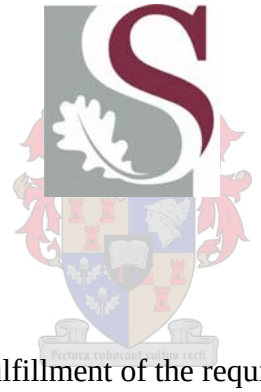
Characterisation of the Malate Transporter and Malic Enzyme from *Candida utilis*

Maryna Saayman



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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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M. Saayman **Date**

SUMMARY

Yeast species differ remarkably in their ability to degrade extracellular dicarboxylic acids and to utilise them as their only source of carbon. The fission yeast *Schizosaccharomyces pombe* effectively degrades L-malate, but only in the presence of an assimilable carbon source. In contrast, the yeast *Saccharomyces cerevisiae* is unable to effectively degrade L-malate, which is ascribed to the slow uptake of L-malate by diffusion. In contrast, the yeast *Candida utilis* can utilise L-malate as the only source of carbon and energy, but this is subject to substrate induction and catabolite repression. Very little research has been done on a molecular level in *C. utilis* and only a few of its genes have been studied.

In this study, we have shown that the yeast *C. utilis* effectively degraded extracellular L-malate and fumarate, but in the presence of glucose or other assimilable carbon sources, the transport and degradation of these dicarboxylic acids was repressed. The transport of both dicarboxylic acids was shown to be strongly inducible by either L-malate or fumarate and kinetic studies suggest that the same transporter protein transports the two dicarboxylic acids. In contrast, *S. pombe* effectively degraded extracellular L-malate, but not fumarate, only in the presence of glucose or other assimilable carbon sources. The *S. pombe* malate transporter was unable to transport fumarate, although fumarate inhibited the uptake of L-malate.

In order to clone the *C. utilis* dicarboxylic acid transporter, a cDNA library from *C. utilis* was constructed using a number of strategies to ensure representativeness and high transformation frequencies. The cDNA library was transformed in a *S. cerevisiae* strain carrying a plasmid containing the *S. pombe* malic enzyme gene (*mae2*) to allow screening for a malate-degrading *S. cerevisiae* clone. However, no positive clones that would indicate the successful cloning of the *C. utilis* malate transporter were obtained.

The *C. utilis* malic enzyme gene, *CuME*, was subsequently isolated from the cDNA library based on conserved sequence homologies with the genes of *S. cerevisiae* and *S. pombe*, and characterised on a molecular and biochemical level. Sequence analysis revealed an open reading frame of 1926 bp, encoding a 641 amino acid polypeptide with a predicted molecular weight of 70.2 kDa. The optimum temperature for the *C. utilis* malic enzyme was 52°C and the enzyme was stable at 50°C for 2 hours. The inferred amino acid sequence showed significant homology with the malic enzymes of *S. pombe* and *S. cerevisiae*. Expression of the *CuME* gene is subject to glucose repression and substrate induction, as was observed for

the dicarboxylic acid transporter from *C. utilis*. The *CuME* gene was successfully co-expressed with the *S. pombe* malate permease gene (*mae1*), resulting in a recombinant strain of *S. cerevisiae* able to effectively degrade L-malate.

OPSOMMING

Daar is ’n merkwaardige verskil in die vermoë van verskillende gisspesies om ektrasellulêre dikarboksielsure af te breek en dit as enigste bron van koolstof te benut. Die splitsingsgis *Schizosaccharomyces pombe* kan L-malaat effektief afbreek, maar slegs in die teenwoordigheid van ’n ander benutbare koolstofbron. In teenstelling hiermee is dit vir die gis *Saccharomyces cerevisiae* onmoontlik om L-malaat effektief af te breek en te benut, wat hoofsaaklik toegeskryf kan word aan die stadige opname van L-malaat deur middel van diffusie. Die gis *Candida utilis* kan egter L-malaat as die enigste bron van koolstof en energie benut, maar dit is onderhewig aan substraat-induksie en kataboliet onderdrukking. Baie min navorsing op molekulêre vlak is tot hede in *C. utilis* uitgevoer en slegs ’n paar gene in hierdie gis is al bestudeer.

In hierdie studie het ons aangetoon dat die gis *C. utilis* L-malaat en fumaraat effektief afbreek, maar dat glukose of ander benutbare koolstofbronne die opname en afbraak van hierdie dikarboksielsure onderdruk. Die opname van beide dikarboksielsure is sterk induseerbaar deur L-malaat óf fumaraat, terwyl kinetiese studies toon dat beide dikarboksielsure deur dieselfde transporter-proteïen vervoer word. In teenstelling hiermee kan *S. pombe* ekstrasellulêre L-malaat, maar nie fumaraat nie, in die teenwoordigheid van glukose of ’n ander benutbare koolstofbron effektief afbreek. Die *S. pombe* L-malaat transporter was nie in staat om fumaraat te vervoer nie, alhoewel fumaraat die opname van L-malaat onderdruk het.

Ten einde die dikarboksielsuur transporter van *C. utilis* te kloneer, is verskeie strategieë gevolg ten einde ’n cDNA-biblioteek van *C. utilis* te konstrueer wat verteenwoordiging en hoë transformasie-frekwensies kan verseker. Die cDNA-biblioteek is getransformeer in ’n *S. cerevisiae* ras wat die *S. pombe* malaatensiem geen (*mae2*) bevat om die sifting van ’n *S. cerevisiae* kloon wat malaat effektief kan afbreek, moontlik te maak. Geen positiewe klone wat dui op die klonering van die *C. utilis* malaat transporter kon egter gevind word nie.

Die *C. utilis* malaatensiem geen, *CuME*, is vervolgens van uit die cDNA biblioteek geïsoleer deur van gekonserveerde DNA-homologie met *S. cerevisiae* en *S. pombe* gebruik te maak, en op molekulêre en biochemiese vlak gekarakteriseer. DNA-volgordebepaling het ’n oopleesraam van 1926 bp onthul, wat kodeer vir ’n 641 aminosuur polipeptied met ’n verwagte molekulêre gewig van 70.2 kDa. Die optimale temperatuur van die *C. utilis* malaatensiem was 52˚C en die ensiem was vir 2 ure stabiel by 50˚C. Die afgeleide

aminosuurvolgorde het beduidende homologie met die malaatensieme van *S. pombe* en *S. cerevisiae* getoon. Die *CuME* geen is suksesvol saam met die *S. pombe* malaat permease geen (*mae1*) uitgedruk om ’n rekombinante *S. cerevisiae* ras te genereer wat in staat is om L-malaat effektief af te breek.

BIOGRAPHICAL SKETCH

Maryna Saayman was born in Cape Town, South Africa, on 15 February, 1972. She attended Bosmansdam High School, Bothasig and matriculated in 1990. She enrolled at Stellenbosch University in 1991 and obtained the B.Sc. (Genetics and Microbiology) degree in 1994 and her Hons.B.Sc. (Microbiology) degree in 1995. She enrolled for a M.Sc. degree (Microbiology) at the University of Stellenbosch in 1996, which was upgraded to a Ph.D. in 1999. She married Johan Saayman in 1997 and is mother to two children, Carla (born 1999) and Anrico (born 2002).

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I planted the seed, Apollos watered it, but God made it grow

~1 Corinthians 3:6 ~

CONTENTS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The metabolism and degradation of extracellular dicarboxylic acids differ remarkably between yeast species. Based upon their ability to utilise tricarboxylic acid (TCA) cycle intermediates such as L-malate, yeast species are classified into two groups: K(+) yeasts utilise one or more TCA cycle intermediates as sole carbon and energy source, while K(-) yeasts cannot utilise TCA cycle intermediates as sole carbon and energy source. The K(-) group includes yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Kuczynski and Radler, 1982; Baranowski and Radler, 1984). The yeast *S. cerevisiae* cannot effectively degrade L-malate, which is ascribed to the slow uptake of L-malate by diffusion (Baranowski and Radler, 1984; Ansanay *et al*., 1996; Volschenk *et al*., 1997[a,b]) and the low substrate affinity of its malic enzyme (K_m of 50 mM) (Fuck *et al*., 1973). Furthermore, Boles *et al.* (1998) reported that the *S. cerevisiae* malic enzyme gene (*MAE1*) is expressed at relatively low, but constitutive levels.

The fission yeast *S. pombe* effectively degrades L-malate, but only in the presence of an assimilable carbon source (Taillandier and Strehaiano, 1991). Cells of *S. pombe* actively transport L-malate via a H^+-symport system (Sousa *et al*., 1992) provided by the malate permease encoded by the *mae1* gene (Grobler *et al*., 1995). L-Malate is decarboxylated 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 (Osothilp and Subden, 1986[a]), resulting in the so-called malo-ethanolic fermentation. The genes encoding the *S. pombe* L-malate transporter (*mae1*) (Grobler *et al*., 1995) and the malic enzyme (*mae2*) (Viljoen *et al*., 1994) have been cloned and characterised on a molecular level.

Previous reports indicated that the active transport of L-malate by *S. pombe* is competitively inhibited by D-malate, succinate, fumarate, oxaloacetate and α-ketoglutarate, suggesting that a general dicarboxylic acid transporter may exist in this yeast (Sousa *et al*., 1992). However, Grobler *et al*. (1995) found that α-ketoglutarate did not inhibit the transport of L-malate by *S. pombe* and Saayman *et al*. (2000) showed that fumarate was not transported by *S. pombe*.

In contrast to K(-) yeasts, the K(+) yeast *Candida utilis* can utilise L-malate as the only carbon source, but this is subject to substrate induction and catabolite repression. Preliminary results indicated a marked difference between the *S. pombe* and *C. utilis* malate transporter proteins, not only with regard to their regulation, but also their substrate affinity (Saayman *et al*., 2000). Whereas *S. pombe* only transports L-malate, the *C. utilis* enzyme is able to transport both L-malate and fumarate. The differences in L-malate metabolism observed between these yeast species suggest unique regulatory mechanisms involved in the regulation of L-malate metabolism in *C. utilis* that required further investigation. However, relatively little is known about carbon metabolism in *C. utilis*, and even less on the metabolism of dicarboxylic acids.

The general aim of this study was to better understand the regulatory mechanisms involved in the differential utilisation of L-malate and its physiological relevance in *C. utilis*, as compared to *S. pombe* and *S. cerevisiae*. Cloning of the *C. utilis* transporter and/or malic enzyme genes will contribute to our understanding of malate metabolism in *C. utilis*. It may also provide us with an alternative dicarboxylic acid transporter and/or malic enzyme for heterologous expression of the appropriate genes for commercial applications.

1.2 AIMS OF THIS STUDY

The specific objectives and approaches were the following:

1. Comparing various yeast species for their ability to transport dicarboxylic acids, with a specific focus on dicarboxylic acid transport in *S. pombe* and *C. utilis*.

2. Constructing a cDNA library from *C. utilis* to enable cloning and characterisation of the genes encoding the *C. utilis* dicarboxylic acid transporter and malic enzyme.

3. Investigate possible industrial applications of the *C. utilis* dicarboxylic acid transporter and malic enzyme, such as:

    a. Co-expression of the *C. utilis* dicarboxylic acid transporter and high copy numbers of the *S. cerevisiae* fumarase gene (*FUM1*) in *S. cerevisiae*.

    b. Co-expression of the *C. utilis* malic enzyme (*CuME*) and the *S. pombe* malate transporter gene (*mae1*) in *S. cerevisiae*.

This dissertation is organised as a number of chapters covering the current literature on the classification and industrial applications of the yeast *C. utilis* (Chapter 2), carbon metabolism in *C. utilis* (Chapter 3), mechanisms for transport of glucose and dicarboxylic acids in *C. utilis*, *S. pombe* and *S. cerevisiae* (Chapter 4) and the general metabolism of L-malate by yeast (Chapter 5). Chapter 6 describes the comparative study on dicarboxylic acid transport in different yeasts. The construction of a cDNA library is described in Chapter 7, together with the strategy envisaged for co-expression of the *S. cerevisiae FUM1* gene. The cloning and regulatory studies on the *C. utilis* malic enzyme gene are discussed in Chapter 8, as well as the co-expression of the *C. utilis* malic enzyme (*CuME*) and the *S. pombe* malate transporter gene (*mae1*) in *S. cerevisiae*. Final conclusions are discussed in Chapter 9, followed by a combined reference list for all the chapters.

Some of the results discussed in this dissertation have been presented in parts at various local and international conferences, i.e.

1. **Saayman, M.**, Viljoen, M., Coton, E.P.N. and H.J.J. van Vuuren. 1998. A comparative study on the transport of dicarboxylic acids in the yeasts *Candida utilis* and *Schizosaccharomyces pombe*. 2nd International Congress of the Federation of African Societies of Biochemistry and Molecular Biology & 15th Congress of the South African Society of Biochemistry and Molecular Biology, Potchefstroom.

2. **Saayman, M.**, Viljoen, M., Coton, E.P.N. and H.J.J. van Vuuren. 1998. Transport of dicarboxylic acids in yeast. The South African Society of Microbiology 10th Biennial Congress, Durban.

3. Viljoen, M., **Saayman**, **M.,** van der Merwe, M., Young, R.A. and H.J.J. van Vuuren. 1998. Regulation of malate degradation in the yeast *Schizosaccharomyces pombe*. The South African Society of Microbiology 10th Biennial Congress, Durban.

4. **Saayman, M.**, van Vuuren, H.J.J. and M. Viljoen. 1999. Differential transport of malate and fumarate in *Candida utilis* and *Schizosaccharomyces pombe*. 19th International Conference on Yeast Genetics and Molecular Biology, Italy.

5. **Saayman, M.**, van Zyl, W.H. and M. Bloom. 2002. Cloning of the *Candida utilis* dicarboxylic acid transporter. The South African Society of Microbiology 12th Biennial Congress, Bloemfontein.

6. **Saayman, M.**, van Zyl, W.H. and M. Bloom. 2004. Regulation of dicarboxylic acid metabolism in the yeast *Candida utilis*. The South African Society of Microbiology 13th Biennial Congress, Stellenbosch.

Chapter 6 has been published as a peer reviewed research article (Saayman *et al*., 2000), while Chapter 8 will be submitted for publication in due course:

1. **Saayman, M.**, Van Vuuren, H.J.J., Van Zyl, W.H. and M. Viljoen-Bloom. 2000. Differential uptake of fumarate by *Candida utilis* and *Schizosaccharomyces pombe*. Appl. Microbiol. Biotechnol. **54**: 792-798.

2. **Saayman, M.**, Van Zyl, W.H. and M. Viljoen-Bloom. Cloning, characterisation and heterologous expression of the *Candida utilis* malic enzyme gene. (to be submitted).

AN INTRODUCTION TO THE YEAST *CANDIDA UTILIS*

2.1 INTRODUCTION

'Yeast' and *Saccharomyces cerevisiae* are frequently used as synonymous terms. However, *S. cerevisiae* is rather exceptional since it is one of the few types of yeast that are able to grow anaerobically (Visser *et al*., 1990). During aerobic growth, this yeast also shows an unusual behaviour. When grown aerobically at a low growth rate, under sugar limitation, cultures tend to spontaneously synchronise their cell cycle (Parulekar *et al*., 1986), which complicates the analysis of growth kinetics.

Molecular biology techniques have allowed the rapid advancement of our understanding of many non-*Saccharomyces* yeasts. In the past decade, yeasts other than *S. cerevisiae* have therefore gained interest as hosts for the industrial expression of heterologous genes. Examples are methanol-utilising yeasts such as *Hansenula polymorpha* and *Picha pastoris*, and the lactose-utilising species *Kluyveromyces lactis* and *Kluyveromyces marxianus* (Romanos *et al*., 1992). Several arguments have been put forward to use 'non-*Saccharomyces*' yeasts as hosts for heterologous gene expression, including broader substrate specificity, availability of strong inducible promoters, absence of aerobic alcoholic fermentation (i.e. the absence of the Crabtree effect), etc. These yeasts possess qualities of both academic and industrial interest, including the ability to use a broad range of carbon sources.

One of these yeasts, *Candida utilis*, is universally recognised as an important experimental model system. Owing to its high protein content (72%, w/w), it is considered to be a fodder yeast and a potential microbial source of protein for animal feed as well as for human consumption. It has been used industrially for the past 70 years in the production of single cell protein (SCP) for food and fodder, waste treatment, and the production of fine chemicals used as flavor enhancers (Klein and Favreau, 1995). It has been approved for use as a foodstuff by the US Food and Drug Administration (Boze *et al*., 1992).

2.2 THE GENUS *CANDIDA*

Meyer *et al*. (1984) described the genus *Candida* "…as an unnatural group of yeasts", containing the wayward species of ascomycetous and basidiomycetous yeast, with species heterogeneity in DNA base composition ranging from 30% to 66% G + C content and a wide diversity of physiological properties. Meyer *et al*. further commented:

> "The taxonomy boundaries of *Candida* still remain broad and essentially any asexual yeasts that does not fit the criteria of some other genus will find its way into *Candida.* In particular, *Candida* acts as a depository for all the asexual yeasts with ascomycetous affinity except those with acetic acid production, bipolar budding on a broad base, triangular cells, blastoconidia formed on sympodulae or on pedicils or denticles, dichotomously branched terminal pseudohyphal cells, needle-shaped terminal conidia, arthroconidia, carotenoid pigments, and extracellular starch-like compounds. In some instances, *Candida* is a temporary repository for a species until ascosporulation is observed and it can be placed in a teleomorphic genus."

