Characterisation of L-malic acid metabolism in strains of *Saccharomyces* and the development of a commercial wine yeast strain with an efficient malo-ethanolic pathway

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

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 accurately adjust wine acidity to obtain the optimum balance with other wine components to produce wine with optimum colour and flavour.

Strains of *Saccharomyces*, in general, rarely degrade L-malic acid completely in grape must during alcoholic fermentation, with relatively minor modifications in total acidity during vinification. The degree of L-malic acid degradation, however, varies from strain to strain. Some strains of *Saccharomyces* are known to be able to degrade a higher percentage of L-malic acid, but the underlying reason for this phenomenon is unknown. The underlying mechanisms of this phenomenon have been partially revealed during preliminary transcriptional regulation research during this study. In contrast, *S. pombe* cells can effectively degrade up to 29 g/l L-malic acid via the malo-ethanolic pathway that converts L-malic acid to pyruvate and CO_2, and ultimately to ethanol under fermentative conditions. A number of reasons for the weak degradation of L-malic acid in *Saccharomyces cerevisiae* have been postulated. Firstly, *S. cerevisiae* lacks the machinery for the active transport of L-malic acid found in *S. pombe* and relies on rate-limiting simple diffusion for the uptake of extracellular L-malic acid. Secondly, the malic enzyme of *S. cerevisiae* has a significantly lower substrate affinity for L-malic acid (K_m = 50 mM) than that of *S. pombe* (K_m = 3.2 mM), which contributes to the weaker degradation of L-malic acid in *S. cerevisiae*. Lastly, the mitochondrial location of the malic enzyme of *S. cerevisiae*, in contrast to the cytosolic *S. pombe* malic enzyme, suggests that the *S. cerevisiae* malic enzyme is inherently subject to the regulatory effects of fermentative metabolism.

The malate permease gene (*mae1*) and the malic enzyme gene (*mae2*) of *S. pombe* was therefore cloned and co-expressed in single or multi-copy under regulation of the constitutive *S. cerevisiae* 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences in a laboratory strain of *S. cerevisiae*. This introduced a strong malo-ethanolic phenotype in *S. cerevisiae* where L-malic acid was rapidly and efficiently degraded in synthetic and Chardonnay grape must with the concurrent production of higher levels of ethanol. Functional expression of the malo-ethanolic pathway genes of *S. pombe* in a laboratory strain of *S. cerevisiae* paved the way for the genetic modification of industrial wine yeast strains of *Saccharomyces* for commercial winemaking.

A prerequisite for becoming an inherited component of yeast is the stable integration of the malo-ethanolic genes into the genome of industrial wine yeast strains. Genetic engineering of wine yeasts strains of *Saccharomyces* is, however, complicated by the homothallic, multiple ploidy and

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prototrophic nature of industrial strains of *Saccharomyces*. Transformation and integration of heterologous genes into industrial strains of *Saccharomyces* require the use of dominant selectable markers, i.e. antibiotic or toxic compound resistance markers. Integration of these markers into the yeast genome is, however, not acceptable for commercial application due to the absence of long-term risk assessment and consumer resistance.

A unique strategy for the integration of the *S. pombe mae1* and *mae2* expression cassettes without the incorporation of any non-yeast derived DNA sequences was. The malo-ethanolic cassette, containing the *S. cerevisiae PGK1* promoter and terminator regions together with the *S. pombe mae1* and *mae2* open reading frames, was integrated into the *URA3* locus of an industrial strain of *Saccharomyces bayanus* EC1118 during co-transformation with a phleomycin-resistance plasmid, pUT332. After initial screening for phleomycin resistance, *S. bayanus* EC1118 transformants were cured of the phleomycin-resistance plasmid, resulting in the loss of non-yeast derived DNA sequences. After correct integration of the *mae1* and *mae2* expression cassettes was verified, small-scale vinification in synthetic and Chardonnay grape must with stable transformants resulted in rapid and complete degradation of L-malic acid during the early stages of alcoholic fermentation. Integration and expression of the malo-ethanolic genes in *S. bayanus* EC1118 had no adverse effect on the fermentation ability of the yeast, while sensory evaluation and chemical analysis of the Chardonnay wines indicated an improvement in wine flavour compared to the control wines, without the production of any off-flavours.

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OPSOMMING

L-Appelsuur en wynsteensuur is die mees prominente organiese sure in wyn en speel 'n kritiese rol in die wynbereidingsproses en organoleptiese wynkwaliteit, insluitende die fisiese, biochemiese en mikrobiese stabiliteit van wyn. Die produksie van hoë-kwaliteit wyne berus op die vermoë van 'n wynmaker om die suurinhoud korrek aan te pas om sodoende 'n gebalanseerde produk met optimale geur en kleur te produseer.

Saccharomyces rasse kan gewoonlik nie appelsuur volledig tydens alkoholiese gisting benut nie en dra dus nie noemenswaardig tot 'n verlaging van die totale suurinhoud van wyn by nie. Die mate van appelsuur afbraak deur *Saccharomyces* wissel egter van ras tot ras. Sekere *Saccharomyces* rasse kan 'n groter persentasie appelsuur afbreek, maar die onderliggende rede vir hierdie verskynsel is onbekend. Die onderliggende meganismes vir hierdie verskynsel is gedurende hierdie studie uitgelig na afloop van voorlopige transkripsionele regulerings studies op die malaatensiemgeen. In teenstelling hiermee kan *S. pombe* tot 29 g/l appelsuur via die malo-alkoholiese padweg afbreek waartydens appelsuur na pirodruiwesuur en CO_2, en uiteindelik na alkohol onder fermentatiewe toestande omgeskakel word. Verskeie redes vir die swak afbraak van appelsuur deur *Saccharomyces cerevisiae* is voorgestel. Eerstens beskik *S. cerevisiae* nie oor 'n meganisme vir die aktiewe transport van appelsuur, soos in die geval van *S. pombe* nie, en is aangewese op die stadige opname van appelsuur deur eenvoudige diffusie. Tweedens het die *S. cerevisiae* malaatensiem 'n baie laer substraataffiniteit vir appelsuur (K_m = 50 mM) in vergelyking met díe van *S. pombe* (K_m = 3.2 mM), wat verder bydra tot die swak afbraak van appelsuur in *S. cerevisiae*. Laastens dra die mitochondriale ligging van die *S. cerevisiae* malaatensiem in teenstelling met die sitoplasmiese ligging van die *S. pombe* malaatensiem, verder by tot die swak afbraak van appelsuur, aangesien die mitochondria onder fermentatiewe toestande negatief gereguleer word.

Die malaatpermease geen (*mae1*) en malaatensiem geen (*mae2*) van *S. pombe* is gevolglik gekloneer en heteroloog in 'n laboratoriumras van *S. cerevisiae* onder die beheer van die konstitutiewe 3-fosfogliseraat kinase (*PGK1*) promoter- en termineerdervolgordes uitgedruk. 'n Sterk malo-alkoholiese fenotipe was duidelik tydens fermentasies met die rekombinante gis in sintetiese en Chardonnay druiwemos, met 'n gepaardgaande verhoging in alkoholvlakke. Funksionele uitdrukking van die malo-alkoholiese gene van *S. pombe* in 'n *S. cerevisiae* laboratoriumras het die weg vir die genetiese modifisering van industriële wynrasse van *S. cerevisiae* vir kommersiële wynfermentasie gebaan.

Om 'n integrale deel van die gis te word, moet die malo-alkoholiese gene stabiel in die genoom van industriële wynrasse geïntegreer word. Genetiese manipulering van industriële wynrasse word egter bemoeilik deur die homotalliese, multi-ploïediese en prototrofiese aard van industriële

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Saccharomyces rasse. Transformasie en integrasie van heteroloë gene in industriële *Saccharomyces* rasse vereis die gebruik van dominante merkers, bv. weerstandbiedendheid teen antibiotika of ander gifstowwe. Integrasie van hierdie merkers in die gisgenoom is egter nie vir kommersiële toepassing aanvaarbaar nie weens die afwesigheid van langtermyn risikobepalings en verbruikersweerstand. Tydens hierdie studie is daar dus gepoog om industriële wynrasse met 'n unieke strategie geneties te verbeter sodat slegs gis-DNA tydens die integrasie van die *S. pombe mae1* en *mae2* uitdrukkingskassette in die gisgenoom opgeneem word. Die Malo-alkoholiese integrasiekasset wat slegs die *S. pombe mae1*, *mae2* oopleesrame en die *S. cerevisiae PGK1* promoter en termineerdervolgordes bevat, is in die *URA3* lokus van *Saccharomyces bayanus* EC1118 geïntegreer tydens parallelle transformasie met 'n 'phleomycin' weerstandbiedendheidsplasmied. Na seleksie van transformante op 'phleomycin'-bevattende media, is die *S. bayanus* EC1118 transformante in nie-selektiewe kondisies opgegroei sodat verlies van die 'phleomycin' plasmied kon plaasvind. Integrasie van die *mae1* en *mae2* uitdrukkingskassette is bevestig en kleinskaalse fermentasies in sintetiese en druiwemos het 'n vinnige en doeltreffende afbraak van appelsuur in die vroeë fases van die alkoholiese fermentasie getoon. Integrasie en uitdrukking van die malo-alkoholiese gene in *S. bayanus* EC1118 het geen nadelige effek op die fermentasievermoë van die gis getoon nie, terwyl sensoriese en chemiese ontleding van die Chardonnay wyne 'n verbetering in aroma relatief tot die kontrole wyne getoon het, met die afwesigheid van enige afgeure.

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A scientific truth does not try to convince its opponents, but rather that its opponents eventually die and a new generation grows up to be familiar with it. – Max Planck

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BIOGRAPHICAL SKETCH

Heinrich Volschenk was born in Bellville, South Africa, on October 1, 1971. He attended Bellville High School and matriculated with distinction in 1989. He enrolled at Stellenbosch University in 1990 and obtained the B.Sc. (Biochemistry and Microbiology) degree in 1992, Hons. B.Sc. (Microbiology) degree *cum laude* in 1993, and the M.Sc. (Microbiology) degree *cum laude* in 1996. In 1997 he enrolled for his Ph.D. (Microbiology) at Stellenbosch University under the supervision of prof. H.J.J van Vuuren and dr. M. Viljoen-Bloom and conducted part of his doctoral research at the Cool Climate Oenology and Viticulture Institute at Brock University, Canada.

Heinrich contributed to the following research articles during his post-graduate studies:

1. **Volschenk, H.**, M. Viljoen, J. Grobler, B. Petzold, F. Bauer, R.E. Subden, R.A. Young, A. Lonvaud, M. Denayrolles and H.J.J. van Vuuren. 1997. Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nature Biotechnol. **15**:253–257.
2. **Volschenk, H.**, M. Viljoen, J. Grobler, F. Bauer, A. Lonvaud–Funel, M. Denyarolles, R.E. Subden and H.J.J. van Vuuren. 1997. Malolactic fermentation in grape musts by a genetically engineered strain of *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. **48**:193-197.
3. Viljoen, M., **H. Volschenk**, R.A. Young and H.J.J. van Vuuren. 1999. Transcriptional regulation of the *Schizosaccharomyces pombe* malic enzyme gene, *mae2*. J. Biol. Chem. **274**:9969-9975.
4. **Volschenk, H.**, M. Viljoen-Bloom, R.E. Subden and H.J.J. van Vuuren. 2001. Malo-ethanolic fermentation of grape must by recombinant strains of *Saccharomyces cerevisiae*. Yeast **18**:1-8.
5. Redzepović, S., S. Orlić, B. Kozina, A. Majdak, **H. Volschenk** and M. Viljoen-Bloom. 2002. Differential malic acid degradation by selected strains *of Saccharomyces* during alcoholic fermentation (*International Journal of Food Microbiology,* **In Press**).

Heinrich received the following publication awards during his post-graduate studies:

1. 1997 – South African Society for Enology and Viticulture (SASEV) Award for best oenology paper published in international journal during 1997 for "*Engineering a pathway for malate degradation in Saccharomyces cerevisiae*. Nature Biotech. **15**:253-257."
2. 1997 – American Society for Enology and Viticulture (ASEV) Award for best oenology paper published in Am. J. Enol. Vitic. during 1997 for "*Malolactic fermentation in grape musts by a genetically engineered strain of Saccharomyces cerevisiae*. Am. J. Enol. Vitic. **48**:193-197."

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

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General Introduction and Project Aims

1.1 INTRODUCTION

Wine acidity and pH play a crucial role in the winemaking process and the final organoleptic quality and shelf-life of wine. L-Tartaric acid and L-malic are the major grape acids, contributing to more than 90% of the titratable acidity in wine (Beelman and Gallander, 1979; Gao and Fleet, 1995; Henick-Kling, 1993; Radler, 1993). The production of premium wines depends on the optimal adjustment of wine acidity in relation to the other wine components to obtain a balanced wine with an optimum flavour and colour profile. In the warmer wine regions of Australia, South Africa, California and southern Europe, acidulating agents such as L-tartaric acid or D/L-malic acid are routinely added prior to fermentation to increase the titratable acidity of must to ensure an optimal acid:sugar ratio in grape must (Beelman and Gallander, 1979; Boulton *et al.*, 1996). In contrast, a number of deacidification methods are employed in the cooler wine regions of northern Europe, eastern United States and Canada to decrease the levels of acid in the final product.

Malolactic fermentation, regarded as the preferred method by the international wine industry for naturally reducing wine acidity, efficiently decreases the acidic taste of wine, improves the microbial stability and modifies to some extent the organoleptic character of wine. Strains of the lactic acid bacterium *Oenococcus oeni* are routinely used to execute malolactic fermentation in wine during which L-malic acid is converted to L-lactic acid and CO_2 (Van Vuuren and Dicks, 1993; Wibowo *et al.*, 1985). However, all the positive attributes of malolactic fermentation may also contribute negatively to wine quality and stability, especially in low-acid wines. Moreover, the recurrent phenomena of stuck or sluggish malolactic fermentation often causes delays in cellar operations such as sulphiting, which may result in the chemical oxidation and spoilage of wine, as well as the production of biogenic amines by spoilage organisms (Lonvaud-Funel and Joyeux, 1994; Straub *et al.*, 1995). Even with the use of starter cultures, malolactic fermentation may only be completed weeks or months after alcoholic fermentation (Henick-Kling, 1995). Factors such as pH, sulphur dioxide, ethanol, temperature, nutritional status of the wine and interactions with other wine flora synergistically influence the onset and completion of malolactic fermentation (Beelman and Gallander, 1979; Boulton *et al.*, 1996; Davis *et al.*, 1985; Henick-Kling, 1993). Furthermore, malolactic fermentation is not always compatible with specific styles of wine. For example, malolactic fermentation is usually undesirable in the production of the fruity-floral cultivars such as Muscat, Sauvignon Blanc, Riesling and Gewurztraminer. The varietal flavours are essential to the aromatic character of these wines and are adversely modified during malolactic fermentation (Radler, 1972; Wagner, 1974).

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Yeast species capable of utilising tricarboxylic acid (TCA) cycle intermediates such as L-malic acid, are classified into two groups: K (+) yeasts utilise TCA intermediates as sole energy and carbon source, while K (−) yeasts can only utilise TCA cycle intermediates when glucose or other assimilable carbohydrates are present. The K (-) group includes yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Zygosaccharomyces balii* (Baranowski and Radler, 1984; Kuczynski and Radler, 1982; Rodriquez and Thornton, 1989). However, wine yeast strains of *Saccharomyces* cannot efficiently utilise L-malic acid during alcoholic fermentation (Ansanay *et al.*, 1996; Baranowski and Radler, 1984; Delcourt, 1995; Fuck and Radler, 1974; Radler, 1993; Rankine, 1966; Rodriques and Thornton, 1990; Salmon, 1987; Subden *et al.*, 1998; Volschenk *et al.*, 2001). The inefficient degradation of L-malic acid by *S. cerevisiae* is ascribed to the slow uptake of L-malic acid by diffusion (Ansanay *et al.*, 1996; Baranowski and Radler, 1984; Volschenk *et al.*, 1997[a,b]) and the low substrate affinity of its malic enzyme (K_m = 50 mM) (Fuck *et al.*, 1973). Furthermore, initial transcriptional analysis of the *S. cerevisiae* malic enzyme gene (*MAE1*), indicated that the gene is expressed at relatively low, but constitutive levels (Boles *et al.*, 1998). The mitochondrial location of the *S. cerevisiae* malic enzyme also contributes to the weak metabolism of L-malic acid under fermentative conditions, since the mitochondrial enzymes are negatively regulated under high glucose conditions (Cho *et al.*, 2001; Dejean *et al.*, 2000; García *et al.*, 1993; Jayaraman *et al.*, 1966; Mattoon *et al.*, 1979; Perlman and Mahler, 1974; Polakis and Bartley, 1965).

In contrast to *S. cerevisiae*, the fission yeast *S. pombe* can efficiently degrade up to 29 g/l of L-malic acid (Taillandier *et al.*, 1988; Taillandier and Strehaiano, 1991). Cells of *S. pombe* actively transport L-malic acid via a H^+-symport system (Sousa *et al.*, 1992) provided by the malate permease encoded by the *mae1* gene (Grobler *et al.*, 1995). Intracellularly, *S. pombe* decarboxylates L-malic acid to pyruvic acid and CO_2 by means of a cytosolic malic enzyme encoded by the *mae2* gene (Viljoen *et al.*, 1994). Under fermentative conditions, pyruvic acid is further metabolised to ethanol and CO_2 (Mayer and Temperli, 1963; Osothsilp and Subden, 1986), resulting in the so-called malo-ethanolic fermentation. Although strains of *S. pombe* have been used for the degradation of L-malic acid in grape must, it is unsuitable for the production of quality wine due to the frequent production of off-flavours or the lack of typical wine flavours and the higher fermentation temperatures required for fermentation (Beelman and Gallander, 1979; Carré *et al.*, 1983; Gallander, 1977; Radler, 1993).

Fundamental knowledge about the regulation and physiological role of L-malic acid and its metabolism in strains of *Saccharomyces* is vital for the successful innovation of genetically engineered strains and the application of these wine yeasts for vinification. Relatively little information is available on malo-ethanolic fermentation and its regulation in wine yeast strains of *Saccharomyces*, i.e. it is a well-known fact that some strains of *Saccharomyces* have an increased ability of up to 45% to degrade L-malic acid during alcoholic fermentation, but the cause of this phenomenon is unknown. Recombinant strains of *Saccharomyces* with the ability to execute

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alcoholic fermentation and simultaneously reduce wine acidity could have a significant influence on the future production of quality wines, especially in cool-climate regions. Since alcoholic fermentation and reduction of excess L-malic acid can be obtained in one single fermentation step, bottling and storage of wine can immediately proceed.

1.2 AIMS OF THIS STUDY

The aim of this study was to develop a genetically engineered industrial strain of *Saccharomyces* with the ability to degrade L-malic acid via the malo-ethanolic pathway during alcoholic fermentation. The specific objectives and approaches were the following:

i) A better understanding of the physiological role of the native malo-ethanolic pathway of *Saccharomyces* by comparing the expression of the malic enzyme gene (*MAE1*) in low malic acid-degrading and high malic acid-degrading strains of *Saccharomyces*.

ii) Introduction of an efficient malo-ethanolic pathway in *S. cerevisiae* by co-expressing the *mae1* and *mae2* genes of *S. pombe* in a laboratory strain of *S. cerevisiae*.

iii) Integration of the *mae1* and *mae2* genes of *S. pombe* in industrial wine yeast strains of *Saccharomyces* containing DNA exclusively derived from yeast.

iv) Characterisation of the malo-ethanolic industrial wine yeast strains in terms of their ability to degrade L-malic acid during vinification, their fermentation kinetics and potential for producing wine of high quality.

The dissertation is organised as a number of chapters covering the current literature on the origin and fate of L-malic acid in grapes and wine, the metabolism of L-malic acid by yeast via the malo-ethanolic pathway and the genetic engineering of industrial strains of *Saccharomyces* (Chapters 2, 3 and 4), followed by the research that addressed the different objectives stated above (Chapters 5, 6 and 7) and general discussion (Chapter 8). Note that Chapters 5, 6 and 7 were written in the style required for the specific journal to which the manuscript was submitted.

The first of the objectives was addressed in **Chapter 5**, which is based on a publication entitled "Differential malic acid degradation by selected strains *of Saccharomyces* during alcoholic fermentation" (Redzepovic *et al*., 2002). This paper describes (i) the isolation and characterisation of a *Saccharomyces paradoxus* strain R088 with the ability to degrade increased levels of L-malic acid during alcoholic fermentation (Vaughan-Martini, 1989); (ii) comparative fermentation and sensory evaluation studies with *S. paradoxus* R088 and other commercially available wine yeast strains; (iii) the expression of the malic enzyme gene (*MAE1*) in these different strains of *Saccharomyces* during the course of alcoholic fermentation; (iv) transient expression studies of the *MAE1* gene from the

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different strains of *Saccharomyces* under artificial conditions, i.e. with or without L-malic acid, high glucose and anaerobic conditions, and (v) cloning and sequence comparison of the *MAE1* promoter region from different strains of *Saccharomyces*. The chapter also includes a brief overview of the PCR cloning strategy of the promoter region of the *MAE1* gene from different *Saccharomyces* strains (Addendum A).

Chapter 6 addresses the second objective and is based on a publication entitled "Malo-Ethanolic Fermentation in Grape Must by Recombinant Strains of *Saccharomyces cerevisiae*", (Volschenk *et al.*, 2001). The paper describes (i) subcloning of the *S. pombe mae1* and *mae2* genes under the regulation of the 3-phosphoglycerate kinase (*PGK1*) promoter and terminator elements; (ii) transformation of the multi-copy plasmid into a laboratory strain of *Saccharomyces cerevisiae*, as well as the single copy integration of the expression cassette into the genome of a laboratory strain of *S. cerevisiae*, and (iii) confirmation and quantification of the malo-ethanolic phenotype in synthetic and actual grape must. This chapter also includes a brief overview of the initial experimental approach and results not included in the paper (Addendum B).

The final two objectives of this thesis are discussed in **Chapter 7,** a draft manuscript to be submitted to *Yeast*. The manuscript describes (i) the construction of the *S. pombe mae1* and *mae2* integration cassette containing DNA exclusively derived from yeast; (ii) the transformation and integration of the *S. pombe mae1* and *mae2* genes in a commercial wine yeast strain of *Saccharomyces bayanus* and screening for malo-ethanolic phenotype; (iii) Southern blot analysis of the integration events and confirmation of stability of the integrated genes; (iv) determination and quantification of the malo-ethanolic phenotype in synthetic and grape must, and (v) sensory evaluation of wine produced by the recombinant malo-ethanolic strain. This chapter also includes a brief overview of the initial experimental approach and results not included in the paper (Addendum C).

The work presented in this dissertation comprises the first report on the construction of a wine yeast strain of *S. bayanus* with the ability to efficiently degrade L-malic acid during alcoholic fermentation via a heterologous malo-ethanolic pathway. The integration strategy developed during this study sets the basis for future genetic engineering of wine yeast without the incorporation of any bacterial selection markers or other foreign DNA, which is essential for commercial application and consumer approval. This study contributes to the daunting challenge of the world-wide wine industry in this new century to understand and anticipate new consumer preferences and to produce wines of enhanced attractiveness while simultaneously developing and implementing sustainable production practices for wine making.

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CHAPTER 2

LITERATURE REVIEW

The origins of L-malic acid in wine and its fate during winemaking

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The origin of L-malic acid in wine and its fate during winemaking

2.1 INTRODUCTION

Acidity in wine originates mainly from two sources, the first being those organic acids that are extracted from grapes into the must during harvesting and crushing. Thus, the development of the grape berry and the chemical composition of mature grapes dictate the composition of grape juice or must at the onset of vinification and ultimately the final quality of the bottled wine. Secondly, the combined metabolism of yeasts and bacteria during subsequent fermentation steps contribute to the pool of wine acids. The net contribution of these microorganisms to wine acidity is, however, the sum of both the degradation of some grape acids and the biosynthesis of some unique organic acids by yeasts and bacteria during and after alcoholic fermentation. Furthermore, several cellar procedures such as maceration, cold stabilisation etc. also influence the final acid composition of wine.

In the wine industry winemakers often experience predicaments when certain wine acids exceed the acceptable concentration ranges. The production of quality wine requires a judicious balance between the sugar, acid and flavour/aroma components of wine. To this end winemakers can only modify the acidity component of wine by the addition or removal of certain acids, since the artificial manipulation of sugars and flavourants in wine is detrimental to wine quality and illegal in most wine producing countries. The adjustment of acidity in must or wine is a complex, since several factors must be taken into account to determine the correct timing and the method of choice for rectifying wine acidity. Modern winemakers routinely employ malolactic fermentation to deacidify wine. Although this step is considered the most natural method for wine acidity adjustment and contributes further to micorbial stability and organoleptic complexity, winemakers face inherent pitfalls associated with this biological process.

This chapter deliberates, with special reference to L-malic acid, on the origin and evolution of organic acids in grapes and wine, the role of acidity in wine and the fate of these organic acids during subsequent fermentation steps and winemaking procedures.

2.2 ORGANIC ACIDS IN GRAPES

The principal organic acids in grapes are L-tartaric and L-malic acid (Table 2.1), accounting for more than 90% of the grape berry's acid content (Amerine and Winkler, 1942; Boulton *et al.*, 1996; Lavee and Nir, 1986; Radler, 1993; Ribéreau-Gayon *et al.*, 1976, Tucker, 1993). Although L-malic and L-

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tartaric acid has similar chemically structures they are synthesised from glucose via different metabolic pathways in grape berries. L-Malic acid is formed via glycolysis and the tricarboxylic acid cycle, while ascorbic acid is the principle intermediary product of L-tartaric acid biosynthesis. The evolution of L-malic and L-tartaric acid differs significantly during grape berry development and maturation. L-Tartaric acid is usually present in grapes at average concentrations of 5 to 10 g/l (Ruffner, 1982), while mature grapes contain between 2 and 6.5 g/l L-malic acid (Boulton *et al.*, 1996; Ribéreau-Gayon *et al.*, 2000[b]). Excessive amounts of malic acid (15 - 16 g/l) may be present in grapes at harvest time during exceptionally cold summers in the cool-climate viticultural regions of the world (Gallander, 1977). Slight differences in grape acidity among different grape varieties are usually found, affecting especially the ratio between L-tartaric acid and L-malic acid in different grape cultivars (Kliewer *et al.*, 1967).

Table 2.1 Organic acids present in grapes and wine (adapted from Boulton *et al.*, 1996)

Fixed acids		Volatile acids	
Major Acids	**Minor Acids**	**Major Acids**	**Minor Acids**
L-tartaric acid (5-10 g/l)	pyruvic acid	acetic acid	formic acid
L-malic acid* (2–6.5 g/l)	α-ketoglutaric acid		propionic acid
L-lactic acid (1-3 g/l)	Isocitric acid		2-methylpropionic acid
citric acid** (0.5-1 g/l)	2-oxoglutaric acid		butyric acid
succinic acid (0.5–1.5 g/l)	dimethyl glyceric acid		2-methylbutyric acid
amino acids	citramalic acid		3-methylbutyric acid
	gluconic acid***		hexanoic acid
	galacturonic acid		octanoic acid
	glucuronic acid		decanoic acid
	mucic acid		
	coumaric acid		
	ascorbic acid		

*15 –16 g/l L-malic acid have been reported in cool climate regions
**> 0.3 g/l when wines are stabilised for metal precipitation
***present in wine with *Botrytis cinerae* infection

Although tartaric acid is often found at higher concentrations than L-malic acid and is the stronger acid of the two, its concentration is relatively constant and it is rather the fluctuating concentration of L-malic acid that poses problems to winemakers (Margalit, 1997; Ribéreau-Gayon *et al.*, 2000[b]). Citric acid, a metabolite of plant photosynthesis, together with all the other intermediates of the tricarboxylic acid (TCA) cycle, is also found in grapes at low concentrations (Ribéreau-Gayon *et al.*, 2000[b]). Other acids that are present in low concentrations in grapes are the phenolic acids, coumaric acid and ascorbic acid, as well as sugar acids, such as gluconic, glucuronic and galacturonic acids from *Botrytis cinerea*-infected grapes (Sponholz and Dittrich, 1984). Grapes also contain several amino acids, of which arginine and proline are the most prominent in wine, while only two inorganic acids are found in wine in the form of dissolved gases, namely SO_2 and CO_2 (Boulton *et al.*, 1996).

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2.2.1 Evolution of organic acids during grape berry development

Vitis vinifera produce non-climacteric grape berries, like strawberry and citrus, since they do not exhibit a significant increase in respiration or ethylene synthesis during ripening (Davies *et al.*, 1997; Koch and Alleweldt, 1978; Palejwala *et al.*, 1985). The development of the grape berry displays a double-sigmoid growth curve, characteristic of all berry fruits (Coombe and Hale, 1973; Kanellis and Roubelakis-Angelakis, 1996). The berry growth pattern is characterised by three successive phases, starting with the green or herbaceous stage immediately after flowering (Fig. 2.1). During this stage the berries are hard and green (Terrier *et al.*, 2001) and undergo a short period of cell division (Kanellis and Roubelakis-Angelakis, 1996; Ribéreau-Gayon *et al.*, 2000[a]) and cell enlargement resulting in rapid expansion of the berry. Characteristic of stage I is the increase in vacuolar size of grape berry cells due to the rapid storage of L-malic and L-tartaric acid (Fillion *et al.*, 1999; Lavee and Nir, 1986; Pratelli *et al.*, 2002; Ruffner, 1982). Stage II comprises a short lag phase during which berry growth ceases and berry acidity reaches a maximum due to continued accumulation of L-malic and L-tartaric acid. Following the lag phase, there is a second period of "berry growth" (Stage III). The entry into Stage III begins with the sudden onset of ripening or "*véraison*", which may occur within 24 hours; in general this starts between 6 to 8 weeks after flowering and lasts for 35 to 55 days depending on the grape cultivar (Coombe, 1992; Pratelli *et al.*, 2002; Ribéreau-Gayon *et al.*, 2000[a]).

Véraison, which seems to be a stress-associated process, is characterised by several drastic physical and biochemical changes in the grape berry (Coombe, 1992; Davies and Robinson, 2000; Vivier and Pretorius, 2000). A rapid accumulation of sugar and amino acids occurs, as well as a decrease in organic acid content. Physical changes to the grape berry during *véraison* include further increase in berry size and softening due to flesh cell expansion rather than cell division, as well as colour changes (especially red cultivars). The softening of grape berries has been linked to changes in the berry cell wall composition, especially polysaccharides and cell wall-associated proteins (Nunan *et al.*, 1998). Colour changes are brought about by the degradation of chlorophyll in the grape and its replacement with the colour pigments anthocyanins and flavonols (in red grapes) after *véraison* (Boss *et al.*, 1996).

The mechanisms of grape berry development, like those of other non-climacteric fruits, are still poorly understood at the molecular level. Most studies have focused on genes whose expression is changed during *véraison* (Davies and Robinson, 2000; Robinson *et al.*, 1997; Tattersall *et al.*, 1997). Particular attention has been given to sugar transporters and enzymes involved in sucrose metabolism (Ageorges *et al.*, 2000; Davies and Robinson, 1996; Fillion *et al.*, 1999; Manning *et al.*, 2001). Recently the role of the vine potassium (K^+) channel during *véraison*, which is involved in ionic homeostasis in the berry, has also been unravelled (Pratelli *et al.*, 2002). K^+ is the most abundant cation in the berry cell and is involved in a number of fundamental functions linked together at the cellular or the whole plant level, *e.g.* control of cell turgor, and thereby control of cell enlargement or guard cell movements, as well as electrical neutralisation of L-malic acid and tartaric acid stored in the

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vacuolar compartment. An increase in grape berry K^+ content, due to *e.g.* increased K^+ levels in the soil, or rootstock efficiency in the uptake and transporting of K^+, leads to decreased vacuolar acidity and changes in relative concentrations of organic acids in the ripe berry, resulting in reduced wine-quality (Delas *et al.*, 1989; Hale, 1977; Pratelli *et al.*, 2002).

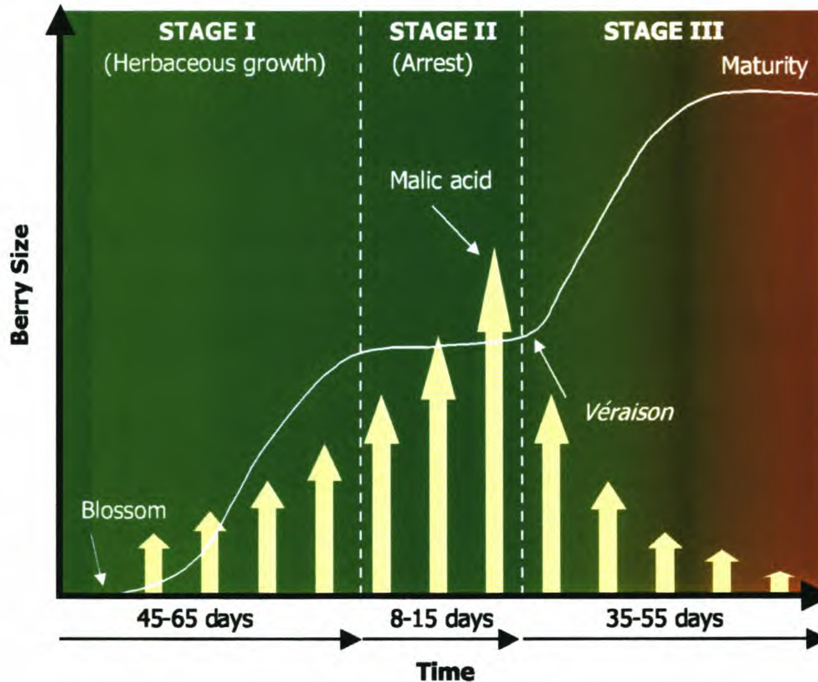

Figure 2.1. The double-sigmoid model of grape berry development indicates the three stages of herbaceous growth, temporary growth arrest and *véraison* (adapted from Coombe, 1973; Jackson and Schuster, 1997). The thick arrows denote the simultaneous pattern of L-malic acid synthesis and accumulation as well as rapid decrease via respiration during grape berry development.

2.2.1 Sugar content changes in grape berries during *véraison*

Ripening of grapes is biochemically characterised by the vacuolar accumulation of grape sugars, glucose and fructose, with the concomitant loss of acidity, mainly L-malic acid (Selvaraj *et al.*, 1977, 1978). The main sugars found in grapes are D-glucose and D-fructose, usually present at near equal concentrations of up to 20% (w/v) in mature grapes (Kliever, 1967; Margalit, 1997). Glucose and fructose are derived from sucrose synthesised in the vine leaves during photosynthesis, which are translocated through the phloem to the grape berry (Hawker, 1969). Upon its arrival in the berry phloem, sucrose can be unloaded into the apoplast and partly cleaved by an apoplastic invertase to form glucose and fructose. The remaining sucrose, glucose and fructose can then be taken up by the berry mesocarp. Once in the cytoplasm of the mesocarp cells, the sugars are transported by tonoplast carriers and accumulate in the berry vacuole. In the berry vacuole the remaining sucrose is rapidly

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converted to glucose and fructose via two putative vacuolar invertases (Davies and Robinson, 1996; Kanellis and Roubelakis-Angelikis, 1996). During the early stages of berry development (stages I and II) glucose and fructose are further metabolised via the normal route of glycolysis and the subsequent respiratory pathways, involving the TCA cycle and oxidative phosphorylation.

The accumulation of glucose and fructose in the grape berry from the start of *véraison* (Stage III, Fig. 2.1) has been partially explained on enzymatic level. The acid invertase enzyme activity increases during *véraison*, producing more glucose and fructose, while simultaneously the rate of glucose and fructose respiration during *véraison* is significantly decreased in the berry vacuole (Davies and Robinson, 1996; Hawker, 1969; Kanellis and Roubelakis-Angelakis, 1996; Takayanagi and Yokotsuka, 1997). As a consequence, carbon flow through respiration is significantly slowed down by the inhibition of the glycolytic pathway, resulting in the accumulation of non-utilised glucose and fructose in the berry vacuole (Robinson *et al.*, 1997). It has been demonstrated that the pyrophosphate : fructose 6-phosphate phosphotransferase enzyme activity, which is directly linked to the glycolytic flux, decreases during *véraison*. The phenomenon of sugar accumulation during *véraison* is, however, only partially understood in terms of fluctuations in enzyme activities. No conclusive evidence for the apparent inhibition of glycolytic enzymes has yet been found to support the decreased flow of carbon via glycolysis during berry maturation. Likewise, the apparent increase in grape berry acid invertase activity, which would theoretically contribute to the accumulation of glucose and fructose, does not correlate with the timing of *véraison* (Coombe, 1989; Coombe, 1992; Davies and Robinson, 1996). It has been suggested that sugar accumulation during berry ripening is more affected by compartmentation of the enzymes involved and the transport of the sugars through the different membranes than the actual enzyme activities in the berry (Davies and Robbinson, 1996; Fillion *et al.*, 1999; Manning *et al.*, 2001; Or *et al.*, 2000; Terrier *et al.*, 2001).

2.2.3 Acidity changes in grape berries during *véraison*

The second most significant biochemical change during *véraison* is the rapid reduction of grape berry acidity, which coincides with the change in sugar composition of the grape berry. During the development of the grape berry, right from the flowering stage up until berry maturity, the metabolism of L-malic acid and tartaric acid follows a predictable pattern of evolution. The final L-malic acid content in the grape berry depends on the balance between the rate of L-malic acid synthesis (Laval-Martin *et al.*, 1977), vacuolar storage (Müller *et al.*, 1996) and mobilisation (Ruffner *et al.*, 1984).

Grape berries respire very actively during the early stages of growth, but the intensity of respiration slows down as they advance in age. Respiration in terms of O_2 uptake decreases, while the respiratory quotient (RQ) continues to increase throughout grape berry development from flowering to maturity (Lutra and Cheema, 1931; Selvaraj *et al.*, 1977). During *véraison* the availability of the respiratory

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substrate, sucrose, via photosynthesis becomes limited due to the degradation of chlorophyll. The berry is forced to shift its metabolism from sugar to L-malic acid respiration, due to the scarcity of respiratory substrate. Prior to the onset of *véraison* L-malic acid is the most abundant organic acid (up to 25 g/l) in the grape berry vacuole, resulting in the low internal pH of 2.5 of grapes (Ribéreau-Gayon *et al.*, 2000[b]; Ruffner, 1982). With the onset of *véraison* the L-malic acid concentration rapidly decreases to between 4 and 6.5 g/l or even as low as 1 to 2 g/l, with a concomitant increase in internal berry pH (pH of ca. 3.5) (Kanellis and Roubelakis-Angelikis, 1996; Ribéreau-Gayon *et al.*, 2000[b]; Ruffner, 1982). Although L-malic acid and tartaric acid are structurally related, only a slight decrease in tartaric acid concentration occurs in grapes during *véraison*, due to the chemical resistance of tartaric acid to respiration (Davies and Robinson, 1996). Padgett and Morrison (1990) also found differences in the exudation of the two acids from the grape berry: L-malic acid was found in berry exudates from the flowering to mature stages of grape berry development, while tartaric acid could not be detected. The accumulation of L-malic and tartaric acid in the berry vacuole and exudation to the berry surface up until the onset of *véraison* may contribute to controlling the growth of *Botrytis cinerea,* the causal agent of an important ripe rot disease that seriously damages the berries between the onset of ripening and harvesting (Vercesi *et al.*, 1997).

The biochemistry behind the accumulation and rapid respiration of L-malic acid in grapes has been studied in detail (Fig. 2.2). L-Malic acid accumulates in the berry vacuole before *véraison* (Stages I and II, Fig. 2.1) via the collective activities of two key enzymes, the phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) enzyme (Blanke and Lenz, 1989; Diakou *et al.*, 2000; Hawker, 1969; Or *et al.*, 2000). The cytosolic PEPC enzyme, well-known for its photosynthetic role in C_4- and CAM-plants, catalyses the β-carboxylation of phosphoenolpyruvic acid to yield oxaloacetic acid and inorganic phosphate. The resulting oxaloacetic acid is further reduced by the NAD-dependent malate dehydrogenase to produce L-malic acid. Oxaloacetic acid and L-malic acid can enter the TCA cycle to produce citrate as well as other metabolites (Diakou *et al.*, 2000). The β-carboxylation of phosphoenolpyruvic acid plays an important role as an anapleurotic CO_2 fixation step that supplies carbon skeletons for other cellular processes such as osmolarity regulation, pH regulation and nitrogen assimilation (Diakou *et al.*, 2000; Latzko and Kelly 1983). Although a high malic enzyme (ME) activity during the accumulation phase of malic acid has been noted, the actual contribution to L-malic acid concentration via the reverse malic enzyme reaction, i.e. pyruvic acid carboxylation, was found to be insignificant (Kanellis and Roubelakis-Angelakis, 1996; Ruffner *et al.*, 1984).

The rapid decrease in L-malic acid concentration inside the grape berry during *véraison* is the result of a significant decrease in L-malic acid biosynthesis synchronised with a sharp increase in L-malic acid degradation via respiration. Initially the concentration of L-malic acid in the berry vacuole is diluted due to the influx of water during berry expansion in the second growth

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phase (Stage III, Fig. 2.1). Secondly, the slowing down of glycolytic carbon flow during *véraison* as described above for sugar accumulation, not only results in the increase of glucose and fructose in the berry vacuole, but also a decrease in L-malic acid synthesis via pyruvic acid in the tricarboxylic acid (TCA) cycle. The biosynthesis of L-malic acid via the PEPC enzyme is also reduced during *véraison* as it has been shown that the disappearance of the PEPC gene transcription and PEPC enzyme activity correlates with the start of *véraison* (Hawker 1969; Or *et al.*, 2000).

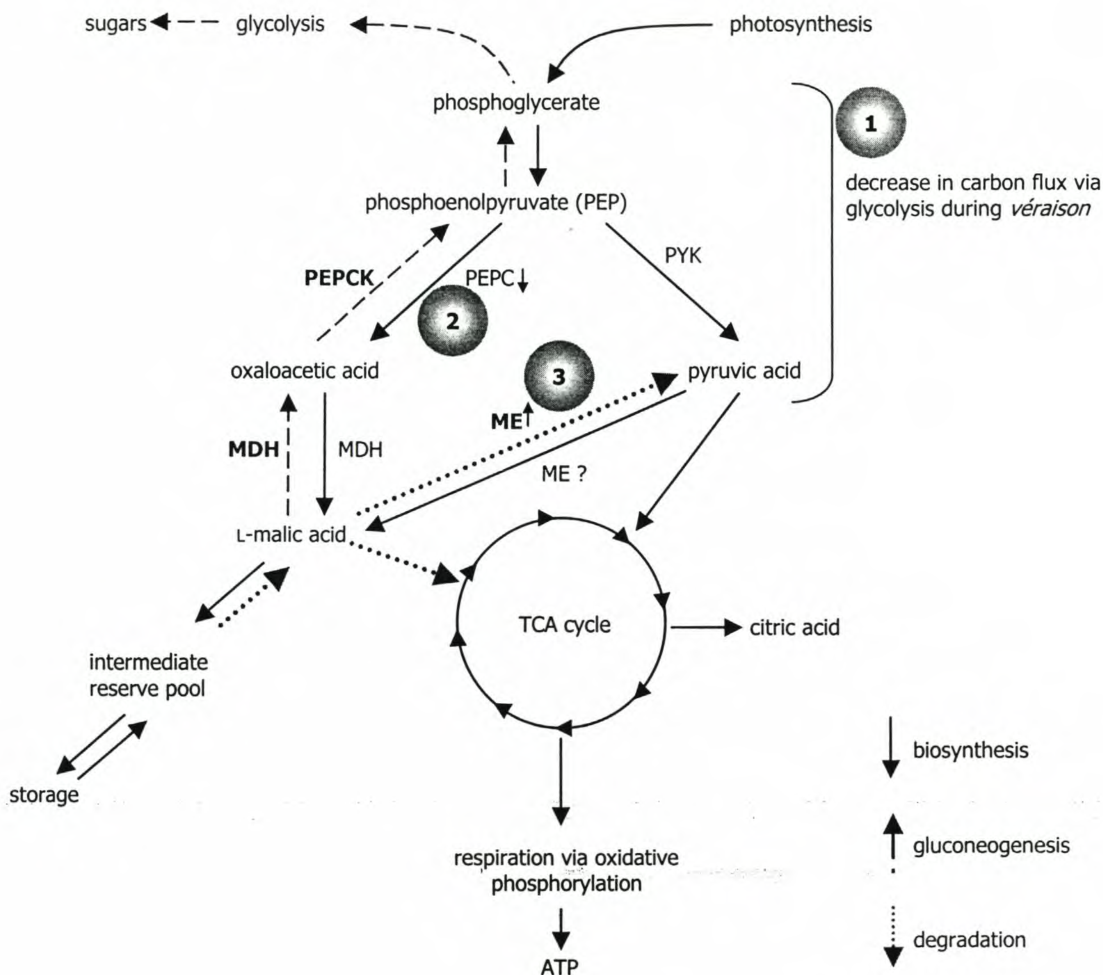

Figure 2.2 The biochemical pathways involved in the biosynthesis, dissipation and regulation of L-malic acid in grape berries (adapted from Kanellis and Roubelakis-Angelakis, 1996). ME = malic enzyme; PEPC = phosphoenolpyruvate carboxylase; PEPCK = phosphoenolpyruvate carboxykinase; MDH = malate dehydrogenase. The decrease in L-malic acid is due to ① a decrease in carbon flux via glycolysis, ② a decrease in L-malic acid biosynthesis via PEPC, and ③ an increase in L-malic acid respiration via the malic enzyme.

The rate of respiration of stored L-malic acid significantly increases during *véraison* due to a higher demand for a respiratory substrate in the grape berry. L-Malic acid is degraded in grape berries via two pathways, mainly the cytosolic NADP-malic enzyme (ME) (Ruffner *et al.*, 1984) and, to a lesser extent, the PEP carboxykinase (PEPCK) (Fig. 2.2) (Ruffner and Kliewer, 1975). There is also

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evidence that the malate dehydrogenase (MDH), especially the mitochondrial iso-enzyme, plays a putative role in the degradation of L-malic acid in the grape berry. Gene expression profiles and enzyme activities of the ME and MDH enzymes increase at the onset of *véraison*, descriptive of the rapid depletion scenario of L-malic acid (Or *et al.*, 2000). L-Malic acid degraded via the NADP-malic enzyme fuels the required biosynthetic (in particular the provision of NADPH) and respiratory pathways (Ruffner *et al.*, 1984), whereas a small percentage of L-malic acid (< 5%) is converted back to phosphoenolpyruvate via MDH and PEPCK for glucose synthesis via gluconeogenesis (Fig. 2.2) (Kanellis and Roubelakis-Angelakis, 1996; Ruffner, 1982; Ruffner and Kliewer, 1975).

2.2.4 Warm climate vs. cool-climate winemaking

Acidity in wine is a function of various exogenous factors, such as the climate or average temperature, the grape cultivar and vineyard practices (Beelman and Gallander, 1979; Ribéreau-Gayon *et al.*, 2000[b]; Zoecklein *et al.*, 1995). The most important factor that influences the final sugar : organic acid and the malic : tartaric acid ratio in grapes is the prevailing climatic conditions and ambient temperature during stage III of berry ripening (Crippen and Morrison, 1986; Kanellis and Roubelakis-Angelakis, 1996; Ruffner, 1982; Zoecklein *et al.*, 1995). The rate of respiration of L-malic acid increases with higher temperatures and *vice versa*. Climatologists recognise three levels of climate, namely macroclimate (regional climate), mesoclimate (vineyard site climate) and microclimate (grapevine canopy climate), of which the mesoclimate plays the most important role in grape composition and wine quality. According to the length of the ripening season and the prevailing climate/temperature during the ripening stage, the wine-producing regions of the world are divided into alpha viticultural zones, i.e. cool-climate regions, and beta viticultural zones, i.e. warm-climate regions (Jackson, 2001; Jackson and Lombard, 1993; Zoecklein *et al.*, 1995;).

The cool-climate regions, which include parts of northern Europe, Canada and northeast USA, are characterised by shorter ripening periods and/or sub-optimal mean temperatures (9 - 15°C) during the ripening season (Jackson, 2001). The rate of respiration of L-malic acid in cold climates is significantly slower, resulting in "immature grapes" at harvesting, containing a high titratable acidity (TA) content and low pH. High acidity in cool-climate grapes is often enhanced by unusually cold and wet seasons, poor vineyard locations, sub-optimal cultivar selections or poor viticultural practices such as overcropping. In these countries L-malic acid can comprise up to 50% of the total acidity in grapes. In contrast, the warm climates have longer ripening seasons and/or higher mean temperatures (16°C and above) and include parts of southern Europe, California, South Africa and Australia (Pretorius, 2000). Since the prevailing temperature of the region directly influences the rate of L-malic acid respiration in grapes, grapes in the warmer climates tend to have a faster rate of L-malic acid respiration compared to those of the cooler climates. Thus grapes from the warmer climates often contain insufficient final titratable acidity values and do not meet desirable pH values at harvest time.

