent and general stress-activated pathways in *Sch. pombe*.

A hypothetical model for the regulation of the malic enzyme expression suggests that there are two possible levels of regulation of the *mae2* gene in response to glucose. The first level of regulation involves a mild carbon-regulated induction of malic enzyme expression in response to increased glucose concentrations and the second, a stronger induction in response to osmotic stress conditions (Groenewald and Viljoen-Bloom 2001; Viljoen et al. 1999). To rectify the osmolarity imbalance in hyper-osmotic conditions, such as high glucose conditions, the yeast responds 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 to maintain the NAD^+/NADH redox balance within the cell. Increased expression of the *Sch. pombe* malic enzyme gene in 30% glucose and 0.8 M KCl (i.e. osmotic stress conditions) could therefore provide a means to reduce the additional NAD^+, to allow for glycerol production (Groenewald and Viljoen-Bloom 2001).

The induced expression of the cytosolic malic enzyme gene under fermentative conditions, when the mitochondria are not fully operational, may also provide pyruvic acid and NADH for essential anapleurotic reactions (Viljoen et al. 1999). Pyruvic acid plays an important role in the provision of α-ketoglutaric acid and oxalacetic acid for the synthesis of amino acids and nucleotides. Both these precursors are synthesised in the mitochondria and transported to the cytosol for biosynthetic reactions. Alternative pathways must therefore be utilised for the synthesis of these precursors when the mitochondria are not functional. These anapleurotic reactions comprise the carboxylation of pyruvic acid to oxalacetic acid via pyruvate carboxylase, the oxidation of L-malic acid to pyruvic acid via the malic enzyme and the production of succinic acid via the glyoxylate cycle. Although earlier biochemical studies indicated that the metabolism of L-malic acid in *Sch. pombe* does not contribute to cell biomass, the induced expression of the *Sch. pombe* malic enzyme under fermentative conditions may provide an important secondary pathway for the provision of pyruvic acid for other metabolic requirements (Groenewald and Viljoen-Bloom 2001).

ME pathway in strains of *Saccharomyces*

Within the five-member *Saccharomyces* sensu stricto group, i.e. *Sac. cerevisiae*, *Sac. paradoxus*, *Sac. pastorianus*, *Sac. uvarum* and *Sac. bayanus* (Pulvirenti et al. 2002), notable variations in the degradation of L-malic acid were observed. The degradation of L-malic acid by strains of *Saccharomyces* also correlated with the optimal growth temperature of the individual strains: cryotolerant species (i.e. *Sac. bayanus*, *Sac. pastorianus*, *Sac. uvarum*) synthesised L-malic acid, while mesophyllic strains of *Saccharomyces* degraded intermediate amounts of L-malic acid during fermentation. The thermotolerant strains of *Sac. cerevisiae* and *Sac. paradoxus* were able to degrade 40–48% L-malic acid (Castellari et al. 1994; Rainieri et al. 1998a, 1998b).

As a K(−) yeast, *Sac. cerevisiae* only utilises L-malic acid in the presence of one or more fermentable carbon sources. However, L-malic acid utilisation in *Sac. cerevisiae* is weak compared with *Sch. pombe*, which seems to be evolutionarily optimised for L-malic acid degradation. Moreover, the malic enzyme is not essential for the survival of *Sac. cerevisiae* cells, as a deletion of the malic enzyme gene does not influence its viability (Boles et al. 1998). Together with the mitochondrial location of the *Sac. cerevisiae* malic enzyme, these characteristics suggest that the malic enzyme fulfils an entirely different role in the metabolism of *Sac. cerevisiae*.

Uptake of L-malic acid by *Sac. cerevisiae*

Previous studies showed simple diffusion of L-malic acid (and other dicarboxylic acids) in *Sac. cerevisiae* (Ansanay et al. 1996; Baranowski and Radler 1984; Camarasa et al. 2001; Rodriquez and Thornton 1990;

Salmon 1987; Salmon et al. 1987; Volschenk et al. 1997a); and the most favourable pH was found to be pH 3.0–3.5, suggesting that only the undissociated form of L-malic acid enters the yeast cell (Salmon 1987; Salmon et al. 1987). The initial L-malic acid concentration influenced the final amount of L-malic acid degraded by *Sac. cerevisiae*, i.e. higher initial L-malic acid concentrations resulted in a faster diffusion rate into the cells, which in turn resulted in higher levels of L-malic acid degradation. However, the initial level of glucose did not have any significant influence on the rate of L-malic acid uptake or the final amount of L-malic acid degraded by *Sac. cerevisiae* (Delcourt et al. 1995).