The genus *Candida* is the largest yeast genus and comprises approximately 200 species. It has been divided into three categories based on the mol% G + C content, morphology and monosaccharide assimilation patterns (vonArx, 1980). The *Candidaceae* includes yeasts of ascomycetous affinity with low mol% G + C ranging from 33% to 40% and low amounts of chitin. These include *Candida albicans*, *Candida boidinii*, *Candida diddensii*, *Candida sake*, *Candida tropicalis* and *C. utilis*. The remaining *Candida* is of basidiomycetous nature and two groups can be distinguished, the *Sporobolomycetaceae* and *Filobasidiaceae*. Membership of the genus *Candida* is continuously changing due to the "refinement' of various criteria based on biochemical and molecular techniques that have recently become available.

Daniel *et al*. (2001) studied the actin gene as a potential phylogenetic marker in order to determine the phylogenetic relationships between *Candida* and related species (Figure 2.1). The chosen outgroup species included *Neurospora crassa*, a member of the Euascomycetes, and *Schizosaccharomyces pombe*, a member of the Archaeascomycetes. The Euascomycetes is the most closely related group to the Hemiascomycetes at this taxonomic level, while the Archaeascomycetes is basal to both of these groups (Hendriks *et al*., 1992; Liu *et al*., 1999).

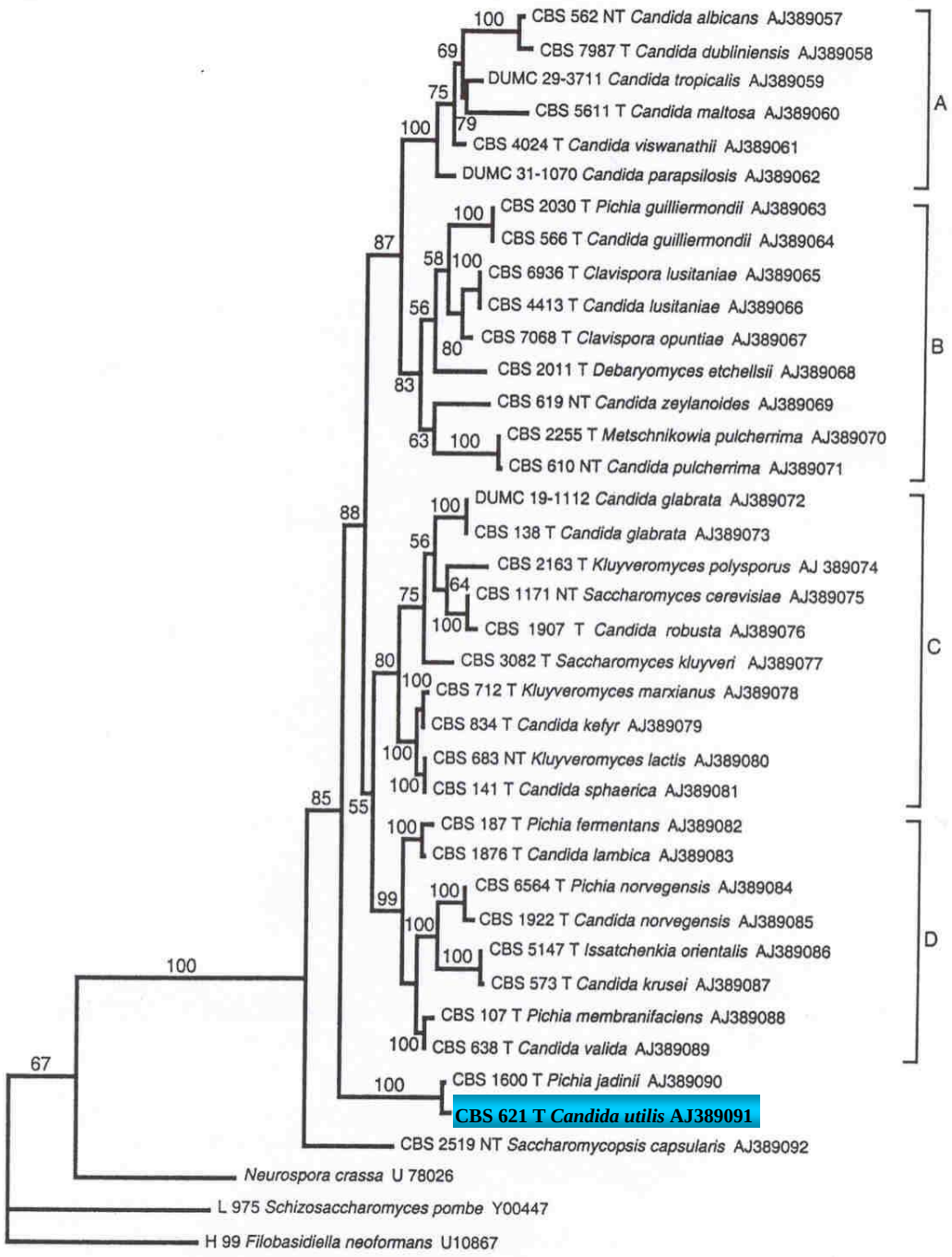

Figure 2.1. Phylogenetic tree produced by weighted parsimony analysis of partial sequences of the actin gene from 39 yeast taxa. *F. neoformans*, *S. pombe* and *N. crassa* was defined as the outgroups. The numbers on branches indicate bootstrap values greater than 50 after 1000 replications (taken from Daniel *et al*., 2001).

A significantly more distant related group, the Basidiomycetes, was represented by *Filobasidiella neoformans*. The phylogenetic tree revealed four major groups, A, B, C and D. Pathogenic *Candida* species were concentrated in, but not confined to, group A. Group C included *S. cerevisiae* and a number of *Candida* and *Kluyveromyces* species. Opportunistic

pathogens, such as *C. albicans* and *C. glabrata*, did not cluster into a single group. According to Daniel *et al.* (2001), *C. utilis* remains ungrouped, with little homology to the above-mentioned groups.

Eight species of *Candida* (Table 2.1) have been reported to be opportunistic pathogens; the major one being *C. albicans*, which is involved in an increasing number of infections. These species are ubiquitous in nature, having been isolated from a variety of environments and, in general, are pathogenic only when an organism's immuno-surveillance system fails. For example, *C. utilis* has been associated with fungemia in patients with an acquired immunodeficiency syndrome (Alsina *et al*., 1988)

Table 2.1. Pathogenic species of *Candida*

Species	Comments	Reference
C. albicans	Most often isolated yeast pathogen	Odds (1988)
C. famata	Rare isolate, clinical features similar to *Propionibacterium acnes* syndrome	Rao *et al*. (1991[a])
C. glabrata	Second most common isolate in vaginitis	Asakura *et al*. (1991) Kobayashi *et al*. (1992)
C. guillermondii	Rare opportunistic pathogen	McQuillen *et al*. (1992) Yagupsky *et al*. (1991)
C. krusei	Implicated in an increasing number of infections of immunocompromised patients (invasive fungemia, indophthalmitis, and in transplants)	McQuillen *et al*. (1992) Tam *et al*. (1992)
C. parapsilosis	Wide distribution in nature, virulent in immunosuppressed mice. Most common *Candida* infecting human nail beds.	Weems (1992)
C. stellatoidea	Some isolates are identical to *C. albicans*, except for sucrose requirement	Kwon-Chung *et al*. (1990)
C. tropicalis	Second most common pathogenic *Candida*	McGuire *et al*. (1992)

The genus *Candida* thus presents a diverse array of organisms that have an affect on human health and welfare (Klein and Favreau, 1995). Representatives include some of the most intractable pathogens known to humans, while others present the hope of supplementing dwindling food supplies by converting industrial waste into SCP, or by providing the enzymatic materials essential for stereo-specific chemical conversions.

2.3 TAXONOMIC CLASSIFICATION OF *CANDIDA UTILIS*

A diagram showing the classification of *C. utilis* is presented in Figure 2.2. The yeast *C. utilis* has a G + C content of 45% (Klein and Favreau, 1995) and was first described by Henneberg in 1926 (cited in Kreger-van Rij, 1984). It was suggested that *C. utilis* and *Hansenula jadinii* are closely related, with *H. jadinii* producing only a few ascospores (one spore per 10 000 vegetative cells) while *C. utilis* produces significantly more ascospores (Kurtzman *et al*., 1979). In addition, *H. jadinii* has been shown to be pathogenic in animals, whereas *C. utilis* is not known to be pathogenic, although characterised species have been isolated from the digestive tract of cows. DNA reassociation studies by Kurtzman *et al*. (1979) showed that *C. utilis* represented the anamorphic form of the teleomorphic species *H. jadinii*. Kurtzman (1984) also demonstrated similarity at DNA level between yeasts of the genus *Hansenula* and those of the genus *Pichia.* Therefore, *C. utilis* can also be designated as *Pichia jadinii*. Other taxonomic synonyms are *Cryptococcus utilis*, *Torula mineralis* and *Torula utilis* (www.cbs.knaw.nl).

2.4 MOLECULAR GENETICS OF *CANDIDA UTILIS*

The yeast *C. utilis* has a highly variable electrophoretic karyotype, as already known for another imperfect yeast species, *Candida albicans*. Karyotype analysis using Pulse-Field Gel Electrophoresis (PFGE) on 13 strains of *C. utilis* revealed the existence of two clearly distinct electrophoretic karyotypes. According to these types, the strains were assigned to group A or group B (Stoltenburg *et al*., 1992). Differing number of chromosomal bands between strains of group B and group A can probably be assigned to ploidy. Apparently, *C. utilis* is at least diploid, since auxotrophs can be obtained by mutagenesis with very low frequencies. If *C. utilis* is diploid or polyploid, strains that possess at least two homologous chromosomes of the same or very similar size can be assigned to group A. The larger number of bands in the strains of group B would then be due to an internal length polymorphism of homologous chromosomes as has been described for polyploid industrial strains of *S. cerevisiae*. This explanation is supported by the number of chromosomal bands, ranging from 5 to 8 in group A, and from 11 to 14 in group B (Stoltenburg *et al*., 1992). In spite of its industrial importance, the molecular genetics of *C. utilis* is not well understood and molecular tools are limited. Studies on *C. utilis* and its use for the expression of heterologous proteins have been limited by the lack of transformation and expression systems. Due to the fact that *C. utilis* is at least diploid and does not have a sexual life cycle, appropriate auxotrophic mutants that can

be used as hosts for transformation have not been available. However, Kondo *et al*. (1995) reported a novel transformation system for *C. utilis* where an endogenous gene encoding the ribosomal protein L41 was used as a selectable marker conferring cycloheximide (CYH) resistance after modification of its sequence by *in vitro* mutagenesis. The gene encoding the L41 protein has a proline residue at the 56th amino acid, which is characteristic of the protein in CYH-sensitive yeasts (Kawai *et al*., 1992). In contrast, CYH-resistant L41 proteins have a glutamine in this position. A marker gene was therefore constructed by converting the 56th codon to a glutamine codon. The gene is particularly useful as a marker gene for the development of a transformation system since the gene is expressed in the host under the control of its own transcriptional and translational machinery. However, the marker needs to be present in multiple copies for selection of CYH resistant transformants since the host possesses endogenous genes encoding a CYH-sensitive L41 protein. A ribosomal DNA (rDNA) fragment was therefore employed as a multicopy target for plasmid integration.

KINGDOM	**Fungi**
DIVISION	**Eumycota**
	Chytridiomycota
	Zygomycota
	Basidiomycota
SUB-DIVISION	**Ascomycota**
	Deuteromycota
CLASS	**Hemiascomycota**
	Archaeascomycota – e.g. *Schizosaccharomyces pombe*
	Euascomycota
ORDER	**Endomycetales**
	Spermophtoraceae
FAMILY	**Saccharomycetoideae**
SUB-FAMILY	**Saccharomycetoideae**
	Saccharomyces – e.g. *Saccharomyces cerevisiae*
GENUS	***Candida***
SPECIES	***Candida utilis***

Figure 2.2. Classification of the yeast *C. utilis* according to Barnett *et al*. (1990).

Kondo *et al*. (1997) also developed an expression system in *C. utilis* using glycolytic promoters. Rodríguez *et al*. (1998) isolated the *URA3* gene of *C. utilis* and developed the first transformation system based on an auxotrophic marker in *C. utilis*. In 2004, Basabe *et al*. cloned the *C. utilis HIS3* gene and its analysis revealed an uninterrupted open reading frame (ORF) of 675 bp, making available a new selectable marker gene to develop an alternative transformation system for further manipulation of this yeast.

2.5 APPLICATION OF *CANDIDA UTILIS*

The yeast *C. utilis* is an industrially important yeast and is widely used for the production of biologically useful materials, such as glutathione, certain amino acids and enzymes. It is also a promising source of nutrients through the large-scale production of single-cell proteins from biomass-derived sugars, such as sugar molasses and spent sulfite liquor (Lawford *et al*., 1979; Boze *et al*., 1994). It is able to utilise a wide range of substrates such as glucose, raffinose, xylitol and saccharose (Lawford *et al*., 1979). It has been approved as a GRAS (General Regarded as Safe) microorganism by the F.D.A. (Food and Drug Administration) (Boze *et al*., 1994). The most important applications of *C. utilis* are summarized in Table 2.2 and a few are discussed in the following sections.

2.5.1 Production of Single-cell Protein (SCP)

The term SCP refers to dried cells of microorganisms such as algae, actinomycetes, bacteria, yeasts, molds and higher fungi grown in large-scale culture systems for use as protein sources in human foods or animal feeds. Although these microorganisms are grown primarily for their protein content in SCP production processes, microbial cells also contain carbohydrates, lipids, vitamins, minerals and non-protein nitrogen materials such as nucleic acids (Litchfield, 1983).

SCP production originated in Germany during World War I when *S. cerevisiae* was grown for consumption as a protein supplement using molasses as the carbon and energy source. In Germany during World War II, *C. utilis* was cultivated on sulfite waste liquor (SSL) from pulp and paper manufacturing processes and on wood sugar derived from the acid hydrolysis of wood and used as a food and fodder supplement (Litchfield, 1979). SSL contains approximately 2.5% fermentable sugars, of which 80% are hexoses and 20% pentoses, in addition to a variety of organic acids. Cells of *C. utilis* can assimilate hexoses and pentoses, as well as many of the organic acids in SSL. Most *C. utilis* fermentations are conducted at

Table 2.2. Industrial applications of *Candida utilis*

Product/Use	Substrate	Reference
SCP	Pectin	Fellows and Worgan (1986)
	Bagasse (sugarcane)	Gamal *et al*. (1985)
	Molasses distillery waste	Azzam and Heikel (1989)
	Corn cob/ corn stalk	Fields *et al*. (1991)
	Apple processing wastes	Fellows and Worgan (1987[a])
	Apple pomace	Gupta *et al*. (1990)
	Ethanol	Imshenetskii *et al*. (1987)
	Defatted mango	Malathi and Laddha (1989)
	Rice straw	Araujo and D'Souza (1986)
	Potato extracts	Davids *et al*. (1986)
	Sucrose	Tub (1986)
	Sugar beets	Wu and Ye (1989)
	Waste Chinese cabbage	Choi *et al*. (2002)
Biodegradation	Wastewater/sauerkraut	Elmaleh *et al*. (1999)
	Sulphite waste liquor (SSL)	Streit *et al.* (1987)
Ethanol	Inulin	Poncet *et al*. (1985)
	Apple pomace	Gupta *et al*. (1990)
Ethyl Acetate	Ethanol waste stream	Kusano *et al*. (1999)
Treatment of silage effluent	Silage effluent	Arnold *et al*. (2000)
Acetylaldehyde	Glucose/ethanol	Armstrong and Yamazaki (1984)
Biofiltration of VOCs	Bakery & distillery waste	Christen *et al*. (2002)
Acetone	Isopropanol	Mueller and Babel (1989)
Carbon & nitrogen removal	Wastewater	Ortiz *et al*. (1997)
Aroma formation in fermented sausages	Valine, Leucine & Isoleucine	Olesen and Stahnke (2000)
Carotenoid Production	Acetyl-CoA	Giovannucci *et al*. (1995)

low pH (4 - 4.5) and at temperatures of 32°C or higher (Klein and Favreau, 1995). Dried *C. utilis* cells, as well as *S. cerevisiae* and *Kluyveromyces fragilis*, have been approved for use as a foodstuff by the U.S. Food and Drug Administration (Boze *et al*., 1992).