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The proper harvesting time of grapes depends mainly on the style of wine to be made. Grapes for dry wine should have high acidity and moderate sugar content. Therefore, such grapes are usually harvested at 20-24° Brix, while grapes for sweet wines are harvested at a higher sugar content of around 24° Brix or higher. To determine the exact time of harvesting, oenologists have to be conscious of the changes in acid content in relation to the sugar content of grapes, especially during the latter stages of berry ripening. Routine maturity evaluation includes the measurement of berry sugar content for alcohol potential, pH and total titratable acidity, where the ratio of sugar : organic acid and sometimes the malic acid:tartaric acid ratios serve as yardsticks or standard quality indexes in the vineyard to predict grape maturity and the optimal time for harvesting (Terrier *et al.*, 2001; Zoecklein *et al.*, 1995).

2.3 ORGANIC ACIDS FOUND IN WINE

Most of the organic acids in wine originate directly from the raw material, grapes. Consequently the predominant acids in wine are also L-tartaric, L-malic acid and citric acid. During the subsequent fermentation of grape must several organic acids are synthesised or metabolised by yeasts and/or bacteria. Wine acids are grouped into two categories, i.e. fixed and volatile acids (Table 2.1). The fixed acids include all the non-volatile carboxylic acids such as L-malic acid, L-tartaric acid and citric acid, as well as the other carboxylic acids produced during fermentation. Succinic acid is the major acid produced by yeast during alcoholic fermentation, with concentrations ranging from 0.5 to 1.5 g/l in wine (Beelman and Gallander, 1979; Ribéreau-Gayon *et al.*, 2000[b]). Pyruvic, α-ketoglutaric, isocitric, citric and fumaric acid are also present in wine as they are TCA cycle intermediates of yeast metabolism. Lactic acid bacteria mainly produce L-Lactic acid during the malolactic fermentation, but small amounts can also be synthesised by yeast. Some yeast strains also produce citramalic acid and variable quantities of L-malic acid in wine during fermentation.

Volatile acids in wine include all the vaporous acids that usually have distinctive odours. The presence of detectable levels of volatile acids in wine is normally associated with wines infected by spoilage yeast or bacteria. The most prevalent volatile acid in wine is acetic acid, which is produced by yeast during fermentation, or by acetic or lactic acid bacteria. Other volatile acids include propionic, formic, butyric, octanoic and decanoic acid. Total acidity in wine combines the sum of both the fixed and volatile acids in wine and is usually expressed in terms of titratable acidity (TA). Titratable acidity is usually lower than the actual total acidity, since it only measures total proton concentration in wine as determined by titration with a strong base. The optimal TA of white wine cultivars lies between 5 and 9 g/l with a corresponding pH range of 3.0 to 3.4 units. Red wines usually have a lower TA range of 4 to 7 g/l with a pH range of 3.3 to 3.7 (Margalit, 1997).

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2.4 THE ROLE OF ORGANIC ACIDS IN WINE

The conversion of grape sugars to ethanol and carbon dioxide is often described as the fundamental biochemical reaction involved in making wine. The chemistry of winemaking involves, however, an intricate ensemble of biological and spontaneous chemical reactions that has been unravelled through decades of research. Besides the chemical importance of flavour and aroma compounds, the presence or the lack of organic acids in wine plays a pivotal role in the production of quality wines. Acidity in wine impacts directly or indirectly on several different levels of wine quality and the success of several winemaking processes. Over and above the apparent contribution of organic acids to the taste of wine, wine acidity ultimately determines wine quality in terms of the perceived organoleptic and aesthetic character of wine. Similarly, wine acidity influences the ageing potential or the shelf life of wine, as it determines the physical, biochemical and microbial stability of wine. In addition, wine acidity and pH affect the timely succession of cellar events and effectiveness of several winemaking techniques applied by modern winemakers.

2.4.1 Organoleptic character of wine

Organic acids can contribute positively to the organoleptic character of wine when in balance with the other wine components. The sour-sweet balance is well-known as a required sensory quality in wine, especially in white wine (Burns and Noble, 1985; Fischer and Noble, 1994; Martin and Revel, 1999; Noble and Bursick, 1984; Vannier *et al.*, 1999). Acid-balanced wines are usually perceived as having refreshing or crisp sensory undertones, while descriptions such as “sharp”, “green”, “acidulous” or “unripe” often refer to wine with too much acidity. When present in excessive concentrations, organic acids leave a uniquely tart or sour taste indicative of the specific acid in wine. For example, excess quantities of L-malic acid are perceived as a sour taste resembling that of unripe apples. Furthermore, aside from the direct detection of acids in wine, wine acidity often disguises or accentuates the perception of other wine tastes. This is often true for the perception of sweetness, where acidity usually masks excess sweetness, while the perception of astringency is emphasised when coinciding with low pH values in wine (Noble, 1998).

The specific organic acid composition of wine determines the specific pH of the wine, which in turn indirectly influences the perception of taste in wine. Since the pH of any given solution is based on the balance between the protonated and deprotonated isoforms of organic molecules, the pH of wine determines the degree of organic acid and amino acid ionisation in a wine solution. The level of ionisation of these organic building blocks influences the ionic state, solubility and biological activity of many complex molecules such as proteins, fatty acids, phenolic compounds, etc. It is thus not inconceivable that a minor change in wine pH (as small as 0.05 units), when coinciding with changes in total acidity (TA) of 0.2-0.5 g/l, significantly modifies the organoleptic perception of wine (Margalit, 1997).

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Organic acids and pH play an important role in the development of specific flavour compounds during vinification. A high TA and low pH in grape juice or must can be linked to the release of floral aroma and other flavour precursors from grapes skins during the crushing stages of vinification. The release of stored organic acids, specifically L-malic acid and tartaric acid, from the grape berry during crushing is responsible for acid hydrolysis of non-volatile flavour compounds like monoterpene glycosides, some phenolic compounds, C_{13}-norisoprenoids, benzyl alcohol and 2-phenylethanol from the berry. These flavour compounds are essential for the development of a healthy, complex flavour profile during vinification and subsequent ageing of wine (Strauss *et al.*, 1987, Winterhalter *et al.*, 1990). Furthermore, most organic acids in wine are involved in the formation of another group of important aroma compounds that contribute to the generic background flavour of wine, namely esters (Lilly *et al.*, 2000; Marais, 1978; Nykänen, 1986; Nykänen and Suomalainen, 1983). Esters are the condensation products of the carboxyl group of an organic acid and the hydroxyl group of an alcohol or phenol. Esters can be synthesised enzymatically during yeast fermentation or chemically during long-term ageing (Ribéreau-Gayon *et al.*, 2000[b]). The main ester produced by yeast is ethyl acetate, but other esters of fusel alcohols, short chain-fatty acids, fatty acid-ethyl esters and long-chain higher alcohol acetates are also produced (Mateo *et al.*, 1999; Mateo *et al.*, 2001; Nykänen and Suomalainen, 1983; Schreier, 1979). The concentration of the various esters produced during fermentation depends on many factors, including the pH of the wine (Lilly *at al.*, 2000; Marais, 1978, 1998).

Acidity in wine, and more specifically wine pH, is also a role player in the preservation of wine aroma and flavour, as the pH of wine influences the rate of oxidation in wine. Wine oxidation entails the reaction of dissolved oxygen in wine with compounds like phenolics, aldehydes, sugars, sulphur dioxide, etc. Oxidation of phenolic compounds causes severe changes in wine aroma and colour. A low pH in must or wine acts as a safeguard in wine that prevents or delays phenolic oxidation by maintaining the phenolic compounds in their non-ionised state, rendering them less susceptible to oxidation. Although only approximately 0.004% of grape phenols are in a readily oxidised state at a pH of 3.5, these compounds are so unstable that even a slight increase in pH could result in significant oxidation (Margalit, 1997). Wines, especially white cultivars, with high pH values (pH $\geq$3.9) are therefore prone to oxidation and lose their fresh aroma and colour easily (Margalit, 1997).

2.4.2 Aesthetic character of wine

As the term aesthetic implies, the acidity and pH of wine greatly influences the visible attributes of wine, such as wine colour and clarity. The colour of white wine is usually affected by pH-dependent phenolic oxidation reactions that lead to browning of the wine's colour. In red wines a low pH, together with free sulphur dioxide, is essential for the development and stability of the red colour. The phenolic compound anthocyanin is responsible for the red colour of red cultivars and occurs in a

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state of dynamic equilibrium between five different molecular states of ionisation in wine (Margalit, 1997). The anthocyanin equilibrium is highly pH-dependent, with the two main isoforms, namely the red flavylium cation and the colourless hemiketal form, most prevalent at normal wine pH levels. Most of the other forms of anthocyanins are colourless at the pH range found in wine (Vivar-Quintana *et al.*, 2002). At low pH values the flavylium cation is more abundant relative to the four other isoforms, resulting in a more intense red colour. Rapid deprotonation of the flavylium cation occurs as the pH increases, with maximum decolourisation between pH 3.2 and 3.5 (Margalit, 1997; Ribéreau-Gayon *et al.*, 2000[b]). Furthermore, the pH of red wine also influences the degree of co-pigmentation of anthocyanins, which in turn determines the red colour density. Co-pigmentation involves complex formation or stacking of the different isoforms of anthocyanin with each other, or with other colourless phenolic compounds, such as coumarins, phenolic acids and flavonols (Ribéreau-Gayon *et al.*, 2000[b]; Somers and Vérette, 1988).

The precipitation of acids, particularly tartaric acid, in wine can also be considered an aesthetic imperfection, especially in young bottled wines. Excess tartaric acid is routinely removed before bottling by cold stabilisation and racking or filtering of the potassium bitartrate crystals to avoid future precipitation of the acid after bottling.

2.4.3 Microbial stability of wine

Wine acidity, but more importantly wine pH, has a profound effect on the microbial stability of wine as it determines the survival and proliferation of bacteria and yeast species during and after vinification. Grape must or juice with a low pH is usually more protected against microbial spoilage at the onset of alcoholic fermentation, as the low pH serves as a natural antimicrobial shield in the must. Although a low pH in must (< 3.5) is not conducive to the growth of most spoilage bacteria and yeast species, it still permits the proliferation of the wine yeast *Saccharomyces cerevisiae*. One of the components in wine responsible for bacterial inhibition under low pH conditions is the fatty acids produced by yeast during alcoholic fermentation (Capucho and San Romao, 1994). The antimicrobial effect of fatty acids against bacteria in wine is enhanced at lower pH values, as the non-ionised form of fatty acids is significantly more toxic to bacteria. It is postulated that at low pH the undissociated isoform of fatty acids enters bacteria cells by diffusion, since it is highly soluble in the phospholipid layer of the plasmamembrane (Warth, 1988). Invasion of these fatty acids into the bacterial plasmamembrane disrupts its normal spatial organisation, which in turn disturbs the selective permeability of the cells and leads to the accumulation of toxic ions. Disruption of the plasmamembrane also leads to the disruption of the transmembrane proton gradient (Δp) (Viegas and Sa-Correia, 1991) required for regulation of intracellular pH and energy production in malolactic bacteria.

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Extreme pH values in wine usually have a negative effect on the growth of yeast and bacteria during vinification. Extremely low pH values (pH < 2.9) in must not only inhibits the growth of spoilage bacteria and yeasts, but also starts to inhibit the growth and rapid population growth of most strains of *S. cerevisiae*. Even with the application of selected *S. cerevisiae* strains in starter cultures, delays in the onset of alcoholic fermentation can be anticipated, leading to scheduling problems in the cellar. Similarly, pH levels below 2.9 will severely affect the success of inoculating wine with malolactic bacteria starter cultures after alcoholic fermentation (Mayer and Vetsch, 1973).

When the pH of must or juice exceeds pH 3.5, the risk of overgrowth of spoilage lactobacilli, pediococci as well as strains of *Oenococcus oeni* during alcoholic fermentation is increased. At elevated pH ranges strains of these lactic acid bacteria can rapidly proliferate to substantial populations before the actual onset of alcoholic fermentation. Premature growth of lactic acid bacteria poses a serious risk to wine quality, since glucose is fermented to acetic acid, resulting in elevated volatile acidity values in wine and reduced ethanol yields. Furthermore, spoilage of this kind could also have serious repercussions further down the line in the winemaking process, often leading to stuck or sluggish alcoholic fermentation (Fugelsang, 1997; Narendranath *et al.*, 1997).

2.4.4 Cellar operations

Several wine-related treatments influence or are influenced by the TA and pH of wine. To be a successful oenologist the winemaker has to take into account all the implications of the acidity and pH of the must or juice before the start of vinification. Skin contact or maceration, for instance, has an important effect on TA and pH of wine before the start of fermentation. Skin contact is often applied in red wines (and some white wines) to extract anthocyanins and other phenolic flavour compounds from the pomace to ensure the optimal colour and flavour development in red wines. However, increased extraction of potassium from the skins coincides with phenolic extraction during skin contact, which normally causes an increase in potassium bitartrate precipitation. Generally skin contact or maceration leads to a slight decrease in TA and an increase in pH in must before the start of fermentation (Darias-Martin *et al.*, 2000; Ferreira *et al.*, 1995; Le Fur, 1992; Singleton *et al.*, 1980). Carbonic maceration is a modified maceration process that can also lead to reduction in grape acidity. In this process the grapes are kept under anaerobic conditions by exposing them to CO_2 gas before crushing or pressing. In the anaerobic environment certain intracellular fermentation reactions are stimulated in the intact berries, one of which is the oxidative decarboxylation of L-malic acid to pyruvic acid, catalysed by a berry NADP-dependent malic enzyme (EC 1.1.1.40) (Beelman and Gallander, 1979).

The use of chemical additives is also reduced in low pH wines. For example, sulphiting (SO_2 addition) of wine is usually necessary before and after alcoholic fermentation as an antimicrobial and antioxidant agent in grape must and wine. Wines with low pH values require lower concentrations of

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SO_2 due to the added protection against oxidation and microbial spoilage supplied by the low pH. The effective precipitation of pectins and heat-unstable proteins during bentonite treatment is also enhanced by a lower pH in wine. Since less bentonite is required at lower pH values for the effective removal of solids and proteins during racking, the loss of flavour compounds during fining due to excessive use of bentonite is minimised (Boulton *et al.*, 1996; Margalit, 1997; Ribéreau-Gayon *et al.*, 2000[b]).

2.5 TECHNIQUES FOR ACIDITY ADJUSTMENT IN WINE

Acidity adjustment in grape juice and must is an essential step during vinification when the TA and/or pH of the must or wine exceed acceptable ranges. The adjustment of acidity in must or wine could be a perplexing decision for winemakers, since several factors need to be considered to determine the timing (before or after alcoholic fermentation) and method of rectifying wine acidity. General guidelines for the timing of acid adjustment exist based on the initial pH of the must or juice. Traditionally, the acidification of low-acid (high pH) grape must in the warm viticultural regions is preferably applied before the start of alcoholic fermentation. Lowering the pH is conducive to optimal wine flavour production during fermentation and prevents the proliferation of spoilage lactobacilli and pediococci during alcoholic fermentation. Similarly, a reduction of TA prior to fermentation is a prerequisite in grape must with a pH below 2.9, since the onset of alcoholic fermentation by strains of *Saccharomyces* will be negatively affected at such low pH extremes.

Except for the two scenarios described above, the adjustment of acidity prior to alcoholic fermentation is not always advisable, especially when the impact of alcoholic fermentation on the final TA and pH of wine is difficult to predict and quantify. As a general rule, the TA of wine is increases by 1 to 2 g/l during alcoholic fermentation via the production of L-malic, succinic, acetic and lactic acid by strains of yeast and bacteria. However, significant variations might occur due to the contribution of the specific yeast strains used for alcoholic fermentation or the success of the malolactic fermentation. Some strains of *S. cerevisiae* have the ability to synthesise significant concentrations of L-malic acid during fermentation (Bhattacharjee *et al.*, 1968; Pines *et al.*, 1996, 1997; Schwartz and Radler, 1988). Under these conditions post-fermentation acid correction is a more reliable treatment option to ensure optimum acid balance in wines.

2.5.1 Vineyard practices

Viticulturists in both cool- and warm-climate regions have several vineyard techniques at hand to preserve or decrease the acidity content of grapes. Winemakers in the warm-climate countries, for example, can alleviate the problem of low acidity wines by selecting the production of specific cultivars with a natural higher tartaric : malic acid ratio. Cultivars like Semillon and Riesling are

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known to have a higher tartaric : malic acid ratio and would ensure an acceptable TA content of the grape must and wine (Lavee and Nir, 1986), while Cabernet Sauvignon is classified as an intermediate-acidity variety (Kliewer *et al.*, 1967) that will to a limited degree relieve high acidity problems in the cool-climate regions.

Canopy management, irrigation control and soil fertilisation has an enormous influence on the acid composition of grapes. For example, nitrogen and potassium fertilisation stimulates the accumulation of L-malic acid due to increased foliage and shading in the bunches. Similarly, several trellising and leaf-pruning practices that increase shading creates cooler bunch and foliage zones that yield grapes with increased potassium concentration and titratable acids in the grapes. Berries maturing in densely shaded canopy interiors are generally associated with low total soluble solids, high titratable acidity, high L-malic acid concentrations, elevated pH, high potassium, low proline, high arginine, low total phenols, low anthocyanin concentrations in red cultivars and high chlorophyll to flavanoid pigment ratio in white cultivars (Kliewer, 1980; Kliewer and Lider, 1968; Morrison and Noble, 1990; Reynolds *et al.*, 1986; Smart, 1985; Zoecklein *et al.*, 1995). The increase in TA is mainly ascribed to an increase in L-malic acid, while the more stable tartaric acid is usually slightly decreased, leading to the elevated pH levels (Archer and Strauss, 1989). Similarly, viticulturists in the cool-climate regions can make use of trellising and leaf-pruning techniques that allows grape bunches to be more exposed to sunlight and increase the microclimate temperature to ensure increased respiration of L-malic acid during *véraison.*

The success of these viticultural methods is, however, limited and more extreme measures, like adapting the harvesting time, could in theory resolve the problem of acidity imbalances. Delaying or shortening the ripening time of grapes in the cool or warm climates to ensure optimal TA content of must is, however, unpractical and generally jeopardises the quality of the final wine. For instance, if grapes were to be harvested earlier than usual to ensure the desirable level of acidity in the warm-climate regions, the grape must would lack the acceptable sugar and varietal aroma content, leading to the production of lower-quality wine (Margalit, 1997). Similarly, extending the ripening period in cool-climate regions will result in both a high pH and high acid concentration due to the increased exchange of protons (H^+) with potassium in the berry (Butzke and Boulton, 1997). The only viable solution for winemakers is therefore to rectify wine acidity artificially either before or after alcoholic fermentation.

2.5.2 Acidification of low-acid wines

Several artificial methods for acidification of grape must are available to winemakers, the most traditional method of which is the use of raw gypsum or plaster (hydrous calcium sulphate), which reacts with potassium bitartrate and releases free tartaric acid. Even though this method is still being used in the Jerez region of Spain during sherry production, it is, however, rarely applied in modern

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winemaking due to the risk of increased hydrogen sulphide production by yeast during fermentation as excess sulphur is added to the wine (Zoecklein *et al.*, 1995). Acidification of wine can also be achieved by adding naturally occurring grape acids to wine, such as tartaric, L-malic, citric or fumaric acid. Although succinic acid is relatively resistant to microbial attack under fermentative conditions, it cannot be utilised as an acidulating agent due to its bitter-salty taste (Ribéreau-Gayon *et al.*, 2000[b]).

Tartaric acid is the preferred acidulating agent in low-acid wines and its application is in accordance with stipulations of the Office International de la Vigne et du Vin (OIV) and the European Community (EC) legislation (Margalit, 1997; Ribéreau-Gayon *et al.*, 2000[b]). Tartaric acid is relatively more resistant to microbial breakdown and can thus be added before the onset of alcoholic fermentation without the risk of off-flavour production. Acidification of grape must with L-malic acid and citric acid can be applied with some degree of success, but degradation of these acids by spoilage malolactic bacteria does pose a risk under winemaking conditions. L-Malic acid does not precipitate like tartaric acid, but can initiate a second round of malolactic fermentation if still present in the wine just before bottling. As a precaution, malic acid is added as a racemic mixture of D/L-malic acid to the must at the beginning of fermentation, which leads to increased TA and lowered pH of the wine. The risk of bottled malolactic fermentation is significantly reduced, since most or all of the L-malic acid isoform is removed from the wine by the first round of malolactic fermentation, leaving only the D-malic acid isoform that is resistant to microbial attack and maintains a low pH in wine. The precise influence of the D-malic acid isomer on malolactic fermentation has not yet been determined in wine, but research in the cider industry showed that D-malic acid has an inhibitory effect on malolactic fermentation. It was found that the D-isomer of malic acid reduced the uptake rate of the L-isomer and also reduced the affinity of the malolactic enzyme for L-malic acid (Ribéreau-Gayon *et al.*, 2000[b]).

2.5.3 Deacidification of high-acid wines

In the cool viticultural regions, the removal of tartaric acid or excess L-malic acid from the wine before or after alcoholic fermentation is usually required to ensure wines with a balanced acid content and sufficient stability. Deacidification of high-acid wines can be achieved through physiochemical methods such as blending, chemical neutralisation and precipitation, or by biological means through the microbial degradation of L-malic acid during or after alcoholic fermentation. However, physiochemical deacidification of wine is often time-consuming, requires increased labour and capital input and is regularly associated with reduced wine quality (Pretorius, 2000). For these reasons, biological deacidification of wine with lactic acid bacteria or yeast is the method of choice for most winemakers as it is a natural or spontaneous process, despite some inherent obstacles associated with its successful execution.

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2.5.3.1 Physiochemical deacidification methods. Blending of grape musts with different TA and pH indexes is one of the most elementary and most effective solutions available to winemakers. However, blending of a low-pH with a high-pH must to neutralise the pH before fermentation is not always practically achievable in a cellar. The main logistical problem with blending is the lack of available musts with significantly opposite characteristics in the same wine-producing region. Another physical method employed to decrease the TA of high-acid musts is the process of amelioration (Kluba and Beelman, 1975). This method is based on the blending concept, but involves the dilution of grape must with water to ultimately reduce the must TA before fermentation. Amelioration, however, does not significantly influence the pH of grape must and requires the addition of sugar to maintain the optimal sugar : organic acid balance. Amelioration has in recent times become an unacceptable winemaking practice due to the detrimental effect that its excessive use has on wine flavour, aroma, body and colour (especially red wines) and has not been legalised in some wine-producing countries (Margalit, 1997).

2.5.3.2 Precipitation-based deacidification methods. Acids become less soluble and their salts precipitate in wine under chilled conditions and increased ethanol concentrations; therefore the TA of wine or grape must can be reduced by precipitating tartaric acid, and to a lesser extent, L-malic acid salts. Tartaric acid usually precipitates in wine at the end of fermentation as potassium bitartrate crystals and it is often considered an aesthetic fault. To prevent the formation of tartaric acid crystals during wine ageing, cold stabilisation, an accepted winemaking procedure, is applied. After alcoholic fermentation the wine is usually chilled to between –4 and 0°C for several days during which tartaric acid crystallisation and precipitation are promoted. Removal of the tartaric acid crystals during subsequent racking, filtration or centrifugation leads to lower tartaric acid concentration, thus a decrease in TA and an increase in pH.

Potassium bicarbonate ($KHCO_3$) and calcium carbonate ($CaCO_3$) are approved neutralisation additives that can be added to wine to assist in tartaric acid precipitation (Faber, 1970). In the presence of these additives, tartaric acid forms the corresponding insoluble salts, potassium bitartrate and calcium tartrate, as well as carbonic acid (H_2CO_3). The carbonic acid eventually breaks down to CO_2 and H_2O, with the concomitant loss of acidity (McKinnon, 1993). One of the disadvantages of the deacidification of wine with $KHCO_3$ and $CaCO_3$ is the fact that only one of the major wine acids, namely tartaric acid, is affected and not L-malic acid, often leading to undesirable tartaric : malic acid ratios in wine (Ribéreau-Gayon *et al.*, 2000[b]).

A very effective method to reduce the TA of high-acid grape musts before fermentation is the double salt-precipitation treatment (also known as Acidex or DICALCIC treatment) (Ribéreau-Gayon *et al.*, 2000[b]). This method is often used in countries like Canada and the USA and is based on the addition of a fine powder of calcium carbonate that contains a 1% calcium tartaric and malic acid salt mixture

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as a seeding agent. At pH values above 4.5 this additive causes the precipitation of supposedly equimolar quantities of both tartaric and L-malic acid (Cole and Boulton, 1989). Usually, a pre-determined fraction of the wine is treated with Acidex and then blended back with the untreated rest to yield a wine with a lower TA and higher pH.

2.5.3.3 Biological deacidification of wine. The most widely accepted winemaking procedure for the removal of excess acidity in wine is the traditional malolactic fermentation. Winemaking regularly involves two sequential vinification steps. First, wine yeast strains initiate the alcoholic fermentation whereby grape sugars, glucose and fructose, are converted into ethanol and various flavour compounds. Secondly, once all of the grape must sugars are depleted, the yeast population rapidly declines, followed by the proliferation of lactic acid bacteria, which execute the depletion of the remaining pentose sugars and performs malolactic fermentation (MLF). Modern winemaking has harnessed the benefits of the naturally occurring lactic acid bacteria in wine by developing pure starter cultures of selected malolactic bacteria for improved efficiency and reliability of malolactic fermentation.

2.6 BIODEACIDIFICATION WITH MALOLACTIC BACTERIA

The organisms responsible for malolactic fermentation in wine were isolated and identified over 40 years ago (Peynaud, 1956). Since then it has become a well-known fact that strains of lactic acid bacteria (LAB) are regularly associated with different food and beverage-related biotopes such as beer, ciders, vegetables, silage, bread (sourdough), cocoa and coffee fermentations (Beech, 1972; Hashizume and Mori, 1990; Henick-Kling, 1993; Salih *et al.*, 1988). As their name suggests, strains of LAB have the ability to produce significant quantities of lactic acid from sugars. The production of lactic acid by LAB is achieved either via a homofermentative metabolic pathway when only lactic acid is produced from glucose, or via a heterofermentative pathway where glucose is fermented to lactic acid, ethanol and acetic acid (Table 2.2). Strains of LAB isolated from wine have the additional unique ability to convert L-malic acid into L-lactic acid and CO_2 by means of the malolactic enzyme (MLE), and have been renamed the malolactic bacteria. The malolactic enzyme is, however, not exclusive to the wine lactic acid bacteria, but is also present in other lactic acid bacteria such as *Leoconostoc*, *Weissella*, *Pediococcus* and *Lactobacillus* species from other ecological niches. The malolactic enzyme is a bi-functional enzyme that executes the conversion L-malic acid into L-lactic acid in the presence of the cofactors NAD^+ and Mg^{2+}, without the generation of any enzymatic intermediates or net cofactor reduction (Fig. 2.3) (Bony *et al.*, 1997).

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Table 2.2 Lactic acid bacteria isolated from wine (Adapted from Fugelsang, 1997)

Genus	Wine-related species	
	Homofermentative species	**Heterofermentative species**
Oenococcus		*O. oeni* (formerly *Leuconostoc oenos*)
Leuconostoc		*L. mesenteroides*
Lactobacillus	*L. casei* ,*L. homohiochii, L. plantarum, L. sake* (or facultative heterofermentative)	*L. brevis, L. hilgardii, L. fructivorans* (formerly *trichodes*), *L. buchneri, L. fermentum*
Pediococcus	*P. damnosus* (formerly *P. cerevisiae*), *P. parvulus, P. pentosaceus*	

Malolactic bacteria are usually present in low numbers (10^2–10^4 colony-forming units (CFU)/g) on grapes and in grape must at the early stages of vinification (Fugelsang, 1997; Lafon-Lafourcade *et al.*, 1983; Lonvaud-Funel, 1999; Radler, 1958; Wibowo *et al.*, 1985). The prevalence of LAB in grape musts is mainly correlated to the must pH: the higher the must pH (pH > 3.5), the higher the total LAB population. However, elevated populations of LAB can also be found in musts from damaged grapes (Lonvaud-Funel, 1999). Mainly four different genera of LAB are present in the must at the beginning of vinification (Table 2.2), i.e. *Oenococcus*, *Lactobacilllus, Pediococcus* and *Leuconostoc* (Dicks *et al.*, 1990; Dicks and Van Vuuren, 1988; Fugelsang, 1997; Irwin *et al.*, 1983; London, 1976; Lonvaud *et al.*, 1977; Lonvaud-Funel, 1999; Stamer, 1979; Wibowo *et al.*, 1985). The *Leuconostoc* group has recently undergone several taxonomic changes due to advances in phylogenetic studies.

The classification of LAB into different genera was originally solely based on their morphology, metabolism and physiological characteristics (Buchanan and Gibbons, 1986; Collins *et al.*, 1987, 1990; Stiles and Holzapel, 1997; Van Damme *et al.*, 1996). With the advent of DNA-DNA hybridisation (Kawai *et al.*, 1996), 16S rRNA sequencing (Collins *et al.*, 1990, 1993; Lane *et al.*, 1985) and soluble protein patterns (Dicks *et al.*, 1996) several changes in the classification of LAB were suggested. For example, *Leuconostoc paramesenteroides* and related species have been reclassified in a new genus, *Weissella* (Collins *et al.*, 1993), on the basis of their 16S rRNA sequences. Phylogenetic studies have also revealed that *Leuconostoc oenos* is distinct from other *Leuconostoc* spp. (Martínez-Murcia and Collins, 1990) and it has been suggested that this organism is an interesting case of a fast-evolving species (Yang and Woese, 1989) that was renamed *Oenococcus* (Dicks *et al.*, 1995).

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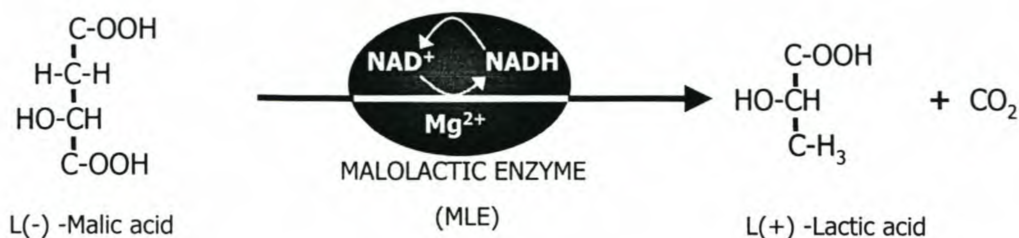

Figure 2.3. The NAD-dependent malolactic enzyme (MLE) transforms the C_4 dicarboxylic acid L-malic acid to the C_3 monocarboxylic acid L-lactic acid without any free intermediates. The conversion is a direct decarboxylation of L(-)-malic acid to L(+)-lactic acid and carbon dioxide (Pilone and Kunkee, 1970).

During the early days of yeast alcoholic fermentation, the LAB population increases to ca. 10^4 CFU/ml, but quickly declines to only a few cells/ml with the onset of ethanol production (Fugelsang, 1997; Van Vuuren and Dicks, 1993). Most importantly, not only do the bacterial numbers diminish but also the diversity of species. The main reasons for the decline in LAB population is a combination of low initial pH values, low temperatures, increased ethanol concentration, competitive interactions with yeasts and possible bacteriophage infections. Furthermore, high concentrations of SO_2 are usually added to the must after crushing to prevent oxidation and uncontrolled growth of wild yeasts, which also severely inhibits the growth of LAB (Van Vuuren and Dicks, 1993). At the end of alcoholic fermentation, mainly strains of *O. oeni* rapidly proceed to proliferate to a population of 10^7 CFU/ml, which coincides with the malolactic fermentation (Costello *et al.*, 1983; Fleet *et al.*, 1984; Kunkee, 1967[b]; Lafon-Lafourcade *et al.*, 1983; Lonvaud-Funel, 1999; Wibowo *et al.*, 1985). Strains of *O. oeni* have the unique ability to survive in a wine milieu at pH values lower than 4.2 and ethanol levels as high as 10% (v/v) (Garvie and Farrow, 1980). Strains of *Pediococcus* and *Lactobacillus* only proliferate in wines with initial high pH levels prior or during alcoholic fermentation, and usually cause spoilage or sluggish alcoholic fermentations in the wines due to the untimely utilisation of glucose (Costello *et al.*, 1983; Fugelsang, 1997; Mayer 1974; Vetsch and Mayer, 1978).

2.6.1 Advantages and disadvantages of malolactic fermentation

Malolactic fermentation is the preferred deacidification method in most of the wine regions of the world. Red wine production in both cold- and warm-climate regions almost always involves the malolactic fermentation, naturally or induced, after yeast alcoholic fermentation. Natural malolactic fermentation occurs less frequently in white wines due to an average lower pH of most white cultivars and higher concentrations of SO_2 employed (Ingraham and Cooke, 1960; Rodriquez *et al.*, 1990), but it can be induced with LAB starter cultures in some styles of wine, e.g. Chardonnay. The malolactic fermentation is crucial in the Champagne wine region of France, where the traditional *méthod*

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champenoise process is used to produce sparkling wine. The grapes used during the production of base wines are usually high in acid content and require the malolactic fermentation as a primary fermentation to deacidify and mature the base wine prior to the yeast fermentation in the bottle (Pool and Henick-Kling, 1991).

The malolactic fermentation affects four different, but interrelated aspects of wine quality: adjustments to wine acidity, influences microbial stability, contributes to the sensory complexity and lastly influences the hygienic quality of wine. Controversy over the benefits of the malolactic fermentation still endures after years of oenological research. Under certain conditions the contributions made by malolactic fermentation improve wine quality, but the same contributions may be considered highly undesirable under a different set of circumstances (Table 2.3). This is especially evident in the cool- and warm-climate wine regions, where its contribution to wine quality differs remarkably.

2.6.1.1 Loss of acidity and increase in pH. Depending on the initial pH of the must, the removal of L-malic acid via MLF can either be advantageous or detrimental to wine quality. In high-acid/low-pH wines typically found in the cool-climate regions, a decrease in excess L-malic acid (2 –10 g/l) is highly favourable for the production of acid-balanced wines (Boulton *et al.*, 1996; Henick-Kling, 1995; Kunkee, 1967[b]; Lonvaud-Funel, 1999). MLF usually leads to a reduction in final TA of 1-3 g/l and an average increase in pH of 0.1 to 0.3 units in wine (Bousbouras and Kunkee, 1971; Margalit, 1997). Subsequently, the rise in pH after MLF often promotes the precipitation of potassium bitartrate, leading to an additional reduction in TA (Beelman and Gallander, 1979; Ribéreau-Gayon *et al.*, 2000[b]). The pH of wine is could also be raised due to the ability of malolactic bacteria to metabolise arginine during MLF. The degradation of arginine leads to the release of ammonia in wine and results in an increase in wine pH (Liu and Pilone, 1998). However, the extent of a pH increase due to arginine metabolism by malolactic bacteria is limited, since it depends on the timing of the bacterial growth during alcoholic fermentation and the buffering capacity of the wine. After alcoholic fermentation, the grape must is depleted of arginine and little if any arginine remains available for malolactic bacteria.

The incidence of MLF in wine with an initial high pH (or low acidity), customarily found in the warmer climatic regions, has the opposite impact on wine quality. MLF in these wines leads to an additional reduction in wine acidity and subsequent increase in pH due to the degradation of L-malic acid and results in undesirable "bland" wines that lack adequate acidity. Under these circumstances, the aesthetics of red wine is often negatively affected in terms of red colour intensity, with a potential loss of ca. 30% in red colour due to the shift in pH (Kunkee, 1967[b]; Vetsch and Lüthi, 1964). Furthermore, as mentioned before, the risk of spoilage by strains of lactobacilli and pediococci is enhanced in these elevated pH wines.

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Removal of excess acidity by malolactic fermentation is thus only a true benefit to winemaking in the cool-climate viticultural regions, where the final wine acidity remains within acceptable ranges to produce balanced wines. This same attribute of MLF, however, complicates rather than alleviates the winemaking process in warmer viticultural regions.

2.6.1.2 Microbial stability. Winemakers have long believed that MLF leads to an increase in microbial stability due to the depletion of essential nutrients in wine and the fastidious growth requirements of LAB, especially *O. oeni*. This might be in part true for high-acid/low-pH wines, where the antimicrobial effect of low pH dominates in inhibiting the growth of spoilage bacteria after the first round of MLF. The apparent depletion of residual nutrients, which includes L-malic acid, citric acid, amino acids, nitrogen bases, vitamins and fermentable sugars left after alcoholic fermentation, prevents the growth of other spoilage bacteria. Furthermore, malolactic bacteria produce antimicrobial compounds such as lactic acid and bacteriocins (Rammelsberg and Radler, 1990) that inhibit the growth of other related bacterial species (Henick-Kling, 1993). However, the depletion of nutrients after the first round of MLF does not completely guarantee total growth inhibition of other bacterial species. Spoilage by strains of malolactic bacteria, including *O. oeni*, is often encountered in wine industries, especially if low concentrations of L-malic acid remain in the wine. In wines with high initial pH levels, the danger exists that the incidence of malolactic fermentation might, contrary to general belief, aggravate the risk of microbial instability of wine. Due to the additional increase in pH (> 3.5), some strains of lactobacilli and pediococci may find it more favourable to proliferate and spoil the wine (Costello *et al.*, 1983; Davis *et al.*, 1986).

The benefit of increased microbial stability due to MLF is therefore also more applicable to the cool-climate viticultural regions, where the low pH of wine remains an inhibitory factor after completion of MLF. Under these circumstances, complete removal of L-malic acid during MLF, which serves as relatively good nutritional resource for *O. oeni*, does minimise the risk of future growth after bottling. In the warmer viticultural regions the use of MLF is tolerated in most red and some white cultivars for the sole purpose of completely removing L-malic acid from the wine. However, winemakers have to take extreme care to adjust the pH of the wine prior to MLF to the accepted range, usually with tartaric acid, to prevent spoilage by LAB and other bacteria.

2.6.1.3 Wine sensory modifications. The role of MLF in improving the sensory complexity of wine is one of the more dubious benefits of MLF when compared to its role in deacidification and microbial stability of wine. The most convincing change in wine taste after MLF is the replacement of the strong "green" taste of L-malic acid with the less aggressive taste of lactic acid (Beelman and Gallander, 1979; Lonvaud-Funel, 1999). Removal of the sharp taste of excess L-malic acid is usually

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described in terms of the mouth feel and extended aftertaste of wine compared to non-MLF control wines (Davis *et al.*, 1985; Henick-Kling, 1993; Henick-Kling *et al.*, 1994; Malík, 1998).

In general wines that underwent MLF, particularly red wines, are often characterised by lower vegetative/herbaceous aromas, while the fruity and floral characters are also reduced due to the degradation of several esters and other flavour compounds (Laurent *et al.*, 1994; McDaniel *et al.*, 1987). Many other flavours such as 'buttery', 'lactic', 'nutty', 'oaky', 'yeasty' and 'sweaty' has been described in wines after MLF (Laurent *et al.*, 1994). The exact sensory contribution of MLF in wine is extremely difficult to evaluate due to the intricate nature of the factors that play a role. The number of flavour compounds synthesised during MLF is greatly influenced by the initial wine pH and the fermentation temperature, which determines the rate of malolactic fermentation. When the rate of malolactic fermentation is fast (high pH and temperature), the production of acetic acid is enhanced, while the production of diacetyl is favoured under low pH and temperature conditions. A second factor that complicates the determination of flavour contribution by MLF in wine is the large diversity of strains and species of malolactic bacteria that are usually involved in MLF (Table 2.3). For example, individual strains of *O. oeni* contribute different flavour changes to the wine during MLF (Henick-Kling *et al.*, 1994; Zeeman *et al.*, 1982).

The improvement of the front-palate volume and roundness in the mouth is not only due to the reduction in acidity of the wine. Certain selected malolactic bacteria can produce metabolites, which improve mouth feel either directly or by binding with bitter and astringent wine compounds. Specific metabolites synthesised during the heterofermentative metabolism of malolactic bacteria, especially strains of *O. oeni*, have been identified as flavour compounds in wine and it is argued that these compounds play a role in improving the sensory complexity of wine (McDaniel *et al.*, 1987; Rodriquez *et al.*, 1990). These metabolites are synthesised at varying concentrations during MLF and include compounds such as acetaldehyde, 2,3-butanediol, acetic acid, acetoin, 2-butanol and various other volatile esters (such as ethyl lactate, isoamyl acetate, ethyl caproate, diethyl succinate and ethyl acetate) (Dittrich, 1987; Meunier and Bott, 1979; Zeeman *et al.*, 1982). Diacetyl, a volatile diketone and end product of citric acid metabolism by LAB, is another compound added to wine during MLF and is often perceived as a desirable buttery or nutty flavour when present at low concentrations (Davis *et al.*, 1985; Shimazu *et al.*, 1985). However, the results of many wine-tasting trials suggest that the sensory changes in wine due to malolactic fermentation cannot always be directly correlated to the production of specific flavour compounds (Davis *et al.*, 1985; Kunkee *et al.*, 1964; Laaboudi *et al.*, 1995; Martineau *et al.*, 1995; Van Wyk, 1976;).

The sensory changes incurred during malolactic fermentation are not always desirable in all wine styles and cultivars. Some delicate European white wines, such as Muscat, Riesling, Sauvignon Blanc and Gewürztraminer, are protected against natural MLF, since the malolactic bacteria degrade

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many terpenes and other flavour molecules that diminish the varietal fruity-floral aromas revealed during alcoholic fermentation (Lonvaud-Funel, 1999; Radler, 1972; Wagner, 1974).

Table 2.3. Influence of malolactic bacteria's metabolism on wine sensory profile.

Bacterial strain	Advantage (positive contribution)	Risk
Selected		
O. œni	• Reduction of total acidity • Reduction of ketone and aldehyde compounds (reducing SO_2 requirement) • Partial microbial stability • Reduction of grassy and vegetative notes • Increase in front-pallet volume • More diacetyl level control • Dominance of feral bacteria	• Production of volatile acidity (especially under high pH conditions, in presence of residual sugars and after L-malic acid degradation) • Small color loss due to the pH increase
Spontaneous		
O. œni	• Reduction of total acidity • Reduction of ketone and aldehyde compounds (reducing SO_2 requirement) • Partial microbial stability • Reduction of grassy and vegetative notes • Increase in front-pallet volume	• Long lag phase involving an increase in the volatile acidity depending on the pH • Significant bacterial growth involving a high production of diacetyl • Production of spoilage aromas and flavours (mousy off-flavour, sweat, sauerkraut) • Reduction of esters (fruity characters) • Loss of varietal aromas • Color loss due to pH increase and by direct action on polyphenols • Production of biogenic amines • Production of ethyl carbamate
L. mesenteroides	• Reduction of total acidity	• Production of viscous compounds (ropy wines) • Production of spoilage aromas and flavours
Lb. plantarum, Lb. casei	• Reduction of total acidity in must or wine • No production of acetic acid from sugar (hexose)	• Sensitive to alcohol over 5% vol. • Sluggish or stuck fermentation in high pH wine at high contamination levels • Production of spoilage aromas and flavours (*Lb.* casei)
P. pentosacceus P. damnosus	• Reduction of total acidity in must or wine • No production of acetic acid from sugar (hexose)	• Production of viscous compounds (ropy wines) • Production of biogenic amines • Risk of sluggish or stuck fermentation, at pH >3.5 with high contamination levels • Risks increase with the pH value
Lb. brevis Lb. hilgardii	• Reduction of total acidity in must or wine	• Production of viscous compounds (ropy wines) • Production of biogenic amines • High production of ethyl carbamate • Production of spoilage aromas and flavours • Production of acetic acid
Lb. kunkeei		• Strong competition with yeasts during the alcoholic fermentation for nutrients • Overproduction of acetic acid

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Furthermore, many of the metabolic end products of malolactic bacteria are usually perceived as spoilage when produced at elevated levels. For example, depending on the type of wine and the spoilage threshold for a specific wine, the synthesis of diacetyl often masks the characteristics of white wines with heavy notes of butter or cheese and can be considered an off-flavour (Martineau *et al.*, 1995). Many other types of severe spoilage by lactic acid bacteria such as mousy off-flavours, "animal" phenolic odours and geranium-like taints have been identified in wine (Cavin *et al.*, 1991; Crowell and Guymon, 1975). Some strains of malolactic bacteria, especially the lactobacilli, have the ability to degrade L-tartaric acid, which could lead to severe deficiencies in wine TA, a spoilage defined as *tourne* (Ribéreau-Gayon *et al.*, 2000[a]). Another type of off-flavour associated with lactobacilli, *amertume*, is caused by the oxidation of glycerol that leads to increased levels of 2,3-butanediol and acetic acid, thus volatile acidity (Siegrist *et al.*, 1983). Some *Lactobacillus* spp. sometimes contributes to the mousy odour in wines due to the production of acetamide in the presence of ethanol or propanol (Heresztyn, 1986).

2.6.1.4 Hygienic quality of wine. The wholesomeness of wine is becoming an ever-increasingly important marketing tool in the wine trade industry. Winemakers are therefore steering clear of procedures that could tarnish the hygienic or health image of their wines, i.e. the excessive use of detrimental chemicals such as SO_2. The synthesis of two undesirable compounds in wine, namely biogenic amines and ethyl carbamate, during the conventional malolactic fermentation have led to a renewed interest in studies on biogenic amines (Table 2.3).

Biogenic amine production during MLF. Lactic acid bacteria are well-known to produce biogenic amines during the process of fermentation of foods and beverages, for example in cheese, sausage, fermented vegetables, beer and wine (Guerrini *et al.*, 2002). Biogenic amines (e.g. histamine) are generated via the decarboxylation of the precursor amino acids (e.g. histidine) through substrate-specific enzymes (Ten Brink *et al.*, 1990). The physiological role of this reaction in lactic acid bacteria is thought to ensure growth and survival in acidic conditions, since it increases the pH. Although the biogenic amine content of fermented foods, i.e. in vegetable and meat products, is usually higher than that found in wine, the presence of alcohol and other biogenic amines has been shown to amplify the toxic effect of certain biogenic amines (Fernandes and Ferreira, 2000; Ibe *et al.*, 1991; Silla Santos, 1996). For example, the presence of putrescine, the most prevalent amine in wine (Soufleros *et al.*, 1998), is known to act as a potentiator of histamine toxicity in humans (Bover-Cid and Holzapfel, 1999; Taylor, 1986).

The concentration of biogenic amines, especially histamine, tyramine and putrescine, in wine is more elevated after the completion of malolactic fermentation (Lonvaud-Funel and Joyeux, 1994; Radler and Fäth, 1991; Soufleros *et al.*, 1998). Other biogenic amines such as methylamine, ethylamine, phenyl-ethylamine, iso-amylamine and diaminopentane (cadaverin) are also synthesised and degraded

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by LAB (Buteau *et al.*, 1984; Cilliers and van Wyk, 1985; Lafon-Lafourcade, 1975; Lonvaud-Funel and Joyeux, 1994; Petridis and Steinhart, 1995). The biogenic amine content of wine is dependent on the amino acid composition of the wine after alcoholic fermentation and the specific microflora present in wine, but more importantly their ability to decarboxylate amino acids. The amino acid content of wine is determined by the composition of the grape must, which in turn is dependent on the specific "terroir", grape variety, vine nutrition and the impact of yeast metabolism during alcoholic fermentation (Soufleros *et al.*, 1998). The metabolic activity of yeasts during alcoholic fermentation leads to modifications in the initial nitrogen content of grape must as some amino acids are utilised while others are synthesised and secreted, especially during yeast autolysis. An increase in biogenic amine content has been noted when wines are maintained in contact with yeast lees for longer periods, as more peptides and free amino acids become available for lactic acid bacteria to hydrolyse and decarboxylate (Lonvaud-Funel, 2001). The decarboxylating capacity of malolactic bacteria was found to differ significantly between strains. The pH of wine is considered to be the governing factor that determines not only the variety of lactic acid bacteria in wine, but also their decarboxylating activity (Coton *et al.*, 1998; Ough *et al.*, 1987). At elevated pH levels biogenic amines are usually found at higher concentrations in wine due to easier total growth and greater bacterial diversity (Lonvaud-Funel and Joyeux, 1994). White wines, which are generally more acidic, therefore contain lower biogenic amine concentrations than red wines (Gerbaux and Monany, 2000; Ribéreau-Gayon *et al.*, 2000[b]).