The complete sequence of the *Sac. cerevisiae* genome was published in 1996 (Goffeau et al. 1996), but no structural gene or functional homologue for the transporter protein of extracellular L-malic acid has yet been identified in this yeast. However, a mitochondrial dicarboxylic acid carrier was purified and characterised and the genes of several mitochondrial dicarboxylic acid transporters were described (Lançar-Benba et al. 1996; Pallotta et al. 1999; Palmieri et al. 1996, 1999, 2000). The lack of a genetic equivalent for the malate transport gene of *Sch. pombe* corroborates the biochemical evidence on the absence of an active transport system for L-malic acid in *Sac. cerevisiae*.

Sac. cerevisiae malic enzyme (MAE1p)

The malic enzyme phenotype was designated to open reading frame YKL029C in the *Sac. cerevisiae* genome, based on amino acid sequence comparison with the known *Sch. pombe* malic enzyme (Boles et al. 1998). The *MAE1* gene, identified as the structural gene for the *Sac. cerevisiae* malic enzyme, encodes a putative protein of 669 amino acids with 47% homology to the *Sch. pombe* malic enzyme. The identity of the malic enzyme gene was confirmed when deletion or over-expression of the gene resulted in a loss or increase in malic enzyme activity, respectively (Boles et al. 1998). Phylogenetic analysis of the *Sac. cerevisiae* malic enzyme indicated a closer relatedness to eubacterial malic enzymes than to malic enzymes of higher eukaryotes.

Initial research by Polakis and Bartley (1965) indicated that *Sac. cerevisiae* lacks malic enzyme activity; but very low malic enzyme activities were reported nearly a decade later in cell extracts of *Sac. cerevisiae* (Fuck et al. 1973). The partially purified *Sac. cerevisiae* malic enzyme (EC 1.1.1.38) was characterised as having a low substrate affinity ($K_m = 50$ mM) that is at least 15-fold weaker than the *Sch. pombe* malic enzyme, which further contributes to the inefficient degradation of L-malic acid in *Sac. cerevisiae* (Fuck et al. 1973; Osothsilp 1987; Salmon 1987; Temperli et al. 1965). As in *Sch. pombe*, malate dehydrogenases contribute little to L-malic acid degradation in *Sac. cerevisiae* during fermentation, since functional mitochondria are absent under these conditions. In contrast to the *Sch. pombe* malic enzyme, the malic enzyme from *Sac. cerevisiae* can use both NAD^+ and $NADP^+$ as an electron acceptor (Fuck et al. 1973) and prefers manganese (Mn^{2+}) as a divalent cation.

The mitochondrial location of the *Sac. cerevisiae* malic enzyme may further contribute to the weak degradation of L-malic acid by strains of *Sac. cerevisiae* under fermentative conditions. The mitochondrial localisation was first suggested by the presence of amino acid-targeting motifs (Allison and Schatz 1986; Von Heijne 1986) and was subsequently confirmed by subcellular fractionation analysis (Boles et al. 1998). Mitochondria, the powerhouse of yeast cells and responsible both for energy generation through oxidative phosphorylation and for the synthesis of haem, pyrimidines, amino acids and many other key metabolites, exist in two different well defined physiological states, depending on the presence or absence of dissolved oxygen. When yeast is grown aerobically on a non-fermentable carbon source, the mitochondria of the fully respiring cells are rich in cristae and up to 50 per cell have been observed. Under anaerobic growth conditions, mitochondria are redundant for respiration, due to the absence of oxygen as a terminal electron acceptor. Nevertheless, the pre-mitochondria present in yeast cells are still responsible for several important functions, including the synthesis and desaturation of fatty acids and membrane lipids, ergosterol biosynthesis, physiological adaptation to stresses caused by ethanol, toxic oxygen radicals and high sugar, modification of cell surface characteristics involved in flocculation and cell partitioning, amino acid and purine/pirimidine biosynthesis, mobilisation of glycogen and production of flavour and aroma compounds (O′Connor-Cox et al. 1996).