Much attention has since been paid to the potential use of microorganisms as a source of SCP, but the production costs were too high for SCP to compete with other sources of protein, such as soybeans. However, *C. utilis* is a promising source of nutrients through the large-scale production of SCP from biomass-derived sugars, such as sugar molasses (Lawford *et al*., 1979; Boze *et al*., 1992). Molasses, a cheap by-product widely available from the sugar industry, consist of water, 47% to 50%, (w/w) sucrose, (the disaccharide most easily utilised by yeast cells), 0.5% to 1% (w/w) nitrogen source, proteins, vitamins, amino acids, organic acids and heavy metals such as iron, zinc, copper, manganese, magnesium, calcium, etc. (Roukas, 1998). It is therefore a very attractive carbon source for yeast production from an economic point of view. Furthermore, *C. utilis* proteins have a relatively high concentration of essential amino acids (Lawford *et al*., 1979) and the ability to metabolise a wide range of saccharides (Shay and Wegner, 1985). The predominantly aerobic metabolism of *C. utilis* and active participation of the pentose phosphate pathway in sugar metabolism predisposes this yeast to carbon balance in favour of biomass production as compared with other yeasts such as *S. cerevisiae*, which are glucose sensitive and largely fermentative (Divjak and Mor, 1973). The production of SCP by *C. utilis* can be done on a number of substrates of which a few will be discussed below.

Starch wastes. Strains of *C. utilis* do not possess enzymes that hydrolyse starch, cellulose or pectic substrates. These substrates must be hydrolyzed by heat (cooking or steaming), acid or biological hydrolyses (addition of purified enzyme mixtures or pretreatment with various microorganisms). In most cases, unless heat or acid treatment is part of an overall production process, additional treatment of starch waste is not considered economical. However, two-step dual fermentation processes have been shown to be economically acceptable, with processing costs recovered in the sale of yeast as a final product (Fellows and Worgan, 1987[a,b]). Starch wastes are used as a substrate for *Saccharomyces fibuliger*, which produces amylases that hydrolyse the starch and allow growth by *C. utilis*. Modifications of the *S. fibuliger* culture conditions permit *C. utilis* to predominate in the final biomass product. This procedure has been economically employed for the production of SCP from starch wastes (Boze *et al*., 1992) and apple processing wastes (Fellows and Worgan, 1986). Conversion was shown to be 45 g cells/100 g of initial substrate, with *C. utilis* comprising

96% of the final biomass. *C. utilis* alone, without pretreatment by *S. fibuliger* (i.e. prehydrolysis), yielded approximately 33 g cells/100 g of initial substrate in the same process.

Cellulosic Waste. Applications of *C. utilis* in single- and multiple-step fermentation processes include the degradation of cellulosic wastes and the reduction of the biological oxygen demand (BOD) of distillery silage from sugarcane molasses production. This distillery effluent has a BOD of 40-50 g/liter and is a major contributor to environmental pollution in some tropical countries. Single-batch fermentations using *C. utilis* alone have been shown to reduce the BOD by as much as 83%, with SCP being a useful by-product (SivaRama *et al*., 1984). Fermenting the waste effluent using *C. utilis*, followed by a fermentation step using *Paecilomyces varioti*, has resulted in a reduction of 92% in the BOD, with the dried biomass exceeding 22 g/liter (Azzam and Heikel, 1989). The SCP produced by this process was low in methionine and cysteine, though the remaining amino acid content showed a favourable comparison to the standards set by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO).

Defatted mango kernels. Defatted mango kernels (DMKs) are a solid waste product of the mango fat industry in India. The DMKs consist of approximately 60% starch. Following hydrolysis by the addition of amylases, glucosidases and glucoamylases, the DMKs have been used as a substrate for cultivation of *C. utilis*. Biomass yields from this process range from 44% to 48%. The resulting cells have a 47% protein and 6% RNA content. The latter is unacceptably high for human consumption, but is acceptable for an animal feed supplement (Malathi and Laddha, 1989).

Aquaculture feed. It is becoming increasingly evident that the development of low-cost, high quality protein foodstuffs is crucial for the future success of the aquaculture industry (Rumsey, 1978). The main protein sources used in aquafeeds are fishmeals, which typically constitute 250-400 g/kg of formulated feeds for carnivorous fish and shrimp. In view of the increasing cost of fish meals and the instability in their supply in the long term, it is essential that alternative protein sources be identified. The use of microbial biomass protein to replace part of the protein required in fish feed could be considered a promising and innovative solution to this problem (Martin *et al*., 1993). Cultivated micro-algae such as *Chlorella, Scenedesmus* and *Spirulina* species have been used as SCP in fish feed. However, the industrial production of micro-algae is still relatively limited and some technological and toxicological problems remain to be solved before they attain a larger role in fish feeding (Beneman, 1992).

It is known that the commercial value of SCP is linked to its protein content. From this point of view, *C. utilis* has been classified among the most interesting microorganisms for their protein content, which can account for up to 50% of the dry weight, the remaining being represented by lipids, polysaccharides, etc. (Ziino *et al*., 1999). Moreover, they can also supply the feed with vitamins, mineral and other components, which could stimulate the disease resistance of fish (Raa, 1990).

Industrial waste streams. Organic acids from dilute industrial waste streams have been shown to be suitable substrates for biomass production in continuous culture. Yields in batch cultures of *C. utilis* vary from 30% to 40%, and in continuous culture they average 44% when using acetic acid or a 55% mixture of propionic, butyric and acetic acid as substrate (Maugeri-Filho and Goma, 1988). Christ (1986) has reported processes for using *C. utilis* to treat the waste effluents from sauerkraut production (cited in Klein and Favreau, 1995). Continuous processes have been developed using high cell density fermentation and have resulted in yields as large as 120 g dry weight cells/liter of sucrose (Shay *et al*., 1987).

2.5.2 Biodegradation of Wastewater

The food industry generates 45% of the total organic industrial pollution with highly loaded effluents whose treatment usually requires many successive steps. When the biodegradable contaminants are very highly concentrated, a yeast reactor followed by an anaerobic bacteria reactor in series, could address the pollution levels to acceptable levels that meet the effluent standards (Elmaleh *et al*., 1999). However, such a yeast reactor is sensitive to bacterial contamination and the anaerobic reactor usually requires a long retention time. An alternative process was proposed by Elmaleh *et al*. (1996) based on an acidogenic reactor followed by a yeast reactor in series. The main organic products of the acidogenic reactor are volatile fatty acids (VFA) such as acetic acid, propionic acid or butyric acid (Dinoupoulou *et al*., 1988).

The first step in the design of such a process for biodegradation includes the identification of a convenient yeast and the determination of kinetic data relating to microbial growth, organic carbon removal and solids production. The yeast *C. utilis* was selected as potential candidate and used for VFA oxidation as early as 1980 by Maugeri-Filho and Goma. A *C. utilis* mixed reactor operated at pH 3.5 to limit bacterial contamination and fed with acetic acid, propionic acid or butyric acid or a mixture of these acids, can oxidise fatty acids with loading rates as high as 30 kg Total Organic Carbon (TOC)/m^3/day with 97% removal efficiency.

2.5.3 Production of Ethyl Acetate

Some yeasts are able to grow and produce volatile compounds of interest from ethanol, a by-product of agro-industries. The *C. utilis* cells are able to assimilate ethanol as sole carbon source (Watteeuw *et al*., 1979) and efficiently convert ethanol to ethyl acetate (Armstrong *et al*., 1984). Factors such as pH (Páca and Votruba, 1990) and dissolved oxygen (Corzo *et al*., 1995) were found to influence the respiration activity of *C. utilis* on ethanol.

2.5.4 Carotenoid Production

Lycopene is a red carotenoid pigment present in tomatoes, watermelon and red grapefruit that have recently received attention due to its health promoting characteristics. For example, lycopene has been shown to have preventative effects against certain cancers, e.g. prostate cancer (Giovannucci *et al*., 1995), and is claimed to be the most effective antioxidant (Miki, 1991).

Cells of *C. utilis* do not synthesise the carotenoid pigment, but do accumulate large quantities of ergosterol (Shimada *et al*., 1998). Like carotenoids, ergosterol is an isoprenoid and biosynthetically related to carotenoids by a common prenyl lipid precursor, farnesyl diphosphate (FPP). In order to increase the carbon flux into lycopene biosynthesis, squalene activity was decreased by disruption of the *C. utilis ERG9* gene encoding squalene synthase. The three carotenogenic genes (*crtE*, *crtB* and *crtI*) required for lycopene synthesis from FPP were introduced under the control of *C. utilis* promoters to produce in a *C. utilis* strain that produces 1.1 mg lycopene per g (dry weight) of cells (Figure 2.3).

Miura *et al*. (1998) also constructed β-carotene-producing and astaxanthin-producing *C. utilis* strains by introducing the metabolic pathway mediated by either four *crt* genes (*crtE*, *crtB*, *crtI* and *crtY*) or six *crt* genes (*crtE*, *crtB*, *crtI*, *crtY*, *crtZ* and *crtW*). The resulting *C. utilis* strains produced 0.4 mg of β-carotene or astaxanthin per g (dry weight) of cells.

2.5.5 Treatment of Silage Effluent

Silage is produced by the controlled fermentation of a crop of high moisture content, such as grass or forage maize, and is used as animal feedstock (Arnold *et al*., 2000). Silage effluent, a by-product of silage production, arises from a combination of surface water and plant juices expelled from the ensiled herbage. This effluent is an extremely powerful pollutant, having a BOD in the region of 30 – 80 g O_2/l (Beck, 1989). The highly acidic silage effluent is

difficult to contain since it is corrosive to steel and concrete (Arnold *et al*., 2000), the materials most commonly used in the construction of silos. Any effluent finding its way into a watercourse would lead to rapid deoxygenation of the water and a decrease in pH, killing fish and other aquatic fauna.

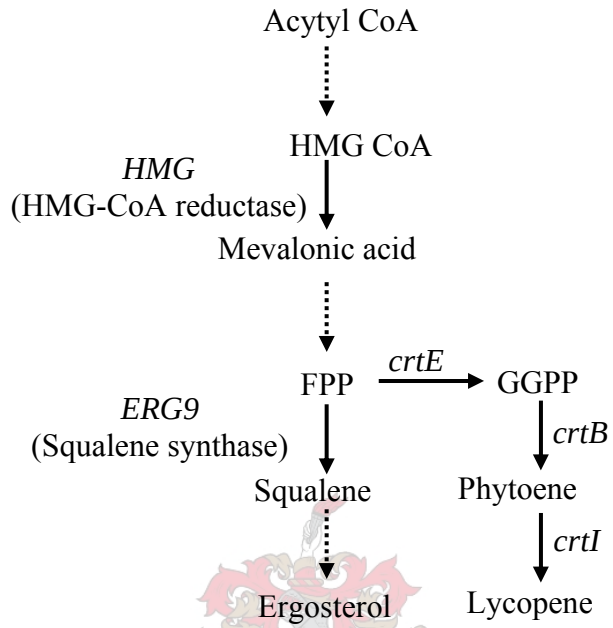

Figure 2.3. Metabolic pathway of endogenous ergosterol biosynthesis and exogenous lycopene biosynthesis in *C. utilis*. The solid arrows show the one-step conversions of the biosynthesis, and the dashed arrows represent a number of sequential steps. The endogenous lycopene synthesis genes are indicated by *crtE*, *crtB* and *crtI*. GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate (Shimada *et al*., 1998).

Silage effluent is usually disposed of by spreading on land or feeding to animals. Spreading on land can lead to scorching of grass or other crops (Burford, 1976) and depletion of oxygen from the surrounding soil (Gross, 1972). The effluent can also find its way into watercourses via land drainage. Only well-preserved silage effluent should be fed to animals, since it deteriorates rapidly. It is important that it is either fed within 3-4 days of production or stored anaerobically (Patterson and Kilpatrick, 1991). Since effluent production cannot be completely eliminated and the disposal methods mentioned above clearly have their disadvantages, other means of effluent disposal or treatment are necessary. Treatment prior to land disposal may reduce the potential environmental problems and it has been shown that a high degree of purification of silage effluent can be achieved by treatment with selected yeast strains. For example, *C. utilis* was effective in reducing the polluting properties of the silage

effluent, with a COD reduction of 74% to 95% (Arnold *et al*., 2000). The pH was increased from 5.7 to 9 pH units, presumably due to removal of lactic acid and volatile fatty acids (VFAs), which are responsible for the distinctive smell of silage effluent. The substantial pH increases would make it easier to contain the effluent since it would no longer cause corrosion of the concrete or steel containers or holding tanks.

CARBON METABOLISM IN *CANDIDA UTILIS*

3.1 INTRODUCTION

Sugars are excellent carbon sources for all yeasts. The different components of the pathways for sugar utilisation in *Saccharomyces cerevisiae* have been studied extensively and it has been assumed that other yeasts utilise sugars in the same way. However, although the pathways of sugar utilisation follow the same theme in all yeasts, important biochemical and genetic variations exist. This chapter provides comparative information on the different steps involved in carbon metabolism as currently known for *Candida utilis*.

Survival of all organisms requires the ability to adapt to changing circumstances. Two physiological regulatory mechanisms influencing carbon metabolism, and thereby helping the organism to adapt to the changing environment, are the Crabtree effect and the Kluyver effect. The yeast *C. utilis* differs from *S. cerevisiae* in that it is a Crabtree negative and Kluyver positive yeast. Therefore, we will also touch on these regulatory mechanisms in order to better understand carbon metabolism in *C. utilis*. Due to the strong catabolite repression exerted on L-malate metabolism in *C. utilis*, general characteristics of catabolite repression will also be discussed, using *S. cerevisiae* as a model with reference to *C. utilis* where applicable. This review will show that basic knowledge on many components of these pathways in *C. utilis* is lacking and that studies on the regulation of critical steps are scarce.

3.2 CARBON METABOLISM IN *CANDIDA UTILIS*

Strains of *C. utilis* can utilise a variety of carbon sources, including mono- and disaccharides such as sucrose, xylose and maltose. However, no growth was observed for *C. utilis* with galactose or lactose as sole carbon source (Meyer *et al*., 1984). The cleavage of sucrose to glucose and fructose is catalysed by invertase (*β*-D-fructofuranosidase, E.C. 3.2.1.26) (Chávez *et al*., 1997). Although the preferred substrate for invertase is sucrose, invertase is also able to catalyse the hydrolysis of raffinose and stachyose in *C. utilis* (Belcarz *et al*., 2002). Furthermore, *C. utilis* appears to be the only yeast strain capable of producing and secreting two different forms of *β*-D-fructofuranosidase (Belcarz *et al*., 2002). While the F-form (Fast-migrating) is a non-glycosylated monomer with a molecular mass of 62 kDa, the S-form (Slow-migrating) is a 280 kDa homodimer that is *N*-glycosylated. The glycoprotein is mainly

formed by a high-mannose oligosaccharide structure and the enzyme is regulated by carbon catabolite repression.

In 1948, Wickerham and Burton reported that many yeasts could grow on intermediates of the TCA cycle by utilising citric, succinic, fumaric and/or malic acid as sole source of carbon. Yeasts may thus be divided into two groups: a 'K(+)' group capable of using one or more intermediates of the TCA cycle for growth or respiration, and a 'K(-)' group of yeasts unable to do so. The difference between K(+) and K(-) yeasts seem not to be one of a major metabolic pathway, but rather the permeability of intact cells for exogenous TCA cycle intermediates. For example, *S. cerevisiae* is considered to be a K(-) yeast, being unable to grow on exogenous intermediates of the TCA cycle (Barnett and Kornberg, 1960), while *C. utilis* is able to utilise various TCA cycle intermediates as sole carbon and energy source. This phenomenon will be discussed in more detail in Chapter 4.

3.2.1 Glycolytic Pathway

The common theme in sugar metabolism in all known yeasts is the conversion of glucose-6-phosphate or fructose-6-phosphate to pyruvate through the glycolytic pathway (Figure 3.1) with the concomitant formation of ATP and NADH. No net oxidation occurs in the process, since the oxidation of some pathway intermediates is balanced by the reduction of NAD^+, which is restored by other metabolic reactions such as the reduction of acetaldehyde to ethanol (Kruckeberg and Dickinson, 2004). The metabolic destiny of pyruvate is, however, different depending on the yeast species and the culture conditions. In *S. cerevisiae*, glucose and related sugars cause a strong impairment in respiratory capacity (Crabtree effect) and therefore, *S. cerevisiae* ferments sugar to ethanol and carbon dioxide in batch cultures even in the presence of oxygen (Fiechter, 1981). Some other yeast species, so-called 'Crabtree positive' yeasts, also behave in this way. However, in most cases, pyruvate is oxidised to CO_2 and water under aerobic conditions through the tricarboxylic acid (TCA) cycle and the electron transport chain, with the formation of more ATP. Glycolysis and the TCA cycle are therefore central metabolic pathways that perform a dual role: (1) to generate energy and reducing equivalents in the form of ATP, NADH or NADPH, and (2) to provide building blocks to synthesise other biomolecules. Glycolysis also plays an important anabolic role, with a number of glycolytic intermediates are utilised by biosynthetic pathways for production of amino acids, nucleotides and lipids (Kruckeberg and Dickinson, 2004).