As biogenic amines are produced in wine by strains of LAB, these bacteria must inherently be equipped with the necessary metabolic pathways, i.e. amino acid decarboxylases and transport proteins. This has indeed been shown for some strains of LAB that are capable of decarboxylating amino acids to form biogenic amines (Straub *et al.*, 1995). Traditionally oenologists have considered strains of *Pediococcus* as the main culprits of histamine production in wine (Aerny, 1985). This genus is always present in wine microflora together with *Lactobacillus*, *Leuconostoc* and *Oenococcus*, but usually at low CFU/ml values. Although many *Pediococcus* spp. are well-known for their ability to produce histamine from histidine by means of a histidine decarboxylase (HDC) enzyme, Lonvaud-Funel and Joyeux (1994) demonstrated that strains of *Oenococcus oeni*, generally regarded as the safe LAB, also contain the histidine decarboxylase gene that may play a role in the production of histamine in wine (Aerny, 1985; Coton *et al.*, 1998; Delfini, 1989; Guerrini *et al.*, 2002; Mayer *et al.*, 1971; Weiller and Radler, 1976).

The presence of biogenic amines is becoming a health concern also for wine consumers, as can be seen by the renewed research interest in biogenic amine formation in wine (Coton *et al.*, 1998; Guerrini *et al.*, 2002; Lasekan and Lasekan, 2000; Lonvaud-Funel, 2001; Moreno-Arribas *et al.*, 2000; Soufleros, *et al.*, 1998). Since amines are part of the natural biochemistry and metabolic functioning of living organisms, it is also an important metabolite in human cells; for example, the

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presence of polyamines is essential for growth, while histamine and tyramine are vital for the correct functioning of the nervous system and control of blood pressure (Lonvaud-Funel, 2001). As could be anticipated, the ingestion of low concentrations of biogenic amines is not considered harmful to humans, as these compounds are natural intermediates of human metabolism. When absorbed in excessive amounts or when the human amine detoxification system is debilitated, e.g. during the simultaneous intake of alcohol and prescription drugs, harmful physiological side effects have been reported (Lonvaud-Funel, 2001). Clinical symptoms of biogenic amine intoxication include headaches, respiratory distress, heart palpitation, hyper- or hypotension, as well as several allergenic disorders (Lonvaud-Funel, 2001; Silla Santos, 1996). Histamine and tyramine are considered to be neurotoxins in humans due to their vasoactive and psychoactive properties (Bover-Cid and Holzapfel, 1999). Many clinical studies have reported on the allergic effect histamine has on humans, especially people with a histamine intolerance due to a deficiency of the diaminoxidase enzyme (Wantle *et al.*, 1993, 1994).

Ethyl carbamate production during MLF. Ethyl carbamate (EC, urethane) is a naturally occurring compound present in all fermented foods and beverages. There is a general agreement that the presence of EC in wine is not desirable and must be maintained as low as possible since it has been proven to be a carcinogen when administered at high dosages in animal tests (Azevedo *et al.*, 2002; Liu and Pilone, 1998). The main source of EC in wine is from the ethanolysis of urea synthesised during yeast metabolism. Arginine, usually one of the most abundant amino acids in grape juice, is metabolised by yeasts to urea, which is released into wine when it accumulates in the yeast cell during or at the end of fermentation. Urea spontaneously reacts with the ethanol in wine to form EC. Some strains of lactic acid bacteria can produce precursors compounds that can form EC via ethanolysis. Most of the commercial *O. oeni* strains are able to breakdown arginine through the arginine deiminase pathway (ADI pathway), during which citrulline and carbamyl phosphate are excreted and react with ethanol to produce ethyl carbamate (Liu *et al.*, 1994). The ADI pathway for arginine metabolism has been identified in strains of *O. oeni* that produce EC precursors in wine (Liu and Pilone, 1998; Lonvaud-Funel, 1999; Tonon *et al.*, 2001).

2.6.2 Control of malolactic fermentation

Although malolactic fermentation is the most widely accepted deacidification method of wine, the bioconversion of L-malic acid to lactic acid is a difficult and time-consuming process that does not always proceed favourably under the natural conditions of wine. It can lead to spoilage of wine when it occurs after bottling and during storage of wine, especially in wines with pH values higher than 3.5 units. The occurrence of spontaneous malolactic fermentation in wineries is unpredictable and irregular (Boulton *et al.*, 1996; Davis *et al.*, 1988; Wibowo *et al.*, 1985). Malolactic fermentation may take place immediately after alcoholic fermentation or only weeks or months later, and sometimes after bottling (Henick-Kling, 1995). The control of malolactic bacteria is crucial during

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winemaking, since their presence at the end of alcoholic fermentation is beneficial, but detrimental to wine quality during maturation. Maturation or ageing of wines is solely based on spontaneous chemical reactions, such as esterification and polymerisation of the chemically active components that result in the modification of wine aroma and colour (Lonvaud-Funel, 1999).

Malolactic fermentation tends to occur spontaneously in low-acid wines where it is least wanted and the outcome of the modified flavours is unpredictable due the proliferation of some *Lactobacillus* and *Pediococcus* spp. (Henick-Kling and Edinger, 1994; Zeeman *et al.*, 1982). After alcoholic fermentation wine cannot be treated by sulphiting until malolactic fermentation is completed. During this period wine is exposed to chemical oxidation and the development of spoilage organisms such as acetic acid bacteria and other spoilage yeasts. Therefore control of malolactic fermentation and an understanding of the favourable and unfavourable conditions for malolactic fermentation are of great importance to winemakers to predict or prevent the occurrence of malolactic fermentation. Numerous physiochemical, chemical and biological conditions in wine influence the development of MLF (Table 2.4); some have individual effects, while others act concurrently (Britz and Tracey, 1990; Vaillant *et al.*, 1995). The most important factors that influence the onset and completion of MLF is the initial wine composition, fermentation and storage temperature, as well as interactions between malolactic bacteria and other wine-related microorganisms (Table 2.4).

2.6.3 Progress in the successful application of malolactic fermentation

Even with a complete understanding of MLF and the factors that play a role in the successful development of MLF, wine remains a hostile environment to malolactic bacteria. MLF is often difficult to control and predict and is also a time-consuming process; therefore winemakers and oenologists continuously investigate and develop new technologies to ensure the reliable and controllable application of MLF in wine. Numerous advances have therefore been made in the field of MLF starter culture production as well as some other alternative technologies for the successful development of MLF in wine.

2.6.3.1 Inducing MLF through bacterial starter cultures. Winemakers can intentionally induce MLF in "resistant" wines by blending them with wine which is undergoing or which has already completed MLF (Castino *et al.*, 1975; Kunkee, 1967[a]). Based on their survival abilities, specific strains of *O. oeni* have been selected for the commercial production of liquid, lyophilised or freeze-dried starter cultures. The must is inoculated with starter cultures to ensure successful MLF (Beelman *et al.*, 1977; Henick-Kling, 1995; Henick-Kling *et al.*, 1989; Ingraham *et al.*, 1960; King, 1984; Lafon-Lafourcade *et al.*, 1983; Pompillo, 1993; Wibowo *et al.*, 1988). Although no official data are available on the prevalence of induced MLF in commercial wine production, roughly 75% of all red wine and 40% of white wines usually undergo induced MLF (Maicas, 2001; Ough, 1992).

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Table 2.4. Factors that influence malolactic fermentation in wine.

Factor	Influence on MLF	Reference
Wine environment		
pH	Low pH values (< 3.5) • Prevents growth of spoilage LAB, not *O. oeni* • Extends lag phase of *O. oeni* • Less volatile acidity due to ▪ slower active growth rate and metabolism ▪ inhibited glucose and fructose catabolism High pH values (> 3.5) • No protection against spoilage LAB • Increased volatile acidity due to ▪ Faster active growth rate and metabolism ▪ Glucose and fructose fermentation by LAB	Asmundson and Kelly, 1990; Henick-Kling, 1988; Kunkee, 1967[b], 1974, 1991; Ribéreau-Gayon *et al.*, 2000[a]
Ethanol	Low ethanol levels (< 6% v/v) • Minor inhibitory effect on MLF High ethanol levels (> 6% v/v) • Inhibits growth of LAB • Toxic to lactobacilli and pediococci, not *O. oeni*	Henick-Kling, 1988; Henick-Kling, 1995; Ribéreau-Gayon *et al.*, 2000[a]
Sulphur dioxide (free form)	No SO_2 addition ▪ MLF occurs naturally ▪ Spoilage by LAB is frequent SO_2 (> 50mg/l) inhibits growth of LAB ▪ Antimicrobial effect is pH dependent ▪ Lower pH = higher ratio of free SO_2 = higher microbial toxicity	Asmundson and Kelly, 1990; Britz and Tracey, 1990; Carreté *et al.*, 2002; Davis *et al.*, 1985; Fang and Dalmasso, 1993; Kunkee, 1967[b]; Ribéreau-Gayon *et al.*, 2000[a]; Wibowo *et al.*, 1985; Van Wyk, 1976
Nutrient Content	LAB have fastidious nutritional requirements: ▪ Limiting concentrations of amino acids, purines, pyrimidines, sugars, organic acids and vitamins inhibits growth ▪ Lactic acid inhibits growth of LAB LAB are micro-aerophilic ▪ Low concentration of dissolved oxygen required ▪ Too much oxygen inhibits growth LAB sensitive to traces of fungicides and insecticides	Cabras *et al.*, 1995; Cox, 1991; Henick-Kling, 1988; Lafon-Lafourcade, 1970; Lonvaud-Funel, 1986; Naouri *et al.*, 1990; Pilone and Kunkee, 1972; Ribéreau-Gayon *et al.*, 2000[a]; Tracey and Britz, 1989
Fermentation and Storage Temperature	5° and 10°C ▪ Complete inhibition of LAB < 18°C ▪ Delayed growth of LAB > 25°C ▪ Growth of spoilage LAB strains	Asmundson and Kelly, 1990; Boulton *et al.*, 1996; Britz and Tracey, 1990; Davis *et al.*, 1985; Henick-Kling, 1988; Lafon-Lafourcade, 1970; Ribéreau-Gayon *et al.*, 2000[a]

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Table 2.4 (continued)

Factor	Influence on MLF	Reference
Microbial Interactions		
Yeast-bacterial	Yeast strains can inhibit LAB growth ▪ Yeast strain dependent ▪ Competition for and depletion of essential nutrients ▪ Production of ethanol and SO_2 ▪ Production of medium-chain fatty acids (C_6-C_{12}) such as octanoic and decanoic fatty Yeast strains can promote LAB growth ▪ Secreted vitamins and amino acids during yeast growth ▪ Yeast autolysis promotes LAB growth ▪ "Yeast ghosts" (yeast hulls) used as additive	Amerine and Kunkee, 1968; Beelman *et al.*, 1982; Capucho and San Ramao, 1994; Dick *et al.*, 1992; Edwards *et al.*, 1990; Edwards and Beelman, 1989; Fornachon, 1968; King and Beelman, 1986; Kunkee and Amerine, 1970; Lafon-Lafoucade *et al.*, 1984; Narendranath *et al.*, 1997; Rankine and Pocock, 1969; Ribéreau-Gayon *et al.*, 2000[a]; Splittstoesser and Stoyla, 1989; Van Wyk, 1976
Bacterial-bacterial	During natural MLF ▪ Related and non-related bacteria inhibits growth of *O. oeni* ▪ Production of antimicrobial substances ▪ Hydrogen peroxide, lactic acid, bacteriocins Bacterial Starter Cultures ▪ Eliminates bacterial-bacterial effect ▪ High rate of successful MLF	Bhunia *et al.*, 1988; Daeschel and Klaenhammer, 1985; Geis *et al.*, 1983; Klaenhammer, 1988; Lücke *et al.*, 1986; Orberg and Sandine, 1984; Rammelsberg and Radler, 1990; Ribéreau-Gayon *et al.*, 2000[a]; Spelhaug and Harlander 1989; Strasser de Saad *et al.*, 1995; Strasser de Saad and Manca de Nadra, 1993; Tagg *et al.*, 1976
Bacterial-bacteriophage	Bacteriophages inhibits MLF ▪ Contaminate, infect and lyse starter cultures of *O. oeni* ▪ Phages are inhibited by SO_2 (>50mg/l) and pH < 3.5, as well as bentonite treatment	Arendt *et al.*, 1991; Daly, 1983; Davis *et al.*, 1985; Henick-Kling *et al.*, 1986; Huggins, 1984; Lonvaud-Funel, 1995; Nel *et al.*, 1987; Olwage, 1992; Sozzi *et al.*, 1976; Ribéreau-Gayon *et al.*, 2000[a]; Sozzi *et al.*, 1982

Starter cultures of malolactic bacteria are selected according to strict criteria (Davis *et al.*, 1985; Vaillant *et al.*, 1995). The strains must have a fast growth rate under winemaking conditions and should not produce any off-flavours or off-odours. Malolactic strains are further selected for their tolerance to low pH (pH < 3.0), high ethanol levels of up to 14% (v/v) and non-lysogenic characteristics after phage infection (Drici-Cachon *et al.*, 1996). Lastly, the strains should have good growth characteristics and be suitable for drying to make their commercial production economically viable in terms of type and cost of culturing media (Kunkee, 1991). Strains of malolactic bacteria can also be selected based on their inability to produce biogenic amines such as histamine and ethyl carbamate precursors. With the help of PCR, DNA probes and activity assays, strains of malolactic bacteria can now be screened for the presence of the amino acid decarboxylase genes (Le Jeune *et al.*, 1995).

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The availability of active bacterial starter cultures to induce MLF has certainly contributed to many successful applications of this secondary fermentation. However, the completion of malolactic fermentation cannot be guaranteed and starter culture delay or failure is not unusual under certain environmental conditions (Beelman and Gallander, 1979; Guerzoni *et al.*, 1995). In addition, unstable strain characteristics due to the presence of plasmids in malolactic bacteria are of some concern to oenologists. Small and large plasmids have been isolated from strains of *O. oeni* (Cavin *et al.*, 1988; Janse *et al.*, 1987; Orberg and Sandine, 1984; Taylor *et al.*, 1990). Although the function of these plasmids is largely unknown, pesticide resistance has been identified as a plasmid-encoded attribute. Genomic rearrangements of selected starter cultures during growth in wine are another source of concern in starter culture development (Henick-Kling, 1995). Reactivation conditions of freeze-dried starter cultures before inoculation into wine also play a role in the successful completion of malolactic fermentation (Nault *et al.*, 1995), since direct inoculation of starter cultures in wine leads to loss of viability (Davis *et al.*, 1985; Nielsen *et al.*, 1996).

2.6.3.2 Alternative technologies to develop MLF in wine. Persistent problems with bacterial starter cultures have driven the search for other alternatives to ensure rapid and reliable evolution of MLF in wine. Alternative strategies are based on the use of high biomasses of free malolactic bacteria cells, immobilised cells, as well as immobilised malolactic enzyme, in a bioreactor-type design. The use of bioreactors with free or immobilised strains of malolactic bacteria, as well as free-cell bioreactors with immobilised malolactic enzyme and cofactors have been evaluated for its application in wine deacidification (Gao and Fleet, 1994; Maicas, 2001; Macais *et al.*, 1999 [a, b],2000; Spettoli *et al.*, 1982, 1984, 1987). The major problems associated with these technologies involve the degree of difficulty to prepare matrixes, the use of unacceptable chemicals in wine and the unsuccessful scaling-up of free or immobilised high-density cell bioreactors for industrial application. Furthermore, free-cell bioreactors using only immobilised enzymes have yet to be 100% effective in converting L-malic acid to lactic acid.

Genetic engineering of strains of *Saccharomyces* with the ability to degrade L-malic acid is an important alternative that has enormous potential in the wine industry. Molecular biologists have attempted to transfer the malolactic activity of malolactic bacteria into *S. cerevisiae* to enable the yeast to execute the alcoholic and malolactic fermentation simultaneously. Williams *et al.* (1984) expressed the gene for malolactic activity of *L. delbrueckii* in *S. cerevisiae*. The recombinant strain of *S. cerevisiae* only managed to metabolise a mere 1% and 1.5% (w/v) L-malic acid in synthetic media and grape must, respectively. In the same year the L-malic acid assimilating activity from *O. oeni* was cloned and expressed in *E. coli* and yeast, but due to DNA stability problems the research was terminated (Lautensach and Subden, 1984). Nearly a decade later the *mle*S genes of *L. lactis* and *O. oeni* were subcloned under the control of the strong, constitutive 3-phosphoglycerate kinase (*PGK1*) promoter and terminator of *S. cerevisiae* and successfully expressed in a laboratory strain of

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S. cerevisiae (Ansanay *et al.*, 1993; Denayrolles *et al.*, 1994, 1995; Labarre *et al.*, 1996). Both *E. coli* and *S. cerevisiae* containing the *mle*S gene, produced malolactic activity, but they were still unable to efficiently degrade L-malic acid under winemaking conditions.

The main inadequacy of the above attempts to genetically engineer *S. cerevisiae* with a malolactic pathway is ascribed to the slow, inefficient uptake of L-malic acid via simple diffusion by the yeast (Grobler *et al.*, 1995). Expression of the malolactic gene (*mle*S) in yeast is not adequate to improve *S. cerevisiae*'s ability to degrade L-malic acid. Ansanay *et al.* (1996) confirmed the lack of efficient L-malic acid transport in *S. cerevisiae* as the sole limiting step in the recombinant malolactic yeast. When the *mle*S gene of *L. lactis* was expressed under the control of the *ADH1* promoter in both *S. cerevisiae* and *S. pombe*, only the recombinant *S. pombe* strain, which contains an active L-malic acid transport mechanism, rapidly converted L-malic acid to lactic acid, while the recombinant *S cerevisiae* strains managed to metabolise only small amounts of L-malic acid (Ansanay *et al.*, 1996).

Pre-empting the possible problems that would be encountered with the genetic engineering of a malolactic strain of *S. cerevisiae* without a functional malate transport mechanism, cloning and characterisation of the malate permease gene (*mae1*) and the malic enzyme gene (*mae2*) of *S. pombe* were deemed a feasible solution (Grobler *et al.*, 1995; Subden *et al.*, 1998; Viljoen *et al.*, 1994). Co-expression of the malate transporter gene (*mae1*) of *S. pombe* and the malolactic enzyme gene of *L. lactis* and *O. oeni* in a laboratory strain of *S. cerevisiae* resulted in a recombinant strain of *S. cerevisiae* that actively transported L-malic acid and efficiently converted it to lactic acid (Volschenk *et al.*, 1997 [a,b]). The recombinant *S. cerevisiae* strain was able to perform alcoholic and malolactic fermentation simultaneously, rendering malolactic fermentation with malolactic bacteria redundant. The malate transporter gene (*mae1*) of *S. pombe* and the malolactic enzyme gene of *O. oeni* were subsequently integrated in the genomes of industrial wine yeast strains, in a pioneering endeavour to develop commercially available wine yeast strains with the ability to degrade L-malic acid during alcoholic fermentation (Husnik, 2003). Currently, the recombinant yeast strains are evaluated for large-scale commercial application and promises to be the first genetically modified yeast available to the wine yeast market.

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CHAPTER 3

LITERATURE REVIEW

Regulation of L-Malic Acid Metabolism in Yeast

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Regulation of L-Malic Acid Metabolism in Yeast

3.1 INTRODUCTION

The conversion of L-malic acid to lactic acid and CO_2 during malolactic fermentation is one of the four possible metabolic conversions found in nature. As a natural compound that serves as a carbon source for many microorganisms, L-malic acid can also be transformed into three other compounds, namely oxaloacetic acid (via malate dehydrogenase), fumaric acid (reversible dehydration/hydration via fumarase) and pyruvic acid (via malic enzyme). Several yeast species have been recognised for their ability to utilise extracellular L-malic acid. Based on yeasts' ability to metabolise L-malic acid and other TCA cycle intermediates as sole carbon or energy source, yeasts are divided into a K (-) or K (+) group (Barnett *et al.*, 1990; Barnett and Kornberg, 1960; Goto *et al.*, 1978; Rodriquez and Thornton, 1990; Saayman *et al.*, 2000; Whiting, 1976).

The K (-) group of yeasts consists of those yeasts capable of utilising TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources (Barnett and Kornberg, 1960). Strains of *Saccharomyces sensu stricto* (including all wine strains), *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, in fact the synthesis of L-malic acid in some strains of *Saccharomyces* has been reported (Ramon-Portugal *et al.*, 1999). In contrast strains of *S. pombe* and *Z. bailii* can degrade extensive amounts of this L-malic acid (Baranowski and Radler, 1984; Kuczynski and Radler, 1982; Osothsilp, 1987; Osothsilp and Subden, 1986[b]; Rodriquez and Thornton, 1989; Taillandier *et al.*, 1988; Taillandier and Strehaiano, 1991).

The K (+) yeast group is comprised of yeasts such as *Candida sphaerica* (Côrte-Real *et al.*, 1989), *Candida 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 genetic and biochemical characterisation of the L-malic acid utilising pathways in several K (-) and K (+) yeast species, including *S. pombe, C. utilis, K. marxianus, Z. bailii* and *Saccharomyces cerevisiae*, have been the focus of several investigations. In agreement with the divergent mechanisms of regulation of metabolic pathways involved in these yeasts, the physiological role and regulation of L-malic acid metabolism differs significant 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, 1986[b]; Rodriquez and Thornton,

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1989). In contrast, the regulation of L-malic acid metabolism in K (+) yeasts typically exhibits strong glucose (or catabolite) repression and rapid substrate induction that leads to a diauxic shift growth pattern where glucose is used prior to the utilisation of L-malic acid (Amador *et al.*, 1996; Cássio and Leâo, 1993; Côrte-Real *et al.*, 1989; Côrte-Real and Leâo, 1990; Queiros *et al.*, 1998).

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 permease, and a L-malic acid-converting enzyme, i.e. fumarase, malolactic enzyme, malate dehydrogenase or a malic enzyme. The focus of this chapter is on the "malic enzymes" that catalyse the oxidative decarboxylation of L-malic acid to pyruvic acid and CO_2, linked to the reduction of the pyridine nucleotides, NAD^+ or $NADP^+$. During fermentative sugar metabolism, pyruvic acid, an important branching point in carbohydrate metabolism in yeast, is further decarboxylated to acetaldehyde by pyruvate decarboxylase and subsequently reduced to ethanol by the alcohol dehydrogenase enzyme in yeast. Since L-malic acid is thus in effect converted to ethanol, this pathway was described as the "malo-ethanolic pathway".

This chapter aims to give an in-depth characterisation of the malo-ethanolic pathways of K (-) and K (+) yeasts, with specific emphasis on *S. pombe* and *Saccharomyces* species. 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 winemaking perspective, K (-) yeasts or their genetically modified counterparts seem to be well suited for the deacidification of wine as an alternative to the bacterial malolactic fermentation. One of the strongest advantages of employing malo-ethanolic K (-) yeast in 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.

3.2 THE MALIC ENZYME

Since the first description of a malic enzyme in pigeon liver almost 50 years ago (Ochoa, 1955), "malic enzyme" activities have been identified in several species, including prokaryotes (*Bacillus subtilis*, *Bacillus stearothermophilus*, *Clostridium thermocellum*, *Pseudomonas putida*, *Sulfolobus solfataricus*, *Rhizobium meliloti* and *Escherichia coli*) (Driscol and Finan, 1996; Kobayashi *et al.*, 1989), parasitic flagellates (*Tritrichomonas foetus)* (Vaňáčová *et al.*, 2001), yeasts, fungi (*Mucor circinelloides*; *Aspergillus nidulans*; *Mortierella alpina* and *Neocallimastix frontalis*) (Song *et al.*, 2001; Van der Giezen *et al.*, 1998; Wynn *et al.*, 1997, 1999; 2000), plants, mammals and humans. Malic enzymes (EC 1.1.1.38-40) (Outlaw and Springer, 1983) differ in their specificity for substrates (L-malic acid and/or oxaloacetic acid), co-factors (either NAD^+ [EC 1.1.1.38 and EC 1.1.1.39] or $NADP^+$ [EC 1.1.1.39 and EC 1.1.1.40]), kinetic constants, oxaloacetate-decarboxylating activity,

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intracellular localisation (cytosolic, mitochondrial or hydrogenosomal), and the degree of reversibility of the decarboxylation reaction (ranging from absent to almost complete) (Voegele *et al.*, 1999). Furthermore, malic enzymes from bacteria to humans exhibit a high degree of amino acid sequence conservation (Viljoen *et al.*, 1994; Xu *et al.*, 1999; Yang *et al.*, 2000). Based on the diversity of regulation of malic enzymes in different organisms or different 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 but 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 pathways where NAD-dependent malic enzymes are involved in oxidative metabolic processes, or 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 energy production 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 those found in the spleen, thymus, mucosal cells of small intestine, as well as tumour cells (Bagetto, 1992; McKeehan, 1982; Sauer *et al.*, 1980).

NADP-dependent malic enzyme isoforms found in bacteria (Gourdon *et al.*, 2000), 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 and Ball, 1966, 1967, 1968[a, b]; Gourdon *et al.*, 2000; Leveille *et al.*, 1968; Nunes *et al.*, 1996; Tanaka *et al.*, 1983; Wynn *et al.*, 1997, 1999; 2000; Xu *et al.*, 1999). Acetyl-coenzyme A is produced in mitochondria through the metabolism of fatty acids and the oxidation of pyruvic acid to acetyl-coenzyme A (Fig. 3.1). When ATP is required, acetyl-coenzyme A can enter the TCA cycle to drive ATP production via oxidative phosphorylation. When ATP supplies are abundant, the acetyl-coenzyme A can be diverted to other purposes like energy storage in the form of fatty acids. The biosynthesis of fatty acids from this acetyl-coenzyme A cannot take place directly, since it is produced inside mitochondria while fatty acid biosynthesis occurs in the cytosol. Also, acetyl-coenzyme A cannot directly be transported out of the mitochondria. To be transported, the acetyl-coenzyme A must be chemically converted to citric acid using a pathway called the tricarboxylate transport system (Fig. 3.1).

Inside mitochondria, the enzyme citrate synthase fuses acetyl-coenzyme A with oxaloacetic acid to produce citric acid. The citric acid can be transported from the mitochondria to the cytosol, thus transporting the acetyl-coenzyme A in the form of citric acid. Once in the cytosol, citric acid is converted back to oxaloacetic acid via the energy-dependent citrate lyase reaction, which is then

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reduced to L-malic acid by means of the malate dehydrogenase enzyme. L-Malic acid can be oxidised to pyruvic acid by the malic enzyme, with the production of NADPH that feeds 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, once inside the mitochondria (Fig. 3.1). The role of the malic enzyme in lipid biosynthesis in filamentous fungi has been studied in depth and strong evidence has been obtained that malic enzyme activity is a key factor in ensuring maximal lipid accumulation (Wynn and Ratledge, 2000). The direct relation between malic enzyme activity and lipid accumulation is still speculative, however, since it is generally agreed that lipogenesis obtains NADPH from the cytosolic NADPH reservoir, which is maintained by a regiment of NADPH-producing enzymes, i.e. the glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and $NADP^+$:isocitrate dehydrogenase, as well as the malic enzyme. Furthermore, fungi possessing a high degree of malic enzyme activity do not necessarily accumulate lipids (Wynn *et al.*, 1999). This finding, together with the presence of multiple isoforms of malic enzyme in some fungi (Savitha *et al.*, 1997; Zink, 1972), suggests that several forms of malic enzyme may exist in fungi, perhaps even encoded by different genes. Specific isoforms of the malic enzyme may therefore be associated with lipid accumulation, whilst others have other cellular functions.

Figure 3.1. The role of the malic enzyme in lipid biosynthesis and desaturation through the provision of cytosolic NADPH in the mouse and humans. 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 (Biocarta, http://www.biocarta.com/).

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In mammals and humans the NADP malic enzyme plays an essential role in microsomal drug detoxification (Sanz *et al.*, 1997) and preventing oxidative damage, i.e. cellular damage by reactive oxygen species (Lee *et al.*, 2002). The NADP-dependent malic enzyme contributes to a pool of NADPH that drives the NADPH-dependent glutathione reductase enzyme in producing small but critical amounts of the well-known antioxidant glutathione (Meister and Anderson, 1983). The antioxidant capacity of a tissue is determined by the constant supply of reducing potentials (Bukato *et al.*, 1995; Izawa *et al.*, 1998; Kochan *et al.*, 1995; Lee *et al.*, 2002; Loeber *et al.*, 1994; McKenna *et al.*, 2000; Vogel *et al.*, 1998).

L-Malic acid metabolism plays multiple roles in plants, including contributing to energy production, reducing power, pH balancing mechanisms, mechanisms of stomatal closure, carbon skeletons for biosynthesis, mineral nutrition, fruit ripening, plant defense and injury responses (Drincovich *et al.*, 2001; Lance and Rustin, 1984; Laporte *et al.*, 2002; Outlaw *et al.*, 1981). However, the quantitative understanding of how the many reactions of L-malic acid metabolism contribute to plant function is rudimentary (Edwards *et al.*, 1998). A well-characterised role of L-malic acid in plants occurs during photosynthesis, i.e. for the transient storage of CO_2 (Hatch, 1971; Leegood, 2002). In C_4- and vascular (CAM) plants, the decarboxylation reaction by the NADP-dependent malic enzyme, in combination with the phosphoenolpyruvate carboxylase (PEP-carboxylase), plays an essential role in delivering CO_2 via the Hatch-Slack pathway for further CO_2 fixation during the Calvin-Benson photosynthetic cycle in chloroplasts (Edwards and Andreo, 1992; Lipka *et al.*, 1999; Wedding, 1989). In C_3 plants that only utilise the Calvin-Benson cycle, the malic enzyme does not play a significant role during photosynthesis (Drincovich *et al.*, 2001; Honda *et al.*, 2000; Lai *et al.*, 2002[a, b]; Stryer, 1995).

3.3 THE MALO-ETHANOLIC PATHWAY OF *S. POMBE*

Since the original isolation of *S. pombe* by Lindner from East African millet beer (pombe beer) in 1890, the remarkable L-malic acid-degrading ability of this yeast under fermentative conditions has attracted the attention of several applied and fundamental scientific studies (Barnett and Lichtenthaler, 2001; Lindner, 1893; Lodder and Kreger-Van Rij, 1952; Osterwalder, 1924; Viljoen *et al.*, 1994; 1998; 1999). The potential application of strains of *S. pombe* for the deacidification of wine was soon realised and has been the subject of numerous oenological studies (Beelman and Gallander, 1979; Carré *et al.*, 1983; Fleet and Heard, 1993; Gallander, 1977; Gao and Fleet, 1995; Maconi *et al.*, 1984; Munyon and Nagel, 1977; Snow and Gallander, 1977; Taillandier *et al.*, 1995; Yang, 1973, 1975; Yokotsuka *et al.*, 1993). Strains of *S. pombe* are occasionally found in spontaneous wine fermentation and exhibit several auspicious characteristics similar to wine yeast strains of *Saccharomyces*, such as a strong alcoholic fermentation capacity and a high SO_2 tolerance. Together with the yeast's unique

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ability to degrade L-malic acid during alcoholic fermentation, strains of *S. pombe* proved to be an ideal candidate for the simultaneous fermentation and deacidification of grape must (Beelman and Gallander, 1979; Benda and Schmitt, 1966; Bidan *et al.*, 1974; Carré *et al.*, 1983; Gallander, 1977; Gao and Fleet, 1995; Magyar and Panyik, 1989; Rankine, 1966; Snow and Gallander, 1977; Taillandier *et al.*, 1995; Yang, 1973, 1975).

Vinification experiments with strains of *S. pombe* yielded a constant 50% decrease in the total acidity (TA) that corresponds to the complete removal of extracellular L-malic acid (Gallander, 1977). The significant increase in wine pH as a result of the complete removal of L-malic acid also leads to a further loss of acidity due to the enhanced precipitation of potassium bitartrate. Although efficient wine deacidification can be achieved with strains of *S. pombe*, some strains, particularly strains of *S. pombe* var. *malidevorans*, are inclined to produce off-flavours in wine with especially high levels of hydrogen sulphide (Dharmadhikari and Wilker, 1998; Gallander, 1977; Rankine, 1966). In other *S. pombe* vinifications, no significant off-flavours were produced, but the wine lacked traditional wine flavours (Gallander, 1977; Munyon and Nagel, 1977). To overcome the adverse sensory effect of *S. pombe* on vinification, co-fermentation studies with *S. pombe* and *S. cerevisiae* were attempted. However, due to *S. pombe*'s higher optimum fermentation temperature and slower doubling time, cells of *S. pombe* grow slower than *S. cerevisiae* in the presence of SO_2 and the low pH (< pH 3.5) associated with wine (Gallander, 1977; Yang, 1973, 1975). Typically, cells of *S. cerevisiae* out-compete *S. pombe* under winemaking conditions and dominate the fermentation, leading to a diminished and insignificant deacidification effect by *S. pombe*.

In pursuit of optimising the deacidification step by *S. pombe*, strains of *S. cerevisiae* were inoculated only after *S. pombe* performed a partial degradation of L-malic acid (Sousa *et al.*, 1995; Taillandier *et al.*, 1995). However, pre-fermentation with *S. pombe* resulted in severe growth inhibition of *S. cerevisiae* due to some unfamiliar inhibitory factors liberated by *S. pombe* (Taillandier *et al.*, 1995). Furthermore, this strategy required the removal of *S. pombe* cells by filtration after a certain desired level of deacidification was obtained before *S. cerevisiae* could be inoculated to complete the alcoholic fermentation (Snow and Gallander, 1977). Implementation of such a strategy in a winery would complicate rather than simplify cellar operations, with an increased risk of oxidation. Deacidification of wine with *S. pombe* after alcoholic fermentation with *S. cerevisiae* has also been ruled out by research indicating severe inhibition of L-malic acid transport in *S. pombe* by low concentrations of ethanol and acetic acid (Sousa *et al.*, 1995). Due to these obstacles, strains of *S. pombe* have not yet been successfully applied in any commercial winemaking operation.

3.3.1 The molecular biology of the malo-ethanolic pathway in *S. pombe*

Genetic analysis of L-malic acid metabolism in *S. pombe* originated in the 1980s when mutants of *S. pombe* defective in L-malic acid metabolism (*mau*$^-$ mutants) were generated and characterised

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(Osothsilp and Subden, 1986[a]). Based on classical genetic analysis, the mutants were found to group into three complementation clusters, namely malate permease, malic enzyme and malate dehydrogenase mutants. Retrieval of the structural genes involved in L-malic acid metabolism was subsequently obtained by complementation of the mutant strains with a genomic library of *S. pombe*, leading to the cloning of the malate transporter (*mae1*) and malic enzyme (*mae2*) genes (Grobler *et al.*, 1995; Subden *et al.*, 1998; Viljoen *et al.*, 1994).

3.3.1.1 The malate permease gene (*mae1*). The structural gene of the malate permease (*mae1*), localised on the *S. pombe* chromosome I, encodes an open reading frame of 1314 bp that translates into a putative protein of 438 amino acids with a theoretical molecular weight of approximately 49 kDa (Grobler *et al.*, 1995). DNA and amino acid sequence analysis of the *S. pombe* malate transport protein did not reveal any significant homologies with other genes or proteins previously sequenced, but computational analysis of the hydrophobic-hydrophilic profile of the putative mae1p indicated some conserved motifs found in other transport proteins. A leucine zipper motif, which normally facilitates protein-protein interaction (Bisson *et al.*, 1993; White and Weber, 1989), a C-terminal PEST motif involved in protein degradation, and several N-linked glycosylation and protein kinase C phosphorylation sites were identified in the mae1p protein (Grobler *et al.*, 1995; Rogers *et al.*, 1986). The biological role of these protein motifs in *S. pombe* has not yet been established and requires more in-depth physiological studies. In addition, the putative mae1p protein did not contain a membrane-targeting signal, usually identifiable at the N-terminal of most membrane proteins, but the existence of an internal membrane signal motif was suggested (Grobler *et al.*, 1995).

3.3.1.2 The malic enzyme gene (*mae2*). The structural gene of the *S. pombe* malic enzyme, *mae2*, was cloned and characterised as an open reading frame of 1695 bp located on the *S. pombe* chromosome III (Viljoen *et al.*, 1994). DNA sequence analysis of the *S. pombe* malic enzyme gene did not indicate the presence of a mitochondrial-targeting signal, suggesting that the malic enzyme functions in the cytosol of *S. pombe* cells. A high degree of homology on the amino acid sequence level (up to 52%) was observed between the putative *S. pombe* mae2p protein and malic enzymes from other organisms (Viljoen *et al.*, 1994). A high degree of homology was also observed between the malic enzyme of eukaryotes and the malolactic enzyme (MLE) of lactic acid bacteria (LAB). The phylogenetic tree obtained with amino acid sequences of the MLE and different malic enzymes showed that these two types of enzymes might have a common ancestor. Fascinatingly, the malic enzyme of *E. coli*, *S. pombe* and *S. cerevisiae* showed a closer phylogenetic link with the MLE of LAB than with malic enzymes from other organisms (Groisillier and Lonvaud-Funel, 1999).

3.3.2 The physiological role of the malo-ethanolic pathway in *S. pombe*

As a K (–) yeast, *S. 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

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Radler, 1972; Rankine, 1966; Magyar and Panyik, 1989; Osothsilp, 1987; Osothsilp and Subden, 1986[b]; Taillandier *et al.*, 1988). *S. pombe* displays an extreme tolerance for high L-malic acid concentrations, as levels of up to 29.0 g/l of L-malic acid 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 production abilities. The fundamental understanding of why *S. pombe* degrades L-malic acid in this manner and the specific metabolic role of MEF in this yeast has been partially revealed through primary biochemical characterisation of the L-malic acid transport and enzymatic conversion to pyruvic acid by *S. pombe* cells. Detailed molecular analysis of the mechanisms involved in regulation of L-malic acid degradation in *S. pombe* has further contributed to our understanding of the physiological role of the MEF pathway in yeast.

3.3.2.1 Uptake of L-malic acid in *S. pombe*. According to the pK_a parameters for L-malic acid dissociation ($pK_{a1} = 3.41$ and $pK_{a2} = 5.1$), 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 *S. pombe* by simple diffusion (Baranowski and Radler, 1984; Camarasa *et al.*, 2001; Osothsilp and Subden 1986[b]; Rodriquez and Thornton, 1990; Sousa *et al.*, 1992; Sousa *et al.*, 1995). Taillandier *et al.* (1988) first demonstrated that the uptake of L-malic acid in *S. pombe* displays saturation kinetic 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, such as oxidative phosphorylation uncouplers and electron transport inhibitors, on L-malic acid transport (Osothsilp and Subden, 1986[b]). Recently, the active mode of transport for the *S. pombe* malate permease protein was confirmed when the *mae1* gene was expressed in *S. cerevisiae* using the regulatory elements of the 3-phosphoglycerate kinase (*PGK1*) gene (Camarasa *et al.*, 2001).

The transport of negatively charged molecules across a plasma membrane is characterised by either anion exchange (antiport), co-transport (symport) with cations or protons involving a single carrier, or separate electrically coupled carriers. Proton flux studies with *S. 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. Uptake of one molecule of L-malic acid corresponds to the uptake of one proton, leading to the formation of a pH gradient between the intracellular and external cell milieu (Camarasa *et al.*, 2001; Osothsilp and Subden 1986[b]; Sousa *et al.*, 1992).

S. pombe has 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 *S. pombe*.

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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 *S. pombe* might act as a general transporter for all these acids (Sousa *et al.*, 1992). Lactic, pyruvic and citric acid, on the other hand, did not inhibit initial uptake rates of L-malic acid, suggesting that they are not transported by the same permease (Sousa *et al.*, 1992). More in-depth investigations into the transport mechanism of the *S. 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 *S. pombe* malate transporter is L-malic acid, other dicarboxylic acids such as succinic, malonic and α-ketoglutaric acid are also weakly transported by this protein (Camarasa *et al.*, 2001; Grobler *et al.*, 1995; Sousa *et al.*, 1992). On the other hand, fumaric acid is not actively transported by mae1p, but competes with L-malic acid during transport by binding to the active site of the protein and thereby blocking L-malic acid transport (Saayman *et al.*, 2000). This stearic occupation by fumaric acid, and possibly some other TCA cycle intermediates and dicarboxylic acids, is due to the structural relatedness of these acids to malic acid.

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 *Kluyveromyces 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 *et al.*, 1989; Côrte-Real and Leão, 1990). In contrast, L-malic acid transport in *Z. bailii* and *S. pombe* were found to be active in the presence of glucose and not induced by the substrate (Baranowski and Radler, 1984; Osothsilp and Subden, 1986[b]). In support of *S. pombe*'s requirement for fermentable carbon sources for L-malic acid utilisation, L-malic acid transport was shown to be insensitive to glucose repression in this yeast. It is postulated that metabolism of sugar(s) supplies 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, 1986[a, b]; Saayman *et al.*, 2000; Taillandier and Strehaiano, 1991). In fact, in heterologous expression studies where the *S. pombe mae1* gene was expressed in *S. cerevisiae* under the regulatory elements of the 3-phosphoglycerate kinase (*PGK1*) gene, L-malic acid transport was characterised by glucose activation of the mae1p permease (Camarasa *et al.*, 2001). These results have, however, not yet been confirmed for the native malate permease in *S. pombe*. On the transcription level, preliminary expression studies of the *mae1* gene in *S. pombe* confirmed that the *mae1* gene is constitutively expressed and not subject to catabolite repression (Grobler *et al.*, 1995; Osothsilp, 1987).

3.3.2.2 Enzymatic conversion of L-malic acid in *S. pombe*. The malic enzyme catalyses the direct 1,4-decarboxylation of L-malic acid to pyruvic acid and CO_2. (Fig. 3.2). The enzyme in *S. pombe* is bifunctional, reacting with either L-malic acid or oxalacetic acid, and requires NAD^+ and the divalent

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cations Mn^{2+} or Mg^{2+} for activity (Osothsilp, 1987; Osothsilp and Subden, 1986[a]). The metal ion in the enzymatic reaction serves as a bridge between L-malic acid and the enzyme and functions to properly position the L-malic acid at the active centre, as well as helping to polarise the C-2 hydroxyl group in the initial stage (Chou *et al.*, 1995). Subsequent decarboxylation of oxaloacetic acid is also catalysed by the metal ion, which acts as a Lewis acid. The metal ion plays a vital role in chelating the negatively charged enolate-pyruvate intermediate (Chang *et al.*, 2002).

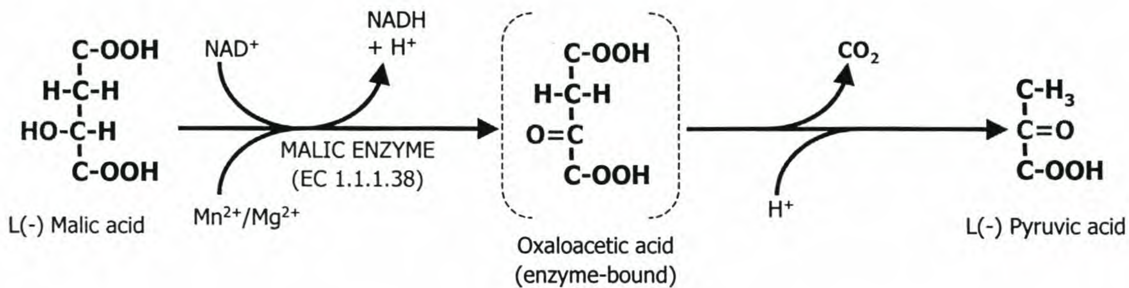

Figure 3.2. The NAD-dependent malic enzyme of *S. pombe* (EC 1.1.38, Malate : NAD^+ oxidoreductase [oxaloacetate decarboxylating]) proceeds by oxidation of L-malic acid to oxaloacetic acid, followed by decarboxylation to pyruvic acid.

The biologically functional form of most eukaryotic malic enzymes is a homotetrameric protein composed of four chemically identical sub-units (Lee and Chang, 1990; Mitsch *et al.*, 1998). In *S. pombe*, however, the malic enzyme was predicted to be a dimer consisting of two identical subunits with a molecular weight of 60 kDa (Temperli *et al.*, 1965). The enzyme has an optimal pH range of 3.5 to 4.0 and has a high substrate affinity for L-malic acid ($K_m = 3.2$ mM) (Temperli *et al.*, 1965).

Molecular analysis of the *S. pombe* malic enzyme gene and its deduced amino acid sequence revealed the presence of eight highly conserved regions, regions A-H, across 27 malic enzymes from various prokaryotic and eukaryotic sources (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, whilst the physiological importance of the other regions remains unknown. Further sequence homology analysis between all known malic enzymes, as well as malolactic enzymes (MLEs), has revealed that the active and cofactor binding sites within these enzymes are highly conserved (Fig. 3.3) (Groisillier and Lonvaud-Funel, 1999). The dinucleotide (NAD^+/$NADP^+$) binding signature motif, i.e. consensus box I, has been identified by Wierenga *et al.* (1985). The consensus box III, identified through site-directed mutagenesis as the metal binding site (Wei *et al.*, 1995), is identical between malic and malolactic enzymes. The importance of a cysteine in malate binding has been demonstrated by SH reagent inhibition studies in several malic enzymes (Chang *et al.*, 1993; Gavva *et*

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al., 1991). This residue is replaced by isoleucine (I) in MLEs (lysine (K) for *Lactobacillus salivarius*). However, the malic enzymes of *S. pombe* and *S. cerevisiae* also have isoleucine, similar to the MLEs (Groisillier and Lonvaud-Funel, 1999). Although the function of only four of the conserved regions found in malic enzyme is known, the importance of the other homologous areas should not be disregarded. This was demonstrated by a single point mutation in the *S. pombe* malic enzyme gene at nt 1331, where a switch from a G to an A changed amino acid 444 from a glycine into an aspartate residue in the conserved H region. The point mutation resulted in the almost complete abolishment of malic enzyme activity in the *S. pombe* mutant (Viljoen *et al*., 1998).

Consensus Malic Acid Binding Site

M	P	I	V	Y	T	P	T	V	G	D	A	C	Q	K	Y	S	S	L	F	R	R	P
L		V	I		**D**		V	I	A	E	**S**	***I***	**K**	Q	F	G	L	I	Y	V	K	S
N								*E*		L	**T**		S	N		N	E	A		L	Q	Y
A																	H			Q	N	
I																	D			T	D	
V																	Y					
																	G					

Consensus Box I (dinucleotide binding site)

V	V	T	D	G	E	R	I	L	**G**	**L**	**G**	**D**	**L**	**G**
		S		A	S	***G***				I			**W**	
				S						M			Q	

Consensus Box III (metal binding site)

F N D D I Q G T

Figure 3.3. Analysis of aligned consensus amino acid sequences of malic and malolactic enzymes. Malolactic specific residues are indicated in bold. Yeast-specific residues are indicated in italics. Adapted from Groisillier and Lonvaud-Funel (1999).

L-malic acid metabolism in *S. 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, 1986[a]). Under fermentative (non-aerated) conditions when functional mitochondria are restricted, the cytosolic malic enzyme of *S. 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, 1986[a]; Subden *et al*., 1998).

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An eminent attribute of the conversion of L-malic acid to ethanol in *S. pombe* is the stoichiometrical 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 *et al.*, 1988; Taillandier and Strehaiano, 1991). An in-depth analysis of the malic enzyme's physiological role in *S. pombe*'s metabolism was prompted by the intriguing question of why *S. pombe* contains such a strong L-malic acid metabolism when it does not contribute to cell biosynthesis. The transcriptional regulation of the *mae2* gene of *S. 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. Further expression studies revealed an increase in transcription of *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 (UAS) and repressor sequences (URS) that play a role in the regulation of the *mae2* gene in *S. 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 *S. 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 a stronger induction in response to osmotic stress conditions (Groenewald and Viljoen-Bloom, 2001; Viljoen *et al.*, 1999). Both these conditions, i.e. high glucose concentrations and osmotic stress, may adversely affect the redox balance inside the cell.

Since the oxidative decarboxylation of L-malic acid coincides with the reduction of NAD^+ to NADH, it is plausible that the malic enzyme may play an important role in maintaining the redox balance under aerobic conditions. Under anaerobic conditions, pyruvic acid produced during this reaction is further metabolised to ethanol with the concomitant oxidation of NADH to NAD^+. Because there is no net gain or loss in NADH during the conversion of L-malic acid to ethanol, the increased expression of the malic enzyme under fermentative conditions should not play a role in stabilising the redox balance. It was therefore suggested by Viljoen *et al.* (1999) that the malic enzyme in *S. pombe* provides additional pyruvic acid for essential anapleurotic reactions under fermentative conditions. 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; therefore alternative pathways have to be utilised for the synthesis of these precursors when the mitochondria are not functional. These

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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 indicate that the metabolism of L-malic acid in *S. pombe* does not contribute to cell biomass, the induced expression of the *S. 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).