Cells of *Sac. cerevisiae* have a strong tendency towards alcoholic fermentation, due to the so-called Crabtree effect. Even under fully aerobic conditions, a mixed respiro-fermentative metabolism is observed when the sugar concentration in the growth medium exceeds a threshold value (typically ca. 1 mM; Verduyn et al. 1984) or when the growth rate exceeds a critical level (usually ca. 66% of the maximum specific growth rate on glucose; Flikweert et al. 1997). In glucose-repressed cells, only a few mitochondria with poorly developed cristae are found and most of the mitochondrial enzyme activities are repressed (Cho et al. 2001; Dejean et al. 2000; García et al. 1993; Jayaraman et al. 1966; Mattoon et al. 1978; Perlman and Mahler 1974; Polakis and Bartley 1965).

Preliminary transcriptional regulation studies of the *MAE1* gene in *Sac. cerevisiae* shed some light on the physiological role of the malic enzyme in this yeast. Expression of the *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). Verifying evidence for the constitutive expression of the *MAE1* gene was obtained by genome-wide expression studies, where the *MAE1* gene

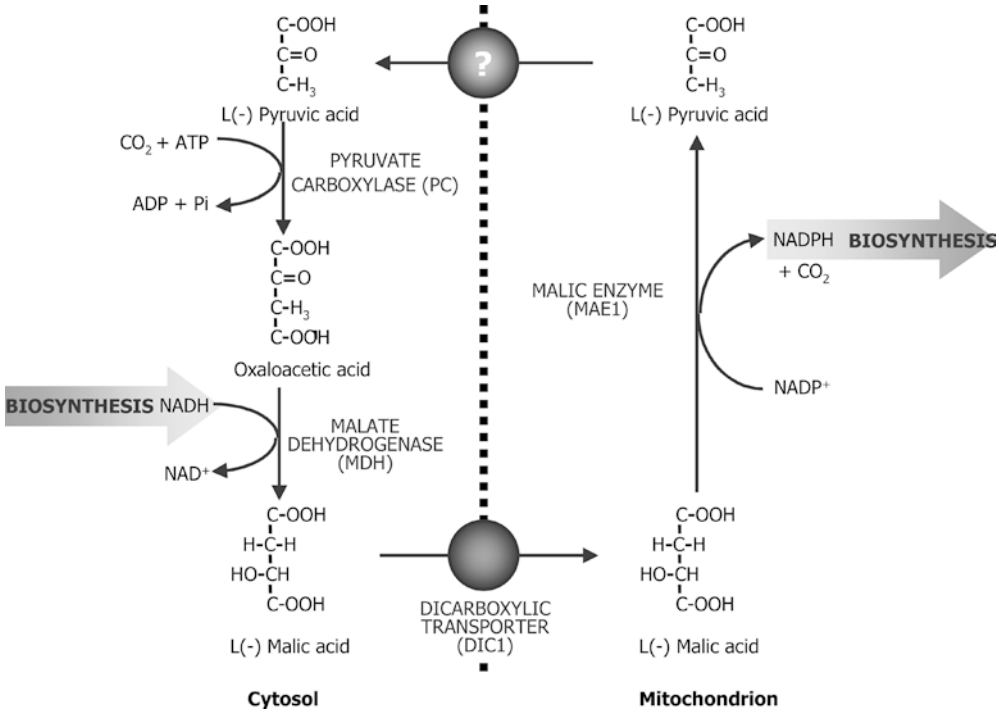


Fig. 3 The predicted transhydrogenase malic acid–pyruvic acid shuttle in *Saccharomyces cerevisiae*, which functions as a recycling process for cytosolic NADH into mitochondrial NADPH. All enzyme activities, except the mitochondrial pyruvate transporter (*indicated by the question mark*) were found in *Sac. cerevisiae* (adapted from Bakker et al. 2001)

expression showed no change during batch growth in a 2% glucose medium until the glucose was exhausted (DeRisi et al. 1997; Ter Linde et al. 1999).