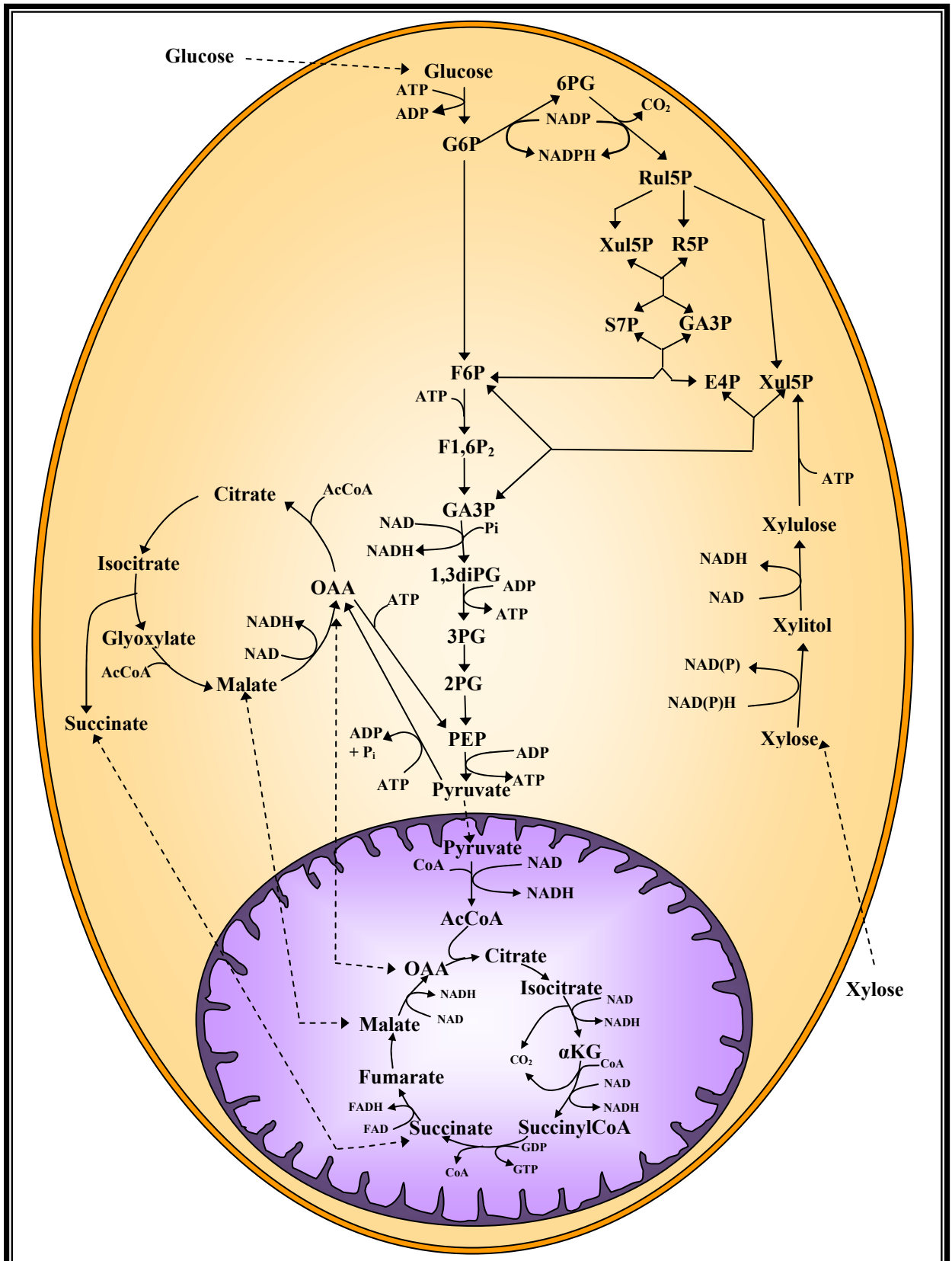

Figure 3.1. Scheme of different pathways implicated in carbon and energy metabolism in yeasts. The figure represents a hypothetical yeast cell with features from *C. utilis* and other yeasts. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; $F1,6P_2$, fructose-1,6-bisphosphate; GA3P, glyceraldehydes-3-phosphate; 1,3diPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphor-enol-pyruvate; 6PG, 6-phosphogluconate; Rul5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; Xul5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; OAA, oxaloacetate (adapted from Flores *et al*., 2000).

The two major quantitative fates of pyruvate produced during glycolysis are either its oxidation to CO_2 or its transformation to ethanol. In most yeast species, oxidation will be predominant under aerobic conditions, while transformation to ethanol takes place under anaerobic conditions or at high glucose concentrations under aerobic conditions in yeasts that present a Crabtree effect (Pronk *et al*., 1996). Complete oxidation of the pyruvate formed during glycolysis via the TCA cycle requires oxidative decarboxylation of pyruvate to acetyl-CoA. This can occur either via the mitochondrial pyruvate-dehydrogenase complex or via the so-called pyruvate dehydrogenase bypass that employs pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase.

The yeast *C. utilis* has an unusual metabolism of glucose. When grown on glucose and ammonium sulfate as only carbon and nitrogen source, respectively, the pH of the cell culture was found to decrease from 5.9 to 2.4 pH units (Cheng and Ma, 1997). The decrease in pH was found to be due to the release of acid compounds such as citrate, succinate, malate and acetate during cell growth. These results indicated that the major metabolic pathway of glucose in *C. utilis* should be via the Embden-Meyerhof pathway to produce two molecules of pyruvate. One of the pyruvate molecules undergoes fermentation to produce ethanol. Under aerobic conditions, the other pyruvate molecule is converted into acetyl-CoA, which is metabolised via the TCA cycle. Very small amounts of glycerol, acetic acid, acetoin and 2,3-butandiol are also formed as byproducts from other minor metabolic pathways, while the accumulation of glyceric acid in the cell culture indicated the existence of a reversible two-step oxidation of glycerol via alcohol dehydrogenase.

3.2.2 The TCA Cycle

In *C. utilis*, the major pathway for carbon metabolism is via the TCA cycle for the synthesis of cell material (amino acids and lipids). Acetyl-CoA, generated by pyruvate dehydrogenase, is the link between glycolysis and the TCA cycle (Figure 3.1). The TCA cycle is ubiquitous in organisms with an oxidative metabolism. Associated with mitochondrial compartmentation of the TCA cycle in eukaryotic cells, is replication of some enzymatic activities in other compartments. Since communication between pathways separated by membrane barriers depends on selective transport of a limited number of common metabolites, the TCA cycle isozyme families are considered to be critical points for control of metabolic flux (McAlister-Henn and Small, 1997). For example, the mitochondrial and cytosolic malate dehydrogenases direct the flux of carbon and reducing equivalents between the TCA cycle and cytosolic pathways.

Isocitrate Dehydrogenase. The oxidative decarboxylation of isocitrate to form α-ketoglutarate is catalysed by mitochondrial NAD^+-specific and differentially compartmentalised $NADP^+$-specific enzymes. This reaction is considered a committed step because it is essentially irreversible under physiological conditions. Furthermore, as expected for a regulatory enzyme, the multisubunit NAD^+-specific enzyme exhibits complex allosteric modulation of activity (McAlister-Henn and Small, 1997).

NAD^+-specific isocitrate dehydrogenase purified from *S. cerevisiae* is an octamer containing four each of two subunits, designated IDH1 and IDH2 (Keys and McAlister-Henn, 1990). The IDH1 and IDH2 polypeptides are very similar, having an overall 42% identity of aligned amino acid sequences. The NAD^+-specific isozymes is key to the TCA cycle, while the $NADP^+$-specific mitochondrial isozymes do not contribute significantly to TCA cycle function.

Fumarase. Fumarase belongs to a family of homologous enzymes that share amino acid sequence conservation and fumarate as a common substrate/product (Weaver *et al*., 1998). Fumarase functions as a component of the Krebs cycle responsible for the interconversion of fumarate and L-malate. In yeast cells, fumarase activity is found in both cytosolic and mitochondrial cellular fractions, with cytosolic activity representing approximately 70% of the total. In *S. cerevisiae*, a single gene harboring two unique start sites is responsible for coding both the mitochondrial and cytosolic forms of fumarase. The *S. cerevisiae FUM1* gene encodes a polypeptide of 53 kDa, the catalytically active form being a homotetramer (Wu and Tzagoloff, 1987) with information for mitochondrial localisation contained within the 17 amino-terminus of the FUM1 polypeptide. The differential localisation of FUM1 polypeptides was linked to transcription of two mRNA species: the longer specie containing codons for the mitochondrial targeting sequence and the shorter containing an alternative downstream site for translation initiation.

Malate Dehydrogenase. There are at least two forms of malate dehydrogenase in most eukaryotic cells; a mitochondrial enzyme that functions in the TCA cycle and a cytosolic enzyme that catalyses the first step in gluconeogenesis from pyruvate. These isozymes also participate in the malate/aspartate shuttle cycle for the indirect exchange of reducing equivalents between cellular compartments. The supply of oxaloacetate, which is compartmentally restricted by transport barriers, is rate limiting for metabolic processes in both compartments (McAlister-Henn and Small, 1997).

Three forms of malate dehydrogenase have been reported for *S. cerevisiae*, namely MDH1, MDH2 and MDH3 (McAlister-Henn and Small, 1997). Whereas MDH1 represents the mitochondrial malate dehydrogenase, a TCA cycle enzyme, MDH2 may be the critical enzyme for glyoxylate metabolism. However, peroxisomal localisation does not appear to be essential for the latter function, since other glyoxylate pathway enzymes, including aconitase and isocitrate lyase, appear to be soluble cytosolic activities. MDH3 plays an important role in reoxidising NADH generated during β-oxidation of fatty acids in peroxisomes. Cellular levels of all three malate dehydrogenases are reduced in yeast cells cultivated with glucose as a carbon source. For MDH1 and MDH3, this appears to be the result of catabolite repression of gene expression, a common effect of glucose on many oxidative functions in yeast. During growth on glucose, over 90% of the much lower total cellular activity for malate dehydrogenase is attributed to MDH1 (Steffan and McAlister-Henn, 1992).

Citrate Synthase. The condensation reaction catalysed by citrate synthase is the rate-limiting step for oxidation via the TCA cycle. The reaction also channels two-carbon units into the biosynthesis of many cellular components, including amino acids, fatty acids and sugars (McAlister-Henn and Small, 1997). The activity is highly regulated; allosterically by ATP, and by alterations in cellular levels in response to environmental conditions.

Intermediates from the TCA cycle are continuously removed during growth for biosynthetic purposes. To prevent a shut down of TCA cycle activity due to depletion of the intermediates, these intermediates are replenished by anapleurotic reactions. In yeast, the key anapleurotic enzyme is pyruvate carboxylase (de Jong-Gubbels *et al*., 1998), responsible for the carboxylation of phospho-*enol*-pyruvate (PEP) or pyruvate to oxaloacetate. The *PYC1* and *PYC2* genes encode isoenzymes of mitochondrial pyruvate carboxylase (PYC) in *S. cerevisiae* (Stucka *et al*., 1991; Walker *et al*., 1991). PYC catalyses the ATP-dependent carboxylation of pyruvate: pyruvate + HCO_3^- + ATP $\rightarrow$ oxaloacetate + ADP + P_i (de Jong-Gubbels *et al*., 1998). In *C. utilis*, these proteins are located in the cytosol, in contrast with the mitochondrial location in other fungi and mammals (Van Urk *et al*., 1989[c]).

Another important anaplerotic route is via the glyoxylate cycle, which is required for growth in minimal medium on carbon sources of less than three carbon atoms, such as ethanol or acetate. The key enzymes of the glyoxylate cycle are isocitrate lyase and malate synthase (Flores *et al*., 2000). Isocitrate lyase catalyses the cleavage of isocitrate to succinate and

glyoxylate, while malate synthase catalyses the condensation of glyoxylate with a molecule of acetyl-CoA to form malate.

3.2.3 The Pentose Phosphate Pathway

The pentose phosphate pathway (also known as the hexose monophosphate pathway) is an important part of the primary carbon metabolism in all living cells (Sundström *et al*., 1993). However, the function of the pentose phosphate pathway (PPP) is not limited to its role in carbon metabolism. Two of its intermediates are also essential starting points for biosynthetic pathways: ribose-5-phosphate is required for biosynthesis of nucleic acid and nucleotide cofactors, while erythrose-4-phosphate is required for biosynthesis of aromatic amino acids (Flores *et al*., 2000). The oxidative part of the PPP converts glucose-6-phosphate to ribulose-5-phosphate and CO_2, while generating NADPH for reductive biosynthesis. The non-oxidative part of the PPP isomerises ribulose-5-phosphate to xylulose-5-phosphate and ribose-5-phosphate, which are then converted into fructose-6-phosphate and glyceraldehyde-3-phosphate by a sugar rearrangement system (Sundström *et al*., 1993).

The first reactions of the PPP in *C. utilis* are two oxidative reactions that are physiologically irreversible, while the others are non-oxidative and reversible. As shown in Figure 3.1, the partition of hexose metabolism between the glycolytic and the PPP occurs at the level of glucose-6-phosphate (Chakravorty *et al*., 1962). Glucose-6-phosphate dehydrogenase directs glucose into the PPP by catalysing the oxidation of glucose-6-phosphate to 6-phospho-δ-gluconolactone, a reaction believed to generate a major part of the cellular NADPH pool (Nogae and Johnston, 1990).

The hydrolysis of 6-phosphate-δ-gluconolactone to 6-phospho-gluconate occurs spontaneously at neutral pH, but at a very slow rate. The oxidative decarboxylation of 6-phospho-gluconate to ribulose-5-phospate is catalysed by 6-phospho-gluconate dehydrogenase. Glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase have been purified from *C. utilis* (Domagk and Chilla, 1975); the latter appears to be a dimer with a high substrate affinity, i.e. K_m of 55 μM for 6-phospho-gluconate and 20 μM for $NADP^+$ (Rippa and Signorini, 1975).

In the non-oxidative, reversible part of the pathway, ribulose-5-phosphate is converted to fructose-6-phosphate and glyceraldehyde-3-phosphate. Ribulose-5-phosphate can be either isomerised to ribose-5-phosphate or epimerised to xylulose-5-phosphate by ribose-5-

phosphate isomerase. The latter enzyme has been purified from *C. utilis* by Domagk and Doering (1975) and Horitsu *et al*. (1976), but its molecular weight and subunit composition vary between 105 kDa with four identical subunits of 26 kDa each (Domagk et *al*., 1973) and 183 kDa with one subunit of 75 kDa and two subunits of 54 kDa each (Horitsu *et al*., 1976).

Since glucose-6-phosphate, fructose-6-phosphate and glyceraldehyde-3-phosphate are also glycolytic intermediates, these two pathways are in close contact. Intermediates of the non-oxidative part of the pentose phosphate pathway are required for the biosynthesis of several molecules. Ribose-5-phosphate is the precursor of phosphoribosyl pyrophosphate, which is required for the biosynthesis of purine and pyrimidine nucleotides, nucleic acids, several coenzymes and the amino acids histidine and tryptophan (Schaaff-Gerstenschläger and Miosga, 1997). Erythrose-4-phosphate, another intermediate of the pathway, is a precursor for the synthesis of the aromatic amino acids tryptophan, phenylalanine and tyrosine, and for the biosynthesis of *p*-aminobenzoate and *p*-hydroxybenzoate. The non-oxidative part is also of enormous industrial interest for utilising the pentose sugar D-xylose as a carbon source (Miosga and Zimmermann, 1996).

Two reactions of the PPP in which a glycolaldehyde moiety (two carbons) is transferred from a ketose donor (xylulose-5-phosphate) to an aldose accepter (either ribose-5–phosphate or erythrose-4-phosphate), are catalysed by a transketolase (Fletcher *et al*., 1992). Transketolase activity is indispensable for the generation of erythrose-4-phosphate, which is needed for the biosynthesis of aromatic amino acids (Schaaff-Gerstenschläger *et al*., 1993). Together with aldolase, transketolase forms a reversible link between the glycolytic and pentose phosphate pathways, thereby enabling the cell to shuttle ribose-5-phosphate and glycolytic intermediates between the two pathways. The transketolase from *C. utilis* has a high affinity for both xylulose-5-phosphate (K_m 80 µM) and ribose-5-phosphate (K_m 430 µM) (Wood, 1981).

An interesting observation by Flores *et al*. (2000) was that the nitrogen source of the medium influences the amount of sugar directed to the pentose phosphate pathway. In *S. cerevisiae*, growth in a medium supplemented with amino acids decreased the flux through the pentose phosphate pathway (Gancedo and Lagunas, 1973). However, in *C. utilis* and other yeasts that use nitrate as nitrogen source, an increase of the carbon flux through the pentose phosphate pathway can be ascribed to the increased NADPH requirement due to the operation of nitrate and nitrite reductase (Bruinenberg *et al*., 1983[a]).