3.4 THE MALO-ETHANOLIC PATHWAY IN STRAINS OF *SACCHAROMYCES*

Strains of *S. cerevisiae* are in general incapable of efficiently utilising malic acid. Relatively minor modifications in L-malic acid concentration, and thus total acidity, are observed under winemaking conditions. Different accounts of the ability of strains of *Saccharomyces* to degrade L-malic acid have been reported in the past. Depending on the strain of *Saccharomyces*, between 0 and 3 g/l L-malic acid can be consumed (Subden *et al.*, 1998), while between 3% and 45% (w/v) of L-malic acid degradation by strains of *Saccharomyces* have been reported by Rankine (1966) and Radler (1993). In contrast, Wenzel *et al.* (1982) described the degradation of between 10% and 20% (w/v) L-malic acid by commercial wine yeast strains of *Saccharomyces*. As a rule, however, strains of *Saccharomyces* rarely degrade all of the L-malic acid in grape must during alcoholic fermentation. Within the five-member *Saccharomyces sensu stricto* group, i.e. *S. cerevisiae*, *S. paradoxus*, *S. pastorianus*, *S. uvarum* and *S. bayanus* (Pulvirenti *et al.*, 2000), notable variations in the action on L-malic acid exist. The degradation of L-malic acid by strains of *Saccharomyces* is correlated to the optimal growth temperature of the individual strains, that is, cryotolerant species (i.e. *S. bayanus*, *S. uvarum* and *S. pastorianus*) were found to synthesise L-malic acid, while mesophylic strains of *Saccharomyces* degraded intermediate amounts of L-malic acid during fermentation. The thermotolerant strains of *S. cerevisiae* and *S. paradoxus* exhibit the strongest L-malic acid degrading phenotype of up to 40 - 48% L-malic acid (Rainieri *et al.*, 1998[a, b]).

Strains of *Saccharomyces* with the ability to synthesise L-malic acid are of important oenological value as they can contribute to improving the total acidity (TA) of wines produced in the warm-climate regions. Strains of *Saccharomyces* produce variable amounts of extracellular carboxylic acids, mainly malic, succinic and citric acid as well as some non-TCA intermediates such as acetic acid, during alcoholic fermentation (Bhattacharjee *et al.*, 1968; Castellari *et al.*, 1994; Ramon-Portugal *et al.*, 1999). The production and accumulation of L-malic acid and other TCA cycle intermediates by some strains of *S. cerevisiae* and *S. uvarum* have been attributed to growth in stress conditions. Depending on the strain of *Saccharomyces*, between 0.1 and 2.6 g/l malic acid is liberated during the active growth phase in conditions of high sugar levels (15-20 % [w/v]), elevated pH levels

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(pH > 5.0) and under limiting nitrogen conditions (< 300 mg/l assimilable nitrogen) (Coggins and Whiting, 1975; Drawert *et al.*, 1965; Fatichenti *et al.*, 1984; Radler and Lang, 1982; Schwartz and Radler, 1988; Whiting, 1976). Although L-malic acid synthesis is regarded as unlikely to proceed via the reverse reaction of the malic enzyme since it would imply fixation of CO_2 (Radler, 1993), the requirement of CO_2 for the production of L-malic acid was suggested by Schwartz and Radler (1988) and confirmed by Pines *et al.* (1996, 1997).

The pathway for L-malic acid synthesis involves three cytosolic enzymes, i.e. the pyruvate carboxylase (PC) responsible for the biotin- and ATP-dependent carboxylation of pyruvic acid to oxalacetic acid, the cytosolic malate dehydrogenase (MDH2) and the cytosolic fumarase enzyme (Pines *et al.*, 1996, 1997) (Fig. 3.4). In *S. cerevisiae*, the two principal pathways for the replenishment of oxalacetic acid are via the carboxylation of pyruvic acid by pyruvate carboxylase (PC) and from the glyoxylate cycle. During growth on glucose, the enzymes of the glyoxylate cycle are, however, repressed and thus PC catalyses the only known reaction to replenish the TCA cycle under these conditions (Bakker *et al.*, 2001).

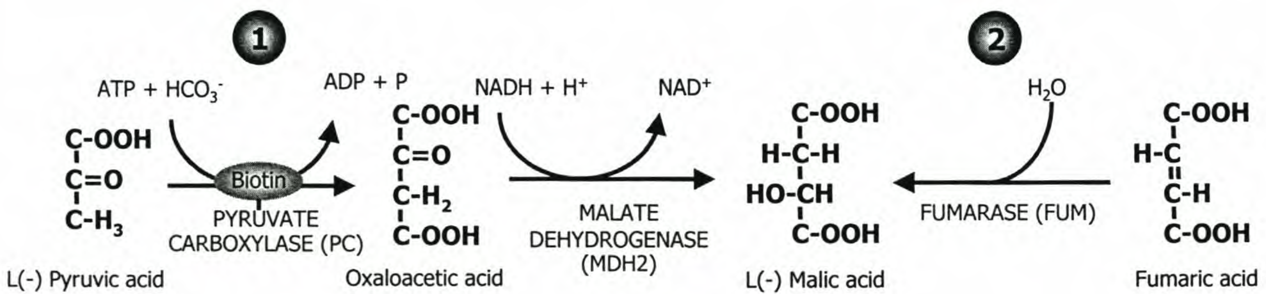

Figure 3.4. The two cytosolic pathways for L-malic acid synthesis in *S. cerevisiae*. 1) L-malic acid synthesis via carbon dioxide fixation by pyruvate carboxylase and malate dehydrogenase, and 2) via the fumarase activity in *S. cerevisiae*.

The decomposition of L-malic acid rather than its synthesis is, however, the norm for commercial wine yeast strains under winemaking conditions due to these yeasts' predominantly mesophylic nature, the prevailing low pH values and sufficient nitrogen levels in grape musts (Radler, 1993). The physiological significance of the liberation of L-malic acid by yeasts and more importantly its significance in cryotolerant strains of *Saccharomyces* is unknown and currently under investigation. The synthesis of L-malic acid in cryotolerant strains may be ascribed to high levels of biotin, the coenzyme for pyruvate carboxylase, present in strains of *S. bayanus*. Interestingly, most strains of *S. cerevisiae* require biotin as an essential growth factor, while *S. bayanus* does not (Rainieri *et al.*, 1988[a]).

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3.4.1 The molecular biology of the malo-ethanolic pathway in *Saccharomyces* strains

Although the complete sequence of the *S. cerevisiae* genome was published in 1996 (Goffeau *et al.*, 1996), no structural gene or functional homologue for the transporter protein of extracellular L-malic acid has yet been identified in this yeast. A mitochondrial dicarboxylic acid carrier has been purified and characterised and the genes of several mitochondrial dicarboxylic acid transporters, i.e. *DIC*1 that catalyse the uptake of L-malic acid in *S. cerevisiae* mitochondria, have been previously described (Lançar-Benba, 1996; Pallotta *et al.*, 1999; Palmieri *et al.*, 1996, 1999, 2000). The lack of a genetic equivalent for the malate transport gene of *S. pombe* corroborates the biochemical evidence on the absence of an active transport system for L-malic acid in *S. cerevisiae*.

3.4.1.1 The malic enzyme gene (*MAE1*). Recently, the malic enzyme phenotype was designated to an open reading frame, YKL029C, in the *S. cerevisiae* genome based on amino acid sequence comparison with the known *S. pombe* malic enzyme (Boles *et al.*, 1998). The *MAE1* gene of *S. cerevisiae* was identified as the structural gene of the *S. cerevisiae* malic enzyme, which encodes a putative protein of 669 amino acids with 47% homology to the *S. pombe* malic enzyme. Further proof of the malic enzyme gene identity was obtained when the gene was deleted or over-expressed in yeast and a loss or increase in malic enzyme activity was observed, respectively (Boles *et al.*, 1998). Phylogenetic analysis of the *S. cerevisiae* malic enzyme indicated a closer relatedness to the eubacteria malic enzymes compared to the malic enzyme of higher eukaryotes. Based on the first 30 amino acids at the amino-terminal of the *S. cerevisiae* malic enzyme, the subcellular location of this protein is predicted to be mitochondrial due the presence of a typical mitochondrial targeting signal, i.e. seven arginine, five leucine, five serine residues and the absence of acidic amino acids. The physiological function of mitochondrial targeting sequences is mainly determined by the overall balance between these basic, hydrophobic and hydroxylated amino acids and the propensity to form amphiphilic helices (Allison and Schatz, 1986; Von Heijne, 1986).

3.4.2 The physiological role of the malo-ethanolic pathway in *S. cerevisiae*

As a K (-) yeast, *S. cerevisiae* only utilises L-malic acid in the presence of one or more fermentable carbon sources. However, L-malic acid utilisation in *S. cerevisiae* is weak compared to *S. pombe*, which seems to be evolutionarily streamlined for L-malic acid degradation. Moreover, the malic enzyme is not essential for the survival of cells of *S. cerevisiae*, as a deletion of the *MAE1* gene does not influence its viability (Boles *et al.*, 1998). Together with the mitochondrial location of the *S. cerevisiae* malic enzyme, these characteristics suggest that the malic enzyme fulfils an entirely different role in the metabolism of *S. cerevisiae*.

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3.4.2.1 Uptake of L-malic acid in *S. cerevisiae*. The ability of yeast to degrade extracellular L-malic acid efficiently is first and foremost dependent on the efficient transport of the dicarboxylic acid, while the efficacy of its intracellular malic enzyme plays an auxiliary role. From previous studies which have shown the simple diffusion of L-malic acid (and other dicarboxylic acids) in *S. cerevisiae* (Ansanay *et al.*, 1996; Baranowski and Radler, 1984; Rodriquez and Thornton, 1990; Salmon, 1987; Salmon *et al.*, 1987) and the absence of an equivalent gene in its genome, it can be concluded that *S. cerevisiae* does not contain an effective method for L-malic acid uptake. The most favourable pH for L-malic acid degradation by *S. cerevisiae* was found to be between pH 3.0 and 3.5, suggesting that only the undissociated form of L-malic acid enters the yeast (Salmon, 1987; Salmon *et al.*, 1987). Further studies on the influence of initial L-malic acid and glucose concentrations, as well as pH, on L-malic acid metabolism in *S. cerevisiae*, revealed some more detail on the nature of simple diffusion of L-malic acid in *S. cerevisiae* (Delcourt, 1995). The initial L-malic acid concentration influenced the final amount of L-malic acid degraded by *S. 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 *S. cerevisiae* (Delcourt, 1995).

3.4.2.2 Enzymatic conversion of L-malic acid in *S. cerevisiae*. Initial research by Polakis and Bartley (1965) indicated that *S. cerevisiae* lacks malic enzyme activity, but very low malic enzyme activities were reported nearly a decade later in cell extracts of *S. cerevisiae* by Fuck *et al.* (1973). The partially purified *S. 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 *S. pombe* malic enzyme and further contributes to the inefficient degradation of L-malic acid in *S. cerevisiae* (Fuck *et al.*, 1973; Osothsilp, 1987; Salmon 1987; Temperli *et al.*, 1965). As in *S. pombe*, malate dehydrogenases contribute little to L-malic acid degradation in *S. cerevisiae* during fermentation, since functional mitochondria are absent under these conditions. In contrast to the *S. pombe* malic enzyme that can only utilise NAD^+ as co-factor (Temperli *et al.*, 1965), the malic enzyme from *S. cerevisiae* has been reported to use both NAD^+ and $NADP^+$ as electron acceptors (Fuck *et al.*, 1973). Manganese cations (Mn^{2+}) are also essential for the malic enzyme activity in *S. cerevisiae*.

An additional feature of the *S. cerevisiae* malic enzyme that may contribute to the weak degradation of L-malic acid by strains of *S. cerevisiae* under fermentative conditions, is the mitochondrial location of this enzyme. The mitochondrial localisation was first suggested by the presence of amino acid targeting motifs and subsequently confirmed by subcellular fractionation analysis (Boles *et al.*, 1998). Mitochondria, the powerhouse of yeast cells responsible for energy generation through oxidative phosphorylation, as well as 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

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absence of dissolved oxygen. When yeast is grown aerobically on a non-fermentable carbon source, mitochondria of the fully-respiring cells are rich in cristae structures and up to 50 have been observed per cell. During anaerobic growth conditions mitochondria are redundant in the respiratory sense, due to the absence of oxygen as terminal electron acceptor. Nevertheless, the premitochondria present in yeast cells are still responsible for 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).

S. cerevisiae has 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 is higher than the so-called critical growth rate (usually ca. two-thirds 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. Glucose plays a crucial role in Crabtree-positive yeasts such as *S. cerevisiae*, since the development of and the enzyme activities in mitochondria are repressed in the presence of glucose (Cho *et al.*, 2001; Dejean *et al.*, 2000; García *et al.*, 1993; Jayaraman *et al.*, 1966; Mattoon *et al.*, 1979; Perlman and Mahler, 1974; Polakis and Bartley, 1965). Under winemaking conditions where high glucose concentrations are present, yeast mitochondria become fewer in numbers and dysfunctional, while many mitochondrial enzymes are down-regulated. It is postulated that this phenomenon will have an adverse effect on the ability of *S. cerevisiae* to degrade malic acid.

Preliminary transcriptional regulation studies of the *MAE1* gene in *S. cerevisiae* have 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). This is in contrast with the biochemical data found for the purified *S. cerevisiae* malic enzyme, which, besides indicating the absence of substrate induction by malic acid, also showed that the malic enzyme of *S. cerevisiae* is sensitive to catabolite repression, which is not the case for the malic enzyme in *S. pombe* (Groenewald and Viljoen-Bloom, 2001; Osothsilp, 1987). Further evidence for the constitutive expression of the *MAE1* gene was obtained by genome-wide expression studies where the *MAE1* gene 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).

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A clear induction of *MAE1* expression was observed during anaerobic growth of *S. cerevisiae* on glucose in continuous culturing, with a ca. 3-fold increase at the transcriptional level and a ca. 4-fold increase of the enzyme activity in cell extracts (Boles *et al.*, 1998). Similar results were obtained during genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *S. 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 *S. cerevisiae MAE1* gene strongly suggests a physiological function of the malic enzyme in anaerobic conditions, possibly in the provision of intramitochondrial NADPH or pyruvate (Boles *et al.*, 1998).

The current proposed physiological role of the malic enzyme in *S. cerevisiae* involves its possible participation as an auxiliary pathway for the regeneration of the main biosynthetic co-factor, NADPH (Boles *et al.*, 1998) (Fig. 3.5). Due to the respiro-fermentative metabolism of *S. cerevisiae*, carbon flow is steered away from biosynthesis towards ethanol production. 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. *S. 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. 3.5), the malic enzyme, pyruvate carboxylase (PC), NAD^+-dependent malate dehydrogenase (*MDH2*) and mitochondrial dicarboxylic carrier (*DIC1*) act as a cyclic transhydrogenase shuttle to convert 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 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 *S. cerevisiae*. Furthermore, the natural direction of flux of pyruvic acid during respiration is from the cytosol, where glycolysis takes place, into the mitochondria. If this shuttle is active in *S. 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).

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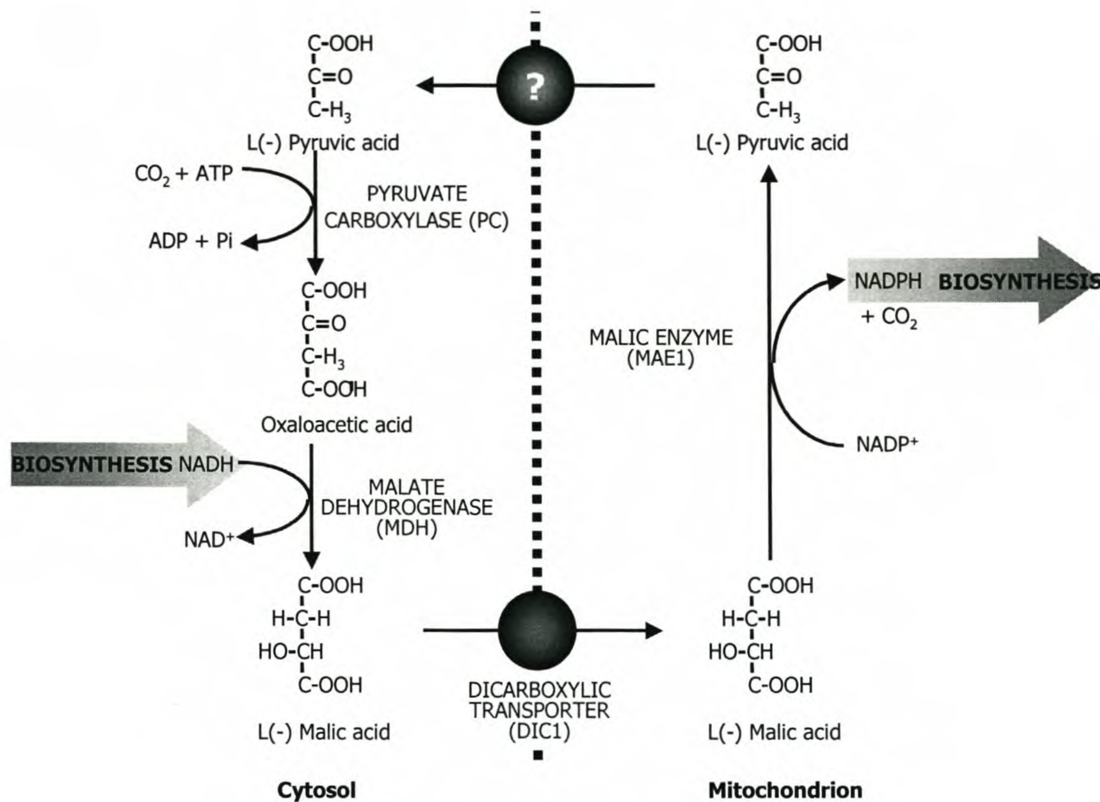

Figure 3.5 The predicted transhydrogenase malic acid-pyruvic acid shuttle in *S. cerevisiae* which functions as a recycling process for cytosolic NADH into mitochondrial NADPH. All the enzyme activities, except the mitochondrial pyruvate transporter (indicated by the question mark) has been found in *S. cerevisiae*. Adapted from Bakker *et al.* (2001).

3.4.3 Comparison of L-malic acid metabolism in *S. pombe* and *S. cerevisiae*

As K (-) yeasts, both *S. cerevisiae* and *S. 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 *S. cerevisiae*. Three main reasons for the weaker degradation of L-malic acid in *S. cerevisiae* have been postulated. Firstly, *S. cerevisiae* lacks the machinery for active transport of L-malic acid (Fig. 3.6) found in *S. pombe* and relies on rate-limiting simple diffusion for the intake of extracellular malic acid. Secondly, the malic enzyme of *S. cerevisiae* has a significantly lower substrate affinity for L-malic acid (K_m = 50 mM) than that of *S. pombe* (K_m = 3.2 mM), which contributes to weaker metabolism of this acid in *S. cerevisiae* (Fuck *et al.*, 1973; Temperli *et al.*, 1965). Lastly, the mitochondrial location of the malic enzyme of *S. 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 *S. cerevisiae*.

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On the basis of the opposing L-malic acid degradation abilities of *S. cerevisiae* and *S. 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 *S. cerevisiae* and *S. pombe* malic enzymes play an almost similar role in the provision of pyruvic acid for cellular biosynthesis. The possibility for the existence of an NADH-NADPH recycling function of the *S. cerevisiae* mitochondrial malic enzyme cannot be ruled out, but additional evidence is still required. On the other hand, 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 *S. pombe* on maintaining the redox balance and energy production in this yeast therefore requires more in-depth investigation.

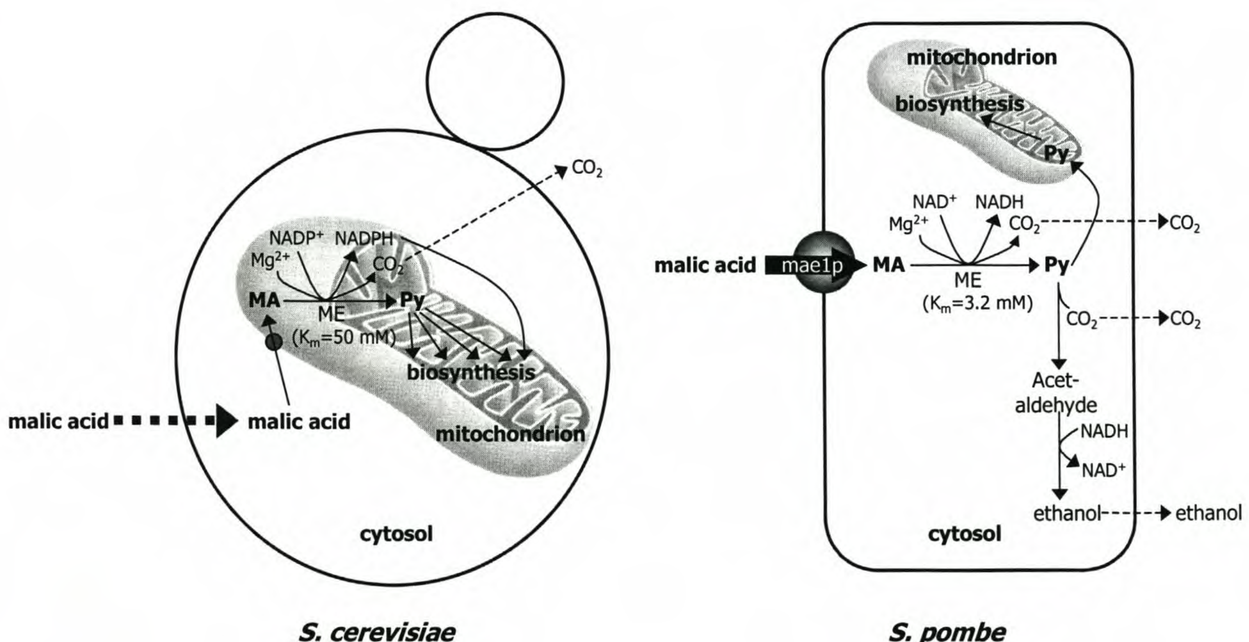

Figure 3.6 The main differences in L-malic acid degradation between *S. cerevisiae* and *S. pombe* involves the transport of malic acid, the substrate affinity of the malic enzyme and the compartmentalisation of the malic enzymes in these two yeast species. Despite these significant differences, the malic enzyme seems to play a similar role in *S. cerevisiae* and *S. pombe*, i.e. to supply pyruvic acid for biosynthesis.

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CHAPTER 4

LITERATURE REVIEW

Genetic engineering of industrial strains of *Saccharomyces*

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Genetic engineering of industrial strains of *Saccharomyces*

4.1 INTRODUCTION

The genetic improvement of wine yeast strains can be obtained in a number of ways to accommodate the requirements of the wine industry. Some techniques alter selective or specific regions of the genome, while others cause the recombination or rearrangement of the entire genome (Barre *et al.*, 1993; Hammond, 1996; Pretorius and Bauer, 2002; Pretorius and Van der Westhuizen, 1991; Querol and Ramon, 1996). Traditionally, since the middle 1980's, development of new industrial wine yeast strains predominantly employed classical genetic techniques such as clonal selection. Variants or mutants with beneficial attributes were selected by harnessing the forces of naturally occurring genetic processes such as spontaneous mutation, genetic drifting (Snow, 1983) and genome renewal (Mortimer *et al.*, 1994) within a wine yeast strain. Forced mutation by means of chemical or radiation mutagenesis and selection for positive phenotypes has increased the frequency of improving wine yeast strains for decades.

Other widely used genetic techniques for the breeding of new wine yeast strains involve several different "shotgun" approaches, including several types of hybridisation methods such as intra-species mating, spore-cell mating, rare-mating, spheroplast fusion and cytoduction. After applying these techniques, the newly constructed strains are usually screened for general beneficial properties such as fermentation capacity, ethanol tolerance, absence of off-flavours, flocculation and carbohydrate utilisation (Barre *et al.* 1993; Dequin, 2001; Pretorius, 2000). Although these classical genetic techniques are extremely valuable in wine yeast development, a major drawback of this approach is the lack of specificity and difficulty to introduce or remove specific properties from a strain in a well-controlled manner without altering the yeast's overall performance. Often a new strain is obtained with desirable new characteristics, but at the risk of compromising other desired characteristics (Barre *et al.*, 1993; Hammond, 1996; Pretorius and Bauer, 2002; Pretorius and Van der Westhuizen, 1991; Querol and Ramon, 1996).

With the advent of recombinant DNA technology in combination with the transformation of foreign DNA into yeast and its application in combination with the classic genetic techniques, especially hybridisation, a new cutting-edge approach to developing improved strains of *Saccharomyces* has emerged. A substantial amount of work took place during the 1990s to develop new strains, which mainly involved recombinant DNA approaches (Barre *et al.* 1993; Blondin and Dequin 1998; Butzke and Bisson 1996; Henschke 1997; Pretorius 2000; Pretorius and Bauer, 2002; Pretorius and Van der Westhuizen 1991; Querol and Ramon 1996). Although natural transformation, defined as the uptake of free DNA from the surrounding medium, has been described in prokaryotes and only recently in

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the yeast *S. cerevisiae* (Dreiseikelmann, 1994; Griffith, 1928; Lorenz and Wackernagel, 1994; Nevoigt *et al.*, 2000; Stewart and Carlson, 1986), yeast cells usually are artificially transformed with DNA. Yeast cells can be forced to take up DNA by chemical treatment, e.g. spheroplast-producing enzymes, polyethylene glycol, thiol compounds, anions or cations, by mechanical treatment, e.g. electroporation or bombardment with microprojectiles, or by a combination of the two treatments (Armaleo *et al.*, 1990; Becker and Guarente, 1991; Beggs, 1978; Gietz *et al.*, 1995; Hinnen *et al.*, 1978; Ito *et al.*, 1983; Schiestl and Gietz, 1989; Thompson *et al.*, 1998). In electroporation, cells are subjected to a short high-voltage electrical pulse that forms pores in their cellular membrane through which macromolecules such as DNA can enter (Wong and Neumann, 1982). Electroporation is currently regarded as the most efficient and versatile method for transforming yeast cells with naked DNA and the method of choice for delivering DNA into industrial wine yeast strains (Manivasakam and Schiestl, 1993; Neumann *et al.*, 1996; Rech *et al.*, 1989; Suga *et al.*, 2000; Thompson *et al.*, 1998).

4.2 GENETIC FACTORS THAT INFLUENCE THE DEVELOPMENT OF NEW INDUSTRIAL WINE YEAST STRAINS

Modern-day industrial strains of *Saccharomyces* have been selected and domesticated over the last 8000 years under conditions that favoured their evolution towards specialised abilities, such as rapid fermentation of high-sugar grape juices, high yield and tolerance of ethanol, resistance to SO_2 and good flavour production (Pretorius, 2000; Querol and Ramón, 1996). As a consequence, wine yeasts have developed a unique genetic composition and have built-up unique physiological traits that distinguish them from laboratory strains as well as other industrial strains, i.e. baker's, brewer's and distiller's yeasts (Dequin, 2001).

4.2.1 Chromosomal make-up of laboratory strains of *Saccharomyces* vs. industrial wine yeasts

The chromosomal composition of industrial strains of *Saccharomyces* shows some unique attributes that complicate the genetic manipulation and improvement of these wine yeast strains. Haploid strains of *S. cerevisiae* have a relatively small genome, containing approximately 12 to 13 megabases (mb) of chromosomal DNA (Pretorius and Van der Westhuizen, 1991). The genome consists of 16 linear chromosomes, ranging in size from 200 to 2200 kilobases and each containing a single DNA molecule. The complete genome of a laboratory strain of *S. cerevisiae* has been sequenced and published in 1996 and original computational analysis showed that it contains approximately 6000 open reading frames (ORF's), as well as 275 tRNA, 140 rRNA and 20 genes encoding small nuclear RNA species (snRNA) (Goffeau *et al.*, 1996). However, according to later estimates up to 20 % of the predicted 6000 ORF's in the Munich Information Center for Protein Sequences (MIPS) Yeast Genome Database (MYGD) were found to be non-coding. A more accurate estimation of 5300–5400

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protein-encoding genes has therefore been suggested (Mackiewicz *et al.*, 2002). Although almost 70 % of the more than 12 million bp comprise open reading frames (ORF's) of which only 4 % contain introns, a high level of apparent genetic redundancy in the genome has been revealed (Delneri *et al.*, 2000; Mewes *et al.*, 1997; Oliver, 1996; Wolfe and Shields, 1997). On the other hand, the *S. cerevisiae* genome is still considered relatively compact relative to the genomes of other yeast and fungi.

In contrast to laboratory strains, industrial and natural (wild) strains of *Saccharomyces* are characterised by karyotype instability, both during meiosis and vegetative growth (Adams *et al.*, 1992; Bakalinsky and Snow, 1990[b]; Carro and Piña, 2001; Codon *et al.*, 1997; Codon and Benitez, 1995; Gasent-Ramirez *et al.*, 1999; Longo and Vezinhet, 1993; Miklos *et al.*, 1997; Mortimer *et al.*, 1994; Nadal *et al.*, 1999). The main karyotype differences between laboratory and industrial strains include the acquisition or loss of whole chromosomes, the presence of chromosomal-length polymorphisms (duplications of 30 – 390 kb as well as deletions of 30 – 50 kb) and the presence of hybrid chromosomes (Adams *et al.*, 1992; Bidenne *et al.* 1992; Dequin, 2001). The most frequent alteration in the karyotype pattern in wine yeast strains is the variation in size of chromosome XII (Chindamporn *et al.*, 1993; Nadal *et al.*, 1996, 1999; Petes *et al.*, 1991; Rustchenko *et al.*, 1993). Genetic heterogeneity in wine yeast strains is due mainly to mitotic recombination during vegetative growth and spontaneous mutation. It has been suggested that these rearrangements occurred by recombination through sub-telomeric repeats and transposable elements (*Ty* elements) (Rachidi *et al.*, 1999). Minor differences, such as point mutations, have also been identified between laboratory and industrial strains of *Saccharomyces*. These variations in DNA sequence also affect strain performance if they are localised inside an open reading frame (ORF) or the regulatory region of a gene, especially if it is a pathway-regulating gene (Cavalieri *et al.*, 2000). Several attempts to elucidate the characteristics and the mechanisms of mitotic instability have been published in recent years (Adams *et al.*, 1992; Codon and Benitez, 1995; Gasent-Ramirez *et al.*, 1999; Puig *et al.*, 2000; Rachidi *et al.*, 1999). However, the question of why some natural strains are more prone to chromosomal rearrangements during vegetative growth than others remains largely unanswered.

Wine yeasts exhibit a greater variety in chromosome number than laboratory strains. Most laboratory strains of *Saccharomyces* are haploid (or sometimes diploid), whereas industrial strains of *Saccharomyces* are predominantly diploid, aneuploid and even occasionally polyploid (Bakalinsky and Snow, 1990[b]; Dequin, 2001; Ibeas and Jimenez, 1996; Johnston, 1990, Kunkee and Bisson, 1993; Mortimer, 2000; Mortimer *et al.*, 1994). Polyploidy and aneuploidy in industrial yeast is linked to the chromosomal centromeres which forms the kinetochore, i.e. a special multiprotein structure located at the chromosomal surface that binds spindle microtubules to form the spindle pole body (SPB) and regulates chromosome movements (Afshar *et al.*, 1995; Ault and Rieder, 1994; Pluta *et al.*, 1995; Zang *et al.*, 2002). The spindle pole body (SPB) in yeast is responsible for the correct segregation of

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chromosomes in early G1 phase. Mutations that prevent SPB duplication lead to monopolar mitosis that gives rise to aneuploidy and polyploidy (Chial *et al.*, 1999). Whole-genome duplication in wine yeast strains of *Saccharomyces* is thought to have played an important evolutionary role, especially in facilitating the evolution of anaerobic fermentation (Mewes *et al.*, 1997; Keogh *et al.*, 1998; Philippsen *et al.*, 1997; Wolfe and Shields, 1997). Multiple copies of a chromosome endow wine yeasts with a selective advantage; increased gene dosage of beneficial genes could partly explain the differences in fermentation kinetics and production of flavour/aroma compounds between different strains (Cavalieri *et al.*, 2000; DiIorio *et al.*, 1987; Mowshowitz, 1979; Stewart *et al.*, 1981; Talbot and Wayman, 1989). Furthermore, multiple ploidy in industrial wine yeasts confers protection against recessive lethal or deleterious mutations (Tavares *et al.*, 1988).

The multiple ploidy phenomenon in combination with the presence of prototrophy, i.e. recessive nature of selectable mutations in amino acid biosynthesis genes (*URA3*, *LEU2*, etc.) or purine and pirimidine genes (*ADE2*, etc.), in industrial strains of *Saccharomyces* severely complicates genetic improvement strategies in industrial strains of *Saccharomyces* (Bakalinsky and Snow, 1990[a]; Beckerich *et al.*, 1984; Rank *et al.*, 1988; Snow, 1983; Spencer and Spencer, 1983, Subden, 1987). The introduction of heterologous genes into a wine yeast strain requires either that the strain be made auxotrophic prior to transformation, or that the plasmid used for transformation carry a marker that is selectable against a wild-type diploid or polyploid background. Only one auxotrophic marker that is based on complementing a pantothenic acid auxotrophy at 35°C with the *ECM331* gene has been reported in industrial sake yeast strains of *S. cerevisiae*, (Shimoi *et al.*, 2000). In addition to the fact that the construction of an auxotrophic polyploid wine yeast strain is a complex and time consuming process, auxotrophic markers have inherent problems in that they frequently lead to secondary growth effects (Baganz *et al.*, 1997; Smith *et al.*, 1995, 1996) and sometimes to undesirable and unpredictable phenotypes (Gaber *et al.*, 1989; Goldstein and McCusket, 1999; Whelan *et al.*, 1979). Auxotrophy also interferes with the two nitrogen-starvation-regulated cellular differentiation processes in *S. cerevisiae*, i.e. sporulation efficiency and pseudohyphal growth as the growth medium must be supplemented with nitrogen-containing compounds such as amino acids and bases (Atkinson *et al.*, 1980; Freese *et al.*, 1984; Goldstein and McCusket, 1999; Schroerder and Breitenbach, 1981; Varma *et al.*, 1985). All these built-in disadvantages of auxotrophic markers therefore contribute to their unsuitability for use in industrial strains of *Saccharomyces*.

As a result of the above, several dominant selectable markers have been developed for the introduction of heterologous genes into industrial strains of *Saccharomyces* (Van den Berg and Steensma, 1997). These dominant selectable markers include resistance to antibiotics such as phleomycin (also known as bleomycin) (*ble* gene) (Wenzel, *et al.*, 1992), cyclohexamide (*cyh2* and *cyh5* genes) (Navas *et al.*, 1991; Del Pozo *et al.*, 1991), geneticin G418 (Tn903/*APT1* gene) (Baganz *et al.*, 1997; Hadfield *et al.*, 1990; Lang-Hinrichs *et al.*, 1989; Wach *et al.*, 1994), nourseothricin,

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bialaphos/phosphinothricin (Goldstein and McCusker, 1999), hygromycin B (*hph* gene) (Goldstein and McCusker, 1999), L-canavanine (*CAN1* gene), chloramphenicol (*cat* gene) (Hadfield *et al.*, 1987), aureobasidin A (*AUR1-C* gene) (Hashida-Okado *et al.*, 1998) as well as other toxic compounds such as *o*-fluoro-DL-phenylalanine (Shimura *et al.*, 1993), trifluoroleucine (*LEU4-1* gene) (Bendoni *et al.*, 1999), cerulenin (*PDR4* gene) (Nakazawa *et al.*, 1993), copper (*CUP1* gene) (Fogel *et al.*, 1983; Henderson *et al.*, 1985), methotrexate (*dhfr* gene) (Zhu *et al.*, 1986), sulfometuron methyl (*SMR1* gene) (Casey *et al.*, 1988), methylglyoxal (Murata *et al.*, 1985), manganese (*MNR1* gene) (Del Pozo *et al.*, 1999), sulfite (*FZF1-4* gene) (Park *et al.*, 1999), formaldehyde (*SFA1* gene) and fluoroacetate (*dehH1* gene) resistance (Van den Berg and Steensma, 1997; Wehner and Brendel, 1993).

4.2.2 Homothallism vs. heterothalism

As a unicellular fungus and member of the *Ascomycetes*, *Saccharomyces* yeasts reproduce asexually through multilateral budding and sexually through the formation of ascospores (Fig. 4.1). Yeasts are termed heterothallic when the ascospores possesses a stable sexual type, either a or α, and the cultures originating from these spores are permanently haploid. Heterothallism is mostly found in laboratory strains of *S. cerevisiae*, while more than 70 % of industrial wine yeast strains have a homothallic life cycle (Haber and Halvorson, 1975; Mortimer, 2000; Mortimer *et al.*, 1994; Pretorius and Van der Westhuizen, 1991; Thornton and Eschenbruch, 1976). Homothallic diploid yeasts yield four haploid ascospores when they sporulate. Due to the presence of the HO gene, these ascospores quickly change their mating type, mate and form homozygous diploids within the first two cell divisions (Haber, 1983; Herskowitz *et al.*, 1992; Nasmyth, 1982; Tamai *et al.*, 2001).

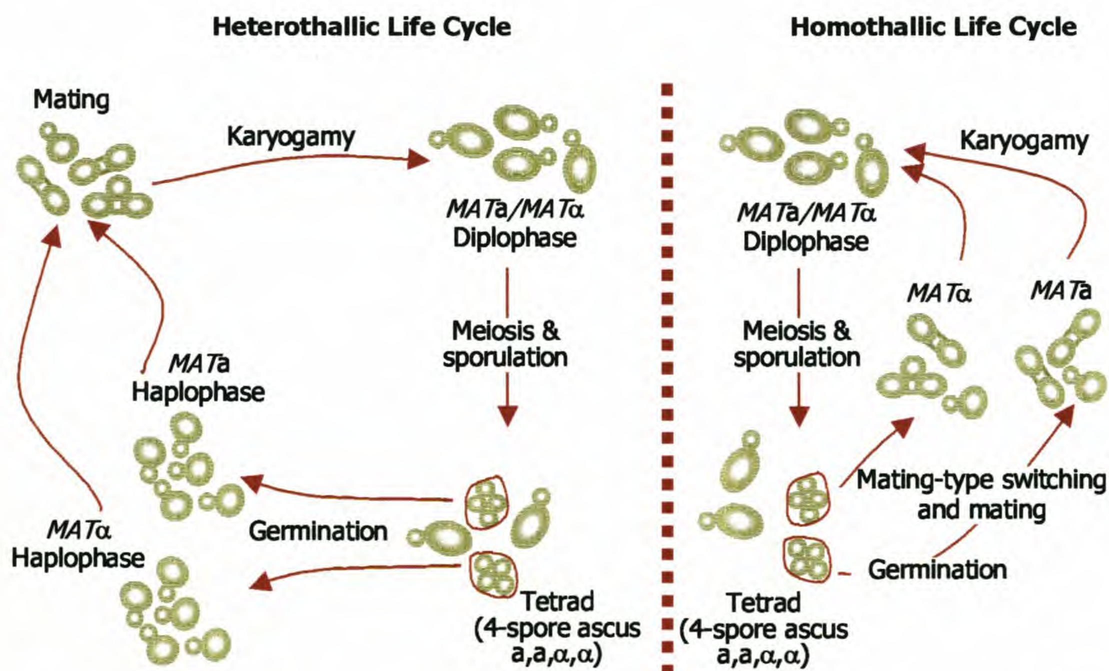

Figure 4.1. Comparison of the heterothallic and homothallic life cycle of the yeast *S. cerevisiae* (adapted from Phaff, 1981).

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The success of hybridisation techniques depends on the generation of stable haploid ascospores for efficient mating between different parent strains. The mating type-switching nature of industrial wine yeast strains complicates this procedure, since true hybrids are obtained at a lower frequency with an increased difficulty to correctly identify them (Bakalinsky and Snow, 1990[a]; Guijo *et al.*, 1997; Jiménez and Benítez, 1994; Mortimer *et al.*, 1994; Romano *et al.*, 1985; Thornton and Eschenbruch, 1976). Furthermore, depending on the ploidy of the parental strain, only between 0 - 25 % of industrial strains are able to mate and sporulate. When mating and sporulation does occur, the production of viable spores varies from 0 - 95 % (Guijo *et al.*, 1997; Johnston *et al.*, 2000; Subden, 1987). Despite these obstacles, different strategies have been developed to improve the success rate of hybridisation between homothallic strains of *Saccharomyces* (Romano *et al.*, 1985; Sebastiani *et al.*, 2002; Winge and Lausten, 1938). Early approaches made use of crossings between laboratory haploid heterothallic strains that contain known markers with the haploid cells of homothallic industrial strains. In these cases, hybrids are easy to detect and the genetic improvement of some industrial yeasts has been described (Guijo *et al.*, 1997; Ibragimova *et al.*, 1994; Jiménez and Benítez, 1994; Thornton, 1985). However, backcrossing is needed to regenerate the exact industrial strain properties that are lacking in laboratory strains. The use of the killer phenotype found in several wine yeast strains has also been used for the successful hybridisation of homothallic strains (Ramírez *et al.*, 1998).

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CHAPTER 5

RESEARCH RESULTS

Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation

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Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation

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ABSTRACT

To produce a high quality wine, it is important to obtain a fine balance between the various chemical constituents, especially between the sugar and acid content. The latter is more difficult to achieve in wines that have a high acidity due to excess malic acid, since wine yeast in general cannot effectively degrade malic acid during alcoholic fermentation. An indigenous *Saccharomyces paradoxus* strain RO88 was able to degrade 38% of the malic acid in Chardonnay must and produced a wine of good quality. In comparison, *Schizosaccharomyces pombe* strain F effectively removed 90% of the malic acid, but did not produce a good quality wine. Although commercially promoted as a malic acid degrading wine yeast strain, only 18% of the malic acid was degraded by *Saccharomyces cerevisiae* Lalvin strain 71B. Preliminary studies on the transcriptional regulation of the malic enzyme gene from three *Saccharomyces* strains, i.e. *S. paradoxus* RO88, *S. cerevisiae* 71B and *Saccharomyces bayanus* EC1118, were undertaken to elucidate the differences in their ability to degrade malic acid. Expression of the malic enzyme gene from *S. paradoxus* RO88 and *S. cerevisiae* 71B increased towards the end of fermentation once glucose was depleted, whereas no increase in transcription was observed for *Saccharomyces bayanus* EC1118 which was also unable to effectively degrade malic acid.

5.1 INTRODUCTION

The relative concentrations of organic and inorganic acids are important factors that determine the quality of wine. The dominant organic acids in wine are L-tartaric and L-malic acid, which represent 70-90% of total grape acidity (Ruffner, 1982). The malic acid concentration in grapes ranges from 1 to 10 g l^{-1} depending on several factors of which the prevailing climate is the most important. The levels of malic acid are usually higher in the cool viticulture regions where the respiration of acids in the grape berry progresses at a slower rate than in the warm climate regions. Excessive amounts of malic acid (15 – 16 g l^{-1}) have been found in grapes at harvest time during exceptionally cold

summers in cool climate viticultural regions (Gallander, 1977). Malic acid not only contributes to the acidic taste of wine, but also serves as a substrate for contaminating lactic acid bacteria that can result in wine spoilage after bottling. It is therefore essential to remove excess malic acid from the wine to ensure the physical, biochemical and microbial stability and quality of the wine (Delcourt *et al.*, 1995; Pretorius, 2000).

There are a number of methods to decrease the acidity of wine, but biological deacidification is most often used. Biological deacidification can be carried out through malolactic fermentation (MLF) or malo-ethanolic fermentation (MEF). During MLF, lactic acid bacteria such as *Oenococcus oeni* convert malic acid into lactic acid and CO_2, whereas MEF is carried out mostly by yeast species such as *Schizosaccharomyces pombe* and strains of *Saccharomyces* that convert malic acid into pyruvate by means of an intracellular malic enzyme. During wine fermentation, the glucose and SO_2 concentration, pH, fermentation temperature, thiamine and biotin content, as well as ethanol concentration, can influence MLF and may result in stuck or sluggish MLF (Tortia *et al.*, 1993). Together with a decrease in acidity, MLF also changes the aromatic characteristics of wine, which can be either desirable or not, depending on the grape cultivar and wine style (Davis *et al.*, 1985; Henick-Kling *et al.*, 1994).

Based on yeasts' ability or inability to metabolise TCA cycle intermediates as sole carbon or energy source, yeasts are divided into a K (+) or K (-) group (Goto *et al.*, 1978; Rodriquez and Thornton, 1990; Barnett *et al.*, 1990). The K (–) group of yeasts comprise those yeasts capable of utilising TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources. According to this definition, strains of *Saccharomyces* (including all wine yeast strains of *Saccharomyces*), *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii*, are all classified as K (-) yeasts. Although grouped together, the yeasts in this group have significant differences in their abilities to degrade malic acid. Typically, strains of *Saccharomyces* are regarded as the most inefficiently metabolisers of extracellular malic acid, whereas strains of *S. pombe* and *Z. bailii* can degrade high concentrations of malic acid (Taillandier and Strehaiano, 1991; Baranowski and Radler, 1984). However, within the species *Saccharomyces*, remarkable differences exist with regard to their ability to decompose malic acid during alcoholic fermentation (from 0 and 3 g l^{-1} malic acid) (Subden *et al.*, 1998). Malic acid metabolism by strains of *Saccharomyces* are linked to the optimal growth temperature of the strains, as cryotolerant strains (i.e. *S. bayanus and S. pastorianus*) were found to synthesise malic acid, while thermotolerant strains (i.e. *S. cerevisiae* and *S. paradoxus*) could degrade significant quantities (up to 48%) of malic acid (Rainieri *et al.*, 1998a, b).

The ability of a yeast strain to degrade extracellular malic acid is dependent on the efficient transport of the dicarboxylic acid, as well as the efficacy of the intracellular malic enzyme (Ansanay *et al.*, 1996; Volschenk *et al.*, 1997). Previous studies have shown that *S. cerevisiae* can import malic acid

and other dicarboxylic acids only via simple diffusion and is therefore unable to effectively degrade or utilise extracellular malic acid (Salmon, 1987). However, the *S. cerevisiae* malic enzyme has a very low substrate affinity (K_m of 50 mM), which further contributes to the inefficient degradation of malic acid by *S. cerevisiae* (Temperli *et al.*, 1965; Osothsilp, 1987). An additional aspect that may contribute to the weak degradation of malic acid by strains of *S. cerevisiae* under fermentative conditions, is the mitochondrial location of the *S. cerevisiae* malic enzyme and the fact that the mitochondria are dysfunctional and present in reduced in numbers under winemaking conditions. In contrast, *S. pombe* can degrade malic acid effectively, but only in the presence of an assimilable carbon source (Osothsilp and Subden, 1986). It has an active transport system for the uptake of malic acid, as well as an intracellular malic enzyme with a very high substrate affinity (K_m of 3.2 mM).

In general, malic enzymes catalyse the oxidative decarboxylation of L-malate and/or oxaloacetate to pyruvate and CO_2 in the presence of the cofactors $NAD(P)^+$ and Mn^{2+} or Mg^{2+}. Malic enzymes exhibit a high degree of homology (Viljoen *et al.*, 1994; 1998), but their metabolic function and regulation vary between different species and types of tissue. The *S. cerevisiae* mitochondrial NAD(P)-dependent malic enzyme, MAE1p, was proposed to play a role in the provision of intramitochondrial NADPH or pyruvate under anaerobic conditions (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 malic acid to ethanol during alcoholic fermentation, but the production of undesired fermentation aroma have been reported (Rankine, 1966; Gallander, 1977; Munyon and Nagel, 1977; Carre *et al.*, 1983; Sousa *et al.*, 1995; Taillandier *et al.*, 1995). Furthermore, *S. pombe* species are not ideally suited for wine fermentation due to their temperature and alcohol sensitivity (Taillandier *et al.*, 1988). In this study, the natural-occurring *Saccharomyces paradoxus* strain RO88 isolated from grapes (unpublished data, S. Redzepovic *et al.*) was evaluated and compared with *S. pombe* strain F, *S. cerevisiae* 71B and *S. bayanus* EC1118 for the biological deacidification of grape must and its influence on the chemical and sensory characteristics of wine. Furthermore, the expression patterns of the malic enzyme gene from the three *Saccharomyces* strains were compared to better understand the underlying mechanisms for the differential degradation of malic acid by *Saccharomyces* strains. Fundamental knowledge about the malo-ethanolic pathways from both K (-) and K (+) yeasts is imperative for our understanding of the regulation and physiological role of malic acid metabolism in yeast and can contribute to innovative applications of improved strains of *Saccharomyces* for the biological deacidification of wine.