A clear induction of *MAE1* expression was observed during anaerobic growth of *Sac. cerevisiae* on glucose in continuous culture, with a ca. 3-fold increase at the transcriptional level and a ca. 4-fold increase in the enzyme activity of cell extracts (Boles et al. 1998). Similar results were obtained during genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Sac. cerevisiae* (Ter Linde et al. 1999). However, a database search with the promoter sequence of the *MAE1* gene did not reveal any significant or relevant transcription factor-binding sites. The expression pattern of *Sac. cerevisiae MAE1* gene strongly suggests an important physiological function of the malic enzyme under anaerobic conditions, possibly in the provision of intramitochondrial NADPH or pyruvate (Boles et al. 1998).

The underlying mechanisms in three different strains of *Saccharomyces* showing varying aptitudes to degrade extracellular L-malic acid during alcoholic fermentation were further investigated by Redzepovic et al. (2003). *Sac. paradoxus* RO88 was able to degrade 28–38% L-malic acid, whereas *Sac. cerevisiae* 71B and *Sac. bayanus* EC1118 degraded only 17% and 8% of the malic acid during alcoholic fermentation, respectively. It was shown that expression of the malic enzyme genes from *Sac. paradoxus* RO88 and *Sac. cerevisiae* 71B increased towards the end of fermentation once glucose was depleted, whereas the level of transcription in *Sac. bayanus* EC1118, a non-degrading strain, decreased towards the end of fermentation. Only *Sac. paradoxus* RO88 showed an increased degradation of malic acid in response to the increase in malic enzyme expression, suggesting that it was able to utilise the malic acid as a secondary carbon source.

These results implicate the native malic enzyme gene as one of the pivotal role players involved in the differential ability of *Saccharomyces* strains to degrade malic acid. The study clearly showed different expression patterns for the three *Saccharomyces* malic enzyme genes that could be ascribed to different regulatory mechanisms employed by the strains. Given the different promoter sequences observed for *Sac. paradoxus* and the other two *Saccharomyces* strains, it is plausible that different transcription regulatory mechanisms exist in *Sac. paradoxus* that could explain this yeast's higher aptitude to degrade L-malic acid.

The current proposed physiological role of the malic enzyme in *Sac. cerevisiae* involves its possible participation in an auxiliary pathway for the regeneration of the main biosynthetic co-factor, NADPH (Boles et al. 1998; Fig. 3). Due to the respiro-fermentative metabolism of *Sac. cerevisiae*, carbon flow is steered away from biosynthesis towards ethanol production in 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 reoxidation of assimilatory NADH. *Sac. cerevisiae* and other yeasts solved this redox dilemma by reducing glucose to

glycerol, with the concomitant reoxidation of NADH (Larson et al. 1998; Nordström 1968; Oura 1977; Van Dijken and Scheffers 1986). According to this model (Fig. 3), malic enzyme, pyruvate carboxylase, NAD^+-dependent malate dehydrogenase (*MDH2*) and the mitochondrial dicarboxylic carrier (*DIC1*) act as a cyclic transhydrogenase shuttle to convert the NADH resulting from biosynthetic metabolism (Van Dijken and Scheffers 1986) to NADPH, to sustain the yeast cell's biosynthetic requirements (Bakker et al. 2001). This role for the malic enzyme in a NADH/NADPH shuttle was also described for the pancreatic islets of rats (MacDonald 1995).