In *C. utilis*, at least 35% to 48% of the available glucose is catabolised via the pentose phosphate pathway, and at most 52% to 65% via glycolysis at various growth rates in continuous cultures (Mian *et al*., 1974). This is in sharp contrast to the minimum quantity of glucose catabolised via the pentose phosphate pathway in *S. cerevisiae* that ranges from 9% to 34%, which suggested that the PPP does not contribute substantially to glucose metabolism in *S. cerevisiae* (Gonzáles Siso *et al*., 1996). However, it should be noted that this represents the maximum estimate of the pentose phosphate pathway participation, since a part of it is presumably converted to biosynthetic intermediates and the overflow converted to fructose-6-phosphate, which in turn is further catabolised by the PPP and glycolysis in the same ratio. It is likely that the difference between the relative importance of the pentose phosphate pathway in *C. utilis* and *S. cerevisiae* is a consequence of different mechanisms for reoxidation of NADPH in these two yeasts.

3.2.4 Oxidation of NAD(P)H and Energy Production

Reducing equivalents in the form of NADPH are required by many enzymes in central biosynthetic pathways, whereas the enzymatic sources of biosynthetic reducing equivalents are believed to be limited in number. Direct (without shuttle mechanisms) oxidation of cytoplasmic NADPH by yeast mitochondria has been reported (Gonzáles Siso *et al*., 1996), although its relative importance in metabolism seems to be species-dependent. In *C. utilis*, oxidation of NADPH via the respiratory chain has been established, and linked to only site II and III phosphorylation. Enzyme studies showed that glucose-6-phosphate dehydrogenase, the enzyme catalysing the rate-limiting step in the PPP, and $NADP^+$-specific isocitrate dehydrogenase are the major sources of NADPH in *C. utilis*. $NADP^+$-specific isocitrate dehydrogenase is structurally and functionally distinct from the mitochondrial NAD^+-specific isocitrate dehydrogenase that functions in the TCA cycle (Minard *et al*., 1998). This is also true for the yeast *S. cerevisiae* (Minard and McAlister-Henn, 2001). High levels of cytosolic NADPH are believed to be important for growth on acetate because of relatively high rates of flux through metabolic pathways, respiration and/or β-oxidation, that generate reactive oxygen species. The pentose phosphate pathway or $NADP^+$-specific isocitrate dehydrogenase is essential for supplying reducing equivalents to support the functions of multiple thiol-dependent peroxidases that utilise reduced gluthathione or thioredoxin as cofactors and that function to protect the cell from oxidative damage.

All available evidence indicates that *C. utilis* is unable to interconvert NADH and NADPH via transhydrogenase or analogous enzyme systems (Bruinenberg *et al*., 1983[a,b]). In

theoretical calculations of the NADPH requirements for biomass formation, it was demonstrated that for growth on glucose as carbon source and ammonium as nitrogen source, depending on the contribution of the $NADP^+$-specific isocitrate dehydrogenase, 2% to 7% of the glucose metabolised has to be oxidised in the PPP to meet the NADPH requirement for biomass formation (Bruinenberg *et al*., 1983[a]). However, radiorespirometric studies revealed that in *C. utilis*, approximately 30% to 50% of the glucose is metabolised via the PPP (Mian *et al*., 1974). This suggests that activities of the PPP exceeding the theoretical minimum NADPH requirement, may point to a mechanism for dissimilatory oxidation of NADPH.

Mitochondria from *C. utilis* grown in carbon-limited continuous cultures exhibit cyanide- and antimycin A-sensitive oxidation of exogenous NADPH (Bruinenberg *et al*., 1985). The occurrence of respiratory control reveals that oxidation of NADPH in the mitochondria may generate energy in the form of ATP, which is delivered to the cytosol via specific ADP/ATP translocators (Figure 3.2). This junction of mitochondrial and cytoplasmic metabolism is also amenable for regulation of the energy flow between the two compartments. The rate of ATP production outside mitochondria depends on the activity of the ADP/ATP translocator (Pallotta *et al*., 1999).

The ADP/ATP translocator in the mitochondrial inner membrane is a major member of the mitochondrial solute-carrier family and mediates the exchange transport of ADP from the cytosol and ATP from the matrix under physiological conditions (Kaplan, 2001). The ADP/ATP translocator is essential for oxidative phosphorylation and has been characterized in *S. cerevisiae* (Hatanaka *et al*., 2001). It appears that the corresponding gene is highly conserved through evolution, as over 50% identity in the amino acid sequences is observed between yeast and human gene products (Cozens *et al*., 1989). Three genes encoding the ADP/ATP translocator has been reported in *S. cerevisiae* (Kolarov *et al*., 1990). *AAC1* is not essential for growth on a respiratory carbon source, although it is preferentially expressed under aerobic conditions. The second gene, *AAC2*, has been shown to encode the bulk of the mitochondrial translocator. The third gene, *AAC3*, which is about 60% homologous to *AAC2*, is expressed almost exclusively under anaerobic conditions.

Oxidation of exogenous NADH and NADPH seems to be a general property of mitochondria from plants and fungi (Palmer and Møller, 1982). In mitochondria from *C. utilis*, the variation in the ratio of NADH and NADPH oxidase activities, as well as the variation in the respiratory control values, indicates that this yeast employs two systems for the oxidation of exogenous NADH and NADPH in mitochondria. The internal dehydrogenase is reported to

be active with both NADH and NADPH (Mackler *et al*., 1980), but the oxidation of cytoplasmic NADH can not be attributed exclusively to the external NADH oxidase (Bruinenberg *et al*., 1985). Various shuttle mechanisms, such as the ethanol-acetaldehyde shuttle (Von Jagow and Klingenberg, 1970), may also contribute to mitochondrial NADH oxidation *in vivo*. Since the NADPH-producing enzymes of the pentose phosphate pathway are located in the cytoplasm (Bruinenberg *et al*., 1985), the external NADPH oxidase activity may be quantitatively more important than the internal NADPH oxidase.

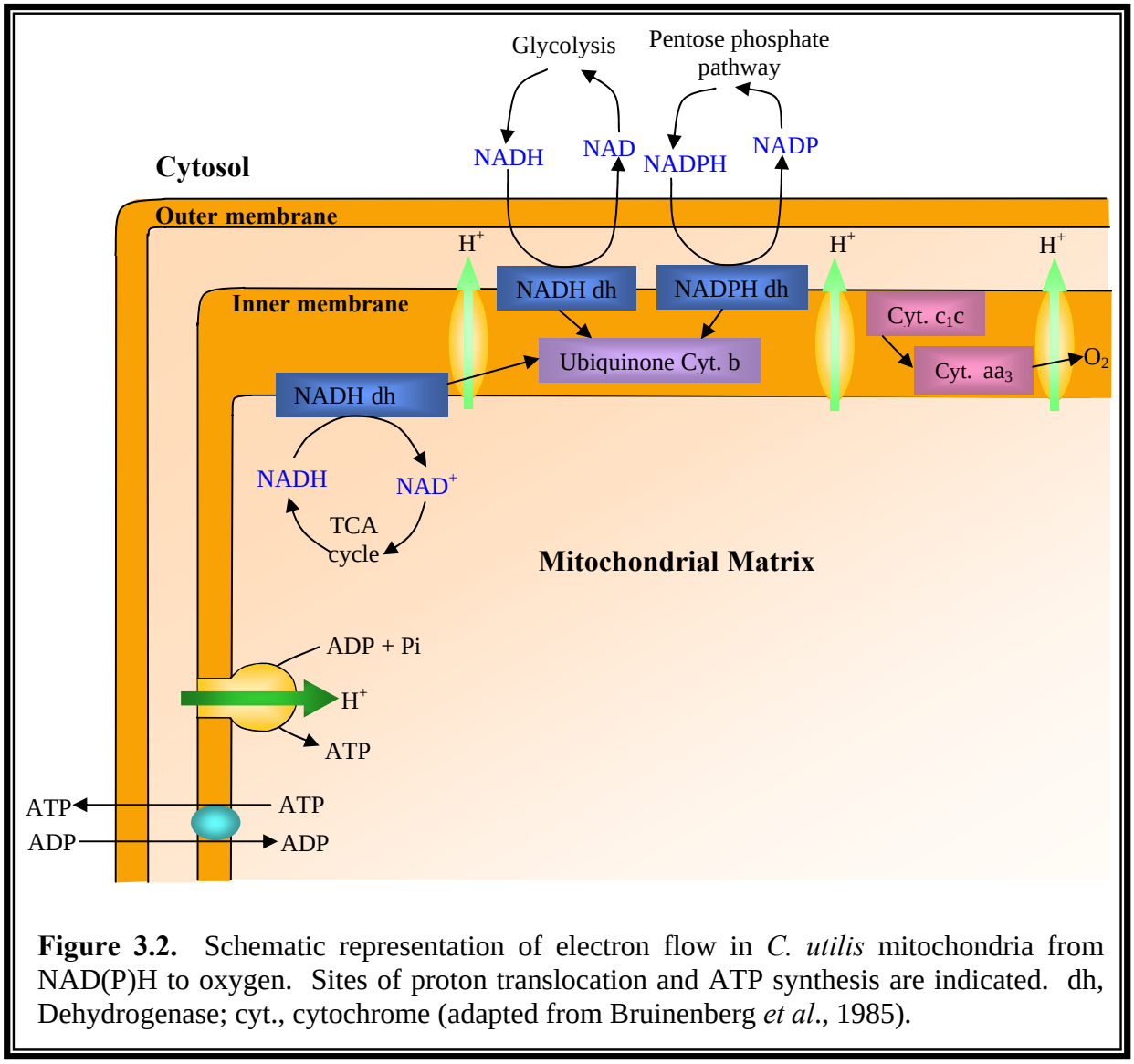

Figure 3.2. Schematic representation of electron flow in *C. utilis* mitochondria from NAD(P)H to oxygen. Sites of proton translocation and ATP synthesis are indicated. dh, Dehydrogenase; cyt., cytochrome (adapted from Bruinenberg *et al*., 1985).

3.3 THE CRABTREE EFFECT

Since the early studies by Crabtree (1929) on the effect of glycolysis on respiration in tumour cells, much information has become available on the interrelation between respiration and fermentation in eukaryotic cells. The Crabtree effect is a regulatory phenomenon which states that when glucose concentrations are high, fermentation predominates over respiration, even in the presence of oxygen (Liti *et al*., 2001). Indeed, many investigators have observed repression of the synthesis of enzymes of the TCA cycle and the respiratory chain (Petrik *et al*., 1983), as well as repression of the synthesis of mitochondria (Neal *et al*., 1971) under conditions of glucose excess.

The Crabtree effect in yeasts has been extensively studied (Fiechter *et al*., 1981) and is used to subdivide facultatively fermentative yeasts on the basis of their physiological response after transition from limiting glucose to excess glucose, as either Grabtree-positive or Crabtree-negative yeasts. The majority of yeasts, for example representatives of the genera *Candida*, *Hansenula, Kluyveromyces* and *Rhodotorula*, are Crabtree-negative; they do not perform aerobic alcoholic fermentation in the presence of high sugar concentrations. In contrast, the yeasts *S. cerevisiae* and *S. pombe* are Crabtree-positive; they ferment glucose when present at high concentrations, even under aerobic conditions. Crabtree-positive and Crabtree-negative yeasts exhibit striking differences with respect to sugar transport and the regulation of fluxes at T-junctions in the metabolism of sugars at the level of sugar phosphates and pyruvate.

3.3.1 Glucose Transport

The Crabtree-negative yeast *C. utilis* has a glucose uptake system with a very high substrate affinity (Postma *et al*., 1988), but sugar transport in this yeast is an energy-requiring process (Van Urk *et al*., 1989[b]). These yeast van therefore restrict the entry of glucose by their regulated H^+-symport systems and prevent the occurrence of overflow metabolism at high sugar concentrations, thus aerobic fermentation characteristic of Crabtree-positive yeast. However, in Crabtree-positive yeasts such as *S. cerevisiae* and *S. pombe*, transport of glucose occurs via facilitated diffusion and the glucose carriers have a low substrate affinity. They can, therefore, not restrict glucose transport at high concentrations, resulting in metabolism overflow, hence aerobic fermentation. Consequently, when a sugar-limited culture of a Crabtree-positive yeasts becomes contaminated with a Crabtree-negative yeast, the former is rapidly out-competed (Postma *et al*., 1989[c]). The higher affinity of Crabtree-negative yeasts for glucose explains problems encountered with so-called ‘wild yeasts’ in industrial sugar-

limited fed-batch cultures of baker's yeast. These contaminants (frequently *Candida* species) tend to outgrow the commercial *S. cerevisiae* strains and negatively affect product quality.

The energy requirement for aerobic glucose-limited growth by *C. utilis* is considerably higher than for *S. cerevisiae*. This is due to the fact that the protein content of *C. utilis* is 25% higher than that of *S. cerevisiae* (Verduyn *et al*., 1991). As a consequence, more energy is required in the synthesis, and especially in the polymerization, of amino acids. In addition, the higher protein content also necessitates an increased ammonium uptake, which is an energy-requiring process. The most significant difference between the two species is that *S. cerevisiae* takes up glucose by facilitated diffusion (Lang and Cirillo, 1987) whereas *C. utilis* exhibits active transport of glucose (Peinado *et al*., 1989). Thus, despite the same biomass yield on glucose, the energy requirements for the production of a given amount of biomass are quite different.

3.3.2 Effects of Oxygen on Growth Kinetics

Facultatively fermentative yeasts refer to those yeasts able to exhibit alcoholic fermentation under appropriate cultivation conditions. In principle, all facultatively fermentative yeasts are able to generate ATP by substrate-level phosphorylation, and therefore do not depend on respiration to drive energy-requiring reactions. In Crabtree-negative yeasts, and in Crabtree-positive yeasts grown under sugar limitation, oxygen is a key parameter determining the rate of alcoholic fermentation (Van Dijken *et al*., 1993). Apparently, alcoholic fermentation by non-*Saccharomyces* yeasts, like *C. utilis*, is confined to a narrow range of oxygen feeds. At very low oxygen feeds, growth (and eventually, fermentation) becomes inhibited due to the intrinsic inability of these yeasts to grow anaerobically. At higher oxygen feeds, the glycolytic flux is preferentially directed towards respiration, thereby lowering the ethanol yield.

When *S. cerevisiae* and *C. utilis* cells grown under glucose limitation, were pulsed with excess glucose, both organisms initially exhibited similar rates of glucose and oxygen consumption. However, striking differences were apparent between the two yeasts with respect to the production of cell mass in the culture and metabolite excretion (Van Urk *et al*., 1988). Upon transition from glucose limitation to glucose excess, *S. cerevisiae* produced much more ethanol but the growth rate remained close to that under glucose limitation. However, *C. utilis* produced little ethanol and immediately started to accumulate cell mass at a high rate. This high production rate of cell mass was probably due to synthesis of reserve

material and not caused by a high rate of protein synthesis. Upon a glucose pulse, both yeasts excreted pyruvate but *S. cerevisiae* also excreted various TCA cycle intermediates, both under steady-state conditions and after exposure to glucose excess. These results and those of theoretical calculations on ATP flows, support the hypothesis that ethanol production as a consequence of pyruvate accumulation in *S. cerevisiae* upon transition from glucose limitation to glucose excess, is caused by a limited capacity of assimilatory pathways.

NADH was oxidised at approximately the same rate by the mitochondria of *S. cerevisiae* and of *C. utilis* (Van Urk *et al*., 1989[a]). In both yeasts, two NADH dehydrogenases are present (Von Jagow and Klingenberg, 1970), one located on the outer side of the inner mitochondrial membrane where it functions in the oxidation of NADH generated in the cytoplasm, and the other located at the inner surface of the inner membrane and functioning in the oxidation of NADH generated in the mitochondrial matrix by the enzymes of the TCA cycle. The oxidation rates of TCA cycle intermediates are also similar in both yeasts. It could therefore be concluded that the rapid alcoholic fermentation in *S. cerevisiae* does not result from a limited capacity of the mitochondrial respiratory system, as respiratory capacities of *S. cerevisiae* are similar to those of *C. utilis*, a yeast which does not perform alcoholic fermentation upon transition to glucose excess.

The yeasts *S. cerevisiae* and *C. utilis* also differ in their anabolic potential. After relief from glucose limitation, *S. cerevisiae* does not increase its growth rate immediately, whereas *C. utilis* instantaneously attains a higher biomass production rate (Van Urk *et al*., 1988). Since *C. utilis* accumulates reserve material in the form of glycogen, the glycolytic flux will be lower in this yeast than in *S. cerevisiae*. After prolonged exposure of *C. utilis* to glucose excess, the glycolytic flux becomes higher than the conversion of pyruvate in assimilatory and dissimilatory pathways. It is probable that the activity of pyruvate decarboxylase, the key enzyme of alcoholic fermentation, is lower in *C. utilis* (Holzer, 1961), therefore resulting in lower ethanol production rates in *C. utilis*.