5.2 MATERIALS AND METHODS

5.2.1 Yeast cultures

Saccharomyces paradoxus strain RO88 was isolated from grapes collected in Jazbina in the Zagreb wine region of Croatia (unpublished data, S. Redzepovic *et al.*, Department of Microbiology, University of Zagreb). The *Saccharomyces bayanus* EC1118 strain and *S. cerevisiae* Lalvin 71B strain are active dry yeast strains from Lallemand Inc. (France) and the *S. pombe* strain F was obtained from Prof. C. Zambonelli (DIPROVAL, University of Bologna, Italy).

5.2.2 Fermentation in synthetic grape must

The *S. bayanus*, *S. cerevisiae* and *S. paradoxus* strains were inoculated at 2 x 10^6 cells ml^{-1} into duplicate sets of 800 ml synthetic grape must (Denayrolles *et al.*, 1995). The synthetic grape must contained either 0.3% or 0.8% L-malic acid (Sigma, St. Louis, MO) and the pH was adjusted with KOH to 3.3. Fermentations were carried out at 20°C without shaking and sealed with fermentation caps filled with 2.5% SO_2 solution for approximately 9 days. Daily samples of 10 ml were taken for total RNA extraction and to determine the malic acid and glucose concentrations. Fermentation was considered to be complete when the glucose was depleted.

5.2.3 Grape must preparation

Chardonnay grapes obtained from the Scientific Research Center of Jazbina (Zagreb, Croatia) were harvested during the 1999 season, destemmed, crushed and pressed. Chemical analysis indicated that the juice contained 191 g l^{-1} total sugars, 3.6 g l^{-1} malic acid, a pH of 3.2 and total acidity of 8.7 g l^{-1}. The must was treated with 50 mg l^{-1} SO_2 and allowed to settle overnight. The juice was racked and the must divided into 16 lots of 15 litre each for four repetitions of four different treatments, i.e. inoculated with *S. pombe*, *S. cerevisiae* 71B, *S. paradoxus* RO88 or with no inoculum (i.e. spontaneous fermentation by the natural yeast population).

5.2.4 Vinification

The Chardonnay grape must was inoculated with pre-cultures of *S. paradoxus* or *S. pombe* to a final concentration of 10% (w/v), and the manufacturer's instructions were followed for the rehydration of strain 71B. Four repetitions of fermentations were carried out at 18°C in 25 litre glass fermentation flasks and the residual sugar concentration was determined every 5 days. After fermentation was completed, the wines were decanted and treated with 30 mg l^{-1} SO_2. Samples were taken for chemical analysis and the wines were bottled after a second decanting and kept at 4°C. An experienced panel of seven judges performed organoleptic analysis of the wines 6 months after bottling. A ranking method was used to determine quality differences between the treatments and statistical significance was determined according to Amerine and Roessler (1976).

5.2.5 Chemical analysis

The concentration of succinic acid, malic acid and lactic acid were determined enzymatically with specialised kits (Roche Biochemicals, Germany) according to the manufacturer's instructions. Standard methods were used to determine the pH, total acidity, volatile acidity, ethanol and sugar concentrations of the must and wines (Ough and Amerine, 1987). Sugar concentrations in synthetic grape must were measured with the Glucose (Trinder) Test Kit (Sigma Diagnostics, St. Louis, USA).

5.2.6 Expression studies with shift assays

Minimal medium (10 ml) containing 0.17 % YNB without amino acids and ammonium sulphate (Difco Laboratories, Detroit, MI), 0.5 % $(NH_4)_2SO_4$, 2 % D-glucose (Saarchem, Midrand, South Africa) and all the required amino acids was inoculated in duplicate with either *S. bayanus* EC1118 or *S. paradoxus* RO88 and grown overnight at 30°C under aerated conditions. The cells were harvested and the pellets resuspended in fresh minimal medium containing either 2% glucose plus 1% malic acid or 10% glucose, and cultured under aerated conditions for an additional 60 min with samples taken every 15 minutes for mRNA analysis. A shift to non-aerated conditions was obtained by resuspending the cultures in fresh minimal medium and covering it with 1-2 cm mineral oil.

5.2.7 RNA isolation and slot blot analysis

Total RNA was isolated with the FastRNA Kit (Bio 101, Carlsbad, CA) and equal amounts were transferred to a Nylon membrane (MSI, Westboro, MA) with slot blotting (Ausubel *et al.*, 1989). Transcripts were visualised with the Chemiluminescent Detection Kit (Roche Biochemicals, Germany) using PCR-generated DIG-labelled fragments of the *S. cerevisiae* malic enzyme gene, *MAE1*, and actin gene, *ACT1*. The PCR primers for the *MAE1* and *ACT1* genes were derived from the corresponding *Saccharomyces* Genome Database gene sequences: i.e. 5'-*MAE1* (5'-TTGCTATCTCCAAATTGGCA-3', 3'-*MAE1* (5'-ATTTTCTTGCGCGCTTCTTC-3', 5'-*ACT1* (5'-TATGGAAAAGATCTGGCATCA-3'), 3'-*ACT1* (5'-CGGTTTGCATTTCTTGTTCG-3'). Relative concentrations of the transcripts were quantified by means of densitometry and expressed in percentage relative to the *S. cerevisiae ACT1* transcripts, which were of comparable concentrations for most of the slots.

5.2.8 DNA sequence analysis of malic enzyme gene promoters

The promoter region of the malic enzyme genes from *S. paradoxus* RO88, *S. bayanus* EC1118 and *S. cerevisiae* 71B was PCR amplified with primers based on the DNA sequence of the *S. cerevisiae* malic enzyme gene, *MAE1*. The 5' primer, SCMAE1-F (5'-CATCGTGCATTGCAAGGTTT-3') binds at nucleotide -594 of the *MAE1* gene promoter and the downstream primer, SCMAE1-R (5'-GAATATAAACGCGATTGCTGA-3'), binds at nucleotide +94 inside the *MAE1* open reading frame. The PCR fragments were subcloned using p-GEM®-T Easy Vector System (Promega Corporation, Madison) and three subclones of each strain was submitted for sequencing. The DNA sequence of the

promoter fragments from *S. paradoxus* RO88, *S. cerevisiae* 71B and *S. bayanus* EC1118 were compared with that of the published sequence of the *S. cerevisiae MAE1* gene (*Saccharomyces* Genome Database).

5.3 RESULTS AND DISCUSSION

5.3.1 Fermentation of Chardonnay and synthetic grape must

A decrease in malic acid concentration in wines with a high total acidity is one of the most important steps in the enhancement of wine quality. The degradation of malic acid by three *Saccharomyces* strains, i.e. *S. bayanus* EC1118 (known for inefficient malic acid degradation), *S. cerevisiae* 71B (reported to degrade up to 35% malic acid) and *S. paradoxus* RO88, an indigenous strain, were evaluated in synthetic grape must containing either 0.3% or 0.8% malic acid. Under both conditions, fermentation by all three strains were completed after 5 days (Fig. 5.1). After being cultured in synthetic must containing 0.3% malic acid for 9 days, *S. bayanus* EC1118 degraded only 5% of the malic acid, whereas *S. cerevisiae* 71B and *S. paradoxus* RO88 degraded 20% and 28%, respectively. Similar results were obtained in the medium containing 0.8% malic acid, i.e. 8%, 17% and 26% of the malic acid was degraded by *S. bayanus* EC1118, *S. cerevisiae* 71B and *S. paradoxus* RO88, respectively. This clearly indicated that *S. paradoxus* RO88 is able to degrade extracellular malic acid under fermentation conditions more efficiently than *S. cerevisiae* 71B.

Based on these results, *S. paradoxus* strain RO88 was compared with *S. cerevisiae* 71B and *S. pombe* strain F for the fermentation of freshly pressed Chardonnay must. Fermentations with either *S. cerevisiae* 71B or *S. paradoxus* RO88 were completed within 30 days (Fig. 5.2), whereas the spontaneous fermentation (no inoculum added) took 40 days. The must inoculated with *S. pombe*, known to be a slow fermenting yeast (Snow and Gallander, 1979; Dharmadhikari and Wilker, 1998; Zambonelli, 1998), took 56 days to complete fermentation. Chemical analyses of the fermented Chardonnay wine (Table 5.1) indicated that *S. paradoxus* RO88 and *S. pombe* degraded approximately 38% and 90% of the malic acid, respectively. In contrast, *S. cerevisiae* 71B reduced the malic acid concentration by only 18%, in accordance with previous reports concerning the weak ability of *S. cerevisiae* to degrade malic acid (Rankine, 1966; Fuck and Radler, 1972; Gandini *et al.*, 1988; Yokotsuka *et al.*, 1993; Pilone and Ryan, 1997). The spontaneous fermentation carried out by the natural microbial population in the Chardonnay must, was the least effective in degrading extracellular malic acid (only 10%).

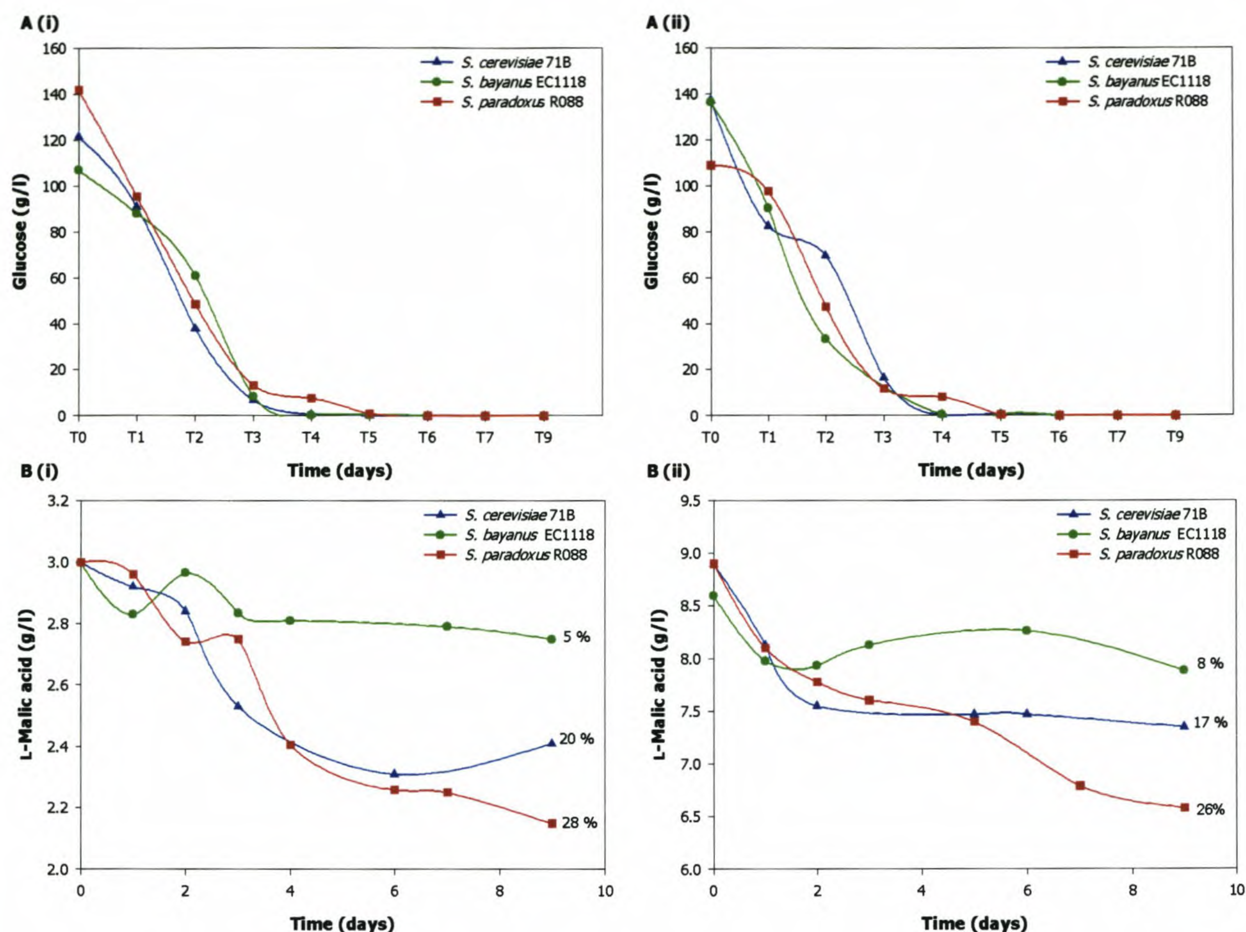

Figure. 5.1. (A) Glucose and (B) malic acid concentrations during fermentation of synthetic grape must containing (i) 0.3 % or (ii) 0.8 % malic acid by *S. bayanus* EC1118, *S. cerevisiae* 71B and *S. paradoxus* RO88.

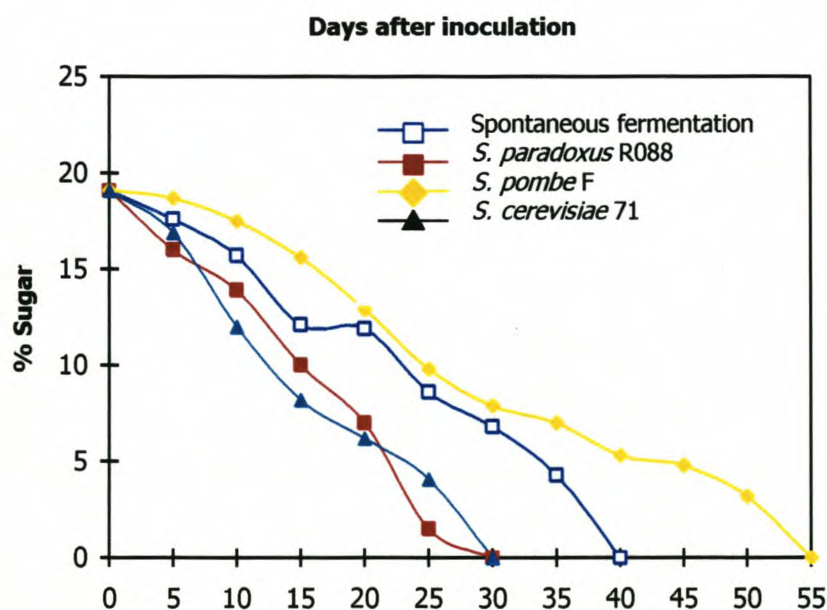

Figure 5.2. Sugar concentration during fermentation of Chardonnay grape must with spontaneous fermentation or inoculated with *S. paradoxus* RO88, *S. pombe* F or *S. cerevisiae* 71B.

Table 5.1. Chemical composition of fermented Chardonnay wines

Treatment	Alcohol (v/v) %	Reducing Sugar ($g\ l^{-1}$)	Total acidity[1] ($g\ l^{-1}$)	Volatile acidity[2] ($g\ l^{-1}$)	Malic acid ($g\ l^{-1}$)	Succinic acid ($g\ l^{-1}$)
Prior to fermentation		191	8.7		3.6	
Spontaneous fermentation	12 ±0.07	1.9 ±0.05	7.8 ±0.05	0.29 ±0.1	3.5 ±0.06	0.98 ±0.05
***S. paradoxus* R088**	13.0 ±0.05	1.9 ±0.06	6.8 ±0.1	0.31 ±0.09	2.2 ±0.06	0.9 ±0.08
***S. cerevisiae* 71B**	13 ±0.06	1.9 ±0.12	7.3 ±0.08	0.29 ±0.05	2.9 ±0.05	1.0 ±0.06
***S. pombe* F**	13.1 ±0.08	2.0 ±0.08	4.2 ±0.13	0.35 ±0.12	0.5 ±0.10	1.3 ±0.09

[1]as tartaric acid, [2]as acetic acid
n.d. = not determined
± = standard deviation

The decrease in malic acid concentration also correlated with the decrease in total acidity (Table 5.1). In the wine fermented with *S. pombe*, total acidity decreased by 4.5 $g\ l^{-1}$, in agreement with almost complete malic acid decomposition. Total acidity was decreased by 1.9 $g\ l^{-1}$ in the wine produced by *S. paradoxus* RO88, by 1.4 $g\ l^{-1}$ for *S. cerevisiae* 71B, and by 0.9 $g\ l^{-1}$ for the spontaneous fermentation. The decrease in acidity was also reflected in the pH of the different wines: the pH of the wine fermented with *S. pombe* increased with 0.16 units, whereas fermentation with *S. cerevisiae* 71B and *S. paradoxus* RO88 resulted in a pH increase of only 0.07 and 0.08 units, respectively.

Organoleptic evaluations of the fermented wines indicated no significant difference between wines produced with *S. cerevisiae* 71B or with spontaneous fermentation (Table 5.2). The lowest score was obtained for the wine fermented by *S. pombe* strain F; no off-flavours were detected as previously described (Rankine, 1968), but a loss in fruitiness was noted. The best results were obtained with the wine fermented by *S. paradoxus* RO88, which confirmed that indigenous strains could be advantageous for the development of wine aroma and improvement of wine quality (Soles *et al.*, 1982; Mateo *et al.*, 1992).

Table 5.2. Results of the organoleptic evaluation of wine

Treatment	order	sum
***S. paradoxus* RO88**	1	8*
***S. cerevisiae* 71B**	2	17
Spontaneous fermentation	3	17
***S. pombe* F**	4	28**

LSD of 1% = 8-20
LSD of 5% = 10-18
* significant at $p<0.05$
** signficant at $p<0.01$

5.3.2 Regulation of malic acid degradation in *Saccharomyces* strains

The ability of a yeast strain to degrade extracellular malic acid is dependent on the efficient transport of the dicarboxylic acid, as well as the efficacy of the intracellular malic enzyme. Little information is available on malic acid transporters from *S. bayanus* or *S. paradoxus*, but it is known that *S. cerevisiae* can import malic acid and other dicarboxylic acids only via simple diffusion and is therefore unable to effectively degrade or utilise extracellular malic acid (Salmon, 1987).

The malic enzyme from *S. paradoxus* has not yet been reported and its structure and function remains unknown. In general, the efficacy of a malic enzyme is determined by its substrate affinity and/or the level of expression of the corresponding gene. In this study, we found that expression of the malic enzyme gene from *S. bayanus* EC1118, the non-degrading strain, slightly increased at the beginning of fermentation in synthetic grape must containing either 0.3% or 0.8% malic acid (Fig. 5.3), but declined towards the end of fermentation. Transcription of the malic enzyme genes from *S. cerevisiae* 71B and *S. paradoxus* RO88 showed a similar increase and decline, followed by a strong increase in transcription towards the end of fermentation. The increase in transcription towards the end of fermentation by both strains was stronger in the presence of 0.8% malic acid, suggesting that the malic enzyme of these yeasts could play an important role in their ability to respond to the varying conditions associated with fermenting wine. Furthermore, our results indicated that the increase in malic enzyme transcription in *S. cerevisiae* 71B and *S. paradoxus* RO88 towards the end of fermentation (Fig. 5.3), correlated with the depletion of glucose (Fig. 5.1). However, only *S. paradoxus* RO88 displayed a marked increase in the degradation of malic acid upon glucose depletion when grown on 0.8% malic acid (Fig. 5.1), with a lesser effect on 0.3% malic acid. It therefore seems likely that malic acid is utilised as a secondary carbon source by *S. paradoxus* RO88 once the glucose is exhausted.

These results support the increase in malic enzyme activity towards the end of fermentation previously reported by Ramon-Portugal *et al.* (1999). As indicated in Fig. 5.4, there are two possible routes for malic acid decomposition inside the yeast cell, i.e. via the malic enzyme or via fumarase (Radler, 1986). Although both routes are utilised during fermentation, succinate production was reported to be more active at the beginning of fermentation, while a large portion of the malic acid is converted into ethanol during the second part of fermentation (Ramon-Portugal *et al.*, 1999). Our results showed differences in the concentration of succinic acid in the wines fermented by the different strains, with that produced by *S. pombe* being the highest (Table 5.1). Of the numerous organic acids produced in wine by yeasts, succinic and acetic acid are the most prominent (Radler, 1986). Wine yeast strains differ in the amount of succinic acid that is produced (Heerde and Radler, 1978; Dharmadhikari and Wilker, 1998) and an increase in malic acid concentration was reported to generally result in a higher production of succinic acid (Ramon-Portugal *et al.*, 1999).

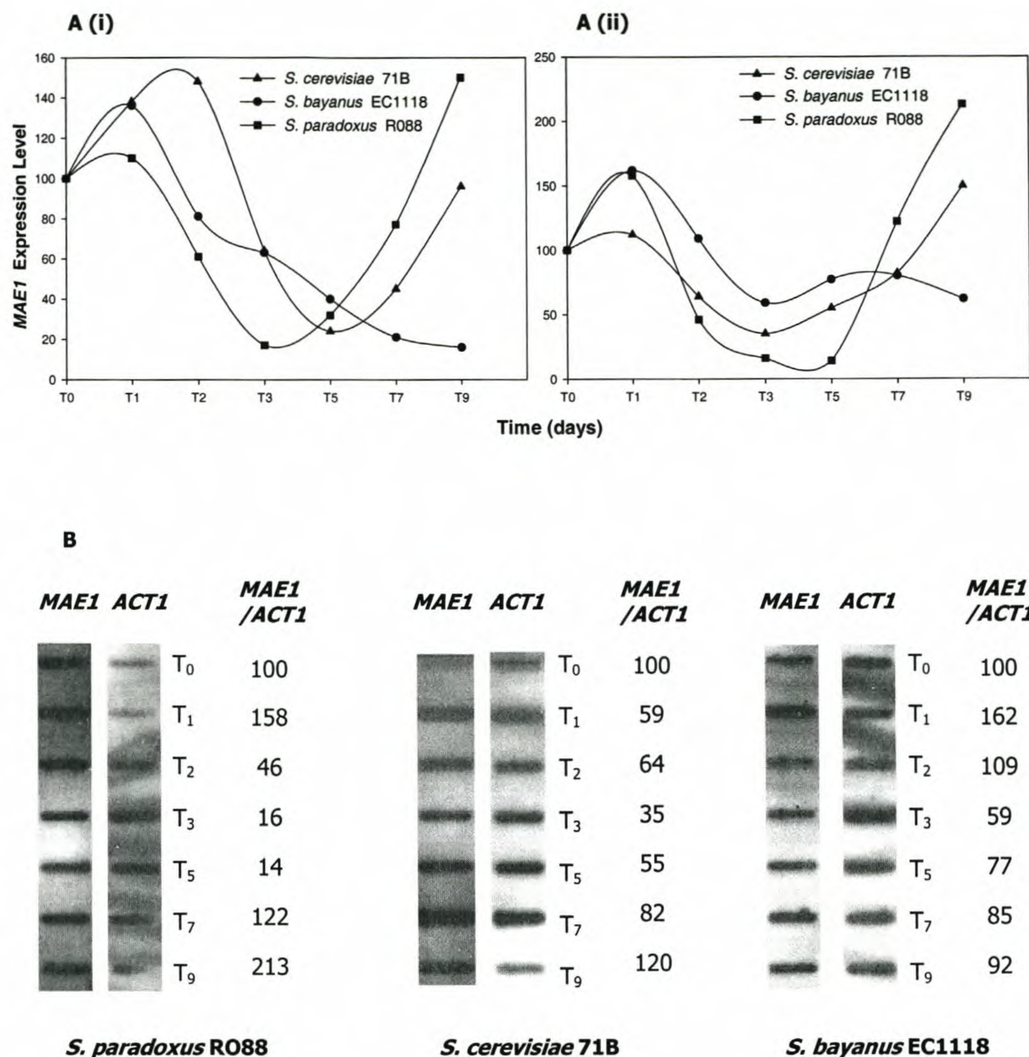

Figure 5.3. (A) Graphic representation of the malic enzyme transcription levels during fermentation of synthetic grape must containing (i) 0.3% or (i) 0.8% malic acid by *S. bayanus* EC1118, *S. cerevisiae* 71B and *S. paradoxus* RO88. The malic enzyme transcript levels are calculated as relative to that of *S. cerevisiae ACT1*. (B) Northern slot blots showing the relative transcript levels for *MAE1* and *ACT1* for *S. paradoxus* RO88, *S. cerevisiae* 71B and *S. bayanus* EC1118 during fermentation of synthetic grape must containing 0.8% malic acid.

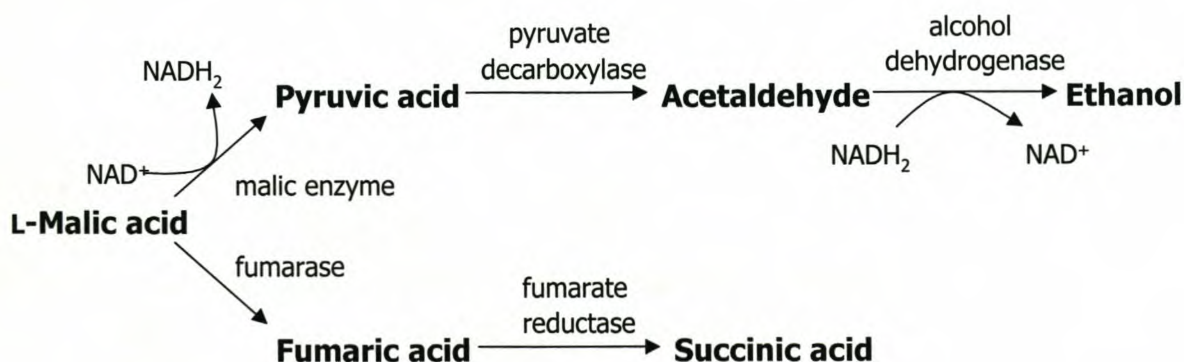

Figure 5.4. Malic acid decomposition in yeast can occur via the malic enzyme or via fumarase to yield either ethanol or succinic acid.

The regulation of the malic enzyme gene from *S. paradoxus* RO88 was further evaluated and compared with that of *S. bayanus* EC1118 (the weak malic acid degrader) under different conditions, i.e. a shift from growth in 2% glucose to 2% glucose plus 1% malic acid, to 10% glucose, or to non-aerated growth (Fig. 5.5). When shifted to 2% glucose plus 1% malic acid, the malic enzyme genes from both *S. bayanus* EC1118 and *S. paradoxus* RO88 showed a rapid, but transient, increase in expression (2 to 2.5-fold) within the first 60 minutes. A shift to 10% glucose also resulted in a transient increase in expression from *S. bayanus* EC1118 (4-fold increase after 45 min), whereas *S. paradoxus* RO88 had a slower, but steady increase in transcription (3.5-fold increase after 60 min). A shift to non-aerated conditions resulted in a decrease in the expression of the mitochondrial malic enzyme in both strains, probably due to a general decrease in mitochondrial activity under these conditions.

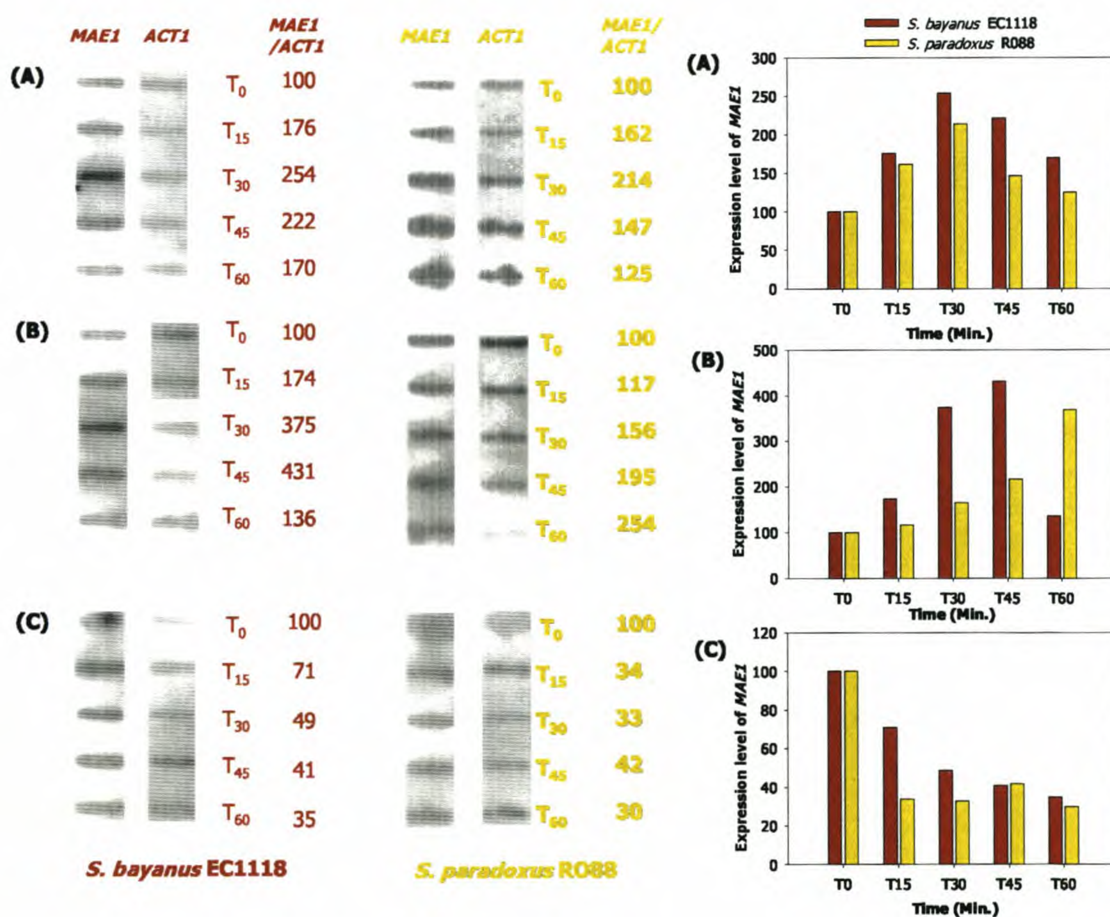

Figure 5.5. Northern slot blots and graphic representation of the expression of the malic enzyme gene of *S. bayanus* EC1118 and *S. paradoxus* RO88 after a shift to media containing (A) 2% glucose plus 1 % malic acid, (B) 10% glucose and in (C) non-aerated conditions.

From these results it is clear that the expression profile of the malic enzyme genes from *S. bayanus* and *S. paradoxus* reacted similarly in the malic acid shift conditions, i.e. both were temporarily induced in the presence of malic acid. This is in contrast to the findings of Boles *et al.* (1998) on the *S. cerevisiae* malic enzyme gene which was constitutively expressed at low levels and not subject to

any substrate induction. A difference in expression pattern of the malic enzyme gene from *S. bayanus* and *S. paradoxus* was, however, observed when cells were shifted to high glucose conditions, which may indicate possible divergent responses to carbon source or osmotic stress by *S. bayanus* and *S. paradoxus*. Increased expression under high glucose conditions was also observed for the cytosolic NAD^+-dependent malic enzyme of *S. pombe* where the transcription of the *S. pombe* malic enzyme gene, *mae2*, seems to be regulated in response to the carbon source, lack of oxygen and osmotic stress conditions, probably to assist in maintaining the intracellular redox balance (Groenewald and Viljoen-Bloom, 2001).

Analysis of the transcriptional regulation of the *S. pombe* malic enzyme gene indicated that two *cis*-acting elements in the *mae2* promoter, UAS1 and UAS2, are required for basal expression whilst three negative-acting elements (URSs) are involved in general derepression of *mae2* (Viljoen *et al.*, 1999). The promoter sequence of the malic enzyme gene from *S. paradoxus* RO88 was therefore compared with that of *S. bayanus* EC1118, *S. cerevisiae* 71B and the published *S. cerevisiae MAE1* sequence to determine whether putative regulatory elements could explain the different expression patterns of the *Saccharomyces* malic enzyme genes. In view of *S. paradoxus* being considered to be the evolutionary parental strain for both *S. cerevisiae* and *S. bayanus* (personal communication, A. Vaughan Martini, Department of Plant Biology and Biotechnology, University of Perugia, Italy), we anticipated a high degree of DNA homology between the promoter regions. However, comparative analysis of the DNA sequences (Fig. 5.6) indicated that the malic enzyme promoter of *S. paradoxus* RO88 differ in a number of nucleotides from those in the other *Saccharomyces* strains which could account for the difference in expression patterns observed. However, further investigation into the promoter sequences and putative regulatory elements of the *S. paradoxus* malic enzyme gene is required to determine their importance in the transcriptional regulation of the malic enzyme gene.

It is difficult to directly compare the expression results obtained in defined minimal medium with the observations from the fermentation studies in Chardonnay or synthetic grape must since two very different questions were addressed, i.e. (1) how do the strains perform in the degradation of malic acid during wine fermentation (i.e. over a number of days), and (2) are there any differences in the immediate response of the malic enzyme genes to changes in their physiological conditions (i.e. within the first 60 min.). For example, the results presented in Fig. 5.1 and 5.3 suggest that expression of the *S. paradoxus* RO88 malic enzyme gene in synthetic grape must with 0.8% malic acid was increased slightly after 24 hours and more profoundly upon glucose depletion after approx. 5 days. The latter suggests that the malic acid was utilised (i.e. converted to pyruvate via the malic enzyme) when glucose was no longer available. The regulatory studies (Fig. 5.5) indicated a transient increase in the *S. paradoxus* RO88 malic enzyme transcription upon a shift to 1% malic acid, which declined after 30 min. This could indicate an initial response to the malic acid, which was subsequently repressed by the 2% glucose present in the medium. If the expression of the

S. paradoxus gene was followed for a longer period of time, it is likely that the same effect would have been observed as in the fermentations studies, i.e. a stronger increase in expression of the malic enzyme gene upon glucose depletion.

```
MAE1_databank     CATCGTGCATTGCAAGGTTTTTCAAATTCTTGCGTCTTGTCAACCGTTCAAGGCGA      -548
MAE1_EC1118       CATCGTGCATTGCAAGGTTTTTCAAATTCTTGCGTCTTGTCAACCGTTCAAGGCGA
MAE1_71B          CATCGTGCATTGCAAGGTTTTTCAAATTCTTGCGTCTTGTCAACCGTTCAAGGCGA
MAE1_RO88         CATCGTGCATTGCAAGGTTTTTtAAATTCTTGCGTtTcGTCcACCGTTCAAGGCGA

MAE1_databank TTGAAAGATATTCACTGCATATCTTCTTATACGAAAAGCATCGAAGAAAAAACACCCATACA    -486
MAE1_EC1118   TTGAAAGATATTCACTGCATATCTTCTTATACGAAAAGCATCGAAGAAAAAACACCCATACA
MAE1_71B      TTGAAAGATATTCACTGCATATCTTCTTATACGAAAAGCATCGAAGAAAAAACACCCATACA
MAE1_RO88     TTaAgAGATATTCACTGCATATCTTCTTATACGAAAAGCATCGAA.AAAAAAgACCCATACA

MAE1_databank ACCAAGTATAGACGGAACAATTCGGGTTTTTACTCTTCCCTAGGCGGTTTAAATCGGATATA    -424
MAE1_EC1118   ACCAAGTATAGACGGAACAATTCGGGTTTTTACTCTTCCCTAGGCGGTTTAAATCGGATATA
MAE1_71B      ACCAAGTATAGACGGAACAATTCGGGTTTTTACTCTTCCCTAGGCGGTTTAAATCGGATATA
MAE1_RO88     ACacAcgccAttCGGAACAATTCGGGgcTTTACTCTTCCCTAGGCGGTTTAAATCGGATATA

MAE1_databank TG.AAAAGAAATCAAAAAAAAAAAAAAAAAAAAAAAAAGAATTGGCGCATTTGAAGTTTTATT    -362
MAE1_EC1118   TG.AAAAGAAATC.......AAAAAAAAAAAAAAAAAAGAATTGGCGCATTTGAAGTTTTATT
MAE1_71B      TG.AAAAGAAATC....AAAAAAAAAAAAAAAAAAAAAGAATTGGCGCATTTGAAGTTTTATT
MAE1_RO88     TGaAAAAGAAATC.tAAgA.AAAAAAAAAtcAAAAAAAGAATTGGCGCATTcGAtaTTTTATT

MAE1_databank ATCGTACGCGTTATTGTTTGCGCTAACATCGCATCGCATCAGCTGACTGAGTGACTGAGTCC    -300
MAE1_EC1118   ATCGTACGCGTTATTGTTTGCGCTAACATCGCATCGCATCAaCTGACTGAGTGACTGAGTCa
MAE1_71B      ATCGTACGCGTTATTGTTTGCGCTAACATCGCATCGCATCAaCTGACTGAGTGACTGgGTCC
MAE1_RO88     ATtGTACGCGTTATTGTTTGCGCTAACATCGCATCGCATCAaCTGACTGAGTGACTGAGTCC

MAE1_databank CCTTGGCTTCGACTCATCATCGCCTTTCTATGGTGAAAAATTTTCGCAATTTCCTATTACTG    -238
MAE1_EC1118   CCTTGGCTTCGACTCATCATCGCCTTTCTATGGTGAAAAATTTTCGCAATTTCCTATTACTG
MAE1_71B      CCTTGGCTTCGACTCATCATCGCCTTTCTATGGTGAAAAATTTTCGCAATTTCCTATTACTG
MAE1_RO88     CCTTGGCTTCGACTCATCATCGCCTTTCTATGGTGAAAAATTTTCGCAATTTCCTATTACTG

MAE1_databank TACCGCGTATGCTCCATTTGACTTCCTTTGGTCTACAGCTTTAGCGCTATAGAGTTTCGAAG    -176
MAE1_EC1118   TACCGCGTATGCTCCATTTGACTTCCTTTGGTCTACAGCTTTAGCGCTATAGAGTTTCGAAa
MAE1_71B      TACCGCGTATGCTCCATTTGACTTCCTTTGGTCTACAGCTTTAGCGCTATAGAGTTTCGAAa
MAE1_RO88     TACtGCGTATaCTCCATTTGACTTCCTTTGGTCTACAGCTTTAGCGCTATAGAcTTTCGAAa

MAE1_databank TCGTACCCGTTACCGGCATGATTGA.CATAT....TATATATATATATATAT..GCGTATCT    -114
MAE1_EC1118   TCGTACCCGTTACCGGCATGATTGA.CATATtataTATATATATATATATAT..GCGTATCT
MAE1_71B      TCGTACCCGTTACCGGCATGATTGA.CATATta..TATATATATATATATAT..GCGTATCT
MAE1_RO88     TCGTACCCGTTACCGGCtTGATTGtgCATATtataTATATATATATATATATagGCGTATCT

MAE1_databank TTATACTTACTCGTATATTGTGTCCAGCTTCGGATA.TTTGTGCTTTTGAAACCTACAACTT    -52
MAE1_EC1118   TTATACTTACTCGTATATTGTGTCCAGCTTCGGATA.TTTGTGCTTTTGAAACCTACAACTT
MAE1_71B      TTATACTTACTCGTATATTGTGTCCAGCTTCGGATA.TTTGTGCTTTTGAAACCTACAACTT
MAE1_RO88     gTATACTTACTCGTATAcTGcaTCtAGCTTttGAattTTTGTGCTTTTaAgAggTACAAaTT

MAE1_databank TTAACGAGTTTAGTGCACATAAAT.ACCAAGACAAAAGGTAGAAATACGGTT ATGCTTAGAA   10
MAE1_EC1118   TTAACGAGTTTAGTGCACATAAAT.ACCAAGACAAAAGGTAGAAATACGGTT ATGCTTAGAA
MAE1_71B      TTAACGAGTTTAGTGCACATAAAT.ACCAAGACAAAAGGTAGAAATACGGTT ATGCTTAGAA
MAE1_RO88     TTAACGAGTaTAcTGCAtATAAAacACCcAGgCAAAAGaTAGAAtTACGGTT ATGCTTAGAA

MAE1_databank CCAGACTATCCGTTTCCGTTGCTGCTAGATCGCAACTAACCAGATCCTTGACAGCATCAAGG    72
MAE1_EC1118   CCAGACTATCCGTTTCCGTTGCTGCTAGATCGCAACTAACCAGATCCTTGACAGCATCAAGG
MAE1_71B      CCAGACTATCCGTTTCCGTTGCTGCTAGATCGCAACTAACCAGATCCTTGACAGCATCAAGG
MAE1_RO88     CCAGACTATCCGTTTCgaTcGCTGCaAGgTCGCAACTAACCAGATCCTTGgCAGCATCtAGG

MAE1_databank ACAGCACCATTAAGAAGATGGCCTAT...TCAGCAATCGCGTTT                      134
MAE1_EC1118   ACAGCACCATTAAGAAGATGGCCTAT...TCAGCAATCGCGTTT
MAE1_71B      ACAGCACC......GAAGATGGCCTAT...TCAGCAATCGCGTTT
MAE1_RO88     ACgGCgCCATTAAaAAGATGGCCTATtcaTCAGCAATCGCGTTT..................
```

Figure 5.6. Alignment of the promoter sequences of *S. cerevisiae* 71B, *S. bayanus* EC1118, *S. paradoxus* strain RO88 and the published sequence of *S. cerevisiae MAE1*.

In this study we have shown that the *S. paradoxus* strain RO88 is able to effectively degrade malic acid in both synthetic grape and Chardonnay must. This strain was able to produce a wine of good quality and may proof to be of value for biological deacidification of wines. In contrast, little malic acid was removed by the *Saccharomyces cerevisiae* Lalvin strain 71B. The mechanism and proteins involved in the degradation of malic acid by *S. paradoxus* strain RO88 is yet unknown, but our results suggest that the malic enzyme may play an important role in enabling the yeast to respond to different physiological conditions, e.g. the available carbon source. Preliminary studies on the transcriptional regulation indicated that expression of the malic enzyme gene from *S. paradoxus* RO88 and *S. cerevisiae* 71B increased towards the end of fermentation once glucose was depleted, whereas no effect was observed with *Saccharomyces bayanus* EC1118, a non-degrading strain. However, only *S. paradoxus* RO88 showed a further 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.

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ADDENDUM A

A.1 INTRODUCTION

Various yeast species found on grapes (Boulton *et al.*, 1996) and on winery surfaces (Amerine and Singleton, 1965) partake in spontaneous wine fermentations. Yeast genera such as *Hanseniaspora*, *Candida* and *Kloeckera* typically prevail during the early stages of wine fermentations, followed by several species of *Metschnikowia* and *Pichia* in the middle stages when the ethanol level increases to 3-4% (Pretorius *et al.*, 1999). Other yeast species such as *Hansenula*, *Brettanomyces/Dekkera*, *Debaromyces*, *Kluyveromyces*, *Torulopsora* and *Saccharomycodes* may also play a role during spontaneous wine fermentations (Boulton *et al.*, 1996; Fleet, 1993; Lafon-Lafourcade, 1983; Snow, 1983). The composition of yeast species in wine originates from the grapes, which in turn is determined by the climate, altitude of the vineyard, the type of nitrogen fertilisation, fungicide control methods, presence of insect vectors and waste disposal practices of wineries (Boulton *et al.*, 1996).

The latter stages of spontaneous alcoholic fermentation are usually dominated by different strains of the *Saccharomyces sensu stricto* group of yeast, which are more resistant to higher levels of ethanol and produce typical wine flavours (Amerine *et al.*, 1972; Boulton *et al.*, 1996; Fleet, 1993). To date, the *Saccharomyces sensu stricto* yeast group contains at least six species, namely *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces paradoxus* and the recently isolated *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*, as well as one hybrid taxon *S. pastorianus* (synonym *S. carlsbergensis*) (Naumov, 1996, 2000; Naumov *et al.*, 2000[a, b]; Vaughan-Martini and Martini, 1998). All the yeasts in the *Saccharomyces senso stricto* group contain at least 16 distinctive nuclear chromosomes of small, medium and large sizes, while each species displays a unique karyotype (Vaughan-Martini *et al.*, 1993). More importantly, it is believed that the chromosomes of the different *Saccharomyces senso stricto* yeasts are homologous, i.e. the order of genes is largely preserved among different species (Hunter *et al.*, 1996; Ryu *et al.*, 1996).

Over the years the classification of the wine yeast has been refined from a wide variety of species or varieties such as *Saccharomyces uvarum*, *Saccharomyces ellipsoideus*, *Saccharomyces vini*, *Saccharomyces fermenti* and the galactose non-fermenting yeast *Saccharomyces oviformis* to only one yeast species, namely *S. cerevisiae* (Kunkee and Goswell, 1977; Lodder, 1970; Vaughan-Martini and Martini, 1998). The majority of commercial wine yeasts are strains of *S. cerevisiae*, while some commercial wine yeast strains belongs to the species *S. bayanus*. Although some confusion exists among winemakers about the identity of *S. bayanus* strains (Masneuf *et al.*, 1996), these strains can

usually be distinguished from *S. cerevisiae* through their distinctive fermentation properties, i.e. some *S. bayanus* strains are cryophilic with a higher growth rate and better fermentation at low temperatures compared to *S. cerevisiae* (Kishimoto and Goto, 1995; Masneuf *et al.*, 1998). Recently, *S. uvarum* has once again been delineated as a subgroup of *S. bayanus*, i.e. *S. bayanus* var. *uvarum*, based on molecular fingerprinting, electrophoretic karyotype profile, distinctive biochemical profile and high sporulation ability (Gouliamova and Hennebert, 1998; Masneuf *et al.*, 1996; Montrocher. *et al.*, 1998; Naumov *et al.*, 2000[a]; Nguyen and Gaillardin, 1997; Pulverenti *at al.*, 2000; Rainieri *et al.*, 1999). Strains of *S. cerevisiae* are almost exclusively associated with man-made fermentation environments, i.e. they are usually absent or rare on grapes, but frequent inhabitants of wineries and have become known as "*wine yeast*" as they are universally favoured for initiating wine fermentations.

In contrast to the strong association of *S. cerevisiae* and *S. bayanus* with winemaking, strains of *S. pastorianus* are usually found in the production of lager beers (Rodrigues de Sousa *et al.*, 1995; Turakainen *et al.*, 1993; Vaughan-Martini and Martini, 1987), while strains of *S. paradoxus* are not associated with either wine or beer production (Redzepovic *et al.*, 2002). Strains of *S. paradoxus*, which are often associated with plant disease, is usually found in exudates of broad-leaved trees, insects and uncultivated soils (Naumov, 1999; Naumov *et al.*, 1992, 1994, 1997, 1998, 2000[b]). However, it was recently isolated in high numbers from Croatian vineyards (Redzepovic *et al.*, 2002). Strains of *S. paradoxus* appear to be present on most continents, but exhibit genetic and reproductive differentiation (Naumov, 1996, 1999), i.e. *S. paradoxus* strains collected from different geographical regions showed relatively little allozyme variability (Naumov *et al.*, 1997), but on a DNA sequence level large variations was found between different strains of *S. paradoxus*, as illustrated by DNA fingerprinting methods (Naumov *et al.*, 2000[b]) and hybridisation profiles (Cliften *et al.*, 2001; Naumov *et al.*, 1992, 1994, 1998, 2000[b]; Redzepovic *et al.*, 2002).

A.2 GENETIC RELATIONSHIP BETWEEN *S. CEREVISIAE* AND *S. PARADOXUS*

Based on DNA re-association studies, *S. paradoxus* is regarded as the natural parent species of *S. cerevisiae* (Nau *et al.*, 1996; Redzepovic *et al.*, 2002; Vaughan-Martini, 1989; Vaughan-Martini and Martini, 1998; Young *et al.*, 2000). The close relation between *S. cerevisiae* and *S. paradoxus* is also indicated by the fact that fructose transport mechanisms are absent in both these yeast, whereas strains of *S. bayanus* and *S. pastorianus* contain proton-symport apparatus for fructose transport (Tornai-Lehoczki *et al.*, 1996).

When the amino acid sequence of the malic enzymes of *S. cerevisiae*, *S. paradoxus* and *S. bayanus* was compared (*Saccharomyces* Genome Database), the protein alignments clearly showed a higher

degree of homology (98 %) between the *S. cerevisiae* and *S. paradoxus* malic enzymes, compared to that of *S. cerevisiae* and *S. bayanus* (94 %) (Fig A1). In-depth analysis of the amino acid sequence of the *S. pombe*, *S. cerevisiae*, *S. paradoxus* and *S. bayanus* malic enzymes, indicated a high degree of conservation between all the *Saccharomyces* malic enzymes with regard to the previously identified homologous regions (Box A-H) found in all malic enzymes (see Chapter 3) (Viljoen *et al.*, 1994). These conserved regions are thought to play essential roles in the enzymatic functioning of the malic enzyme, while the linker regions between regions A-H are conserved in length, but not in amino acid composition. A closer look at these homologous regions indicates that there is an almost 100% homology between the malic enzymes of *Saccharomyces* species, with the exception of two amino acid changes in the malic enzyme from *S. bayanus* within region E and H. Furthermore, the amino acid variations between the *S. cerevisiae* and *S. paradoxus* malic enzyme are positioned within the linker regions of the protein and not the functional domains of the protein. Strains of *S. paradoxus* and *S. cerevisiae* has been shown to have divergent abilities to degrade L-malic acid, however, this is not corroborated in the amino acid sequences of these two proteins (Fig. A.1).