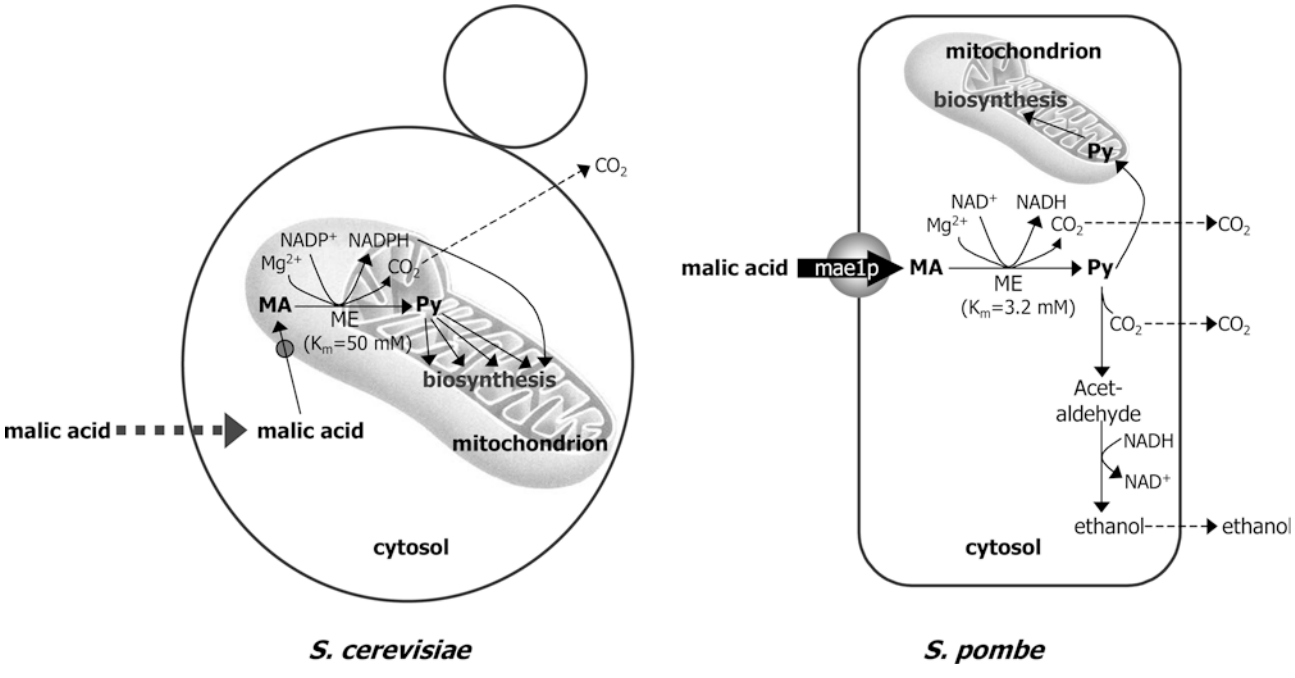
One of the major shortcomings in this model is that the actual existence of a mitochondrial pyruvate transporter has not yet been established through the identification of its structural gene in the genome of *Sac. cerevisiae*. Furthermore, the natural direction of pyruvic acid flux during respiration is from the cytosol, where glycolysis takes place, into the mitochondria. If this shuttle is active in *Sac. cerevisiae*, it is therefore unlikely to operate as a complete shuttle. The physiological role of the presumed malic acid–pyruvic acid shuttle is thus considered to be a complementary, but nevertheless important, pathway for the provision of pyruvic acid for biosynthesis purposes in the yeast's mitochondria (Bakker et al. 2001; Gombert et al. 2001).

Fig. 4 The main differences in L-malic acid degradation between *Sac. cerevisiae* and *Sch. pombe* involves the transport of malic acid, the substrate affinity of the malic enzyme and the compartmentalisation of the malic enzymes in these two yeast species. Despite these significant differences, the malic enzyme seems to play a similar role in *Sac. cerevisiae* and *Sch. pombe*, i.e. to supply pyruvic acid for biosynthesis