3.4 THE KLUYVER EFFECT

Yeast species can grow on various sugars, but growth on certain sugars (especially oligosaccharides) occurs in many cases only under aerobic conditions when fermentation is completely blocked. This apparent dependence of sugar utilisation on respiration has been called the Kluyver effect, and such ‘respiration-dependent’ species are referred to as Kluyver-positive (Fukuhara, 2003). A yeast may be Kluyver-positive for some sugars and not for

others. Of the 215 glucose-fermenting yeast species, 96 exhibit the Kluyver effect for at least one disaccharide (Barnett *et al*., 1990). The yeast *S. cerevisiae*, which has a predominantly fermentative mode of life, is Kluyver effect negative on most sugars (Fukuhara, 2003). However, Van Rooijen *et al*. (1994) observed that *S. cerevisiae* is Kluyver effect positive for trehalose. This observation was confirmed by Malluta *et al.* (2000) who showed that *S. cerevisiae* could not grow on trehalose since the trehalose influx was probably too low to sustain fermentative growth.

During growth of facultatively fermentative yeasts (such as *C. utilis*) on glucose, oxygen-limited growth conditions invariably result in the occurrence of alcoholic fermentation. Because the ATP yield from alcoholic fermentation is much lower than that from respiration, this leads to a reduction in the biomass yield on glucose. Alcoholic fermentation further negatively affects biomass yields due to the accumulation of toxic fermentation products (Castrillo *et al*., 1996). However, many facultatively fermentative yeast species show a peculiar behaviour with respect to the utilisation of certain disaccharides. When disaccharides are used as a carbon source for the cultivation of facultatively fermentative yeasts, oxygen limitation does not always result in alcoholic fermentation. Depending on the yeast species, some disaccharides cannot be fermented, although respiration of the disaccharides and fermentation of the component hexose(s) are both possible (Weusthuis *et al*., 1994[a]).

The Kluyver effect must somehow be related to differences in the metabolism of monosaccharides and disaccharides. Target reactions at which control of disaccharide metabolism may be exerted, are sugar uptake and/or disaccharide hydrolysis. The failure to utilise some disaccharides anaerobically appears to be due to a slowing down of the active sugar transporter, probably because in anaerobiosis there is a lower ATP concentration within the cell, which is insufficient to supply the proton pump optimally and to sustain the proton symport (Rolim *et al*., 2003). Also, the possibility that specific kinases are involved in transport-associated phosphorylation of hexose molecules generated from disaccharide hydrolysis (Clifton *et al*., 1993), cannot be ruled out as a possible cause of the Kluyver effect. Pyruvate decarboxylase and alcohol dehydrogenase, the two key enzymes of fermentative sugar metabolism, are present at high activities in extracts of cells from maltose-grown, oxygen-limited chemostat cultures (Weusthuis *et al*., 1994[b]). Apparently, the absence of alcoholic fermentation in maltose-grown cells is not caused by regulation of the synthesis of these fermentative enzymes (Kaliterna *et al*., 1995). Independent of the nature of the responsible molecular signal, various mechanisms can be involved in the regulation of

maltose metabolism under oxygen-limited conditions. An important discrimination that can be made in this respect is between regulation at the level of enzyme synthesis and regulation of the activity of an existing enzyme, i.e. allosteric modification of enzyme activity or post-translational modification.

This poorly understood phenomenon can be clearly illustrated by studying the utilisation of maltose by *C. utilis*, a facultatively fermentative, Crabtree-negative yeast. In aerobic cultures, *C. utilis* exhibits rapid growth on glucose and maltose. In oxygen-limited chemostat cultures, however, glucose is readily fermented to ethanol. When an aerobic, glucose-limited culture was shifted to oxygen limitation, ethanol formation did not set in immediately, but was preceded by a lag phase, required to induce the fermentative key enzyme pyruvate decarboxylase (Kaliterna *et al*., 1995). In contrast, alcoholic fermentation does not occur in maltose-grown, oxygen-limited chemostat cultures of *C. utilis*. The amount of maltose that can be metabolised is limited by the amount of oxygen available for respiration, leading to incomplete utilisation of the disaccharide. It therefore seems that oxygen availability is not a key factor in the Kluyver effect for maltose utilisation by *C. utilis*, but rather the yeast's intrinsic inability to ferment the particular disaccharide.

Weusthuis *et al*. (1994[a]) reported that *C. utilis* cells express all the enzymes required for alcoholic fermentation during oxygen-limited growth on maltose. Indeed, when oxygen-limited cultures of *C. utilis* grown on maltose were pulsed with glucose, alcoholic fermentation set in almost immediately. It was suggested that alcoholic fermentation which occurs after the addition of glucose, inhibits or suppresses maltose metabolism via a feed-back mechanism involving ethanol or a related metabolite (Figure 3.3). Build-up of disaccharide fermentation products to a critical level results in inhibition of disaccharide uptake, which can be relieved by the respiratory degradation of the inhibitory metabolites. This mechanism tunes the rate of disaccharide uptake to a value that is sufficiently low not to result in alcoholic fermentation.

3.5 CATABOLITE REPRESSION

S. cerevisiae and many other types of yeast may thrive on a variety of carbon sources, but glucose and fructose are the preferred ones. The presence of glucose in the environment represses the transcription of genes involved in certain metabolic pathways. When one of these sugars is present, the enzymes required for the utilization of alternative carbon sources are synthesised at low rates or not at all. This phenomenon is known as carbon catabolite

repression, or simply catabolite repression. The term "glucose repression" has also been proposed, since no "catabolite" derived from glucose and involved in the repression has been identified yet. This metabolic regulatory response affects the genes involved in the assimilation of alternative sugars, such as the *GAL* and *SUC* genes, as well as genes involved in gluconeogenesis, the tricarboxylic acid cycle and respiration (Gancedo, 1998). Since very little is known about catabolite repression in *C. utilis*, information available on *S. cerevisiae* will be discussed in the following sections, with reference to *C. utilis* where applicable.

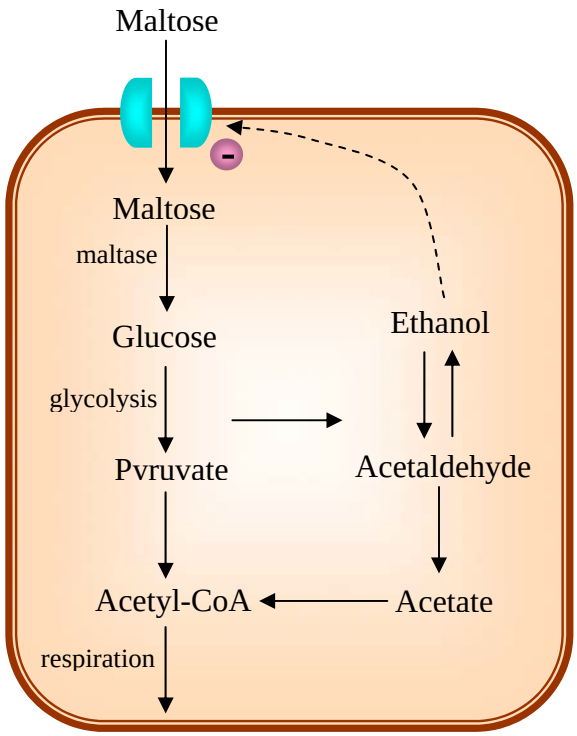

Figure 3.3. Feedback inhibition of disaccharide utilisation as a possible cause of the Kluyver effect in yeasts (adapted from Weusthuis *et al*., 1994[a]).

The yeast *C. utilis* shows different characteristics to *S. cerevisiae* with regards to the regulation of carbon source consumption. It is a Crabtree-negative and Kluyver-positive yeast and catabolite repression effects seems to be more limited than in *S. cerevisiae*, since oxidative metabolism is not affected in *C. utilis* (Weusthuis *et al*., 1994). Both yeasts have similar enzymes (two hexokinases, one of them containing more than 90% of total fructose kinase activity, and the other a glucokinase) (Espinel and Peinado, 1994), with similar substrate affinities for glucose, fructose and ATP, but a high affinity for mannose in the case of *C. utilis* hexokinases. However, some differences between the hexokinases of *C. utilis* and

S. cerevisiae have been observed: (1) *C. utilis* hexokinases were insensitive to xylose effects, either inhibition or inactivation; and (2) physiological Mg-free ATP concentrations did not affect enzyme activity in *C. utilis*. Unlike some other yeast species, *C. utilis* hexokinases do not show a higher activity under repressing conditions, but exhibit a constant fructose/glucose phosphorylation ratio in any conditions used. It was concluded that in *C. utilis*, high hexokinase activity is not related to glucose repression. In fact, hexokinase activity seemed inversely proportional to glucose repression, since cells growing in a chemostat at low concentration of glucose had a higher level of hexokinase than in repressed cells (Espinel *et al*., 1996).

The next question is how extensively glucose should be metabolised to be able to repress transcription. It has been concluded that for catabolite repression, glucose signaling does not require any metabolic step in the glycolytic pathway beyond phosphorylation (Rose *et al*., 1991). Glucose is known to trigger an immediate, transient increase in the intracellular concentration of cAMP in derepressed cells of *S. cerevisiae* (Wigler *et al*., 1988), but cAMP only affects the repression of some genes in *S. cerevisiae* and even for those genes, redundant regulatory mechanisms exist. In contrast, catabolite repression of the *S. pombe fbp*$^{+}$ gene, encoding fructose-1,6-bisphosphatase, is dependent on a cAMP signaling pathway. Several genes (named *git* for glucose insensitive transcription) participate in the repression process (Byrne and Hoffman, 1993). It is less widely appreciated that glucose also has a long-term effect on cAMP levels. The fact that the intracellular levels of cAMP are higher in the presence of glucose and other sugars than under derepressed conditions in some yeasts (Eraso and Gancedo, 1984), could suggest a role for cAMP in catabolite repression.

Similar to *S. cerevisiae*, the yeast *C. utilis* can use sucrose as an alternative carbon source. The *INV1* gene, encoding the *C. utilis* invertase enzyme (EC 3.2.1.26) responsible for the cleavage of sucrose to glucose and fructose, has been purified and characterised (Chávez *et al*., 1998). The synthesis of this enzyme decreased drastically when glucose concentrations in the medium were higher than 1% in the medium (Chavez *et al*., 1997). Putative binding sites for the Mig1p repressor have been found in the *INV1* gene from *C. utilis*, suggesting that Mig1p could repress the gene in the presence of high glucose concentrations. The *C. utilis MIG1* gene has been characterised; there is only one gene copy that encodes a polypeptide of 345 amino acids with a molecular weight of 37.2 kDa (Delfin *et al*., 2001).

The Mig1 Complex. A generalised scheme for the role of Mig1p in catabolite repression is shown in Figure 3.4 (Gancedo, 1998). The *MIG1* gene, an important element in glucose repression, was identified in a search for genes that would turn off the *GAL1* promoter of *S. cerevisiae* (Nehlin and Ronne, 1990). Mig1p is a C_2H_2 zinc finger protein that is able to bind to the promoters of a variety of genes repressed by glucose. DNA binding requires a GC box with the consensus sequence (G/C)(C/T)GGGG, but it also requires an AT-rich region 5' to the GC box (Lundin *et al*., 1994).

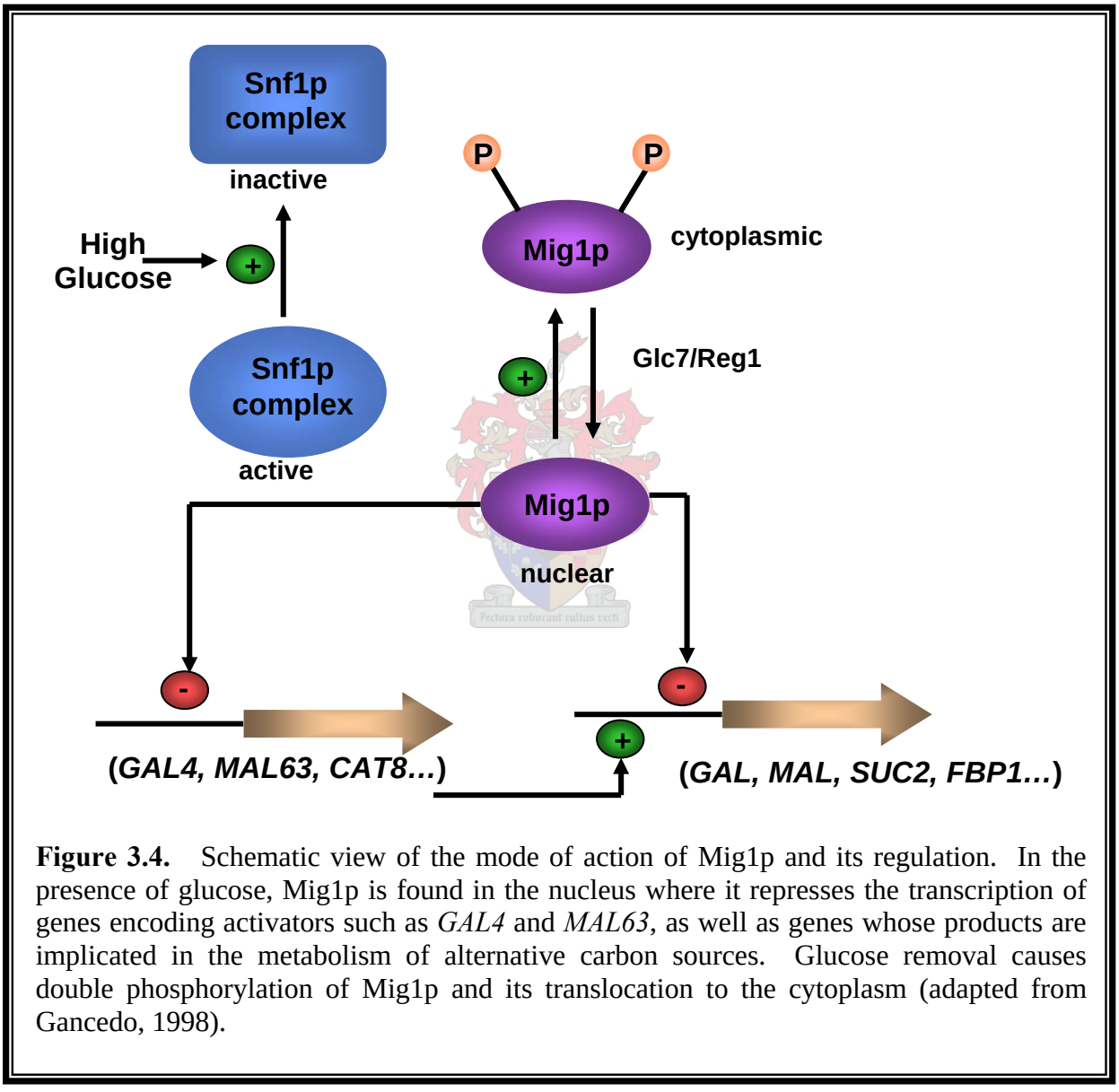

Figure 3.4. Schematic view of the mode of action of Mig1p and its regulation. In the presence of glucose, Mig1p is found in the nucleus where it represses the transcription of genes encoding activators such as *GAL4* and *MAL63*, as well as genes whose products are implicated in the metabolism of alternative carbon sources. Glucose removal causes double phosphorylation of Mig1p and its translocation to the cytoplasm (adapted from Gancedo, 1998).

The operation of Mig1p appears to be controlled by the protein kinase Snf1p (Östling *et al*., 1996). Mig1p is phosphorylated to different extends in repressed and derepressed cells. Relief of Mig1p repression requires the protein kinase Snf1p, which suggests a role for

phosphorylation of Mig1p in the control of repression (Treitel and Carlson, 1995). Mig1p is localised in the nucleus in repressed cells, but after glucose removal from the medium, Mig1p is both phosphorylated and translocated to the cytosol (DeVit *et al*., 1997).

Our knowledge of glucose signaling is still limited. It is likely that different rates of phosphorylation are correlated with different concentrations of intracellular metabolites and that the activation or inhibition of specific regulatory proteins is carried out by these metabolites. While glucose-6-phosphate, fructose-6-phosphate and cAMP could be some of the candidates for signaling metabolites and Snf1p is likely to be one of the final targets, other key intermediary elements remain to be identified.

TRANSPORT IN THE YEAST *CANDIDA UTILIS*: A COMPARISON WITH OTHER YEASTS

4.1 INTRODUCTION

One of the most challenging problems facing a cell is the regulation and integration of its metabolism in response to varying internal needs and a changing external environment. For cells to survive the demands of a changing environment, they constantly have to regulate their metabolism. One way of regulating the metabolism is to control the transport of metabolites across the semi-permeable membrane that separates the cell from its surroundings. The cells must therefore possess highly specialised transport systems in their plasma membranes to import the necessary metabolites from the extracellular environment and to export waste products from the cell. In addition, energy must be supplied to keep the intracellular concentrations at a level that may differ significantly from the external concentrations. Transport processes also play an important role in cellular organelles such as the nucleus, vacuole and mitochondrion whose membranes employ a variety of transport mechanisms. The following discussion will, however, focus on transport systems applicable to plasma and mitochondrial membranes, in particular those systems involved in the transport of dicarboxylic acids. In view of the effect that glucose has on the regulation of dicarboxylic acid transport in *C. utilis*, relevant information on the general mechanisms involved in glucose transport in yeast will also be discussed in a subsequent section.