Recently, the close phylogenetic relationship between *S. cerevisiae* and *S. paradoxus* has become the motivation for evaluating strains of *S. paradoxus* for their enological characteristics and potential as a winemaking yeast (Majdak *et al.*, 2002). Strains of *S. paradoxus* were found to have excellent vinification characteristics such as a vigorous fermentation of grape sugars, high ethanol tolerance, lack of off-flavour production, as well as low H_2S production. The major differences in wine produced by *S. paradoxus* and *S. cerevisiae* were the amount of volatile components, i.e. comparative analysis between wine made by *S. paradoxus* and *S. cerevisiae* revealed that *S. paradoxus* strains are prone to produce lower amounts of higher alcohols but higher amounts of volatile esters, which has a major influence on the wine aroma (Majdak *et al.*, 2002; Redzepovic *et al.*, 2002).

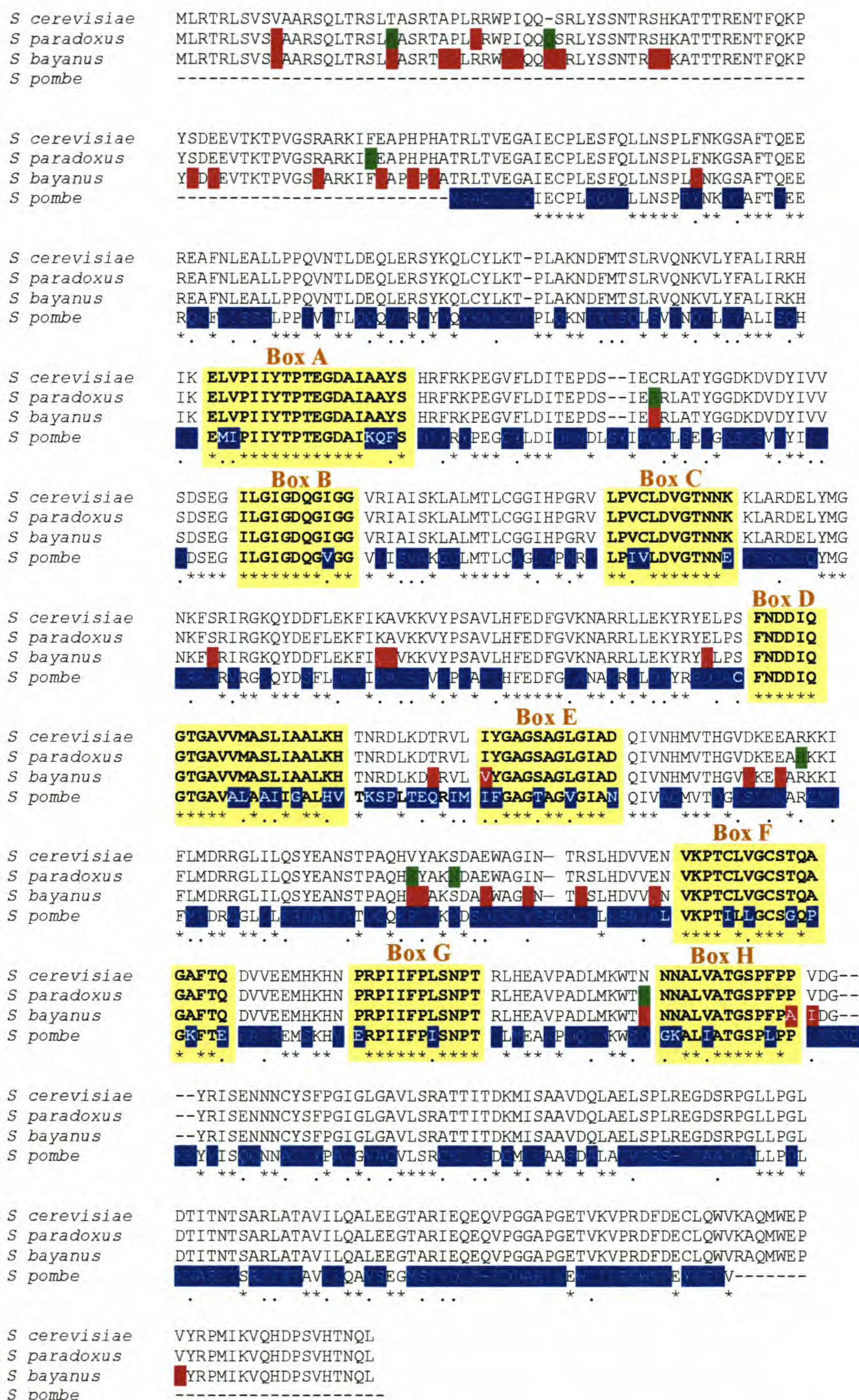

Figure A.1. Amino acid sequence alignment of the malic enzymes from *S. cerevisiae*, *S. paradoxus*, *S. bayanus* and *S. pombe*. Homologous regions A-H are indicated by the yellow boxes, while amino acid changes are indicated in blue for *S. pombe*, in green for *S. paradoxus*, and in red for *S. bayanus*.

A.3 PCR-AMPLIFICATION TO ISOLATE THE *MAE1* PROMOTER REGION FROM SELECTED *SACCHAROMYCES* STRAINS

The promoter region of the malic enzyme gene (*MAE1*) from *S. paradoxus* RO88, *S. bayanus* EC1118 and *S. cerevisiae* 71B were PCR amplified based on the nucleotide sequence of the *MAE1* gene of *S. cerevisiae* (*Saccharomyces* Genome Database) (Fig. A.2). The 5' primer, SCMAE1-F (5'-CATCGTGCATTGCAAGGTTT-3') anneals to nucleotide -594 of the *MAE1* gene promoter and the downstream primer, SCMAE1-R (5'-GAATATAAACGCGATTGCTGA-3'), anneals to nucleotide +94 inside the *MAE1* open reading frame. Genomic DNA from *S. paradoxus* RO88, *S. bayanus* EC1118 and *S. cerevisiae* 71B was isolated using a glass bead-phenol extraction method (Hoffman and Winston, 1987), and approximately 1 μg was used as template for PCR amplification. The PCR program consisted of an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of 94°C for 45 seconds, 58°C for 1 minute and 72° for 1.5 minutes.

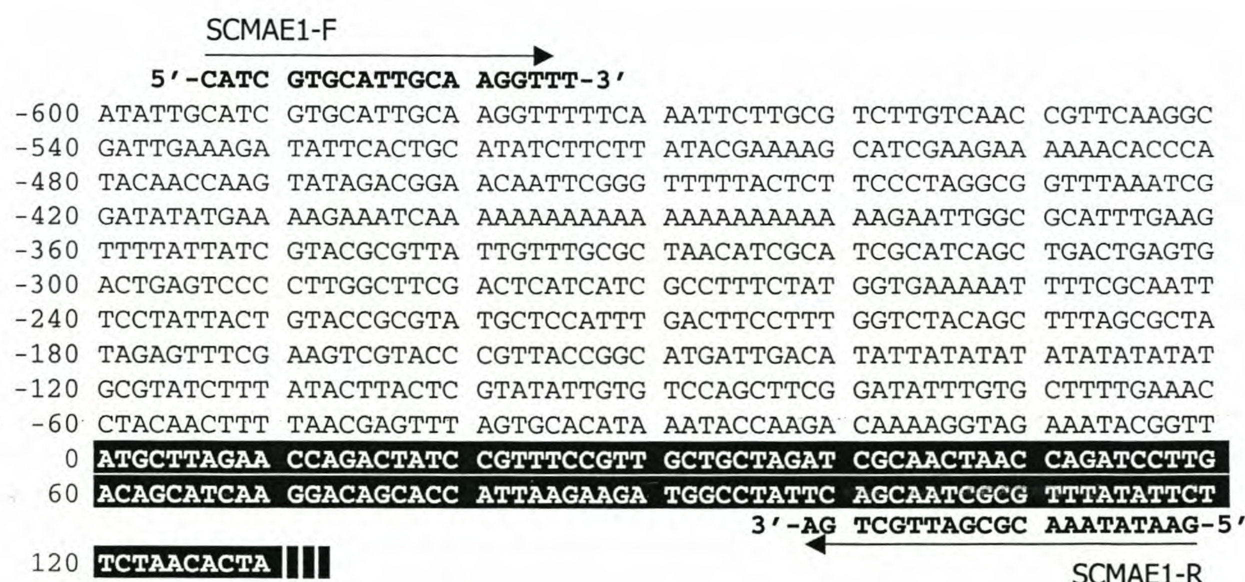

Figure A.2. Nucleotide sequence of the promoter region of the *S. cerevisiae MAE1* gene used for designing the PCR amplification primers, i.e. SCMAE1-F and SCMAE1-R, for subcloning and sequencing of the *MAE1* promoter region of *S. paradoxus* R088, *S. cerevisiae* 71B and *S. bayanus* EC1118. The *MAE1* open reading frame is indicated by shaded area.

A 710 bp fragment was obtained from all three *Saccharomyces* strains and these fragments were purified by gel electrophoresis and subcloned using the p-GEM®-T Easy Vector System (Promega Corporation, Madison). Three subclones of each strain were submitted for automated sequencing using the M13 forward and M13 reverse primers. The DNA sequence of the promoter fragments from

S. paradoxus RO88, *S. cerevisiae* 71B and *S. bayanus* EC1118 were compared with that of the published sequence of the *S. cerevisiae MAE1* gene using DNAMAN version 4.13 (Lynnon Biosoft.).

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CHAPTER 6

RESEARCH RESULTS

Malo-Ethanolic Fermentation in Grape Must by Recombinant Strains of *Saccharomyces cerevisiae*

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Malo-Ethanolic Fermentation in Grape Must by Recombinant Strains of *Saccharomyces cerevisiae*

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ABSTRACT

Recombinant strains of *Saccharomyces cerevisiae* with the ability to reduce wine acidity could have a significant influence on the future production of quality wines, especially in cool climate regions. L-Malic acid and L-tartaric acid contribute largely to the acid content of grapes and wine. The wine yeast *S. cerevisiae* is unable to effectively degrade L-malic acid, whereas the fission yeast *Schizosaccharomyces pombe* efficiently degrades high concentrations of L-malic acid by means of a malo-ethanolic fermentation. However, strains of *S. pombe* are not suitable for vinification due to the production of undesirable off-flavours. Heterologous expression of the *S. pombe* malate permease (*mae1*) and malic enzyme (*mae2*) genes on plasmids in *S. cerevisiae* resulted in a recombinant strain of *S. cerevisiae* which efficiently degraded up to 8g/l L-malic acid in synthetic grape must and 6.75 g/l L-malic acid in Chardonnay grape must. Furthermore, a strain of *S. cerevisiae* containing the *mae1* and *mae2* genes integrated in the genome efficiently degraded 5 g/l of L-malic acid in synthetic and Chenin Blanc grape must. The malo-ethanolic strains produced higher levels of ethanol during fermentation which is important for the production of distilled beverages.

6.1 INTRODUCTION

Wine acidity and pH play an important role in the organoleptic quality and shelf life of wine. L-Tartaric acid and L-malic are the most prominent grape acids, contributing to more than 90% of the titratable acidity in wine (Beelman and Gallander, 1979; Radler, 1993; Henick-Kling, 1993; Gao and Fleet, 1995). The production of premium wines depends on the oenologist's skill to accurately adjust wine acidity to obtain a balanced wine with optimum flavour and colour profile. In the warmer wine regions of Australia, South Africa, California and southern Europe, acidulating agents such as L-tartaric acid and D/L-malic acid are routinely added prior to fermentation to increase the titratable acidity of must (Beelman and Gallander, 1979; Boulton *et al.*, 1996). However, in the cooler wine regions of northern Europe, eastern United States and Canada, cold stabilisation and malolactic fermentation are necessary to decrease the levels of L-tartaric and L-malic acid in the final product.

Strains of the lactic acid bacterium *Oenococcus oeni* are used to perform the malolactic fermentation in wine during which L-malic acid is converted to L-lactic acid and CO_2 (Wibowo *et al.*, 1985; Van Vuuren and Dicks, 1993). Malolactic fermentation sufficiently decreases the acidic taste of wine, improves the microbial stability and modifies the organoleptic profile of the wine. However, stuck or sluggish malolactic fermentation often causes delays in cellar operations such as sulphiting, which may result in the chemical oxidation and spoilage of wine as well as the production of biogenic amines by spoilage organisms (Lonvaud-Funel and Joyeux, 1994; Straub *et al.*, 1995). Even with the use of starter cultures, malolactic fermentation may only be completed weeks or months after alcoholic fermentation (Henick-Kling, 1995). Factors such as pH, sulphur dioxide, ethanol, temperature, nutritional status of the wine and interactions with other wine flora synergistically influence the onset and completion of malolactic fermentation (Beelman and Gallander, 1979; Boulton *et al.*, 1996; Davis *et al.*, 1985; Henick-Kling, 1993). Furthermore, malolactic fermentation is usually undesirable in the production of the fruity-floral cultivars such as Sauvignon Blanc, Riesling and Gewurztraminer. The varietal flavours in these wines are essential to their aromatic character and are adversely modified during malolactic fermentation (Radler, 1972; Wagner, 1974).

Yeast species capable of utilizing tricarboxylic acid (TCA) cycle intermediates, such as L-malic acid, are classified into two groups: K (+) yeasts utilize TCA intermediates as sole energy and carbon source, while K (–) yeasts can only utilise TCA cycle intermediates when glucose or other fermentable carbohydrates are present. The K (-) group includes yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Zygosaccharomyces balii* (Baranowski and Radler, 1984; Kuczynski and Radler, 1982; Rodriquez and Thornton, 1989). However, wine yeast strains of *S. cerevisiae* cannot efficiently utilise L-malic acid during alcoholic fermentation (Subden *et al.*, 1998; Volschenk *et al.*, 1997[b]). The inefficient degradation of L-malic acid by *S. cerevisiae* is ascribed to the slow uptake of L-malic acid by diffusion (Ansanay *et al.*, 1996; Baranowski and Radler, 1984; Volschenk *et al.*, 1997[a,b]) and the low substrate affinity of its malic enzyme (K_m = 50 mM) (Fuck *et al.*, 1973). Furthermore, transcriptional analysis of the *S. cerevisiae* malic enzyme gene (*MAE1)*, indicated the gene is expressed at relatively low but constitutive levels (Boles *et al.*, 1998).

In contrast to *S. cerevisiae,* the fission yeast *S. pombe* can efficiently degrade up to 29 g/l of L-malic acid (Taillandier *et al.*, 1988; Taillandier and Strehaiano, 1991). Cells of *S. pombe* actively transport L-malic acid via a H^+-symport system (Sousa *et al.*, 1992) provided by the malate permease encoded by the *mael* gene (Grobler *et al.*, 1995). Intracellularly, *S. pombe* decarboxylates L-malic acid to pyruvate and CO_2 by means of a cytosolic malic enzyme encoded by the *mae2* gene (Viljoen *et al.*, 1994). Under fermentative conditions, pyruvate is further metabolised to ethanol and CO_2 (Mayer and Temperli, 1963; Osothsilp and Subden, 1986), resulting in the so-called malo-ethanolic fermentation. Although strains of *S. pombe* have been used for the degradation of L-malic acid in grape must, it is unsuitable for the fermentation of wine due to the production of off-flavours and the higher

fermentation temperatures required (Beelman and Gallander, 1979; Carré *et al.*, 1983; Gallander, 1977; Radler, 1993).

We have cloned and co-expressed the *mae1* and *mae2* genes responsible for the malo-ethanolic fermentation in *S. pombe* in a laboratory strain of *S. cerevisiae*. Since the native promoters of these genes are not recognised by *S. cerevisiae*, the genes were expressed under control of the constitutive 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences of *S. cerevisiae*. The recombinant yeast strain rapidly degraded L-malic acid in synthetic and Chardonnay grape must and consistently produced higher levels of ethanol in the presence of malic acid, relative to the control yeast strain. The *mae1* and *mae2* genes were also integrated in the *ILV2* locus of *S. cerevisiae* genome to overcome possible artefacts due to the instability of 2μm plasmids. Recombinant strains of *S. cerevisiae* containing a single genomic copy of the *mae1* and *mae2* genes effectively degraded L-malic acid in synthetic and Chenin Blanc grape must and compared well with the malolactic yeast strains previously reported (Volschenk *et al.*, 1997[a]).

6.2 MATERIALS AND METHODS

6.2.1 Strains and maintenance

The bacterial and yeast strains and plasmids used in this study are listed in Table 6.1. Cells of *E. coli* JM109 were transformed by electroporation and selected on LB medium supplemented with ampicillin (Ausubel *et al.*, 1995). Cells of *S. cerevisiae* were cultured in liquid YPD media at 28°C and competent cells (LiOAc method) were transformed or co-transformed with plasmids pHVX2, pHV3 and pHV7 (Table 6.1). Transformants were isolated on selective YNB agar plates [Difco Laboratories, Detroit, MI] supplemented with amino acids as required. The transformants were cultured to high cell density in 50 ml selective YNB media (0.17% Yeast Nitrogen Base [Difco Laboratories, Detroit, MI], 0.5% $(NH_4)_2SO_4$, 2% D-glucose, 0.13% of drop-out amino acid pool [Ausubel *et al.*, 1995] at 28^{0}C, harvested by centrifugation and resuspended in 5 ml sterile grape juice before inoculation into grape must.

6.2.2 Plasmid construction

Standard recombinant DNA techniques were performed essentially as described by Ausubel *et al.* (1995). All subcloning and DNA manipulations, except the construction of the integration plasmid pHVS2, were performed in the 2μ-based plasmids YEplac181 and YEplac195 (Gietz and Sugino, 1988). The construction of the expression vectors pHVX2 and pHV3 (Table 6.1) were previously described (Volschenk *et al.*, 1997[a,b]). The *PGK1*p-*mae2*-*PGK1t* expression cassette from pHV4 (Volschenk *et al.*, 1997[b]) was subcloned as a *Hin*dIII fragment into YEplac195, resulting in pHV7.

Table 6.1. Strains and plasmids used in the genetic construction of malo-ethanolic strains of *S. cerevisiae*.

Strains	Description	Reference
E. coli **JM109**	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 [r_k-, m_k+], *relA*1, *supE*44, λ^-, Δ(*lac-proAB*), [F', *traD*36, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15]	(Yanisch-Perron, 1985)
S. cerevisiae **YPH259**	*MAT*α, *ura3-52*, *lys2-801*^{amber}, *ade2-101*^{ochre}, *his3*Δ*200*, *leu2*-Δ*1*	(Sikorski and Hieter, 1989)

Plasmids	Description	Reference
pHVX2	YEplac181 (*LEU2* marker gene) containing the *PGK1* promoter and terminator sequences..	(Volschenk *et al.*, 1997 [a,b])
pHV3	pHVX2 containing the *mae1* ORF subcloned between the *PGK1* promoter and terminator sequences.	(Volschenk *et al.*, 1997[a,b])
pHV7	YEplac195 (*URA3* marker gene) containing the *mae2* ORF subcloned between the *PGK1* promoter and terminator sequences.	This study
pHVS2	pBluescript KS+ containing the *SMR1*-410 gene. The *PGK1*p-*mae1-PGK1*t and *PGK1*p-*mae2-PGK1*t expression cassettes were subcloned in the terminator region of the *SMR*1 gene.	This study

6.2.3 Integration of *mae1* and *mae2* genes in *S. cerevisiae*

Integration of the *mae1* and *mae2* genes into *S. cerevisiae* genome was obtained by selecting for resistance to the herbicide sulfometuron methyl (SMM) via the *SMR1-410* gene (Casey *et al.*, 1988). The *SMR1-410* gene was subcloned from pWX509 (Casey *et al.*, 1988) by *Kpn*I-*Bam*HI digestion into pBluescript KS+ to yield pDLG42 (provided by Dr. DC la Grange, Dept of Microbiology, University of Stellenbosch). The *PGK1*p-*mae2-PGK1*t cassette from pHV7 was subcloned as a *Hin*dIII fragment into the *SMR1* terminator region in pDLG42 (Fig. 6.1), while *PGK1*p-*mae1-PGK1*t was subcloned as a *Pvu*II fragment from pHV3 into the blunt-ended *Nde*I site of the *SMR1* terminator region in pDLG42 to yield pHVS2 (Fig. 6.1). pHVS2 was linearised with *Apa*I and transformed into LiOAc competent cells of *S. cerevisiae* YPH259. Transformants were selected on YNB agar plates [Difco Laboratories, Detroit, MI] containing 200μg/ml SMM and supplemented with all amino acids except isoleucine and valine. Transformants were maintained on YPD plates for more than 200 generations.

6.2.4 Pulse-field gel electrophoresis and Southern blotting

S. cerevisiae YPH259 and the integrated malo-ethanolic strain of *S. cerevisiae* was grown in 200 ml YPD overnight. Chromosomal DNA plugs were prepared by lyticase enzyme treatment (Boehringer Mannheim, Germany) and the chromosomes separated by counter-clamped homogeneous electric field (CHEF) electrophoresis as described by van der Westhuizen and Pretorius (1992). Standard

procedures (Ausubel *et al.*, 1995) were used to prepare the gel for Southern blotting and to transfer the DNA to a positively charged nylon membrane (Boehringer Mannheim, Germany). An internal 622 bp *Xho*I DNA fragment of *mae1*, 864 bp *Eco*RI/*Eco*RV DNA fragment of *mae2* and a internal 900 bp *Bgl*II DNA fragment of *ILV2* was labelled with [α-^{32}P]dCTP by using the random-primed DNA-labelling kit (Boehringer Mannheim, Germany) and used as probes to detect the *mae1*, *mae2* and *ILV2* genes.

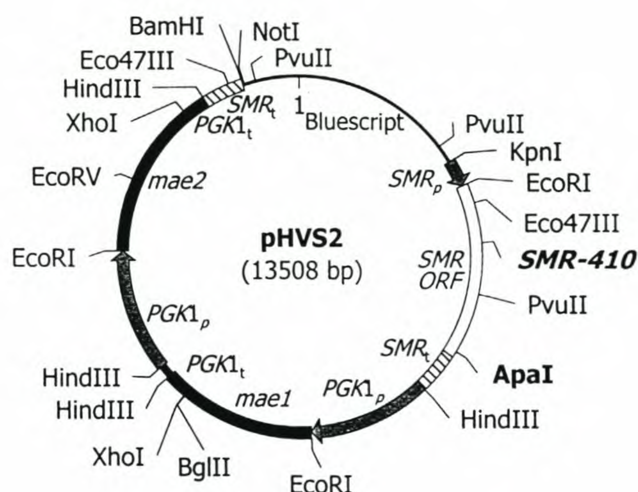

Figure 6.1. Plasmid map of integration plasmid pHVS2. The *PGK*1$_p$–*mae2*–*PGK*1$_t$ fragment was cloned as a *Hin*dIII fragment into the *SMR*1 terminator region in pDLG42. The *PGK*1$_p$–*mae1*–*PGK*1$_t$ fragment was cloned as *Pvu*II fragment in the *Nde*I site (blunt-ended) in the terminator region of *SMR*1 in pDLG42. Digestion with *Apa*I linearised the plasmid in the *SMR*1 ORF region.

6.2.5 Malo-ethanolic fermentation in grape must

The synthetic grape must consisted of 0.17% YNB (without amino acids and ammonium sulphate), 0.5% $(NH_4)_2SO_4$, 10% glucose, 8 or 5 g/l L-malic acid and amino acids supplemented as required. The pH was adjusted to 3.3 with 1N KOH. The recombinant strains of *S. cerevisiae* were inoculated to a final concentration of 2 X 10^6 cells/ml in 100 ml synthetic grape must in 250 ml Erlenmeyer flasks and incubated at 28°C while shaking.

Chardonnay must (6.75 g/l L-malic acid, pH 3.29) and Chenin Blanc must (5 g/l L-malic acid, pH 3.42) were also inoculated with 2 X 10^6 cells/ml into 800 ml must in 1l flasks and incubated at 22°C without aeration. The Chardonnay and Chenin Blanc grape must were supplemented with 0.075% diammonium phosphate before inoculation to ensure a sufficient nitrogen source during fermentation. The concentration of L-malic acid, D-glucose and ethanol were measured at regular intervals during the fermentation using enzymatic assays (Roche Diagnostics, Germany).

6.3 RESULTS AND DISCUSSION

Although both *S. cerevisiae* and *S. pombe* are classified as K (-) yeasts, their ability to degrade L-malic acid differs significantly. The method of L-malic acid uptake contributes largely to this phenomenon: cells of *S. cerevisiae* rely on simple diffusion for the uptake of L-malic acid, whereas *S. pombe* actively transports L-malic acid via a H^+-malate symporter. The malic enzyme of *S. cerevisiae* has strong homology with the malic enzyme of *S. pombe*, but they differ significantly in their co-factor specificity, substrate affinity and subcellular localisation (Boles *et al.*, 1998). The malic enzyme of *S. cerevisiae* requires either $NADP^+$ or NAD^+ as electron acceptor and is located in the mitochondria whereas the cytosolic malic enzyme from *S. pombe* is NAD^+-specific (Fig. 6.2). Furthermore, the affinity (K_m = 50 mM) (Fuck *et al.*, 1973) of the *S. cerevisiae* malic enzyme is much lower for the substrate L-malic acid than the malic enzyme from *S. pombe* (K_m = 3.2 mM) (Temperli *et al.*, 1965).

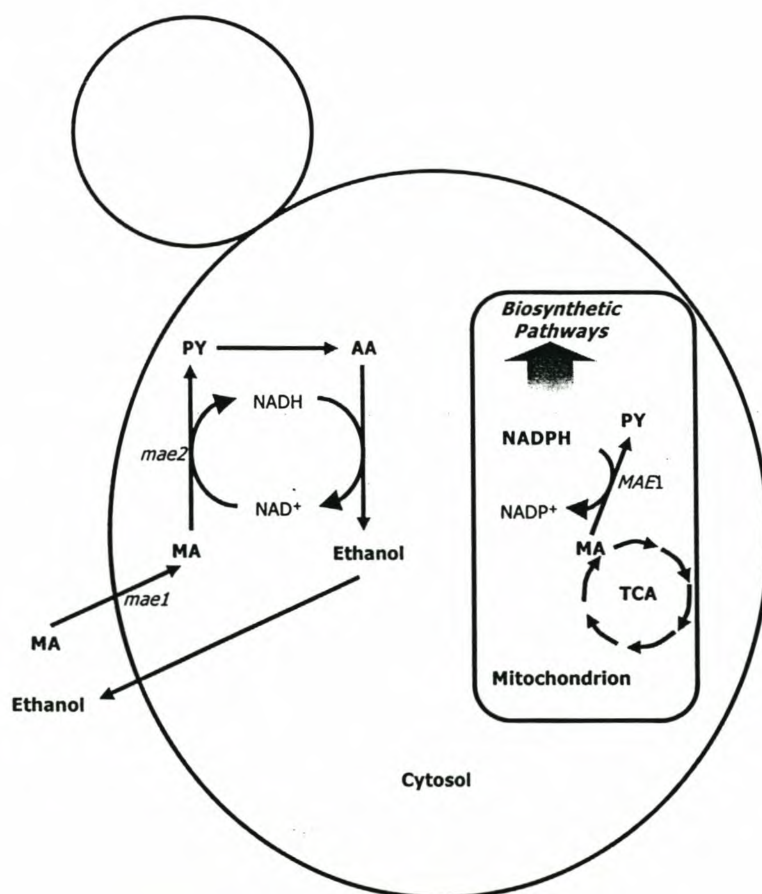

Figure 6.2. A schematic representation of the pathway for L-malic acid degradation in *S. cerevisiae* with the newly introduced malo-ethanolic pathway from *S. pombe*. *MAE1*, malic enzyme gene of *S. cerevisiae*; *mae1*, *S. pombe* malate permease gene and *mae2*, *S. pombe* malic enzyme gene. MA = malic acid, PY = pyruvic acid, AA = acetaldehyde, TCA = tricarboxylic acid cycle.

These data strongly suggest that the malic enzyme of *S. cerevisiae* plays an entirely different physiological role than that of *S. pombe*. In *S. cerevisiae*, malic acid is mainly metabolised through malate dehydrogenase and the oxidative reactions of the TCA cycle. It has been suggested that the *S. cerevisiae* malic enzyme plays a role in providing intra-mitochondrial NADPH or pyruvate for biosynthetic pathways under anaerobic conditions (Fig. 6.2) (Boles *et al.*, 1998). The role of the highly efficient degradation of L-malic acid by the yeast *S. pombe* is somewhat enigmatic, since L-malic acid is not incorporated into biomass, but stoichiometrically converted to ethanol and CO_2 under anaerobic conditions (Taillandier *et al.*, 1988; Taillandier and Strehaiano, 1991; Subden *et al.*, 1998). Analysis of the transcriptional regulation of the malic enzyme gene of *S. pombe* suggests that this enzyme may help to maintain the redox potential under fermentative conditions (Viljoen *et al.*, 1999).

Winemakers rely on malolactic fermentation to balance the acidity levels of wine after alcoholic fermentation. However, the malolactic fermentation is often erratic and difficult to manage. The use of genetically improved strains of *S. cerevisiae* with the ability to reduce L-malic acid levels during alcoholic fermentation could be of great benefit to winemakers. We previously reported the construction of a malolactic strain of *S. cerevisiae* able to efficiently carry out malolactic fermentation by co-expressing the malate permease gene (*mae1*) of *S. pombe* and the malolactic enzyme gene (*mleS*) of *Lactococcus lactis* (Volschenk *et al.*, 1997[a]). Since malolactic fermentation is, however, not always desired in certain cultivars we investigated an alternative pathway to reduce the levels of L-malic acid in these wines. This pathway uses the *S. pombe* malate permease and malic enzyme to perform a so-called malo-ethanolic fermentation.

Recombinant strains of *S. cerevisiae* containing both the *S. pombe mae1* and *mae2* genes were constructed by co-transformation of plasmid pHV3 and pHV7 into *S. cerevisiae* YPH259. The malo-ethanolic fermentation by the recombinant *S. cerevisiae* strain (MEF) was completed within 3 days in synthetic grape must containing 8 g/l of L-malic acid, while 6.75 g/l of L-malic acid was fully degraded within 11 days in Chardonnay grape must (Fig. 6.3). In contrast, the control yeast strain containing only the *PGK1*-expression cassette (plasmid pHVX2) was not able to degrade the malic acid present in the media. Furthermore, the recombinant strain containing only the *mae2* expression cassette (plasmid pHV7) had no effect on the levels of L-malic acid (results not shown). This confirmed the essential contribution of the *S. pombe* malate permease for the effective degradation of malic acid in *S. cerevisiae*.

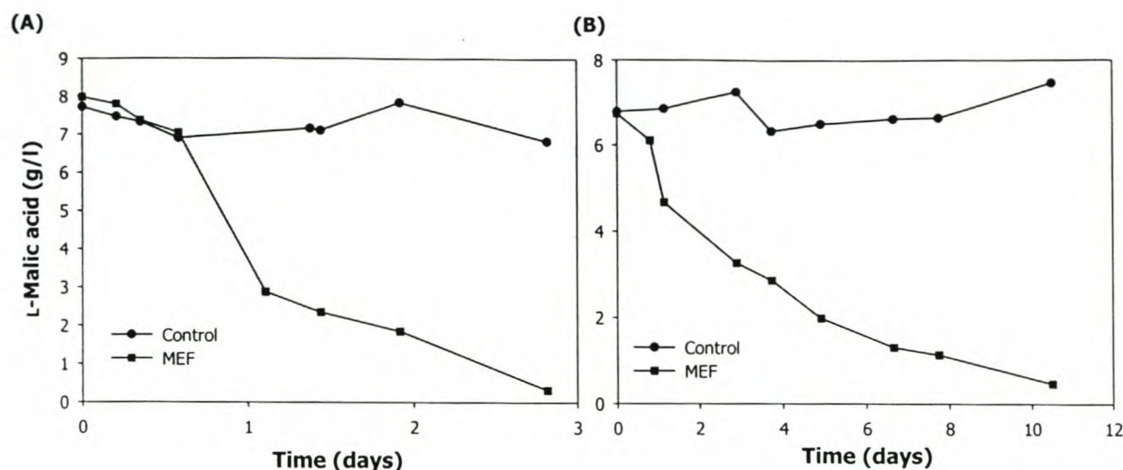

Figure 6.3. Malo-ethanolic fermentation in (A) synthetic grape must containing 8 g/l L-malic acid, or (B) Chardonnay grape must containing 6.75 g/l L-malic acid. The recombinant strain of *S. cerevisiae* (MEF) contained the *S. pombe mae1* and *mae2* genes (■), whereas the control yeast contained only the pHVX2 expression vector (●).

In *S. pombe*, the malic enzyme catalyses the oxidative decarboxylation of L-malic acid to L-pyruvate. Under fermentative conditions, pyruvate is further metabolised to ethanol and CO_2 by alcohol dehydrogenase (Maconi *et al.*, 1984). The malo-ethanolic strain of *S. cerevisiae* (MEF) containing the malate transport (*mae1*) and malic enzyme (*mae2*) genes from *S. pombe*, consistently produced higher levels of ethanol, relative to the control strain (Fig. 6.4). This confirmed that the two *S. pombe* genes enabled cells of *S. cerevisiae* to metabolise the extracellular L-malic acid to ethanol under fermentative conditions. The *S. pombe mae1* and *mae2* expression cassettes were successfully integrated in the *S. cerevisiae* genome.

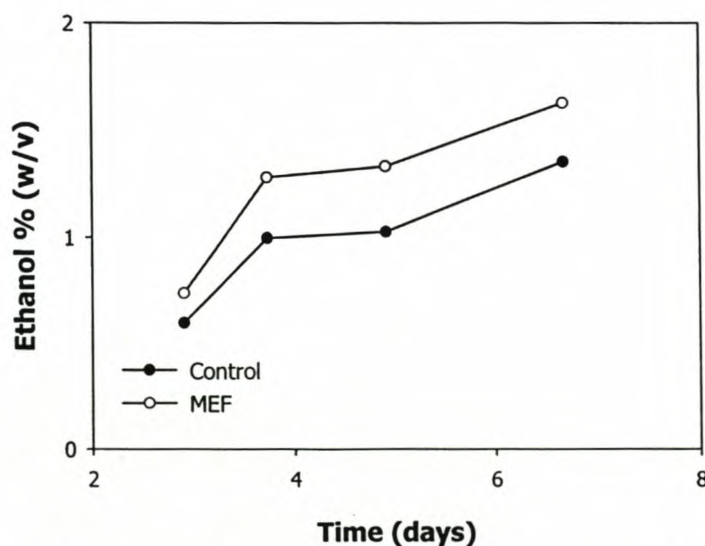

Figure 6.4. Ethanol production by the malo-ethanolic strain of *S. cerevisiae* containing the *S. pombe mae1* and *mae2* genes (O) in a Chardonnay grape must, compared to the control yeast strain containing only plasmid pHVX2 (●).

Southern analysis of the CHEF gel confirmed that the *mae1* and *mae2* genes are located on the same chromosome (Chr.XIII) as the *ILV2* gene (Fig 6.5). Cells of *S. cerevisiae* (MEF) containing the integrated *mae1* and *mae2* genes efficiently degraded 5g/l L-malic acid within 34 hours and 10 days in a synthetic and Chenin Blanc grape must, respectively (Fig. 6.6). Cells of *S. cerevisiae* containing single copies of the *mae1* and *mae2* genes compared well with those containing multiple copies of the same genes. Results suggest that single copy expression of the *mae1* and *mae2* genes of *S. pombe* under the control of the *S. cerevisiae PGK1* promoter and terminator is sufficient for rapid degradation of L-malic acid and have the potential to be applied in commercial wine yeast strains.

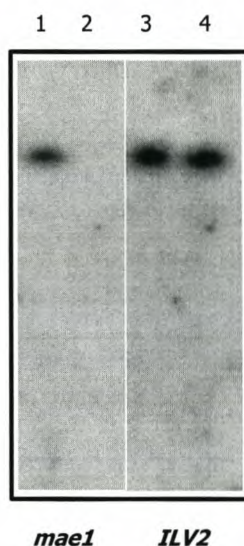

Figure 6.5. Chromosomal blotting of the *mae1*, *mae2* and *ILV2* genes. The chromosomes of *S. cerevisiae* YPH259 (lane 2, 4) and the integrated malo-ethanolic strain of *S. cerevisiae* (MEF) (lane 1, 3) were separated on a CHEF gel and probed with the internal 622 bp *Xho*I DNA fragment of *mae1*, an internal 864 bp *Eco*RI/*Eco*RV DNA fragment of *mae2* and a internal 900 bp *Bgl*II DNA fragment of *ILV2*.

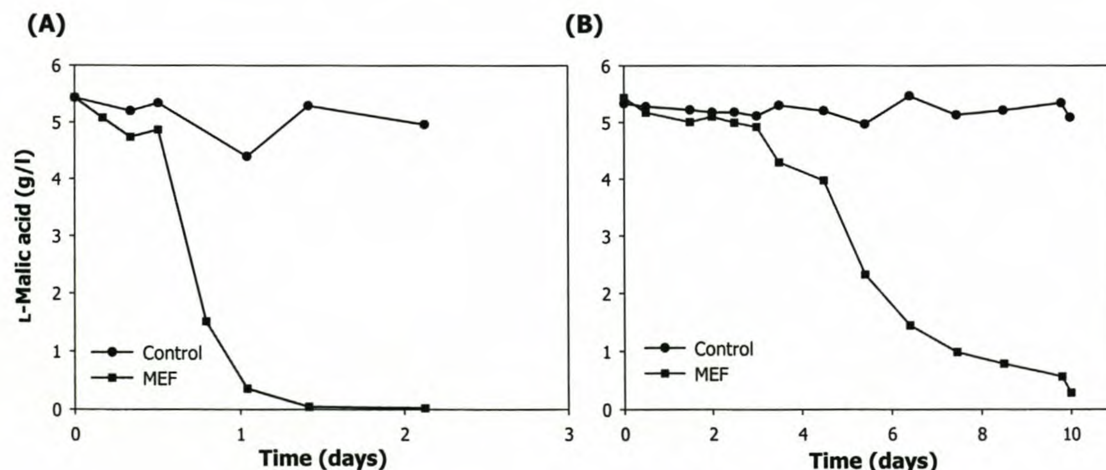

Figure 6.6. Malo-ethanolic fermentation in (A) synthetic grape must or (B) Chenin Blanc grape must containing 5 g/l L-malic acid. The recombinant strain of *S. cerevisiae* (MEF) contained the integrated *S. pombe mae1* and *mae2* genes (■), whereas the control yeast is the parent strain (*S. cerevisiae* YPH259) (●).

We have successfully introduced a malo-ethanolic pathway into a strain of *S. cerevisiae* on multicopy plasmids enabling this yeast to degrade ~7 g/l of L-malic acid during fermentation of grape must. The degradation of L-malic acid by the malo-ethanolic yeast was as efficient as the conversion of L-malic acid to L-lactic acid by the malolactic yeast strain previously reported (Volschenk *et al.*, 1997[a]). Stable expression of the malo-ethanolic genes in *S. cerevisiae* was also obtained by integration in the *ILV2/SMR1* locus. Strains of *S. cerevisiae* containing a single copy of the malo-ethanolic genes could efficiently degrade ~5g/l L-malic acid during grape must fermentation.

Strains of *S. cerevisiae* able to conduct the malo-ethanolic fermentation in grape musts will be well-suited for the production of aromatic wines such as Sauvignon Blanc, Riesling and Gewurztraminer where the reduction of malic acid is required, without the negative effects that the malolactic fermentation has on the organoleptic profile of these wines.

Strains of *S. cerevisiae* with the capacity to produce higher levels of ethanol during vinification are of particular importance to the distilled beverage industry for the production of a higher-alcohol rabate wines for distillation purposes. To ensure stable expression of the genes under the non-selective conditions associated with wine, current research is focused on the integration of the *mae1* and *mae2* genes into the genomes of selected commercial wine yeast strains. Once industrial malo-ethanolic strains of *S. cerevisiae* are obtained, we will determine their fermentation kinetics and evaluate the organoleptic quality of wines produced by the recombinant yeasts.

6.4 ACKNOWLEDGEMENTS

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ADDENDUM B

B.1 OPTIMISED HETEROLOGOUS EXPRESSION OF THE *S. POMBE MAE1* AND *MAE2* GENES *S. CEREVISIAE*

To create efficient genetically engineered industrial wine yeast strains, the functional expression of the malo-ethanolic genes (*mae1* and *mae2*) of *S. pombe* was first evaluated on multicopy plasmids in a laboratory strain of *S. cerevisiae*. Once the functionality of the genetic constructs and the efficacy of the malo-ethanolic phenotype in a laboratory strain of *S. cerevisiae* are satisfactory, the heterologous genes can be integrated into the genomes of commercial strains of *Saccharomyces*.

B.1.1 *ADH1/PGK1* expression cassettes

The main objective for subcloning the *mae1* and *mae2* open reading frames (ORF's) of *S. pombe* was to over-express these open reading frames (ORF's) in *S. cerevisiae* to ensure a strong malo-ethanolic phenotype. The native *S. pombe* promoters of the *mae1* and *mae2* genes were shown to be non-functional in *S. cerevisiae* (Viljoen *et al.*, 1999). A combination of expression vectors (Table B.1) containing either the 3-phosphoglycerate kinase (*PGK1*) or alcohol dehygrogenase (*ADH1*) expression cassettes was used to subclone the *mae1* and *mae2* ORF's through a series of DNA manipulations to form an ensemble of multicopy expression vectors for transformation into S. *cerevisiae* (Table B.1) (Volschenk *et al.*, 1997[a, b]; Volschenk *et al.*, 2001).

Expression from the *mae1* and *mae2* genes under the regulation of the *ADH1* promoter was not optimal (Volschenk *et al.*, 1997[a]) and the recombinant strains showed a very weak malo-ethanolic phenotype (8 g/l malic acid degraded in 19-22 days under aerobic conditions) (Volschenk *et al.*, 1997[a]). A hypothesis for the weak expression from the *ADH1* expression cassette was formulated based on Western blot results that indicated a possible glucose inactivation of the *ADH1* promoter. In addition, we suspect that the tandem configuration of the *PGK1* and *ADH1* expression cassettes in plasmid pHV5 and pHV6 might contribute to the weak expression from the *ADH1* promoter. Strong constitutive expression from the *PGK1* promoter correlates with a high rate of transcriptional initiation by RNA polymerase machinery and might lead to the channelling of the RNA polymerase initiation complex away from the *ADH1* promoter, resulting in lower levels of expression from the *ADH1* expression cassette. The heterologous expression strategy was therefore modified to overcome these obstacles, rather than to unravel the fundamental aspects of promoter disfunctioning in heterologous expression systems.

Table B.1. Summary of strains and plasmids used in the genetic construction of malo-ethanolic strains of *S. cerevisiae*.

Strains	Description	Reference
E. coli JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 [r_{k}-, m_{k}+], *relA*1, *supE*44, λ^{-}, Δ(*lac-proAB*), [F', *traD*36, *proA*$^{+}$*B*$^{+}$, *lacI*^{q}ZΔM15]	Yanisch-Perron, 1985
S. cerevisiae YPH259	*MAT*α, *ura3-52*, *lys2-801*^{amber}, *ade2-101*^{ochre}, *his3*Δ*200*, *leu2*-Δ*1*	Sikorski and Hieter, 1989

Plasmids	Description	Reference
pHVX1	Shuttle vector YEplac181, containing the $ADH1_p$-$ADH1_t$ expression cassette	Volschenk *et al.*, 1997[a]
pHVX2	Shuttle vector YEplac181 (*LEU2* marker gene), containing the $PGK1_p$-$PGK1_t$ expression cassette	Volschenk *et al.*, 1997[a]
pHV1	pHVX1 with *mae1* ORF ($ADH1_p$-*mae1*-$ADH1_t$)	Volschenk *et al.*, 1997[a]
pHV2	pHVX1 with *mae2* ORF ($ADH1_p$-*mae2*-$ADH1_t$)	Volschenk *et al.*, 1997[a]
pHV3	pHVX2 with *mae1* ORF ($PGK1_p$-*mae1*-$PGK1_t$)	Volschenk *et al.*, 1997[a, b]
pHV4	pHVX2 with *mae2* ORF ($PGK1_p$-*mae2*-$PGK1_t$)	Volschenk *et al.*, 1997[a]
pHV5	YEplac181-based vector containing the $ADH1_p$-*mae1*-$ADH1_t$: $PGK1_p$-*mae2*-$PGK1_t$ expression system	Volschenk *et al.*, 1997[a]
pHV6	YEplac181-based vector containing the $ADH1_p$-*mae2*-$ADH1_t$: $PGK1_p$-*mae1*-$PGK1_t$ expression system	Volschenk *et al.*, 1997[a]
pHV7	YEplac195-based vector containing the $PGK1_p$-*mae2*-$PGK1_t$ expression cassette	Volschenk *et al.*, 2001

B.1.2 Double plasmid *PGK1* expression cassettes

The strategy for improved expression of the *S. pombe mae1* and *mae2* genes in a laboratory strain of *S. cerevisiae* required that both the *mae1* and *mae2* ORF's are expressed under the regulation of the *PGK1* promoter on two independent multicopy plasmids (also referred to as the double plasmid or co-transformation strategy). The co-transformation of two plasmids into a laboratory strain of *S. cerevisiae* requires the use of two different auxotrophic marker genes to select for transformants. The previous strategy only involved the YEplac181 plasmid backbone, which contains the *LEU2* auxotrophic marker gene. Subsequently, the $PGK1_p$-*mae2*-$PGK1_t$ expression cassette was subcloned as a *Hin*dIII fragment from plasmid pHV4 (Table B.1) into YEplac195 (a *URA3*-based vector) to create plasmid pHV7 (Fig. B.1) (Volschenk *et al.*, 2001). *S. cerevisiae* strain YPH259 was subsequently transformed by both plasmids (pHV3 and pHV7) using the lithium acetate procedure (Ausubel *et al.*, 1995) in two consecutive transformation steps and the transformants were analysed for their ability to degrade L-malic acid.

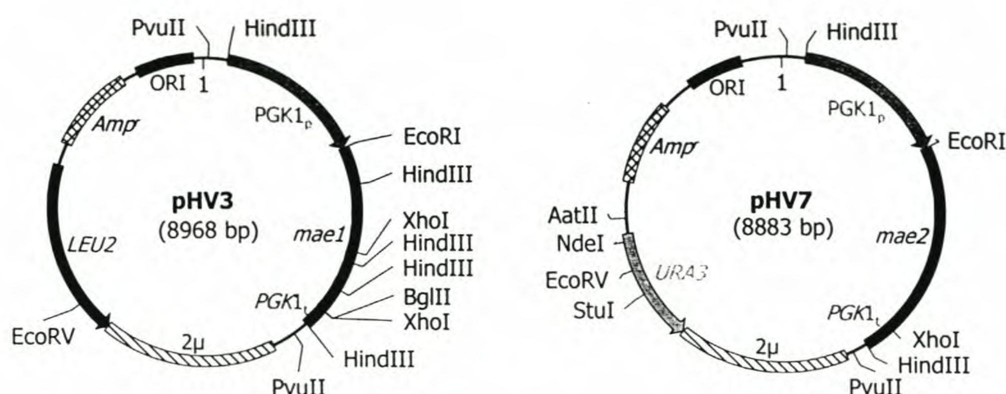

Figure B.1. Plasmid pHV3 and pHV7 contain the *PGK*1_p-*mae1*-*PGK*1_t and *PGK*1_p-*mae2*-*PGK*1_t expression cassettes, respectively. The construction of pHV3 was previously described in Volschenk *et al.* (1997). pHV7 was obtained by subcloning the *Hin*dIII fragment from pHV4 into YEplac195.

Malic acid degradation by these engineered strains of *S. cerevisiae* containing both the *mae1* and *mae2* genes under control of the *PGK1*-promoter and terminator sequences, was more effective compared to the previously results obtained with the *ADH1*-promoter (Volschenk *et al.*, 1997[b]). Efficient degradation of 8 g/l malic acid was obtained in a 10% glucose synthetic grape must in less than three days (see Fig. 6.3), indicating that the malo-ethanolic fermentation in *S. cerevisiae* can be as efficient as malolactic fermentation in *S. cerevisiae*.