Comparison of ME pathways in *Sch. pombe* and *Sac. cerevisiae*

As K(−) yeasts, both *Sac. cerevisiae* and *Sch. pombe* are unable to utilise L-malic acid as only energy or carbon source. L-Malic acid is oxidatively decarboxylated to pyruvic acid and CO_2 by a malic enzyme in both yeasts, but the efficiency of L-malic acid degradation is significantly weaker in *Sac. cerevisiae*. Three main reasons for the weaker degradation of L-malic acid in *Sac. cerevisiae* have been postulated. First, *Sac. cerevisiae* lacks the machinery for active transport of L-malic acid (Fig. 4) found in *Sch. pombe* and relies on rate-limiting simple diffusion for the intake of extracellular malic acid. Second, the malic enzyme of *Sac. cerevisiae* has a significantly lower substrate affinity for L-malic acid ($K_m = 50$ mM) than that of *Sch. pombe* ($K_m = 3.2$ mM), which contributes to the weaker metabolism of this acid in *Sac. cerevisiae* (Fuck et al. 1973; Temperli et al. 1965). Third, the mitochondrial location of the malic enzyme of *Sac. cerevisiae* suggests that this enzyme is inherently submitted to the regulatory effect of fermentative glucose metabolism, such as mitochondrial deterioration, which is a well documented phenomenon in Crabtree-positive yeast and which may amplify the already weak L-malic acid metabolism of *Sac. cerevisiae*.

On the basis of the opposing L-malic acid degradation abilities of *Sac. cerevisiae* and *Sch. pombe*, one can argue that L-malic acid metabolism should play distinct physiological roles in these yeast species. However, biochemical and genetic evaluation of the enzymes and genes involved in this pathway from both yeasts concluded that the *Sac. cerevisiae* and *Sch. pombe* malic enzymes play an almost similar role in the provision of pyruvic acid for cellular biosynthesis. The possibility for



the existence of a NADH–NADPH recycling function of the *Sac. cerevisiae* mitochondrial malic enzyme cannot be ruled out, but additional evidence is still required. Furthermore, since the NADP-malic enzyme is implicated in lipogenesis in higher eukaryotes, like fungi, mammals and humans, it is arguable that the *Sac. cerevisiae* NADP-malic enzyme gene might also be directly involved in the increased synthesis of certain phospholipids, especially in the yeast cell's protective response against hyper-osmotic stress. However, it has been postulated that the conversion of L-malic acid to pyruvic acid to ethanol is a redox-neutral process. The exact influence of the strong cytosolic malic enzyme of *Sch. pombe* on maintaining the redox balance and energy production in this yeast therefore requires more in-depth investigation.

Future research and biotechnological applications

The physiological role and regulation of the ME pathway in yeasts are still relatively poorly understood. Recently, significant advances were made to unravel the underlying mechanisms for malic acid degradation in *Sch. pombe* and *Saccharomyces* strains. However, no clear-cut explanation for the highly effective ME pathway in *Sch. pombe* has yet been found. Furthermore, variations in malic acid degradation between strains of the same species, such as *Saccharomyces* spp, have yet to be satisfactorily described, since direct comparison of sequence data only provides some vague explanations. In-depth analysis of the ME pathways in yeasts from different genetic backgrounds by means of DNA microarrays, multidimensional protein chromatography and metabolic profiling approaches will illuminate the cardinal physiological importance and regulation of this pathway in yeasts.

Fundamental knowledge about the regulation and physiological role of L-malic acid and its metabolism in yeast is imperative for the successful application of innovative genetic engineering strategies for *Saccharomyces*. From a wine-making perspective, K(−) yeasts or their genetically modified counterparts seem to be well suited for the de-acidification of wine as an alternative to the bacterial malolactic fermentation. One of the strongest advantages of employing malo-ethanolic K(−) yeast in the biodeacidification of wine is the production of the primary end-product of alcoholic fermentation, ethanol, without the introduction of any other unnatural metabolic intermediates to wine. A strong malate-degrading phenotype was therefore introduced into a *Sac. cerevisiae* laboratory strain in which the *Sch. pombe mae1* and *mae2* genes were functionally co-expressed under the constitutive regulation of the *Sac. cerevisiae* 3-phosphoglycerate kinase (*PGK1*) promoter and terminator elements (Volschenk et al. 1997b). This introduced two new enzymatic activities into *Sac. cerevisiae* that yielded recombinant strains with the ability to: (1) actively transport L-malic acid into the cell and (2) convert the malic acid to ethanol under fermentative conditions (Volschenk et al. 2001).

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