4.2 MECHANISMS OF TRANSPORT

Metabolic compounds may be transported across the plasma membrane by three different mechanisms, namely diffusion, facilitated transport and active transport (Figure 4.1). The way in which cellular energy, which is primarily stored in chemical bonds (e.g. ATP), is supplied directly or indirectly to the transport systems is tightly related to the mechanism in which solutes are translocated across the cell membrane.

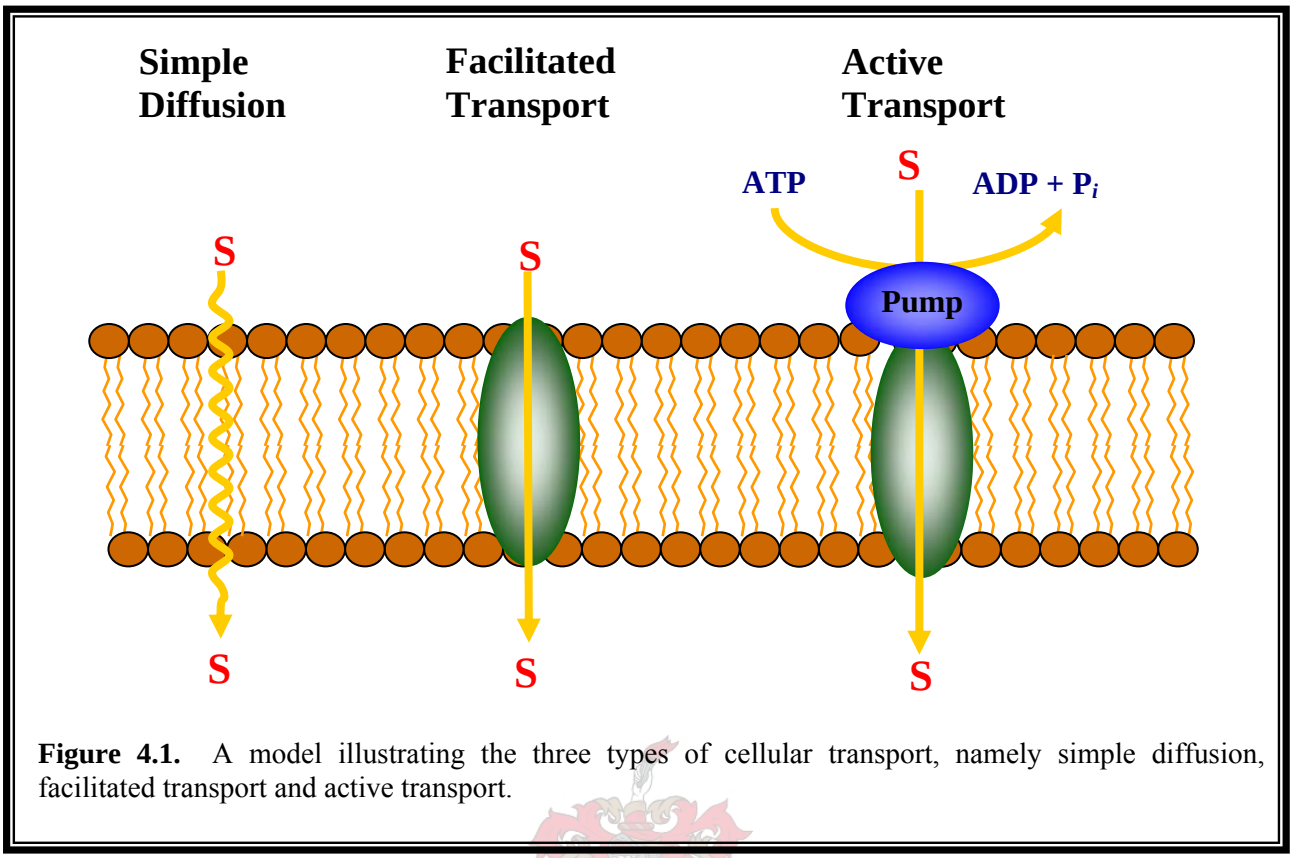

Figure 4.1. A model illustrating the three types of cellular transport, namely simple diffusion, facilitated transport and active transport.

4.2.1 Diffusion

Diffusion is the process of random movement of molecules across the membrane with a characteristic dependence upon the size of the diffusing molecule (Stein, 1986). Simple chemical diffusion is rarely observed in yeasts as a means of metabolite uptake for a number of reasons: (1) diffusion is governed by the law of mass action and metabolites in the cell never exceed those in the surrounding medium, (2) the process is not saturable with respect to substrate concentration, and (3) neither temperature nor metabolic inhibitors play a role in the diffusion process (Cooper, 1982).

Substances with a hydrophilic character enter the cell rapidly via passive diffusion with the largest permitted diameter of penetrating molecules being approximately 0.4 nm. This limitation excludes the cyclic forms of the saccharides, but allows the penetration of the linear or acyclic forms (Kockova-Kratochvilova, 1990). Amino acids with a diameter of less than 0.4 nm may also enter the cells by free diffusion. Ethanol, H_2O, O_2, CO_2 and hydrophobic molecules such as

undissociated carboxylic acids, can cross the membrane by passive diffusion without the participation of either permeases or channel proteins (Stein, 1986).

4.2.2 Facilitated Diffusion

For many substances, the slow transport provided by passive diffusion is insufficient for the functional and metabolic requirements of the cell and other means are required to increase the transport rate. In facilitated diffusion systems where translocation of the substrate across the membrane is mediated by a carrier protein, the driving force for solute translocation is provided exclusively by the concentration gradient of the substrate over the membrane (Solomon *et al*., 2005). Therefore, the uptake of substrates by facilitated diffusion does not require any metabolic energy. Since the driving force becomes zero when internal and external substrate concentrations are equal, this process does not allow the uptake of substrates against a concentration gradient. It is important to remember that although facilitated diffusion is sometimes very fast and very selective, it only represents a specialised form of diffusion.

4.2.3 Active Transport

Transport against a concentration gradient is called active transport and requires a free energy source that usually comes from the hydrolysis of ATP (Figure 4.2). In particular, during growth at very low extracellular sugar concentrations, the intracellular accumulation of sugars may be required to allow the cytoplasmic sugar kinases and disaccharide hydrolases to function optimally (Lagunas, 1993). This can be accomplished by coupling the uptake of a sugar molecule to the uptake of one or more protons via proton symport systems. Thus, the proton motive force over the plasma membrane can be used to drive the intracellular accumulation of sugar. This proton motive force is generated mainly by the plasma membrane H^+-ATPase complex, which couples the hydrolysis of ATP to ADP + P_i to the outward translocation of protons (Weusthuis *et al*., 1994[a]).

Depending on the way in which solute translocation across the plasma membrane is energised, one generally discerns primary active transport and secondary active transport. Primary transport forms the basis of transport by generating voltage and ion gradients across the membrane, whereas secondary active transporters are designed to utilise such gradients to drive solute transport (Gennis, 1989).

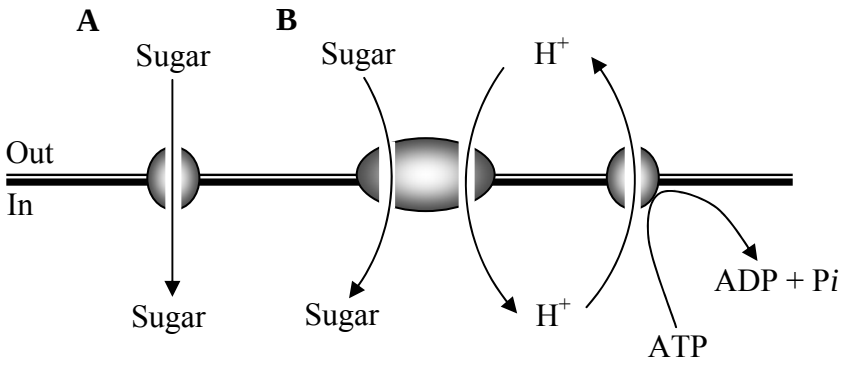

Figure 4.2. Schematic representation of two mechanisms of sugar transport in yeast. (A) Facilitated transport driven by the concentration gradient of the sugar. (B) Proton-sugar symport driven by the proton motive force and the sugar concentration gradient. ATP hydrolysis is required to expel the protons that enter the cytosolic compartment together with the sugar (adapted from Weusthuis *et al*., 1994[a]).

Primary active transport. In primary active transport processes, chemical energy is supplied directly to the transport system via hydrolysis of energy-rich chemical bonds catalysed by an enzyme located in the plasma membrane. During this process, the enzyme may undergo a conformational change by which ions are translocated from one side of the membrane to the other side. In yeast, the extrusion of protons from the cell is probably a primary active transport process that is mediated by a membrane bound ATPase. The ion pumps not only create a concentration gradient across the membrane, but also an electrical potential difference between the cytoplasma and the medium due to the diffusion potentials of the actively translocated ions (Van Winkle, 1999). In addition, if the active transport process translocated a net charge across the membrane, an extra electrogenic component is added to the membrane potential. In yeast cells, the membrane potential (negative inside the cell) is generally believed to originate mainly from the electrogenic extrusion of protons from the cells. By primary active translocation of protons, the energy stored originally in the chemical bonds is thus transformed into an electrochemical potential difference of protons across the membrane, known as the proton motive force (PMF).

Secondary active transport. Secondary transport proteins can be divided into two classes: symporters simultaneously translocate two different solutes across the membrane, for example protons and malate by the malate permease system in *S. pombe* (Sousa *et al*., 1992). In contrast,

antiporters involve the coupled transport of two different molecules that move in opposite directions across a membrane, for example the mitochondrial dicarboxylate and succinate/fumarate antiports in *S. cerevisiae*. In secondary active transport, solute translocation is energised not directly by hydrolysis of the chemical bonds, but by coupling of the solute transport to the electrochemical potential difference created via the primary active transport processes. In the case of cation uptake, the coupling may be quite simple via the electrical part of the driving force. Due to the membrane potential, which is generally negative inside the cell with respect to the medium, cations can be accumulated inside the cell to concentrations that are higher than those in the surrounding environment. Intracellular concentrations of neutral solutes that are higher than the medium concentrations can be obtained by coupling neutral solute transport to the total PMF. This may be achieved via mechanisms that transport one or more protons along with the neutral solute. In a similar way, anion uptake can be energised via co-transport of the anion with two or more protons. The membrane potential therefore plays an important role in the translocation of various solutes.

4.3 CARBOXYLIC ACID TRANSPORT IN YEAST

The concerted function of the cytosolic and mitochondrial compartments requires the presence of specific carrier proteins that catalyse the transport of various metabolites. Therefore, transport of carboxylic acids occurs on two different levels in yeast cells, i.e. transport across plasma membranes and transport across mitochondrial membranes. This section will focus on the transport of carboxylic acids across the plasma membrane, while transport across mitochondrial membranes will be discussed in a following section.

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

1984; Osothsilp and Subden, 1986[b]).

Strains of the K(-) yeast *S. cerevisiae* lack the machinery for the active transport of L-malate and rely on rate-limiting simple diffusion for the uptake of extracellular L-malate (Delcourt *et al*., 1995). However, *S. cerevisiae* is able to utilise short-chain mono-carboxylic acids, such as lactate and pyruvate, as sole carbon and energy sources under aerobic conditions. This is done by means of a proton symport process via a monocarboxylate permease (lactate/pyruvate permease, Jen1p), encoded by the *JEN1* gene. The gene is repressed by glucose and induced by non-fermentable substrates such as ethanol and lactate (Bojunga and Entian, 1999; Casal *et al*., 1999). *JEN1* is negatively regulated by the repressors Mig1p and Mig2p, and requires Cat8p for full derepression during a shift from fermentative to respiratory growth (Bojunga and Entian, 1999). The Hap2/3/4/5 complex interacts specifically with a CAAT-box DNA element in the *JEN1* promoter to exert transcriptional derepression of the gene (Lodi *et al*., 2002). Cat8p and the Hap2/3/4/5 complex are both essential for growth on non-fermentable carbon sources, but have different targets: Cat8p controls the glyoxylate cycle and gluconeogenesis, whereas the Hap2/3/4/5 complex activates genes involved in respiratory pathways.

The Jen1 lactate/pyruvate permease is the only permease reported to be involved in the transport of carboxylic acids in *S. cerevisiae* (Casal *et al*., 1999). However, monocarboxylate proton symporters with different specificities have been described in *S. cerevisiae*: acetate or ethanol-grown cells contain a permease that is shared by acetate, propionate and formate (Casal *et al*., 1996), while cells grown in lactate express an additional permease that transports lactate, pyruvate, acetate and propionate (Cássio *et al*., 1987; Casal *et al*., 1995). The first system is constitutively expressed in cells growing on non-fermentable carbon sources, whereas the lactate system was specifically induced by lactate. Both systems are completely repressed in the presence of glucose (Makuc *et al*., 2001) when monocarboxylates can cross the plasma membrane only by simple diffusion of the undissociated form of the acids.

The presence of multiple carriers may be the basis of the versatility of "non-conventional" yeasts in the utilisation of organic acids as carbon sources. The *KlJEN1* and *KlJEN2* genes from *Kluyveromyces lactis* (Lodi *et al*., 2004) were reported to encode a monocarboxylate permease and dicarboxylic acid transporter, respectively. Transcription of *KlJEN1* is glucose repressed and induced by lactate, while *KlJEN2* is expressed in ethanol, acetate and succinate (but not lactate)

and repressed in the presence of glucose. Both transporters are linked to oxidative metabolic processes: succinate, fumarate and malate (substrates of KlJen2p) are intermediates of the Kreb's cycle, while lactate (substrate of KlJen1p) is oxidised by means of an enzymatic pathway strictly connected to the respiratory chain. Mono- and dicarboxylic acids also have an additional metabolic role as gluconeogenetic precursors, which may explain the glucose repression observed for the expression of *KlJEN1* and *KlJEN2*.

4.3.1 Dicarboxylic Acid Transport in *S. pombe*

Although *S. pombe* cannot use externally added L-malate as the only carbon and energy source, it is able to metabolise L-malate if glucose is present in the culture medium. The dicarboxylic acid carrier of *S. pombe* was shown to be a proton dicarboxylate symport system that allows mediated transport and accumulation as a function of ΔpH (Sousa *et al*., 1992). However, in the presence of high malate concentrations and low pH values, simple diffusion of the undissociated acid occurs through the lipid bilayer.

The *S. pombe* proton-malate symport is constitutive and remains active in the presence of high glucose concentrations (Sousa *et al*., 1992), but the transport of L-malate was inhibited by ethanol or acetate. In support of *S. pombe*'s requirement for fermentable carbon sources for L-malate utilisation, it was postulated that sugar metabolism provides the required energy by inducing the proton motive force for active transport of L-malate (Taillandier and Strehaiano, 1991; Camarase *et al*., 2001).

Competitive inhibition of the initial uptake rates of both labeled L-malate and labeled succinic acid by D-malate, fumarate, oxaloacetate, α-ketoglutarate, maleate and malonate strongly suggested that these acids share the same carrier in *S. pombe* (Osothsilp and Subden, 1986[b]; Sousa *et al*., 1992). All of these acids induce proton uptake that follows Michaelis-Menten kinetics as a function of the concentration of the acids. It was therefore concluded that the negatively charged form of L-malate (probably the mono-anionic form) is transported by a proton-symport mechanism and that the carrier is a common 'dicarboxylate transport system' (Sousa *et al*., 1992). However, Grobler *et al*. (1995) and Saayman *et al*. (2000) observed that although α-ketoglutarate and fumarate acted as competitive inhibitors of L-malate transport, neither α-ketoglutarate nor fumarate were transported by *S. pombe* cells.

The structural gene for the *S. pombe* malate permease (*mae1*) was cloned and characterised by Grobler *et al*. (1995). It encodes an open reading frame of 1 314 bp that is translated into a putative protein of 438 amino acids with a calculated molecular mass of approximately 49 kDa. A hydropathy profile of the predicted amino acid sequence revealed a protein with ten membrane-spanning or associated domains and a similar structure to models proposed for integral membrane proteins from both prokaryotes and eukaryotes. Although the putative mae1p protein did not contain a N-terminal membrane-targeting signal, the presence of an internal membrane signal motif was suggested.

4.3.2 Carboxylic Acid Transport in *C. utilis*

The yeast *C. utilis* can utilise short-chain carboxylic acids as sole source of carbon and energy (Barnett *et al*., 1990; Côrte-Real and Leão, 1990). When cells of *C. utilis* are grown in media with lactate, L-malate, citrate or other metabolisable carboxylic acids, they are able to transport mono-, di- and tricarboxylic acids across the plasma membrane by two mediated transport systems: a proton symport specific for mono-, di- and tricarboxylates and a facilitated transport system able to accept the undissociated forms of the acids (as well as some amino acids). Both transport systems are inducible and subject to glucose repression (Leão and Van Uden, 1986; Cássio and Leão, 1991, 1993). Lactate in *C. utilis* is transported by a monocarboxylate proton symport that is also inducible and subject to glucose repression (Leão and Van Uden, 1986). This system is also able to accept pyruvate, acetate, propionate and other monocarboxylic acids, but not di- and tricarboxylic acids or amino acids.