B.2 MALO-ETHANOLIC FERMENTATION IN VIDAL GRAPE MUST

In addition to the fermentations in synthetic and Chardonnay grape must, the recombinant strain of *S. cerevisiae* containing the *S. pombe mae1* and *mae2* genes on plasmid pHV3 and pHV7 (Table B.1) was also evaluated in a Vidal grape must from a cool viticultural region (Ontario, Canada). Grapes from cool climate regions characteristically contain higher concentrations of L-malic acid due to the slower rate of acid respiration during the ripening stages of the grape berry. The Vidal grape must (8.4 g/l L-malic acid, pH 3.12) was inoculated with 2 X 10^6 cells/ml into 200 ml must in 250 ml flasks and incubated at 20°C without aeration. The Vidal grape must was also supplemented with 0.075% diammonium phosphate before inoculation to ensure a sufficient nitrogen source during fermentation. The concentration of L-malic acid, D-glucose and ethanol were measured at regular intervals during the fermentation using enzymatic assays (Roche Diagnostics, Germany).

Malic acid degradation by the recombinant *S. cerevisiae* strain in the Vidal grape must was rapid and efficient with complete degradation of extracellular L-malic acid in less than 7 days (Fig. B.2 A). In contrast, the control yeast strain containing only the *PGK1*-expression cassette (plasmid pHVX2) was not able to degrade the malic acid present in the media. Furthermore, the recombinant *S. cerevisiae* strain also produced higher levels of ethanol compared to the control yeast strain (Fig. B.2 B).

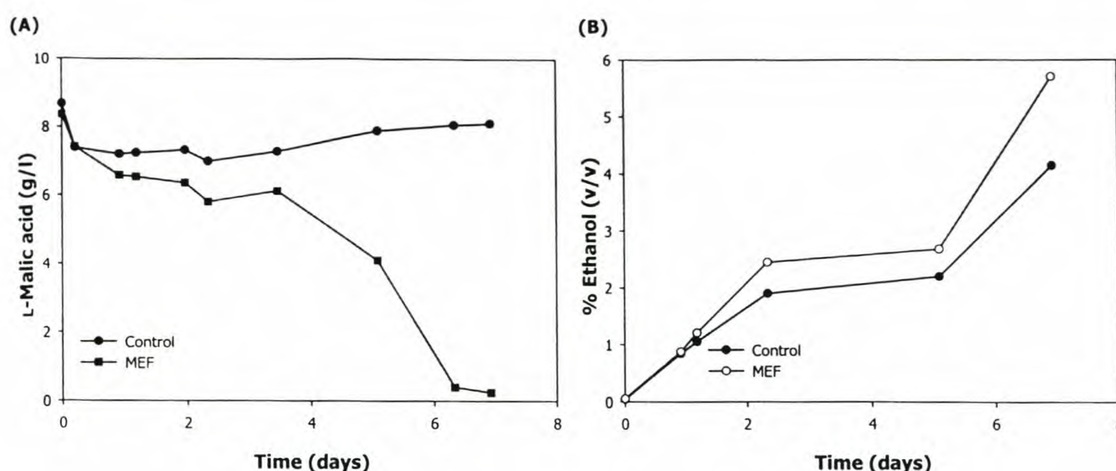

Figure B.2. (A) Malo-ethanolic fermentation in Vidal grape must containing 8.4 g/l L-malic acid. The recombinant *S. cerevisiae* strain contained the integrated *S. pombe mae1* and *mae2* genes (■), whereas the control yeast is the parent strain, *S. cerevisiae* YPH259, containing pHVX2 (●). (B) ethanol production by the malo-ethanolic strain of *S. cerevisiae* containing the *S. pombe mae1* and *mae2* genes (O) compared to the control yeast strain containing only plasmid pHVX2 (●).

The effective malo-ethanolic fermentation by the recombinant *S. cerevisiae* strain in a high-acid Vidal grape must demonstrates the importance of a malic acid degrading yeast strain in cool climate regions where acid imbalances are regularly found in grapes and wine.

B.3 INTEGRATION WITH *SMR1-410* GENE AS DOMINANT SELECTABLE MARKER

Sulfometuron methyl (SM), *N*-[(4,6-dimethyl-pyrimidin-2-yl) aminocarbonyl]-2-methoxycarbonyl-benzenesulfonamide, is the active compound of the sulfonylurea herbicide, Oust (Dupont), and a powerful inhibitor of growth of several species of bacteria, yeasts and higher plants (Chauleff and Mauvais, 1984; Falco and Dumas, 1985; Falco *et al.*, 1995; LaRossa and Schloss, 1984; Ray, 1984; Xie and Jiménez, 1996). SM inhibits the enzymatic activity of the acetolactate synthase enzyme (EC 4.1.3.18), encoded by the *ILV2* gene (Chromosome XIII) in *S. cerevisiae*, which catalyses the first step in the biosynthesis of the branch chain amino acids isoleucine and valine (Falco and Dumas, 1985).

Genetic analyses of several phenotypically distinct mutations of the *ILV2* gene of *S. cerevisiae* (named *SMR1*) that confer resistance to SM have been previously characterised. The *SMR1-410* mutation was found to be dominant in heterozygous diploids and has been applied successfully as a dominant selectable marker in *S. cerevisiae* (Casey *et al.*, 1988; Gasent-Ramínez *et al.*, 1995; Kitamoto *et al.*, 1991; Marín *et al.*, 2001; Xiao and Rank, 1989, 1990). Sequence comparison between *SMR1-410* and the wild-type *ILV2* genes has shown that *SMR1-410* carries a single C to T transition mutation at nucleotide position 1086 (nucleotide 574 of the *ILV2* open reading frame) of the

coding sequence of *ILV2*, which results in a proline to serine exchange at amino acid 192 (Fig B.3) (Xie and Jamínez, 1996; Yadav *et al.*, 1986).

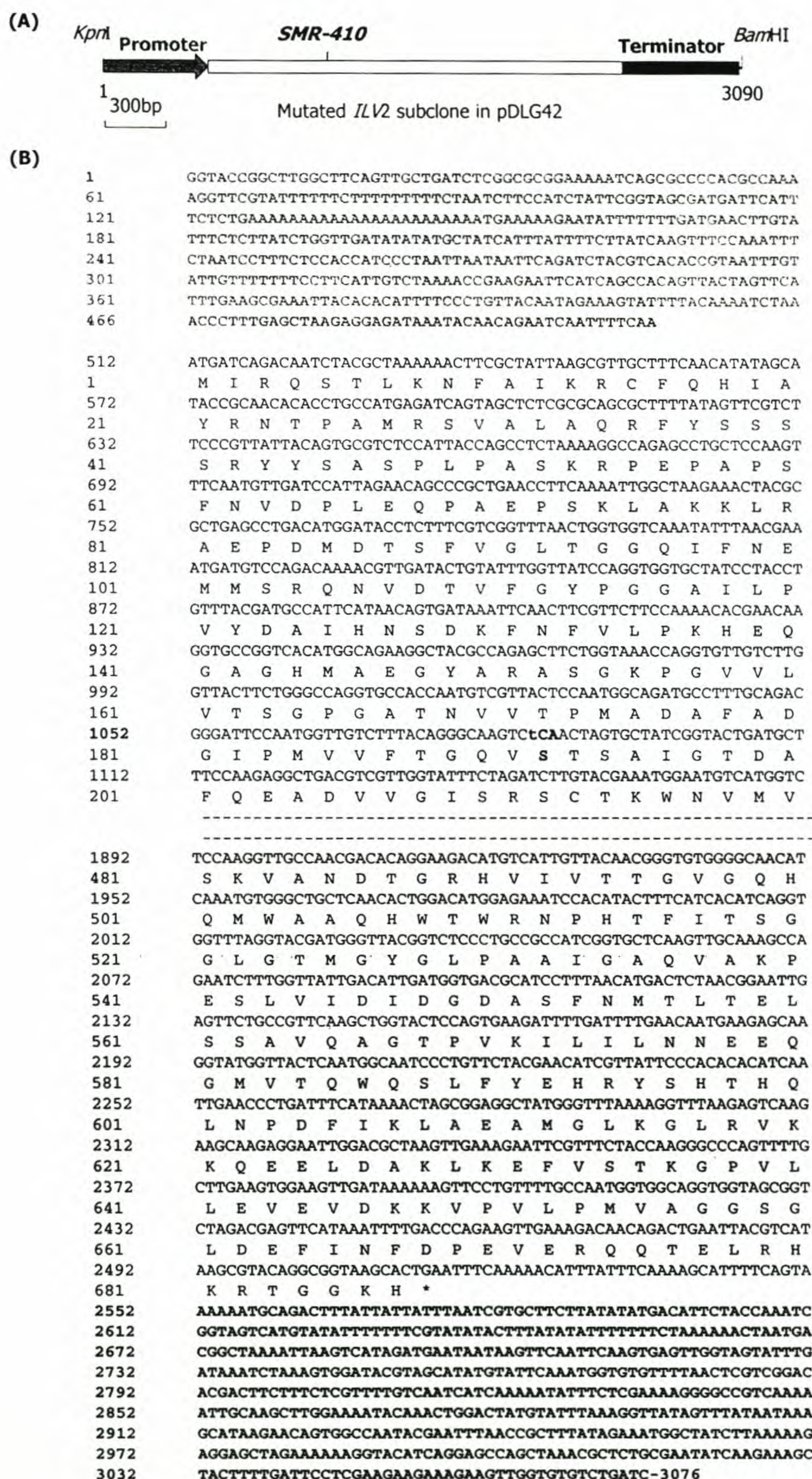

Figure B.3. Schematic representation (A) and nucleotide sequence (B) of the *SMR1-410* allele of the *S. cerevisiae ILV2* gene. *The SMR1-410* point mutation is indicated on the sequence at nucleotide 1086. The promoter, open reading frame and terminator regions are also indicated (adapted from Yadav *et al.*, 1986).

For the purpose of integrating the *S. pombe mae1* and *mae2* genes into *S. cerevisiae*, the *SMR1-410* gene was subcloned from pWX509 (Casey *et al.*, 1988) by *Kpn*I-*Bam*HI digestion into pBluescript KS+ to yield pDLG42 (Dr. D.C. la Grange, Dept. of Microbiology, Stellenbosch University) (Fig. B.4). The *PGK1*p-*mae2*-*PGK1*t cassette from pHV7 was subcloned first as a *Hin*dIII fragment into the *SMR1* terminator region in pDLG42 to yield pHVS1 (Fig. B.4), while *PGK1*p-*mae1*-*PGK1*t was subcloned as a *Pvu*II fragment from pHV3 into the blunt-ended *Nde*I site of the *SMR1* terminator region of pHVS1 to yield pHVS2 (Fig. B.4, see also Fig. 6.1). pHVS2 was linearised with *Apa*I digestion, purified and concentrated with the High Pure PCR Purification Kit (Roche Diagnostics, Germany) and transformed into LiOAc competent cells of *S. cerevisiae* YPH259 (Ausubel *et al.*, 1995).

The use of SM as a dominant selectable marker in *S. cerevisiae* requires the use of minimal media or the absence of either valine or isoleucine, which acts as feedback inhibitors on the expression of *ILV2* and ultimately leads to restored growth on SM if present (Chauleff and Mauvais, 1984; Falco *et al.*, 1985; Falco and Dumas, 1985). SM was dissolved in N'N'-dimethyl formamide at a concentration of 2mg/ml and added to minimal media (at a final concentration of 200 μg/ml) containing 0.17% Yeast Nitrogen Base [Difco Laboratories, Detroit, MI], 0.5% $(NH_4)_2SO_4$, 2% D-glucose and 0.13% of a drop-out amino acid pool without valine and isoleucine (Ausubel *et al.*, 1995). Transformants were isolated after 3-4 days of incubation at 28^0C in the dark.

After 4 days, 30-40 transformants appeared on the SM plates. Transformants were picked and cultured in rich media (YPD) for 2 days and spotted onto fresh SM selection plates to confirm stable integration of *PGK1*p-*mae1*-*PGK1*t and *PGK1*p-*mae2*-*PGK1*t cassettes into the genome of *S. cerevisiae* YPH259.

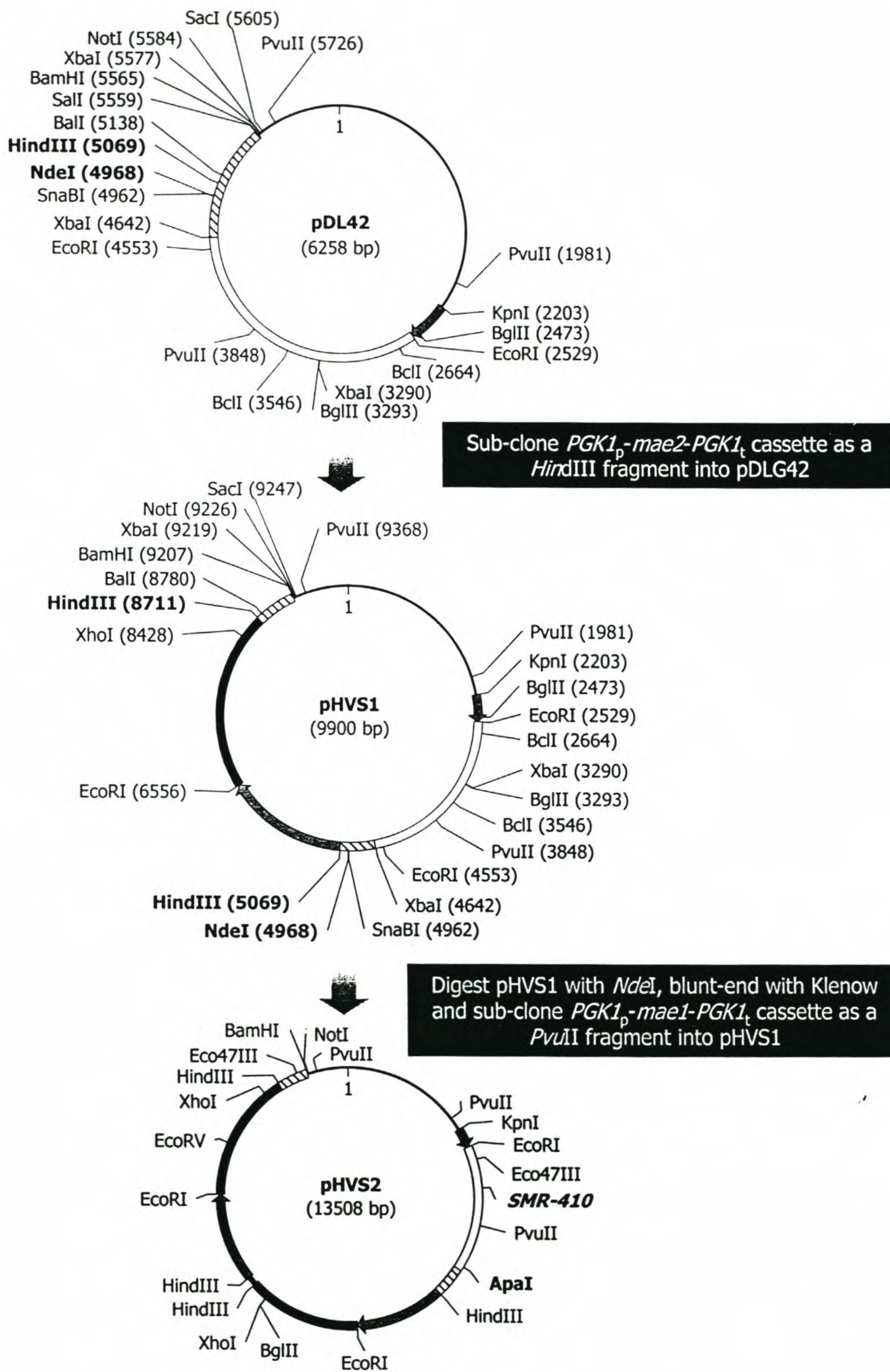

Figure B.4. Subcloning strategy of the *PGK1*p-*mae2-PGK1*t and *PGK1*p-*mae1-PGK1*t cassettes into the terminator region of the *SMR1-410* gene in pDLG42 to yield pHVS2.

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CHAPTER 7

RESEARCH RESULTS

Genetic engineering of an industrial strain of *Saccharomyces bayanus* for L-malic acid degradation via an efficient malo-ethanolic pathway

To be submitted to "Yeast" for publication

Genetic engineering of an industrial strain of *Saccharomyces bayanus* for L-malic acid degradation via an efficient malo-ethanolic pathway

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ABSTRACT

The optimal ratio of L-malic and L-tartaric acid in relation to other wine components is one of the most important aspects that ultimately determine wine quality during winemaking. Winemakers routinely rely on the judicious use of malolactic fermentation (MLF) after alcoholic fermentation to deacidify and stabilise their wines. However, due to the unreliability of the process and unsuitable sensory modifications in some cultivars, especially fruity-floral wines, MLF is often regarded problematical and undesirable. Alternative methods for reducing L-malic acid in wine will contribute to the production of quality wines in the future, especially in cool-climate regions. Most wine yeast strains of *Saccharomyces* are unable to effectively degrade L-malic acid, whereas the fission yeast *Schizosaccharomyces pombe* efficiently degrades high concentrations of L-malic acid by means of malo-ethanolic fermentation. However, strains of *S. pombe* are not suitable for vinification due to the production of undesirable off-flavours. The *S. pombe* malate permease (*mae1*) and malic enzyme (*mae2*) genes were previously successfully expressed under the 3-phosphoglycerate kinase (*PGK1*) regulatory elements in *S. cerevisiae*, resulting in a recombinant laboratory strain of *S. cerevisiae* with an efficient malo-ethanolic pathway. Stable integration of the *S. pombe* malo-ethanolic pathway genes has now been obtained through the construction of a unique integration strategy in a commercial wine yeast strain. Co-transformation of the linear integration cassette containing the *mae1* and *mae2* genes and *PGK1* regulatory elements and a multi-copy plasmid containing the phleomycin-resistance marker into a commercial *Saccharomyces bayanus* strain resulted in the successful transformation and integration of the malo-ethanolic genes. The recombinant *S. bayanus* strain was successfully cured of phleomycin-resistance plasmid DNA in order to obtain malo-ethanolic yeast containing only yeast-derived DNA. The integrated malo-ethanolic genes were stable in *S. bayanus* and during synthetic and grape must fermentation, L-malic acid was completely fermented to ethanol without any negative effect on fermentation kinetics and wine quality.

7.1 INTRODUCTION

The conversion of L-malic acid to lactic acid and CO_2 during MLF is one of the four possible metabolic conversions found for L-malic acid in nature. L-Malic acid can also serve as a carbon

source for yeast, which can transform L-malic acid into one of three other compounds, i.e. oxaloacetic acid (via malate dehydrogenase enzyme), fumaric acid (via reverse reaction of fumarase enzyme) and pyruvic acid (via malic enzyme). Based on yeasts' ability to utilise L-malic acid and other TCA cycle intermediates as sole carbon or energy source, yeasts are divided into a K (-) or K (+) group (Barnett and Kornberg, 1960). Strains of *Saccharomyces sensu stricto,* including commercial wine yeast strains, as well as *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii,* are all classified as K (-) yeasts that can utilise TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources (Barnett and Kornberg, 1960).

Strains of *Saccharomyces*, cannot degrade L-malic acid completely in grape must during alcoholic fermentation, resulting in relatively minor modifications in total acidity during vinification (Rankine, 1966; Radler, 1993; Subden *et al.*, 1998). A number of reasons for the weak degradation of L-malic acid in *S. cerevisiae* have been postulated. Firstly, *S. cerevisiae* lacks the machinery for active transport of L-malic acid found in *S. pombe* and relies on rate-limiting simple diffusion for the uptake of extracellular L-malic acid. Secondly, the malic enzyme of *S. cerevisiae* has a significantly lower substrate affinity for L-malic acid (K_m = 50 mM) than that of *S. pombe* (K_m = 3.2 mM), which contributes to weaker metabolism of this acid in *S. cerevisiae* (Fuck *et al.*, 1973; Temperli *et al*, 1965). Lastly, the compartmentalisation of the malic enzyme in mitochondria may contribute to the weak L-malic acid degradation in *S. cerevisiae*. The mitochondrial location of the malic enzyme of *S. cerevisiae*, in contrast to the cytosolic location of the *S. pombe* malic enzyme, suggests that this enzyme is inherently subject to the regulatory effect of glucose (Redzepovic *et al.*, 2002). Furthermore, mitochondrial deterioration a well-documented phenomenon in Crabtree-positive yeast may amplify the already weak L-malic acid metabolism of *S. cerevisiae.*

The ability yeast to efficiently degrade extracellular L-malic acid depends on an efficient uptake system for L-malic acid, i.e. active transport via a malate permease, and an effective L-malic acid converting enzyme, such as the malic enzyme. The malate permease gene (*mae1*) and the malic enzyme gene (*mae2*) of *S. pombe* were therefore cloned (Grobler *et al.*, 1995; Viljoen *et al.*, 1994) and co-expressed in multi-copy and single copy under the *S. cerevisiae* constitutive 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences in a laboratory strain of *S. cerevisiae* (Volschenk *et al.*, 2001). A strong malo-ethanolic phenotype was introduced in *S. cerevisiae* where L-malic acid was rapidly and efficiently degraded in synthetic and Chardonnay grape must with the concurrent production of higher ethanol levels (Volschenk *et al.*, 2001). Functional expression of the malo-ethanolic pathway genes of *S. pombe* in a laboratory strain of *S. cerevisiae* paved the way for the genetic modification of industrial wine yeast strains of *Saccharomyces* for commercial winemaking application.

Stable integration of the malo-ethanolic pathway genes into the genome of industrial wine yeast strains is a prerequisite for becoming an inherited component of yeast genome. Genetic engineering of wine yeasts strains of *Saccharomyces* is, however, complicated by the homothallic, multiple ploidy and prototrophic nature of industrial strains of *Saccharomyces* (Pretorius, 2000). Transformation and integration of heterologous genes into industrial strains of *Saccharomyces* require the use of dominant selectable markers, i.e. antibiotic or toxic compound resistance markers that when integrated into the yeast genome, are not acceptable for commercial application mainly due to the absence of long-term risk assessment and consumer disapproval.

In this study, we report on a unique integration strategy for the *S. pombe mae1* and *mae2* expression cassettes without incorporation of any non-yeast derived DNA sequences. The malo-ethanolic cassette, containing only the *S. cerevisiae PGK1* promoter and terminator regions together with the *S. pombe mae1* and *mae2* open reading frames, was integrated into the *URA3* locus of an industrial strain of *S. bayanus* EC1118 during co-transformation with a phleomycin-resistance plasmid. After initial screening for phleomycin resistance, *S. bayanus* EC1118 transformants were cured of the phleomycin-resistance plasmid, resulting in the loss of any non-yeast derived DNA sequences. After correct integration of the *mae1* and *mae2* expression cassettes was verified, small-scale vinification in synthetic and actual grape must with stable transformants resulted in rapid and complete degradation of L-malic acid and increased ethanol production during the early stages of alcoholic fermentation. Integration and expression of the malo-ethanolic genes in *S. bayanus* EC1118 had no adverse effect on the yeasts fermentative ability, while sensory evaluation and chemical analysis of Chardonnay wine indicated an improvement in wine flavour perception compared to the control wines, without the production of any off-flavours.

7.2 MATERIALS AND METHODS

7.2.1 Strains and maintenance

The bacterial and yeast strains and plasmids used in this study are listed in Table 7.1. Cells of *E. coli* JM109 were transformed by electroporation and selected on LB agar medium supplemented with 200 mg/l ampicillin (Ausubel *et al.*, 1995). *S. bayanus* EC1118 was maintained on YPD agar, while transformants were plated onto YEG media containing 0.5 % yeast extract, 2% glucose, 3 % Pastagar B (Difco Laboratories, Detroit, MI) and 250 μg/ml phleomyc.

7.2.2 Plasmid Construction

Standard recombinant DNA techniques were employed essentially as described by Ausubel *et al.* (1995). Restriction enzymes, modification enzymes and DNA purification kits were used as

prescribed by the manufacturer (Roche Diagnostics, Germany). All PCR reactions were executed with Takara Ex Taq (Takara Bio Inc, Japan). All subcloning and DNA manipulations were performed in the 2μm-based plasmid YEp352 (Hill *et al.*, 1986). Prior to any subcloning, the *Kpn*I restriction site located in the multiple cloning region of YEp352 was eliminated by *Kpn*I digestion, filled to blunt-ends with Klenow enzyme and religated to yield pHV9. A 944 bp upstream *URA3* fragment was PCR amplified from *S. bayanus* EC1118 genomic DNA using primer set 5'-XBASFRURA3 and 3'-URA3KPN (Table 7.2), while a 959 bp downstream *URA3* fragment was PCR amplified using primer set 5'-KPNNOTURA3 and 3'-URA3SFRXBA.

Table 7.1. Strains and plasmids used for the integration of the *S. pombe* malo-ethanolic genes into industrial strains of *Saccharomyces*

Strains	Description	Reference
E. coli **JM109**	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 [r_{k}-, m_{k}+], *relA*1, *supE*44, λ^-, Δ(*lac-proAB*), [F', *traD*36, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15]	Yanisch-Perron, 1985
S. bayanus **EC1118**	*Commercial wine yeast*	Lallemand Lalvin®
Plasmids	**Description**	**Reference**
YEp352	Yeast/*E. coli* shuttle vector with a *URA3* marker	Hill *et al.*, 1986
pUT332	Yeast episomal plasmid containing the *Tn5ble* gene for selection of phleomycin resistance	Gatignol *et al.*, 1990; Wenzel *et al.*, 1992
pHV3	pHVX2 containing the *mae1* ORF subcloned between the *PGK1* promoter and terminator sequences.	Volschenk *et al.*, 1997[a, b]
pHV7	YEplac195 (*URA3* marker gene) containing the *mae2* ORF subcloned between the *PGK1* promoter and terminator sequences.	Volschenk *et al.*, 2001
pHV9	YEp352 without the *Kpn*I restriction site	This study
pHVJH1	pHV9 containing the mutated *URA3* gene	This study
pHV11	pHVJH1 containing the $PGK1_p$-*mae1*-$PGK1_t$ expression cassette subcloned into the *Kpn*I site in the mutated *URA3* gene	This study
pHV13	pHV11 containing the $PGK1_p$-*mae2*-$PGK1_t$ expression cassette subcloned into the *Not*I site in the mutated *URA3* gene	This study

Both the upstream and downstream *URA3* fragments were digested with *Kpn*I and fused by T_4 DNA ligation. The resulting linear product, which was isolated after 1% agarose gel electrophoresis and purified by the High Pure Gel Extraction Kit, served as template for PCR amplification with primer set 5'-XBASFRURA3 and 3'-URA3SFRXBA. The modified *URA3* fragment containing unique cloning sites (*Kpn*I, *Not*I) and excision sites (*Srf*I and *Xba*I) was subcloned in the *Xba*I restriction site of pHV9, resulting in pHVJH1 (Fig. 7.1).

Table 7.2. List of PCR primers used in this study to construct the linear integration cassette containing the *PGK1*$_p$-*mae1*-*PGK1*$_t$ and *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassettes flanked by *URA3* sequences

Primer name	Primer sequence
5'-XBASFRURA3	5'-GATC*TCTAGA*GCCCGGGCAACGGTTCATCATCTCATGGATCTGC-3'
3'-URA3KPN	5'-GATC**GGTACC**TACTTCTTCCGCCGCCTGCTTCAAACCGCT-3'
5'-KPNNOTURA3	5'-GATC**GGTACC***GCGGCCGC*ACAAAGGAACCTAGAGGCCTTTTGATGTTAG-3'
3'-URA3SFRXBA	5'-GATC*TCTAGA*GCCCGGGCTACACCAGAGATACATAATTAGATAT-3'
5'-KPNPGK	5'-GATC**GGTACC**AACCTTTCTAACTGATC-3'
3'-PGKKPN	5'-GATC**GGTACC**AAGCTTTAACGAACGCA-3'
5'-NOTPGK	5'-GATC*GCGGCCGC*AACCTTTCTAACTGATCTATCCAAAACTG-3'
3'-PGKNOT	5'-GATC*GCGGCCGC*AAGCTTTAACGAACGCAGAATTTTCG-3'
5'-mae1	5'-GATCGAATTCATGGGTGAACTCAAGGAAAT-3'
3'-mae1	5'-GATCAGATCTTTAAACGCTTTCATGTTCACT-3'
5'-mae2	5'-GATCGAATTCATGCCTGCAGGAACCAAAGAA-3'
3'-mae2	5'-GATCCTCGAGTTATACAAAAGGCTTGTATTC-3'
5'-mae1DIG	5'-CTTTCAATATCCACGTTCATCGACA-3'
3'-mae1DIG	5'-GAGACAGTAACACCAAGCAGCAAGA-3'
5'-mae2DIG	5'-GAACCAAAGAACAAATCGAGTGTCC-3'
3'-mae2DIG	5'-GAGAACAATGGGCAAGAATCGATTA-3'

TCTAGA = *Xba*I, GCCCGGGC = *Srf*I, **GGTACC** = *Kpn*I, *GCGGCCGC* = *Not*I

The construction of the expression vectors and pHV3 and pHV7 (Table 7.1) was previously described (Volschenk *et al.*, 1997[a,b], Volschenk *et al.*, 2001). The *PGK1*$_p$-*mae1*-*PGK1*$_t$ expression cassette was PCR amplified using primer set 5'-KPNPGK and 3'-PGKKPN with plasmid pHV3 as template, while primers 5'-NOTPGK and 3'-PGKNOT was used for PCR amplification of the *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassette from plasmid pHV7 (Volschenk *et al.*, 2001). The *PGK1*$_p$-*mae1*-*PGK1*$_t$ PCR product was subcloned as a *Kpn*I fragment into pHVJH1 to yield pHV11 (Fig. 7.2). Similarly, the *PGK1*$_p$-*mae2*-*PGK1*$_t$ PCR product was subcloned as a *Not*I fragment into pHV11 to yield pHV13. *Srf*I digestion of pHV13 resulted in the excision of a linear *PGK1*$_p$-*mae1*-*PGK1*$_t$-*PGK1*$_p$-*mae2*-*PGK1*$_t$ fragment flanked by ca. 500-600 bp *URA3* sequences, which excludes any vector-derived DNA sequences.

6.2.3 Phleomycin and geneticin resistance of industrial wine yeast strains

The minimum inhibition concentration (MIC) of phleomycin and geneticin for *S. bayanus* EC1118 was determined. Yeast cells were cultured overnight in 10 ml YPD broth and plated with or without electroporation (in the absence of any DNA) onto YPD plates with Pastagar B (Difco Laboratories,

Detroit, MI), containing a range of 5 μg/ml to 500 μg/ml phleomycin or geneticin. A minimum concentration of 100 μg/ml geneticin was required for complete inhibition *S. bayanus* EC1118 prior to electroporation. However, electroporated cells of *S. bayanus* EC1118 cells gave rise to background colonies (false positives) even at a concentration of 500 μg/ml geneticin. The minimum inhibitory concentration of phleomycin was determined at 250 μg/ml for electroporated cells of *S. bayanus* EC1118.

7.2.4 Adaptation of GMIA media for optimised malo-ethanolic phenotype selection

A plate assay method was developed to simplify the selection of positive transformants with a malo-ethanolic phenotype after electroporation and integration. The Glucose-Malate-Indicator Agar (GMIA) selection media was previously developed for the malo-ethanolic yeast *S. pombe* (Osothsilp *et al.*, 1986). The plates produce blue colonies with a surrounding blue halo when L-malic acid in the media is degraded by *S. pombe* due to a shift in pH (pH 3.3 to 5.2) when L-malic acid is converted to pyruvic acid. Initial attempts with a recombinant strain of *S. cerevisiae* containing the malo-ethanolic genes on a multi-copy plasmid (Volschenk *et al.*, 2001), did not produce a clear phenotype for L-malic acid degradation on the GMIA plates. The original GMIA media was therefore modified to contain 0.17 % Yeast Nitrogen Base (Difco Laboratories, Detroit, MI), 0.5 % $(NH_4)_2SO_4$, 10 % glucose (simulate glucose levels in grape must), 10 % L-malic acid, 0.01 % bromocresol green and 2 % Noble agar (Difco Laboratories, Detroit, MI, instead of the Bacto-agar). The pH of the optimised GMIA media was adjusted to 3.3 with KOH.

The optimised GMIA media allowed for the effective screening of the transformants with the integrated malo-ethanolic cassette after the initial screening of transformants on phleomycin-containing medium. Phleomycin-resistant transformants were transferred to GMIA plates and screened accordingly for the malo-ethanolic phenotype. The antibiotic resistance-plasmid, pUT332, was lost during subsequent non-selective growth of transformants, resulting in recombinant yeast containing only the integrated malo-ethanolic cassette without the requirement of integrating the antibiotic marker. Genetically engineered yeasts produced in this manner should be more acceptable for industrial application, since no antibiotic markers are present in the recombinant yeast strain.

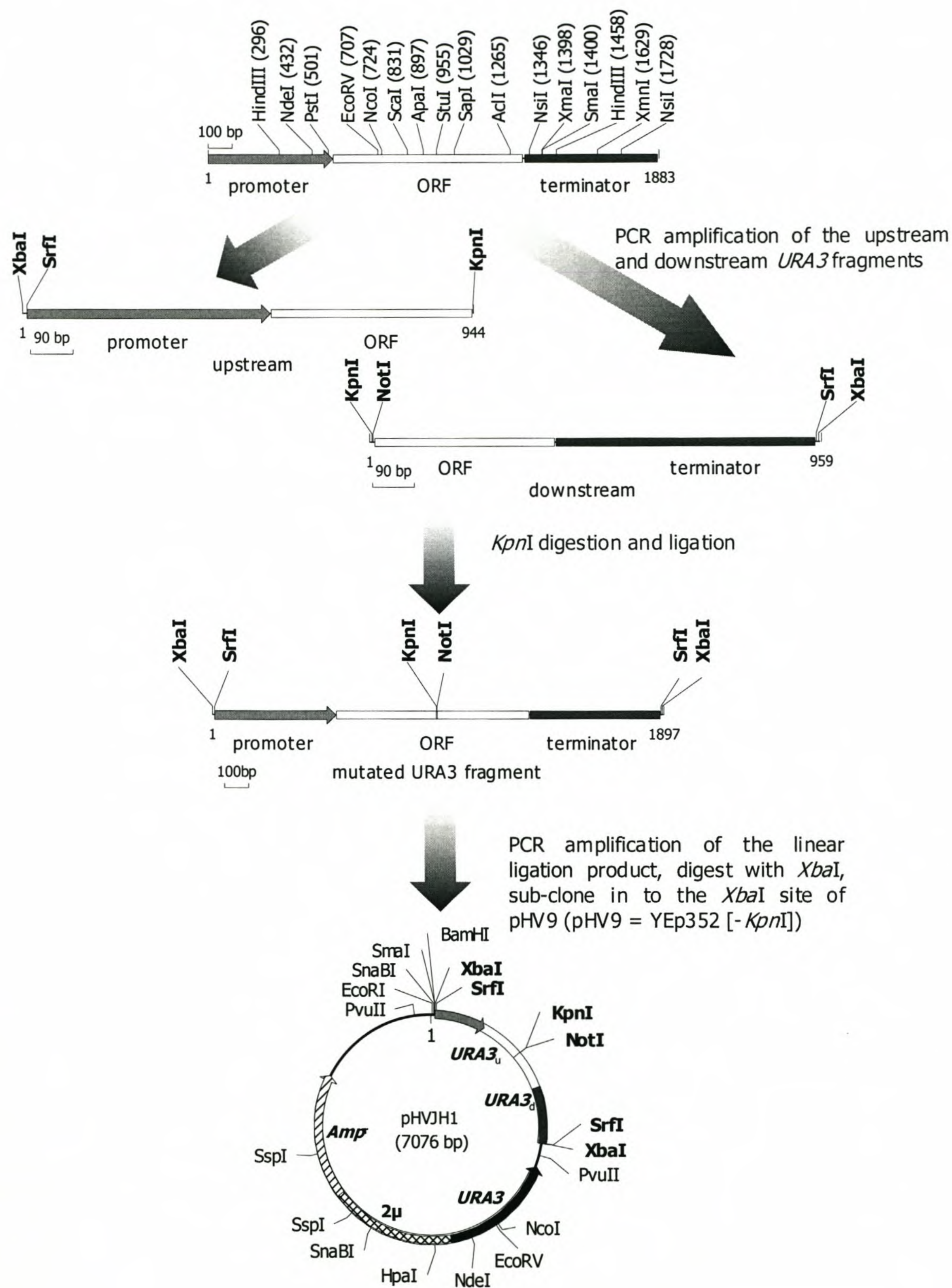

Figure 7.1. Construction of plasmid pHVJH1 by subcloning an upstream and downstream region of the *URA3* gene synthesised by PCR amplification to create unique restriction sites (*Kpn*I and *Not*I) for subcloning and excision sites (*Srf*I and *Xba*I).

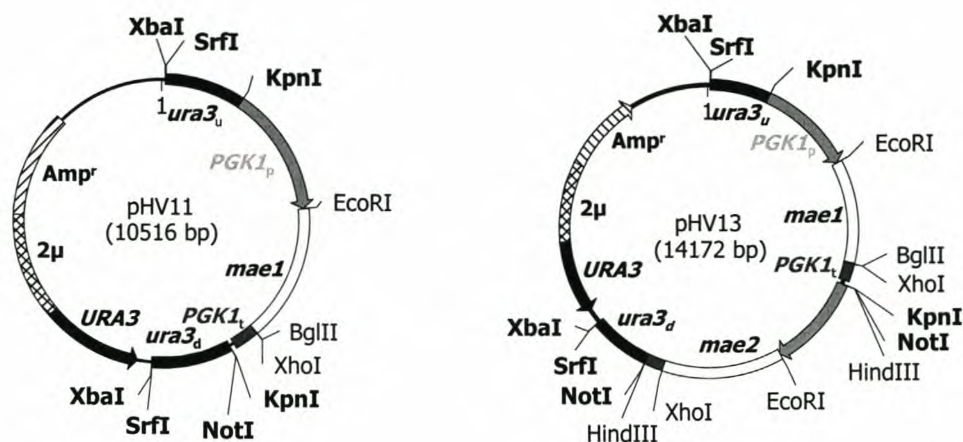

Figure 7.2. Plasmid maps of pHV11 containing the *PGK1*$_p$-*mae1-PGK1*$_t$ expression cassette and plasmid pHV13 containing both the *PGK1*$_p$-*mae1-PGK1*$_t$ and *PGK1*$_p$-*mae2-PGK1*$_t$ expression cassettes. *Srf*I digestion of pHV13 yielded a linear integration cassette without any vector, bacterial or other foreign DNA sequences that were used for co-transformation with plasmid pUT332.

7.2.4 Co-transformation and integration of *mae1* and *mae2* genes in *S. bayanus* EC1118

Integration of the *mae1* and *mae2* genes into the genome of commercial wine yeast strains was obtained by co-transformation of the linear *URA3*-flanked *PGK1*$_p$-*mae1-PGK1*$_t$-*PGK1*$_p$-*mae2-PGK1*$_t$ integration cassette and plasmid pUT332, which contains the *Tn5ble* gene for selection of phleomycin resistance (Gatignol *et al.*, 1990; Wenzel *et al.*, 1992). Initial screening on phleomycin-containing media was required to select for successful transformation and to minimise the amount of colonies to be screened for the malo-ethanolic phenotype.

An adapted electroporation method was used in this study for the transformation of industrial wine yeast strains. Yeast cells were precultured overnight in 10 ml YPD at 30°C followed by 500 ml YPD in a 2 l flask to an OD_{600} = 0.1. The culture was shaken vigorously at 30°C until an OD_{600} of 1.3 - 1.5 was reached. Yeast cells were harvested by centrifugation at 4000 x g at 4°C and resuspended in 80 ml sterile ddH_2O. While swirling, 10 ml 10 X TE buffer (pH7.5) was added, followed by 10 ml 1M LiOAc. After incubation for 45 minutes at 30°C with gentle agitation, 2.5 ml fresh 1M DTT was added to the yeast suspension while swirling, with a continued incubation for 15 minutes at 30°C with gentle agitation. The yeast suspension was subsequently diluted to a volume of 500 ml with ddH_2O, washed and concentrated three times at 4000 x g, 4°C. Cell pellets were resuspended first in 250 ml ice-cold ddH_2O, then in 30 ml ice-cold 1M Sorbitol and finally in 0.5 ml ice-cold 1M Sorbitol. This yielded a final volume of 1 – 1.5 ml cells with an approximate OD_{600} of 200. After the cell pellet was resuspended, 40 µl of the concentrated yeast cells was mixed with 5µl DNA in a sterile, ice-cold 1.5 ml tube. A 10:1 molar ratio of linear : plasmid DNA was used, with ideally 50 ng of pUT332 and

an appropriate 10-fold molar increase of linear DNA. After the cell-DNA mixtures were transferred to an ice-cold 0.2 cm gap electroporation cuvette (Biorad, South Africa) and subjected to a pulse of 1.5kV, 25 μF and 200 ohms (Gene Pulser II Electroporator, Biorad, South Africa). Immediately after the pulse was administered, 1ml ice-cold YPD (1% yeast extract, 2% peptone and 2% glucose) was added to the cuvette followed by a gentle mix for 2-4 hours at 30°C. Aliquots of 250 μl yeast suspension were spread directly onto YEG plates containing 250 μg/ml phleomycin. Transformants were incubated for 3-4 days at 30°C. Putative transformants were inoculated in 10 ml YPD (non-selective conditions) and cultured successively for > 200 generations at 30°C to cure the yeast of plasmid pUT332. After the loss of plasmid pUT332 was confirmed on phleomycin media (data not shown), transformants were streaked onto modified GMIA plates.

7.2.5 PCR confirmation of integration and Southern blotting

Initial proof of the integration of the linear $PGK1_p$-*mae1*-$PGK1_t$ -$PGK1_p$-*mae2*-$PGK1_t$ fragment was obtained through PCR amplification of the entire *mae1* and *mae2* open reading frames using primer sets 5'-mae1/3'-mae1 and 5'-mae2/3'-mae2, respectively (Table 7.2). Integration of the linear $PGK1_p$-*mae1*-$PGK1_t$-$PGK1_p$-*mae2*-$PGK1_t$ fragment in the genomic *URA3* locus was confirmed through Southern blot analysis. Standard procedures (Ausubel *et al.*, 1995) were used to prepare the gel for Southern blotting and to transfer the DNA to a positively charged nylon membrane (Roche Diagnostics, Germany). Genomic DNA was isolated from *S. bayanus* (Hoffman and Winston, 1987), digested with *Hpa*I and separated on a 1% agarose gel. An internal 944 bp *URA3* fragment corresponding to the upstream *URA3* region used for construction of the linear integration cassette, was DIG-labelled (PCR Probe Synthesis Kit, Roche Diagnostics, Germany) using primer set 5'-XBASFRURA3 and 3'-URA3KPN. The presence of the *URA3* gene was visualised with the Chemiluminescent Detection Kit (Roche Biochemicals, Germany).

7.2.6 Malo-ethanolic fermentation in grape must

Synthetic grape must. The parental yeast strain *S. bayanus* EC1118 and three transformants containing the integrated *mae1* and *mae2* genes were inoculated at 2 x 10^6 cells /ml into duplicate sets of 200 ml synthetic grape must in 250 ml Erlenmeyer flasks (Denayrolles *et al.*, 1995). The synthetic grape must contained 0.94% L-malic acid (Sigma, St. Louis, MO) and the pH was adjusted with KOH to 3.3. Fermentations were carried out at 20°C, without shaking and sealed with fermentation caps filled with 2.5% SO_2 solution, for approximately 15 days. Yeast cell growth was monitored spectrophotometrically at OD_{600}.

Small-scale grape must fermentation. Small-scale fermentations were also performed in Chardonnay (3 g/l L-malic acid, pH 3.40), Cabernet Sauvignon (2.5 g/l L-malic acid, pH 3.77), Colombard (4.5 g/l L-malic acid, pH 3.42) and Ruby Cabernet (3.5 g/l L-malic acid, pH 3.54) grape must, which were inoculated at 2 x 10^6 cells/ml into 400 ml must in 500 ml flasks and incubated at 20°C, without

shaking and sealed with fermentation caps filled with 2.5% SO_2 solution for approximately 5 days. White and red grape must were supplemented with 50 ppm and 30 ppm SO_2, respectively, while 0.075% diammonium phosphate was added to all flasks before inoculation to ensure a sufficient nitrogen source during fermentation. The weight of the fermentation flasks was measured at regular intervals as an indication of fermentation speed by indirectly measuring CO_2 production and evaporation.

Large-scale vinification for sensory evaluation. Chardonnay grapes (23.7°B) were harvested during the 2001 season, destemmed, crushed and pressed. The must was treated with 50 mg/l SO_2 and allowed to settle overnight. Chemical analysis indicated that the Chardonnay juice contained 2.97 g/l L-malic acid, a pH of 3.53 and total acidity of 6.27 g/l. Similarly, the Cabernet Sauvignon grapes (21.8°B) were destemmed and crushed, treated with 30 mg/l SO_2 and divided into 12 lots of 15 l each, followed by direct inoculation with yeasts as described above. The Cabernet Sauvignon must contained 3.5 g/l L-malic acid, a pH of 3.77 and total acidity of 7.16 g/l. After three days of skin contact, the must was pressed and returned to fermentation flasks for further alcoholic fermentation.

The Chardonnay and Cabernet Sauvignon juice was divided into 12 lots of 15 l each for three repetitions of two different treatments, i.e. (1) inoculation with the parental yeast strain, *S. bayanus* EC1118 as a control fermentation, or (2) three positive transformants containing the integrated malo-ethanolic genes to a final concentration of 2 X 10^6 cells/ml. Fermentations in Chardonnay were carried out at 15°C, while Cabernet Sauvignon must was fermented at 23°C. After alcoholic fermentation was completed, one set of control wines (*S. bayanus* EC1118) from both Chardonnay and Cabernet Sauvignon was inoculated with Viniflora Oenos (Chris Hansen, Denmark) for MLF according to the manufacturers recommendations, while the rest of the wines were decanted and treated with 30 mg/l SO_2 and stored at 0°C for seven days for cold stabilisation. An experienced panel of 15 judges performed organoleptic evaluation of the Chardonnay wine six months after bottling. A ranking method was used to determine quality differences between the treatments and statistical significance was determined according to Amerine and Roessler (1983).

7.2.7 Chemical analysis

The concentrations of L-malic acid, D-glucose, glycerol and ethanol were determined using enzymatic assays (Roche Diagnostics, Germany). In-depth analysis of large-scale fermented wines were done by Capillary Electrophoresis (HP3D CE system, Hewlett-Packard) and GrapeScan 2000 (FOSS Electric A/S, Denmark) to determine glucose, fructose, glycerol, ethanol, tartaric, malic, citric, succinic, acetic and lactic acid concentrations. CE analysis was carried out with a diode array detector. The CE detector wavelength was fixed at 200 nm with 350 nm as the reference wavelength. A bare silica capillary with an internal diameter of 50 μm (total length = 80.5 cm and effective length = 72 cm) was

used for wine analysis and samples were injected hydrodynamically (50 mbar for 2 seconds). A constant voltage of –25 kV was applied during the separation run and the temperature of the column was set at 25°C. The "HP organic acid buffer" was used as the separation buffer. Wine samples were centrifuged (8 minutes x 12 000 rpm) before diluting them 20-fold in MilliQ water. A standard solution of L-tartaric acid (60 mg/l), L-malic acid (40 mg/l), citric acid (20 mg/l), succinic acid (20 mg/l), acetic acid (20 mg/l) and lactic acid (20 mg/l) was prepared freshly and were run between samples to create valid calibration curves for each component. HP Chemstation Software was used to calculate the concentrations of L-tartaric, L-malic, citric, succinic, acetic and lactic acids in the wine using data obtained from the standard and sample runs. The commercial calibration for the Grapescan 2000 was verified and adjusted to South African wines and conditions to ensure the correct intercepts. Standard methods were used to confirm the results obtained for residual sugar, final ethanol content, pH, total and volatile acidity of the finished wines (Ough and Amerine, 1987).

7.3 RESULTS AND DISCUSSION

7.3.1 Transformation of *S. bayanus* EC1118 with integration cassette

Electroporation of competent cells of *S. bayanus* with the linear malo-ethanolic integration cassette and pUT332 resulted in 100-200 phleomycin resistant transformants/µg of linear DNA. The transformants were individually picked and cultured in non-selective conditions (YPD broth) for more than 200 generations to obtain a phleomycin sensitive phenotype that corresponded to the loss of pUT332 carrying the resistance marker gene, *Tn5ble*. Transformants cured of pUT332 were subsequently spotted onto GMIA media to screen for the presence of a malo-ethanolic phenotype (Fig.7.3).

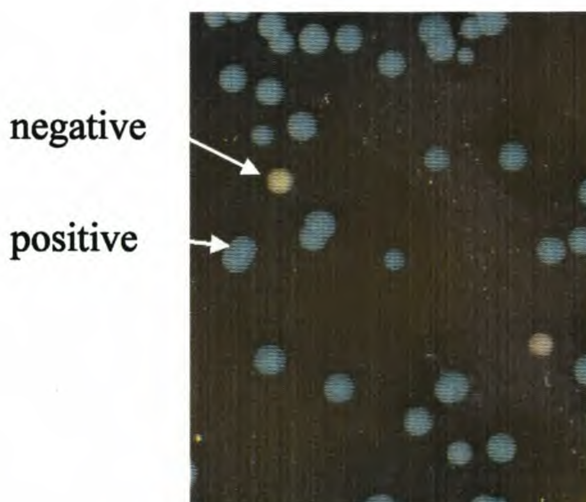

Figure 7.3. Transformants of *S. bayanus* EC1118 containing the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ expression cassettes (blue colonies) showed a clear malo-ethanolic phenotype on GMIA plates and could be distinguished from transformants lacking the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ expression cassettes (yellow/brown colonies).

Transformants with the ability to degrade L-malic acid appeared as blue-coloured colonies that could be easily distinguished from transformants lacking the malo-ethanolic phenotype (yellow/brown colonies). Colonies with a positive malo-ethanolic phenotype were re-inoculated into non-selective media and repeated in triplicate on GMIA media to determine the stability of the malo-ethanolic phenotype. The malo-ethanolic phenotype was considered to be stable in transformants if less than 1/10 000 revertant colonies appeared after each round of non-selective growth.

7.3.2 PCR amplification and Southern blot analysis of integration

The presence of the *mae1* and *mae2* open reading frames in the genome of *S. bayanus* EC1118 transformants (MEF) was confirmed by PCR amplification of a 1317 bp and 1698 bp fragment, corresponding to the complete open reading frame of the *mae1* and *mae2* genes, respectively (Fig. 7.4A). The parent strain (wt) did not produce any PCR products under the same conditions.

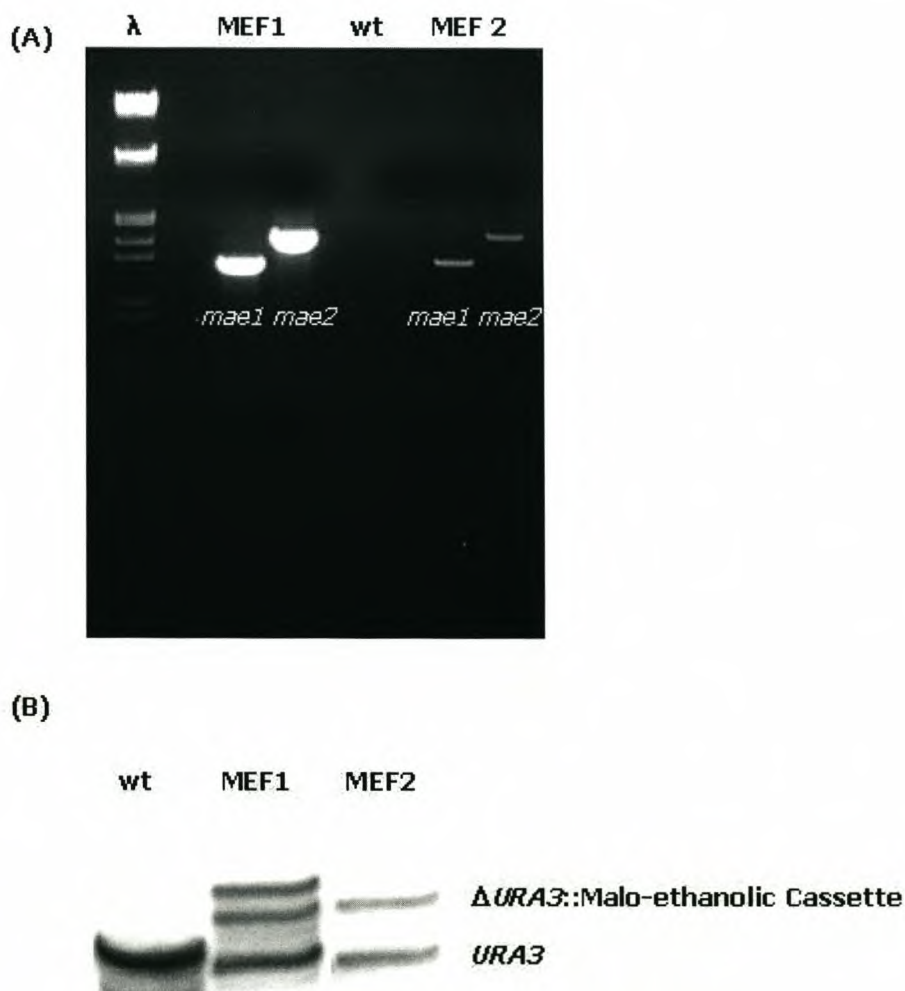

Figure 7.4. (A) PCR amplification of the *mae1* (1317 bp) and *mae2* (1698 bp) open reading frames using genomic DNA from *S. bayanus* EC1118 and selected transformants as template. (B) Southern blot results showing single (MEF2) or multiple (MEF1) integration of the malo-ethanolic cassette in the *URA3* locus. wt = parent strain *S. bayanus* EC1118.

The PCR product yield was significantly higher in transformants where multiple integrations of the malo-ethanolic cassette has occurred (MEF1), compared to the single integration events (MEF2). Integration of the malo-ethanolic cassette in the *URA3* gene was also confirmed with Southern blot analysis that clearly demonstrated single (MEF2) or multiple integration (MEF1) events into the *URA3* locus (Fig. 7.4B). MEF2 transformants containing a single integration of the malo-ethanolic cassette were used for subsequent fermentation and sensory evaluation experiments.

7.3.3. Malo-ethanolic fermentation in synthetic and actual grape must

Rapid and efficient degradation of ca. 9.5 g/l L-malic acid within 5 days was obtained in synthetic grape must during small-scale fermentations by a recombinant strain of *S. bayanus* EC1118 (MEF2), which contains a single integrated copy of the $PGK1_p$-$mae1$-$PGK1_t$ and $PGK1_p$-$mae2$-$PGK1_t$ expression cassettes (Fig. 7.5A). The parental strain (*S. bayanus* EC1118) showed no significant degradation of L-malic acid during the first 5 days of fermentation, but after 15 days, almost 32% of the total L-malic acid was degraded by this strain. This reduction in L-malic acid concentration by the control yeast strain is not ascribed to the active metabolism of L-malic acid by the yeast cells, but rather to the release of intracellular enzymes, i.e. malate dehydrogenases and the native malic enzyme during yeast autolysis at the late stationary phase of fermentation. Comparison of the growth rate and the rate of glucose consumption between the MEF2 and parental strain, showed no significant aberrations (Fig. 7.5B and C). This suggested that the introduction of the heterologous genes had no adverse effect on the recombinant yeast's growth ability and fermentation capacity.

During fermentative sugar metabolism, pyruvic acid is further decarboxylated to acetaldehyde by pyruvate decarboxylase and subsequently reduced to ethanol by the alcohol dehydrogenase enzyme in yeast. Theoretically, the introduction of an efficient malo-ethanolic pathway in yeast should contribute additional pyruvic acid to the existing intracellular pool, promoting the production of elevated levels of ethanol. As previously reported for laboratory strains (Volschenk *et al.*, 2001), the MEF2 strain consistently produced higher levels of ethanol relative to the parental strain (Fig. 7.5 B), confirming that the two *S. pombe* genes enabled cells of *S. cerevisiae* to metabolise the extracellular L-malic acid to ethanol under fermentative conditions.

The ability of the malo-ethanolic MEF2 wine yeast strain to degrade L-malic acid during alcoholic fermentation was also investigated during small-scale fermentations in Cabernet Sauvignon, Chardonnay, Colombard and Ruby Cabernet grape must (Fig 7.6 A). Rapid and complete degradation of extracellular L-malic acid degradation was observed for MEF2 within 1, 1.5, 2 and 5 days in Cabernet Sauvignon, Ruby Cabernet, Colombard and Chardonnay grape must, respectively. The parental yeast strain did not contribute significantly to the degradation of L-malic acid in the corresponding control fermentations. Furthermore, the fermentation rate measured as the loss of weight (CO_2 evaporation) was almost identical for MEF2 and the parental yeast strain (Fig. 7.6 B),

confirming that the expression of integrated *mae1* and *mae2* genes did not adversely affect the alcoholic fermentation capacity in the recombinant yeast.

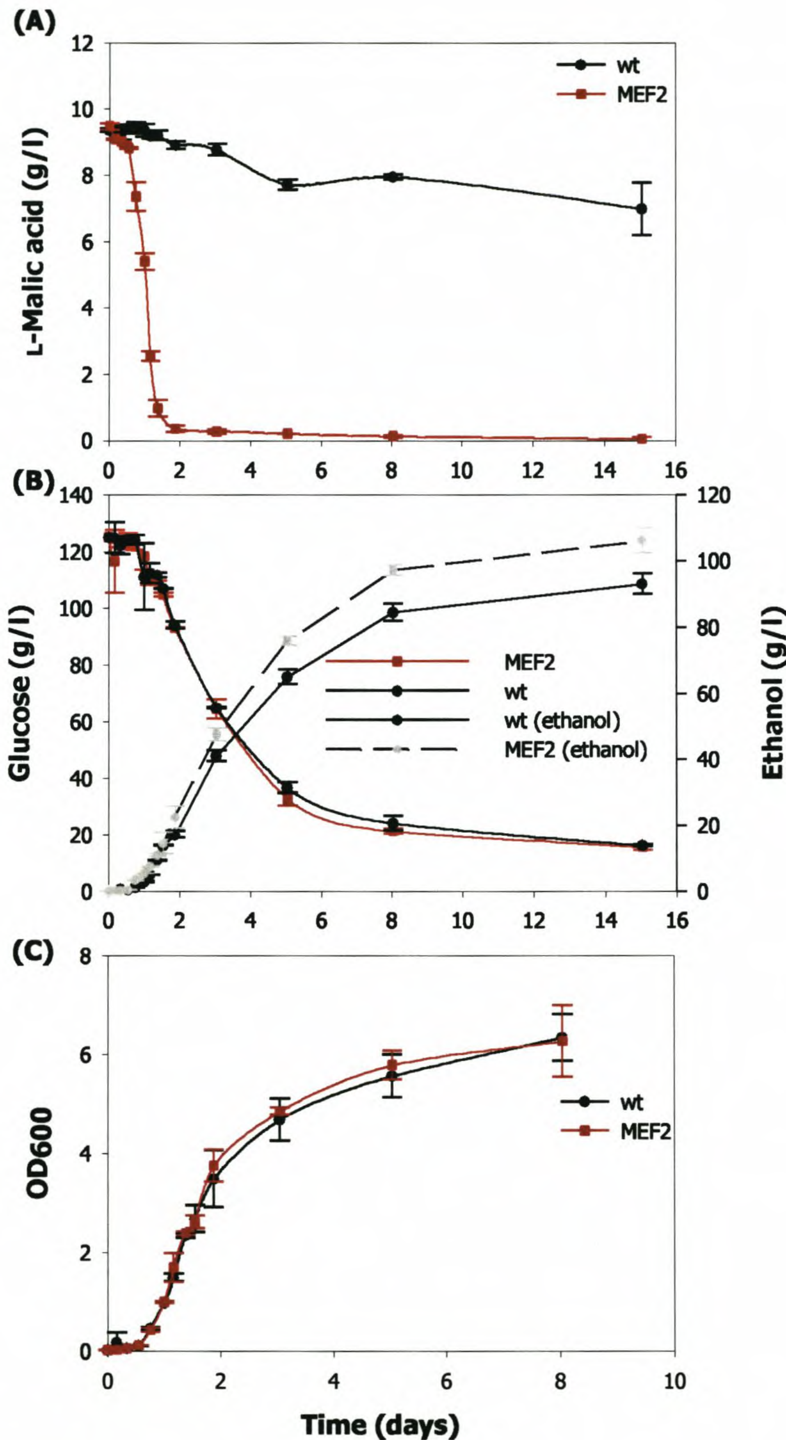

Figure 7.5. (A) L-Malic acid degradation by MEF2 compared to the control yeast (*S. bayanus* EC1118, wt); (B) Glucose utilisation and ethanol production by the MEF2 strain compared to the control yeast during alcoholic fermentation, and (C) growth curve of the malo-ethanolic yeast (MEF2) and control strain in synthetic grape must as measured by cell density at OD_{600}.

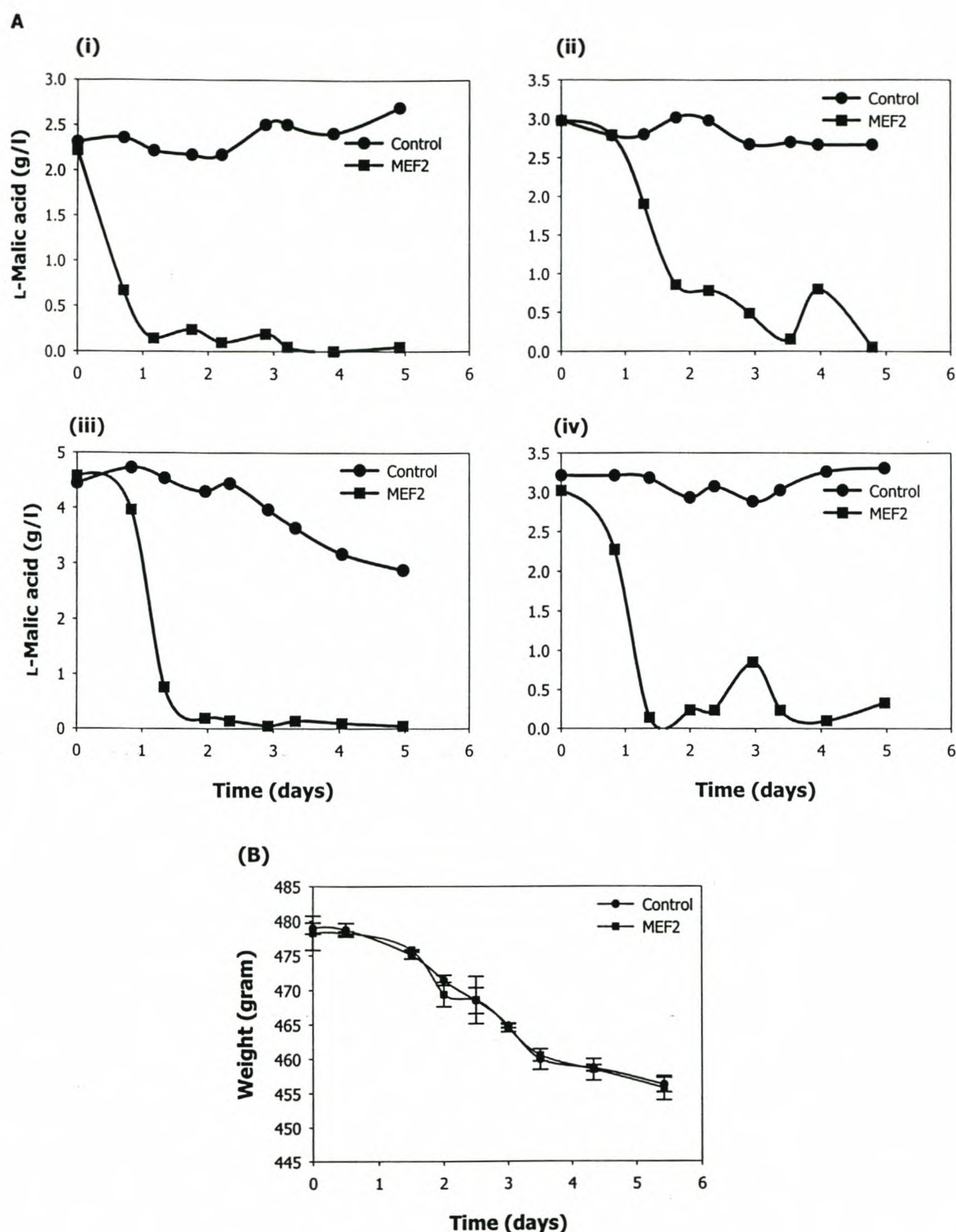

Figure 7.6. (A) Malo-ethanolic fermentation in (i) Cabernet Sauvignon, (ii) Chardonnay, (iii) Colombard and (iv) Ruby Cabernet grape must by MEF2 and the control yeast strain *S. bayanus* EC1118. (B) Fermentation rate of MEF2 and the control yeast strain in Chardonnay grape must as measured by the loss of CO_2 during alcoholic fermentation.

7.3.4. Effect of malo-ethanolic fermentation on organoleptic quality of wine

The ability of the MEF2 recombinant strain to produce a wine of quality was also evaluated during larger scale vinification of Chardonnay and Cabernet Sauvignon grape must. Standard winemaking practices were employed during the vinification, including the inoculation of the malolactic

bacterium, *O. oeni*, after alcoholic fermentation with *S. bayanus* EC1118 was completed. The malo-ethanolic yeast (MEF2) efficiently degraded all the L-malic acid in both Chardonnay and Cabernet Sauvignon grape must, whereas the parental strain, *S. bayanus* EC1118, had little effect on the L-malic acid concentration (Fig.7.7). The decrease in L-malic acid concentration also correlated with the decrease in total acidity (Table 7.3). In the wine fermented with MEF2, total acidity decreased by 2.3 g/l, in agreement with complete L-malic acid decomposition.

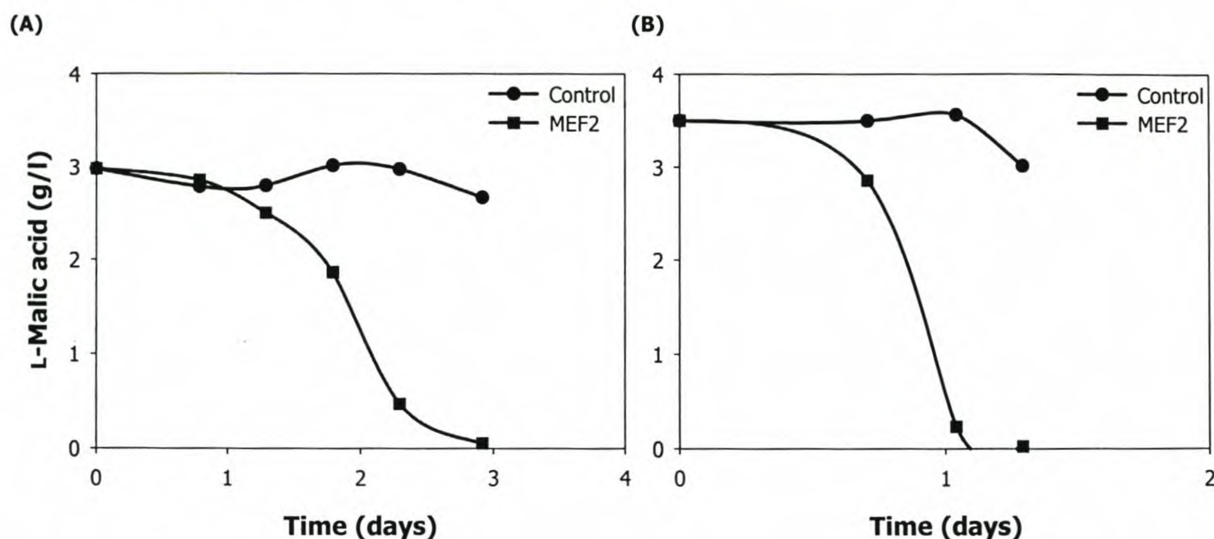

Figure 7.7. L-Malic acid degradation during larger scale fermentation of (A) Chardonnay and (B) Cabernet Sauvignon grape must with the recombinant MEF2 yeast containing the integrated malo-ethanolic expression cassette. The control fermentation was performed using the parental yeast *S. bayanus* EC1118.

Total acidity was decreased by 1.34 g/l in the wine that underwent MLF after alcoholic fermentation by *S. bayanus* EC1118, and by 0.54 g/l for wine produced by *S. bayanus* EC1118 without MLF. The decrease in acidity was also reflected in the pH of the different wines: the pH of the wine fermented with MEF2 increased with 0.46 units, whereas fermentation with *S. bayanus* EC1118 with and without MLF resulted in a pH increase of only 0.28 and 0.16 units, respectively. Chemical analysis of the final wines indicated no significant changes to the other organic acids in the wine fermented with MEF2 and the control yeast. The tartaric, citric and succinic acid concentrations remained relatively unchanged for the different treatments (Table 7.3). The concentration of lactic acid remained relatively constant for the control and MEF2 wine, while a significant increase in lactic acid could be seen in the wine that underwent MLF. Furthermore, volatile acidity as measured by the acetic acid concentration, was slightly increased in the wine fermented with MEF2, compared to the control yeast fermentation, but still within the threshold value for acetic acid in wine.

Table 7.3 Chemical composition of fermented Chardonnay wine

	L-Malic acid 9g/l)	Tartaric acid (g/l)	Citric acid (g/l)	Succinic acid (g/l)	Acetic acid (g/l)	Lactic acid (g/l)	Glycerol (g/l)	Ethanol (g/l)	Residual Glucose (g/l)	Residual Fructose (g/l)	Total acidity (g/l)	pH
Prior to fermentation	2.97 ±0.03								115 ±0.6	n.d.	6.27 ±0.02	3.53 ±0.01
***S. bayanus* EC1118**	2.82 ±0.2	1.26 ±0.01	0.36 ±0.01	0.26 ±0.02	0.38 ±0.01	0.09 ±0.1	6.5 ±0.1	14.3 ±0.1	0.44 ±0.6	3.13 ±0.3	5.73 ±0.6	3.69 ±0.01
***S. bayanus* EC1118 + MLF**	0.29 ±0.2	1.16 ±0.2	0.24 ±0.1	0.27 ±0.01	0.38 ±0.02	1.32 ±0.2	6.4 ±0.1	14.48 ±0.1	1.31 ±0.5	1.75 ±0.5	4.93 ±0.4	3.81 ±0.06
MEF2	0.22 ±0.1	1.47 ±0.02	0.44 ±0.18	0.27 ±0.01	0.56 ±0.01	0.13 ±0.2	7.2 ±0.2	14.6 ±0.2	1.24 ±0.4	2.64 ±0.2	3.97 ±0.2	3.99 ±0.2

n.d. = not determined
± = standard deviation

Table 7.4 Results of the organoleptic evaluation of Chardonnay wine

Treatment	Aroma order	Palate order
***S. bayanus* EC1118**	3*	1**
***S. bayanus* EC1118 + MLF**	2	2
MEF2	1**	3*

* -significant at p<0.05
** signficant at p<0.01

Comparison of the final ethanol values of the wines indicated an insignificant increase in ethanol concentration of the wine fermented with MEF2 in relation to the control wine fermented with *S. bayanus* EC1118. Glycerol production in yeast acts as a mechanism to rectify any possible NAD^+/NADH imbalances during yeast metabolism. Therefore, changes in the redox balance in yeast metabolism, for example during oxidative or osmotic stress conditions, is associated with changes in the amounts of glycerol produced by yeast (Larson *et al.*, 1998; Nordström, 1968; Oura, 1977; Van Dijken and Scheffers, 1986). Since the conversion of L-malic acid to pyruvic acid by the malic enzyme involves the reduction of NAD^+ to NADH, the level of glycerol in wine was also determined after alcoholic fermentation. Wine fermented by the MEF2 strain showed an increase of ca. 1 g/l glycerol relative to the wine fermented by the control yeast.

Organoleptic evaluations of the fermented Chardonnay wine indicated a significant difference between wines produced with *S. bayanus* EC1118, the MEF2 recombinant strain and wines that underwent MLF (Table 7.4). Based on the perceived aroma of the wines, the lowest score was obtained for the wine fermented by *S. bayanus* EC1118, while wine made by MEF2 scored the highest, even higher than the wine that underwent MLF. No off-flavours were detected, while an increase in fruitiness was noted. These results indicate that the malo-ethanolic strain was more successful in producing fruity-floral aromas in wine, a definite advantage in the production of cultivars such as Muscat, Riesling, Sauvignon Blanc and Gewürztraminer. However, based on the perceived palate of the wine, the best results were obtained with wine fermented by the control yeast, *S. bayanus* EC1118, while wine produced with MEF2 scored the lowest. The taste panel detected an imbalance in the acid : sugar ratio in the wine made by the malo-ethanolic yeast (MEF2). This could be expected, since all the L-malic acid was completely removed from the wine and resulted in a sub-optimal final total acidity (Table 7.3). The Chardonnay and Cabernet Sauvignon wines used in this study were harvested from a warm-climate viticultural region and contained low levels of L-malic acid in the grape must, i.e. 2.97 and 3.5 g/l L-malic acid, respectively. Future evaluation of the malo-ethanolic yeast in high-acid wines from a cool-climate viticultural region will be required to determine the actual organoleptic influence of this recombinant yeast on wine.

In this study, the commercial wine yeast *S. bayanus* EC1118 was successfully transformed through integration of a malo-ethanolic cassette containing the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ linear integration cassette flanked by large *URA3* homologous sequences. A single genomic copy of the malo-ethanolic cassette in *S. bayanus* EC1118 was sufficient to yield a strong malo-ethanolic phenotype, i.e. the conversion of L-malic acid to ethanol, in the recombinant yeast in synthetic and grape must fermentations. Sensory evaluation and chemical analysis of a Chardonnay wine produced by the malo-ethanolic yeast indicated an improvement in wine aroma compared to the traditional MLF. Commercial availability of malo-ethanolic wine yeast will be especially beneficial in the

production of fruity-floral wines and the deacidification of high-acid wines in the cool-climate viticultural regions of the world.

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Addendum C

C.1 INITIAL STRATEGIES DEVELOPED FOR INTEGRATION OF MALO-ETHANOLIC CASSETTE IN *SACCHAROMYCES*

C.1.1 Linear *URA3*- *$PGK1_p$-mae1-$PGK1_t$* - *$PGK1_p$-mae2-$PGK1_t$-URA3* cassette

During the course of this study, the integration strategy for the malo-ethanolic cassette has evolved through several steps of development. The initial strategy involved the PCR amplification of the *$PGK1_p$-mae1-$PGK1_t$* and *$PGK1_p$-mae2-$PGK1_t$* expression cassettes from plasmid pHV3 and pHV7 as two separate cassettes, which were ligated to form a linear integration cassette of 7500 bp after digestion at the *Kpn*I-site (Fig. C.1). Based on homology to the *URA3*, *PGK1* promoter and terminator regions, PCR primers were designed (Table C.1) to add a 50 bp upstream *URA*3 sequence at the 5'-end and a *Kpn*I restriction site at the 3'-end of the *$PGK1_p$-mae1-$PGK1_t$* expression cassette (5'-PGKURA50 and 3'-PGKKPN). Similarly the *$PGK1_p$-mae2-$PGK1_t$* expression cassette was PCR amplified with primer set 5'-PGKKPN and 3'-PGKURA50 to create a *Kpn*I restriction site at the 5'-end and a 50 bp downstream *URA3* sequence at the 3'-end.

Figure C.1. PCR amplification of the linear integration cassettes containing the *$PGK1_p$-mae1-$PGK1_t$* and *$PGK1_p$-mae2-$PGK1_t$* expression cassettes. The PCR primers added a *URA3* flanking region on one side of the linear construct in such a way that when the fragments were joined together by ligation at the *Kpn*I site, the *URA3* sequences flanked the cassette on both sides.

PCR conditions were optimised by changing the *Taq* DNA polymerase enzyme (Expand High-fidelity, Roche Diagnostics, Germany), primer and $MgCl_2$ concentrations, as well as the annealing temperature and extension time to obtain sufficient amounts of PCR product for *Kpn*I digestion and T_4 DNA ligation. However, ligation of the two individual cassettes yielded low amounts of DNA that had to be amplified again to generate sufficient quantities of the 7.5 kb linear integration cassette for transformation of industrial strains of *Saccharomyces*.

Several co-transformations of industrial wine yeast strains, including *S. bayanus* EC1118, with the purified 7.5 kb linear fragment together with ether phleomycin-resistance plasmid pUT332 or the G418 geneticin-resistance plasmid pKX34 (Lang-Hinrichs *et al.*, 1989), were attempted by using electroporation. This yielded 200-300 transformants/μg linear DNA with phleomycin and large amounts of background colonies with G418 geneticin. The use of geneticin selection in combination with electroporation of the yeast cells always resulted in undesirable high levels of background colonies, but not when the LiOAc transformation method was used. However, all transformants generated via the LiOAc or electroporation methods yielded a negative malo-ethanolic phenotype on GMIA agar. Since the transformation efficiency with electroporation of the linear integration cassette was relatively high, the absence of a malo-ethanolic phenotype in the transformants was ascribed to a lack of integration events. A possible explanation for unsuccessful integration of the malo-ethanolic cassette could be that the 50 bp *URA3* flanking regions were insufficient for homologous recombination in the genome of the industrial wine yeast strains.

Table C.1. PCR primers used for the amplification of the *URA3* and *LEU2* flanking regions and *Kpn*I restriction site in the *PGK1*$_p$-*mae1*-*PGK1*$_t$ and *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassettes

Primer	Sequence
5'-PGKURA50	5'-CCCAGTATTC TTAACCCAAC TGCACAGAAC AAAAACCTGC AGGAAACGAA **GAAAGCTTTC TAACTGATCT ATCC**-3'
3'-PGKURA50	5'-TTTTGCTGGC CGCATCTTCT CAAATATGCT TCCCAGCCTG CTTTTCTGTA ACGTTCACCC **AAGCTTTAAC GAACGCAGAA TTTTCG**-3'
5'-PGKKPN	5'-GATCGGTACC **AACCTTTCTA ACTGATC**-3'
3'-PGKKPN	5'-GATCGGTACC **AAGCTTTAAC GAACGCA**-3'
5'-PGKURA100	5'-CTTAGATTGG TATATATACG CATATGTAGT GTTGAAGAAA CATGAAATTG CCCAGTATTC TTAACCCAAC TGCACAGAAC AAAAACCTGC AGGAAACGAA **GAAAGCTTTC TAACTGATCT ATCC**-3'
3'-PGKURA100	5'-TAATTTGTGA GTTTAGTATA CATGCATTTA CTTATAATAC AGTTTTTTAG TTTTGCTGGC CGCATCTTCT CAAATATGCT TCCCAGCCTG CTTTTCTGTA ACGTTCACCC **AAGCTTTAAC GAACGCAGAA TTTTCG**-3'
5'-PGKLEU100	5'-GAGAAGCGTT CATGACTAAA TGCTTGCATC ACAATACTTG AAGTTGACAA TATTATTTAA GGACCTATTG TTTTTTCCAA TAGGTGGTTA GCAATCGTCT **GAAAGCTTTC TAACTGATCT ATCC**-3'
3'-PGKLEU100	5'-CCCTATGAAC ATATTCCATT TTGTAATTTC GTGTCGTTTC TATTATGAAT TTCATTTATA AAGTTTATGT ACAAATATCA TAAAAAAAGA GAATCTTTTT AAGC**AAGCTT TAACGAACGC AGAATTTTCG**-3'

Bold sequences correspond to the ***PGK1*** promoter and terminator regions
Underlined sequences correspond to the *Kpn*I restriction site

In an attempt to increase the homologous *URA3* flanking regions, new PCR primers were designed which contained 100 bp *URA3* extensions (Table C.1, 5'-PGKURA100 and 3'-PGKURA100). The new PCR primers were used to amplify the *PGK1*$_p$-*mae1-PGK1*$_t$ and *PGK1*$_p$-*mae2-PGK1*$_t$ expression cassettes using a similar strategy as for the 50 bp *URA3* flanking regions (Fig C.1). Transformation of the new 7.7 kb linear cassette in combination with the phleomycin resistance plasmid (pUT332) again did not result in integration of the malo-ethanolic cassette into the genome of the industrial strains. Similar to the 50 bp *URA3* flanking integration strategy, efficient transformation efficiency was obtained based on phleomycin resistance selection, but no malo-ethanolic phenotype was observed on GMIA media. It was concluded that the molecular size of the linear integration cassette (7.5 –7.7 kb) might be responsible for the inability to integrate the cassette into the genomes of the industrial wine yeast strains.

C.1.2 Linear *URA3-PGK1*$_p$*-mae1-PGK1*$_t$*–URA3* and *LEU2-PGK1*$_p$*-mae2-PGK1*$_t$*-LEU2* cassettes

A second integration strategy was therefore designed for the integration of the malo-ethanolic cassette in industrial strains of *Saccharomyces*. This strategy involved the PCR amplification of shorter linear integration cassettes containing the individual *PGK1*$_p$-*mae1-PGK1*$_t$ and *PGK1*$_p$-*mae2-PGK1*$_t$ expression cassettes with PCR primers (Table C.1) that added 100 bp flanking sequences of either the *S. cerevisiae URA3* or *LEU2* genes (Fig. C.2). PCR amplification of the individual expression cassettes was accomplished by using previously optimised conditions for long-template amplification, resulting in high product yields. The linear PCR fragments were purified using the High-Pure PCR purification kit (Roche Diagnostics, Germany) and co-transformed with the phleomycin resistance plasmid, pUT332, by electroporation into industrial yeast strains of *Saccharomyces*. Transformants were selected on phleomycin-containing medium at the optimum concentrations of the antibiotic as previously determined. Phleomycin-resistant transformants were selected and transferred to GMIA plates and non-selective liquid medium (YPD).

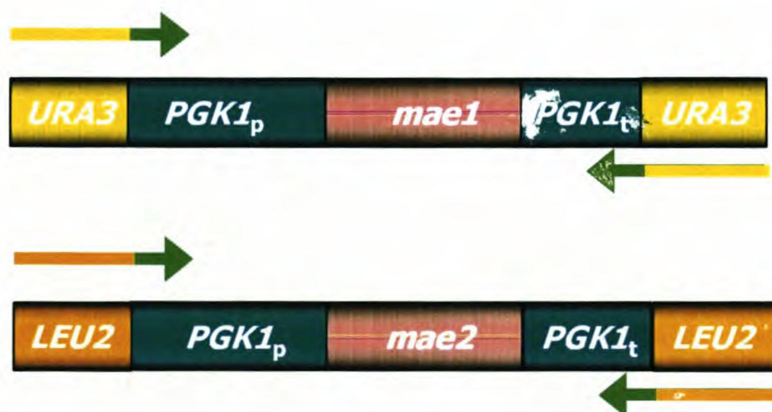

Figure C.2. PCR-amplification of the *URA3-PGK1*$_p$*-mae1-PGK1*$_t$*-URA3* and *LEU2-PGK1*$_p$*-mae2-PGK1*$_t$*-LEU2* expression cassettes for transformation into wine yeasts.

Analyses of transformants by PCR indicated that the *mae1* and *mae2* genes were not retained, which implied that stable integration of the linear expression cassettes did not occur. Typically, the *mae1* and *mae2* genes could be detected by PCR amplification during the first few generations after growth in non-selective media, but would subsequently disappear. It was concluded that the problem lies with the ineffective homologous recombination of the linear integration cassette into the yeast genome and not the transformation efficiency, since an average transformation frequency of 100 to 300 colonies/μg of DNA was be obtained.

C.1.3 Sub-cloning of the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ expression cassettes into a mutated *URA3* gene

The next strategy was therefore developed to increase the *URA3* flanking regions to ca. 500-600 bp to enhance homologous recombination of the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ expression cassettes in industrial yeast genome. Furthermore, this strategy will ensure a high degree of fidelity and integrity of the original $PGK1_{pt}$, *mae1* and *mae2* sequences, since one of the disadvantages of the above PCR-based strategies is the inevitable introduction of random mutations in the DNA sequence of the expression cassettes due to repetitive amplification cycles. The *URA3* gene was PCR amplification and subsequent cloning yielded a mutated *URA3* gene containing unique restriction sites for subcloning of the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ expression cassettes and excision restriction sites for generation of a linear integration cassette (see research results, Chapter 7).

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

General Discussion and Conclusions

The production of good quality wines often requires the proper adjustment of wine acidity in relation to the other wine components to create a well-balanced bottled product. The traditional method to deacidify wine involves the conversion of L-malic acid to L-lactic acid and CO_2 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. Although wine yeast strains of *Saccharomyces* have different abilities to utilise extracellular L-malic acid, they are generally regarded as weak degraders of L-malic acid in grape must during alcoholic fermentation. A number of reasons for the weak degradation of L-malic acid in *Saccharomyces cerevisiae* have been postulated, of which the most significant is the lack of active transport of L-malic acid, the low substrate affinity of its malic enzyme, and the fact that the malic enzyme is located in the mitochondria.

The aim of this study was in part to obtain a better understanding of the fundamental differences between strains of *Saccharomyces* with diverse malic acid-degrading abilities, and ultimately to develop a genetically engineered industrial strain of *Saccharomyces* with the ability to efficiently degrade L-malic acid to ethanol during alcoholic fermentation.

8.1 REGULATION OF THE NATIVE MALIC ENZYME GENE IN *SACCHAROMYCES* SPP.

In this study, we investigated the underlying mechanisms in three different strains of *Saccharomyces* showing varying aptitudes to degrade extracellular L-malic acid during alcoholic fermentation. *Saccharomyces paradoxus* strain RO88, an indigenous strain isolated from Zagreb vineyards, was able to degrade 28% and 38% L-malic acid in a synthetic and Chardonnay must, respectively. *S. cerevisiae* strain 71B, although marketed as a malate-degrading strain, and *Saccharomyces bayanus* strain EC1118, a commercial wine yeast strain known for its poor malic acid degradation, degraded only 17% and 8 % of the malic acid during alcoholic fermentation, respectively.

The reason(s) for the varying degrees of malic acid degradation by *Saccharomyces* strains are not known and has not yet been described on a molecular level. Since *Saccharomyces* strains do not have a transport system for the uptake of malic acid, the answers are most likely to be found inside the cell, i.e. the intracellular enzymes involved in the degradation of malic acid. In *Saccharomyces,* intracellular malic acid is usually degraded via the malic enzyme or malate dehydrogenase, with the malic enzyme playing the major role. The focus of our further investigations was therefore the malic

enzyme gene and its regulation in the three *Saccharomyces* strains, i.e. *S. paradoxus* R088, *S. cerevisiae* 71B and *S. bayanus* EC1118.

The highly conserved amino acid sequence of the malic enzyme proteins from the three *Saccharomyces* strains suggested that the difference in malic acid degradation by these strains was most likely due to transcriptional regulation rather than enzyme activity. Sequence analysis of the promoter region of the malic enzyme genes from the three strains indicated that the malic enzyme promoter sequence of *S. paradoxus* R088 differs significantly from that of *S. cerevisiae* 71B and *S. bayanus* EC1118 (see Chapter 5). This suggested that the ability of *S. paradoxus* R088 to degrade malic acid more efficiently could possibly be ascribed to a higher rate of transcription of its malic enzyme gene.

An mRNA expression profile of the malic enzyme (*MAE1*) genes from *S. paradoxus*, *S. bayanus* and *S. cerevisiae* during alcoholic fermentation in a synthetic grape must was used to determine possible divergent regulation scenarios (Chapter 5). The results suggested that the malic enzyme may play an important role in enabling the yeast to respond to different physiological conditions, such as the available carbon source. Preliminary transcriptional regulation studies indicated that expression of the malic enzyme genes from *S. paradoxus* RO88 and *S. cerevisiae* 71B increased towards the end of fermentation once glucose was depleted, whereas the level of transcription in *S. bayanus* EC1118, a non-degrading strain, decreased towards the end of fermentation. Only *S. paradoxus* RO88 showed 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 *S. paradoxus* and the other two *Saccharomyces* strains, it is plausible that different transcription regulatory mechanisms exist in *S. paradoxus* that could explain this yeast's higher aptitude to degrade L-malic acid.

8.2 INTEGRATION AND EXPRESSION OF THE MALO-ETHANOLIC GENES OF *S. POMBE* IN AN INDUSTRIAL WINE YEAST STRAIN OF *SACCHAROMYCES*

In contrast to *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* can effectively degrade extracellular L-malic acid via the malo-ethanolic pathway due to the presence of a malate permease for the active transport of malic acid, as well as a malic enzyme with a high substrate affinity. In our

search for an alternative approach to deacidify wine, we investigated the expression of the *S. pombe* malate permease (*mae1*) and malic enzyme (*mae2*) genes in strains of *Saccharomyces*.

A strong malo-ethanolic phenotype was introduced into a *S. cerevisiae* laboratory strain when the *S. pombe mae1* and *mae2* genes were functionally co-expressed under the constitutive regulation of the *S. cerevisiae* 3-phosphoglycerate kinase (*PGK1*) promoter and terminator elements (Chapter 6). Although the regulatory elements of the *S. pombe mae1* and *mae2* genes were not functional in *S. cerevisiae*, the results clearly showed that the malate transport protein and malic enzyme was active in *S. cerevisiae* when expressed under the *PGK1* promoter and terminator elements. This approach allowed us to introduce two new enzymatic activities into *S. cerevisiae* to yield recombinant strains with the ability to (1) actively transport L-malic acid into the cell, and (2) convert the malic acid to pyruvic acid and CO_2. The effective functioning of the malo-ethanolic pathway was confirmed when higher levels of ethanol were produced by the malo-ethanolic strains during fermentation. This is of particular importance to the distilled beverage industry where an improved ethanol yield is highly desirable.

The introduction of a heterologous malo-ethanolic pathway in a laboratory strain of *S. cerevisiae* was regarded as a major biotechnological breakthrough in the wine industry. However, the stable integration of the *S. pombe* malo-ethanolic genes into the genome of commercial wine yeast strains of *Saccharomyces* was essential to provide a recombinant yeast strain that can be used for commercial wine fermentation. Several integration strategies were developed during the course of this study with the underlying principle being the generation of linear fragments containing the *mae1* and *mae2* expression cassettes flanked by *URA3* regions (Chapter 7).

Initial efforts to generate the linear integration cassettes were based on a PCR amplification procedure whereby 50 to 100 bp of flanking *URA3* sequences are added, proved to be unsuccessful. To increase the odds of homologous recombination at the *URA3* locus, the *mae1* and *mae2* expression cassettes were sub-cloned into a modified *URA3* fragment that provided 500-600 bp *URA3* flanking regions on the linear fragment. Integration of the *mae1* and *mae2* genes was achieved in *S. bayanus* EC1118 and verified with Southern blot analysis as being a single copy integration in the *URA3* locus of the recombinant yeast (Chapter 7). The initial problems associated with the integration of the malo-ethanolic cassette in industrial strains of *Saccharomyces* suggested that, although 50-100 bp flanking regions are sufficient for homologous recombination in laboratory strains of *S. cerevisiae*, the same process requires much larger homologous flanking regions for efficient integration in industrial strains of *Saccharomyces*.

The single integrated copy of the malo-ethanolic genes in *S. bayanus* EC1118 was sufficient to yield a strong malo-ethanolic phenotype, i.e. the conversion of 9.5 g/l of L-malic acid to ethanol within 5

days (Chapter 7). The efficiency of the malo-ethanolic phenotype in the recombinant *S. bayanus* industrial strain also compared well with that obtained for the laboratory strain of *S. cerevisiae*, suggesting that the copy number of the genes had little effect on the efficiency of the malo-ethanolic phenotype. Sensory evaluation and chemical analysis of a Chardonnay wine produced by the malo-ethanolic yeast indicated an improvement in wine aroma compared to the traditional malolactic fermentation. These results underline the prediction that the newly introduced malo-ethanolic pathway should not have any negative influence on the fermentation capacity and metabolic activities of the yeast, since the malo-ethanolic pathway is an electro-neutral process, i.e. all the NADH produced during the oxidative decarboxylation of L-malic acid is re-oxidised to NAD^+ during the final conversion to ethanol. However, a slight increase in glycerol and acetic acid concentrations indicate that the recombinant yeast is under some degree of metabolic stress due to the over-expression of the *mae1* and *mae2* genes.

8.3 GENERAL CONCLUSIONS

It has been well documented that strains of *Saccharomyces* display different abilities to degrade extracellular L-malic acid, but the underlying reasons for the phenomenon were unknown. This study has provided insight into the regulation of the malo-ethanolic pathway in strains of *Saccharomyces* with different abilities to degrade L-malic acid. It is the first report on the possible role of transcriptional regulation of the malic enzyme (*MAE1*) gene in the differential degradation L-malic acid by different strains of *Saccharomyces* during wine fermentation. However, a further in-depth analysis of the molecular machinery and physiological effectors governing the regulation of malic acid metabolism in *Saccharomyces* is required to fully elucidate this phenomenon.

The deacidification of wine can be achieved through one of several physiochemical or biological methods. Physiochemical deacidification of wine is, however, often time-consuming, requires increased labour and capital input and is regularly associated with reduced wine quality. For these reasons, biological deacidification of wine with malolactic fermentation is the method of choice for most winemakers as it is a perceived to be a natural or spontaneous process. However, due to inherent problems associated with malolactic fermentation and its unsuitability in some fruity-floral cultivars, alternative biological methods for the deacidification of wine will be of great benefit to the wine industry. The commercial availability of a malo-ethanolic wine yeast will be especially beneficial to the production of fruity-floral wines, such as Gewürtzraminer and Riesling, as well as the deacidification of high-acid wines in the cool-climate viticultural regions of the world. Deacidification of wine with a malo-ethanolic 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-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.

8.3.1 Commercial application of genetically engineered wine yeast: Ethical Perspectives

Genetic engineering of wine yeast is considered an expansion of modern biotechnology where new and improved yeast strains are constructed in a highly regulated and specific fashion as opposed to the arduous process of artificial selection and breeding employed by humans for more than 10 000 years. Although genetic engineering by definition harnesses the basic power of natural selection and breeding, it has not gained worldwide acceptance due to stigmatisation by lobbying groups, consumer mistrust, obvious regulatory oversights and mistrust in government bureaucracies. All these factors fueled debates for over 20 years about the environmental and public health safety of genetically modified organisms (GMO's).

One of the foremost consumer concerns is the human and environmental safety aspect of GMO's, and whether or not GMO's are safe in the immediate and long-term. Some of the prerequisites include a complete and exact knowledge of the function(s) of the incorporated or modified genetic material. Substantial proof must be provided that this new function is correctly expressed and the complete nucleotide sequence of the incorporated DNA has to be determined. Unwanted spreading of the inserted DNA to other organisms' genomes through horizontal gene transfer should be evaluated and controlled. The performance of recombinant yeast strains in beverages and in the environment should be anticipated and eventually evaluated. Lastly, the absence of any toxicity or pathogenicity must be proved.

One of the negative effects from genetically modified food and beverages could be the risk of increasing potential sources of allergies in various products. However, there is no reliable method of foreseeing potential allergic reactions that may be introduced by a newly acquired enzyme activity. Most of the public health risks essentially revolve around antibiotic resistance, since many GMO's contain bacterial plasmid elements which may include genes encoding for resistance to ampicillin.

In complying with consumer safety concerns, a unique strategy was developed in this study for the stable integration of an efficient malo-ethanolic pathway in a commercially used *Saccharomyces* wine yeast strain that would adhere to the above consumer safety requirements. The genes that were integrated into the commercial wine yeast strain, have a known enzymatic function and were introduced into the yeast in a judicious manner, i.e. only yeast-derived DNA was integrated at a known chromosomal location. Any possible toxicity or pathogenicity associated with foreign or non-

yeast DNA elements, such as antibiotic resistance markers, were therefore avoided. The commercial production of wine with a malo-ethanolic yeast strain will in fact have a tendency to enhance the health benefits of wine, since the risk of the production of biogenic amines and carcinogenic ethyl carbamate will be significantly reduced. Furthermore, evaluation of the recombinant yeast during wine fermentation indicated that the recombinant yeast did not deviate from the parental yeast strain in terms of fermentation performance or metabolic activities. The integration strategy developed in this study could therefore serve as a model technique for future construction of genetically engineered strains of *Saccharomyces*.

Commercial application of the recombinant malo-ethanolic yeast will require further characterisation and performance evaluation under large-scale fermentation conditions. Specific care should be taken to determine the complete sequence of the integrated fragment to account for every single base pair introduced into the yeast. DNA micro-array analysis could be used to determine the effect of the two integrated malo-ethanolic genes on the transcription of other yeast genes on a genomic scale. In addition, a long-term study on the potential environmental consequences of the recombinant malo-ethanolic yeast strain in a vineyard and winery environment will be required to determine the stability of the malo-ethanolic phenotype in terms of "horizontal gene transfer" to other yeasts via the natural process of mating.