Citrate-grown cells of *C. utilis* induced two transport systems for citrate, presumable a proton symport and a facilitated diffusion system for the charged and the undissociated form of the acid, respectively (Cássio and Leão, 1991). Both systems could be observed simultaneously at pH 3.5 with a V_{max} = 1.14 nmol/s/mg cells and K_m = 0.9 mM for the undissociated acid in the low-affinity system, and a V_{max} = 0.38 nmol/s/mg cells and K_m = 0.056 mM for citrate in the high-affinity system. At pH values above 5.0 units, the low-affinity system was absent or not measurable. The two transport systems exhibited different substrate specificities: isocitrate and citrate showed competitive inhibition for the high-affinity system, while the low-affinity system seems to be involved in the transport of isocitrate, lactate and L-malate. The two transport systems were repressed by glucose and inducible by citrate, lactate and L-malate, but the

induction was not dependent on the relative concentration of the anionic form(s) and of undissociated citrate in the culture medium. The passive diffusion of undissociated citrate in *C. utilis* is subject to opposite pH influences: an increase in diffusion due to the relative increase of undissociated acid with decreasing pH, and a decrease in diffusion due to decreasing permeability with decreasing pH. Similar behaviour was observed with respect to passive diffusion across the plasma membrane for protons in *S. cerevisiae* (Leão and Van Uden, 1984), undissociated lactate in *C. utilis* (Leão and Van Uden, 1986) and *S. cerevisiae* (Cássio *et al*., 1987), and undissociated L-malate and succinate in *C. sphaerica* (Côrte-Real *et al*., 1989) and *H. anomala* (Côrte-Real and Leão, 1990), respectively.

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

A significant difference in the transport of fumarate and L-malate has been observed for *C. utilis* and *S. pombe*. Cells of *S. pombe* were unable to transport fumarate, although fumarate acted as a competitive inhibitor, whereas cells of *C. utilis* were able to actively transport both L-malate and fumarate (Saayman *et al*., 2000). The uptake of both dicarboxylic acids by *C. utilis* was induced by either of the substrates. Furthermore, kinetic data suggested that fumarate and L-malate was transported by the same carrier protein in *C. utilis*, which explains the similar regulatory mechanisms observed for the transport of the two substrates. Degradation of either fumarate or L-malate by *C. utilis* was sensitive to the presence of glucose, supporting previous reports that the utilisation of L-malate in *C. utilis* was subject to glucose repression (Cássio and Leão, 1993). Transport of either fumarate or L-malate by *C. utilis* was also insignificant in the presence of

other carbon sources such as raffinose and glycerol/ethanol (Saayman *et al*., 2000), confirming that the dicarboxylic acids are only being transported in the presence of either of the inducers and when no alternative carbon source is available. In contrast, the *S. pombe* proton-malate symport system is constitutive and can only metabolise L-malate in the presence of glucose or another assimilable carbon source (Sousa *et al*., 1992).

4.4 MITOCHONDRIAL TRANSPORT OF TCA CYCLE INTERMEDIATES

In addition to providing most of the cellular energy in the form of ATP by oxidative phosphorylation, mitochondria are also involved in several metabolic processes that require the participation of both intra- and extra-mitochondrial enzyme reactions. Therefore, metabolite movement across the mitochondrial membrane is essential to both mitochondrial and cytosolic metabolism. Hence, in facultative aerobic eukaryotes such as *S. cerevisiae*, mitochondria are indispensable for growth even under anaerobic conditions. The combined function of the cytosolic and mitochondrial compartments requires the presence of specific carrier proteins that catalyse the transport of various metabolites across the inner mitochondrial membrane. The transport of metabolites through the inner mitochondrial membrane is accomplished by related proteins belonging to the mitochondrial carrier superfamily (Palmieri, 2004). Their sequences are characterised by three tandem related sequences of about 100 amino acids, each of them probably being folded into two anti-parallel transmembrane α-helices linked by an extensive hydrophilic sequence (Palmieri *et al*., 1996). The three repeats are joined together by shorter hydrophilic sequences. This arrangement was first identified in the sequence of the ADP/ATP translocase, and subsequently in the uncoupling protein from brown fat mitochondria, as well as in the mitochondrial phosphate, oxoglutarate-malate, citrate and carnitine transporters (Aquila *et al*., 1982, 1985; Runswick *et al*., 1987, 1990; Kaplan *et al*., 1993; Indiveri *et al*., 1997).

Although metabolite transport across the mitochondrial inner membrane has been widely investigated over the last 30 years, our knowledge of the function of individual mitochondrial transporters in cell metabolism is still limited. The genome of *S. cerevisiae* encodes 35 putative mitochondrial carriers (Palmieri, 1994), including three isoforms of the ADP/ATP translocase (Lawson and Douglas, 1988, Kolarov *et al*., 1990), the phosphate and ornithine carriers and four transporters involved in C_2/C_3 transport (Wohlrab and Briggs, 1994; Kaplan *et al*., 1995). From a

metabolic point of view, the presence of 23 yet-unidentified mitochondrial transporters in *S. cerevisiae* indirectly points to the existence of metabolic pathways involving transport processes across the mitochondrial membrane that have so far escaped discovery. It is likely that the identification of these genes will provoke interesting bioenergetic and metabolic research.

In the following sections, the four known transporters involved in the mitochondrial transport of C_2/C_3 compounds in *S. cerevisiae* will be discussed in more detail. None of the mitochondrial or plasma membrane transporters have yet been identified in *C. utilis*, with little known about mitochondrial transport in *S. pombe*.

4.4.1 Succinate-Fumarate Transporter

The *SFC1* gene encoding the succinate-fumarate transporter in *S. cerevisiae* is responsible for the transport of succinate, produced in the cytosol by the glyoxylate pathway, into the mitochondrial matrix in exchange for fumarate (Palmieri *et al*., 1997). As succinate dehydrogenase is only accessible to succinate in the mitochondrial matrix, succinate produced in the cytosol has to be imported into the mitochondrial matrix (Figure 4.3). The succinate-fumarate transporter therefore connects the production of succinate by the glyoxylate cycle in the cytosol with the tricarboxylic acid cycle. Fumarate exported to the cytosol in exchange with succinate is first converted to malate and then to oxaloacetate, which is funneled into the gluconeogenic pathway that is indispensable for *S. cerevisiae* growth on ethanol or acetate (Gancedo and Serrano, 1989). Cells of *S. cerevisiae* lacking the *SFC1* gene, encoding the succinate-fumarate transporter, are therefore unable to grow on ethanol or acetate as the sole carbon source (Fernandez *et al*., 1994). Bojunga *et al*. (1998) and Redruello *et al*. (1999) demonstrated that the induction of *SFC1* is strictly co-regulated with the genes encoding key enzymes involved in the glyoxylate cycle (isocitrate lyase and malate synthetase) and gluconeogenesis (phosphoenolpyruvate carboxykinase and fructose-1,6-bi-phosphatase).

4.4.2 Dicarboxylate Transporter

The *S. cerevisiae* dicarboxylate transporter (DIC) is an integral membrane protein that catalyses a dicarboxylate-phosphate exchange across the inner mitochondrial membrane (Palmieri *et al*., 1999[b]). Accumulation of the dicarboxylates succinate and malate in the mitochondrial matrix was found to be dependent on the pH gradient across the mitochondrial membrane. Since the

dicarboxylate-phosphate exchange is electroneutral, the pH dependence of the dicarboxylate uptake into the mitochondria is due to the re-uptake of phosphate (as P_i^-/H^+ symport or P_i^-/OH^- antiport via the phosphate carrier), whose distribution across the membrane is in direct equilibrium with the pH gradient. Subsequent studies on intact *S. cerevisiae* cells confirmed the accumulation of phosphate and dicarboxylates in the mitochondria with respect to the cytosol (Palmieri *et al*., 2000[b]). Thus, the function of the dicarboxylate transporter was clearly to catalyse the entry of Krebs cycle intermediates into the mitochondria.

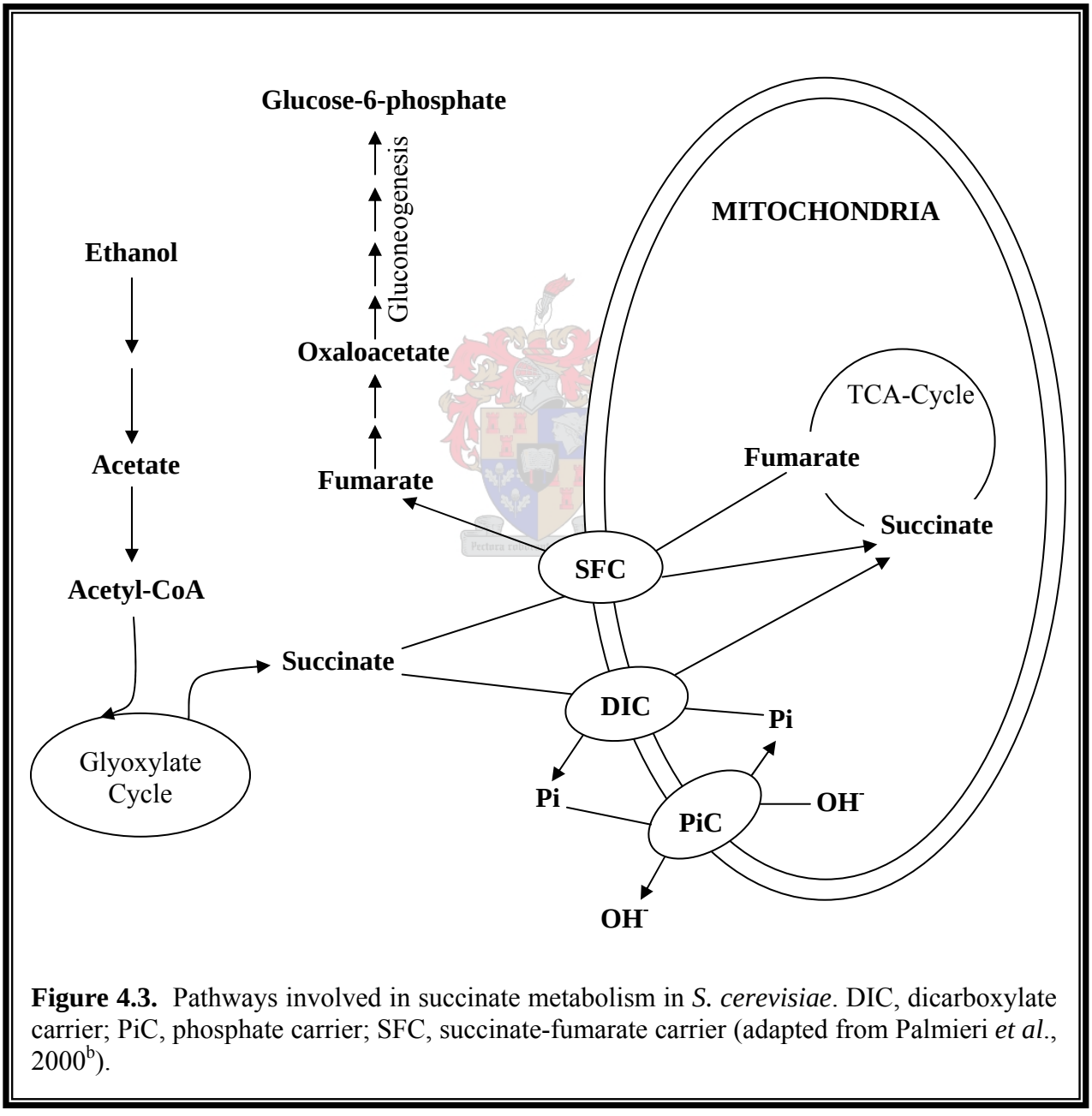

Figure 4.3. Pathways involved in succinate metabolism in *S. cerevisiae*. DIC, dicarboxylate carrier; PiC, phosphate carrier; SFC, succinate-fumarate carrier (adapted from Palmieri *et al*., 2000[b]).

In *S. cerevisiae*, two mitochondrial transport systems for succinate were identified: the DIC (Palmieri *et al*., 1996) and the succinate-fumarate carrier (SFC) (Palmieri *et al*., 1997). As discussed above, the SFC protein requires fumarate as counter-substrate, whereas the DIC catalyses the import of succinate into the mitochondria in exchange for internal phosphate. As the latter is recycled in the mitochondria by the phosphate carrier, the combined activity of the DIC and phosphate carrier leads to a net uptake of succinate.

In *S. cerevisiae* growing on ethanol or acetate, the dicarboxylate transporter catalyses the import of succinate in exchange for internal phosphate (Palmieri *et al*., 2000[a]). The conversion of succinate to fumarate and oxaloacetate within the mitochondria allows the oxidation of acetyl-CoA produced from ethanol or acetate and triggers the activity of the succinate-fumarate carrier. Findings by Palmieri *et al*. (1996, 1997, 1999[a]) indicated that the primary function of the dicarboxylate transporter is to catalyse the entry of cytoplasmic dicarboxylates into the mitochondrial matrix and calls into question the generally-accepted view that this transport is involved in gluconeogenesis by exporting malate from the mitochondria (Meijer and Van Dam, 1974). Clearly a lower activity of the dicarboxylate transporter would favour the utilisation of cytosolic succinate by the succinate-fumarate transporter and thereby the gluconeogenic pathway.

4.4.3 Oxaloacetate Transporter

The *S. cerevisiae* gene *OAC1* encodes a member of the mitochondrial transporter family responsible for the transport of oxaloacetate and sulfate, while the main substrates of the oxoglutarate and the dicarboxylate transporters, i.e. oxoglutarate, malate, succinate and phosphate, are poorly transported (Palmieri *et al*., 1999[a]). The Oac1p catalyses both uni-directional transport and counter-exchange of substrates. As the *S. cerevisiae* pyruvate carboxylase is cytoplasmic, a physiological role of Oac1p is probably to catalyse the uptake of oxaloacetate into mitochondria, a role supported by the higher transcript level observed in synthetic medium than in rich medium (Richard *et al*., 1997). Since the *OAC1* gene is not essential for growth of *S. cerevisiae* cells, it is likely that in the absence of Oac1p, cytosolic oxaloacetate is converted into malate, which enters the mitochondrion via the dicarboxylate carrier in exchange for phosphate. A role in anaplerosis for both the oxaloacetate and dicarboxylate transporters is consistent with the failure of a yeast strain lacking both transporter genes to grow on non-fermentable carbon sources (Palmieri *et al*., 1999[a]). Another possible role

for Oac1p may be to transfer reducing equivalents from the mitochondrial matrix to the cytosol by catalysing a malate/oxaloacetate exchange when the intramitochondrial concentrations of NADH and L-malate are high.

4.4.4 Citrate Transporter

The *S. cerevisiae* citrate transport protein, CTP, catalyses the efflux of the tricarboxylate citrate plus a proton across the mitochondrial inner membrane in exchange for another citrate, malate, isocitrate, succinate or phosphoenolpyruvate molecule (Sandor *et al*., 1994). Following diffusion through the outer membrane voltage-dependent anion channels, the resulting cytoplasmic citrate fuels both fatty acid and sterol biosyntheses and generates NAD^+ for glycolysis (Endemann *et al*., 1982; Conover, 1987). The CTP transporter has been kinetically characterised in mitochondria isolated from *S. cerevisiae* where it displays a K_m of 0.36 mM (Kaplan *et al*., 1995), and substrate preference for citrate, isocitrate, and to a considerably lesser extent, malate.

4.5 SUGAR TRANSPORT IN YEAST

All known yeasts are able to utilise one or more sugars as their principal source of carbon and energy. The Crabtree-positive yeasts, including *S. cerevisiae*, have a strong tendency toward alcoholic fermentation where high rates of sugar uptake result in alcoholic fermentation, even when oxygen is present in excess (Van Dijken and Scheffers, 1986). In contrast, low sugar concentrations result in a low rate of sugar uptake where sugar metabolism is fully respiratory. However, the high K_m values for sugar transport that are characteristically found in *S. cerevisiae* strains, are not typical of yeasts, since many species appear to be well equipped for growth at low sugar concentrations.

The most intensively studied case of sugar transport in yeasts is that of glucose transport in *S. cerevisiae* (Lagunas, 1993). Glucose is the preferred carbon and energy source for most cells and in addition to being a major nutrient, glucose can act as a "growth hormone" to regulate several aspects of cell growth, metabolism and development. How a eukaryotic cell senses glucose and signals its presence, how this signal affects cellular processes, and how optimal utilisation of the sugar is achieved, are fundamental questions of which many are still unanswered.

Since transport constitutes the first step in the metabolism of a large number of sugars, transport is likely to have a substantial impact on the regulation of the glycolytic flux (Weusthuis *et al*., 1994[a]). Glucose can be transported into yeasts via different uptake mechanisms such as facilitated diffusion (Romano, 1982) or active transport through a proton-glucose symporter as shown for species of *Candida* (Spencer-Martins and Van Uden, 1985). Since sugars are highly polar molecules, free diffusion across the membrane lipid bilayer probably does not contribute significantly to their rate of entrance into the cell at low sugar concentrations (Lengeler, 1993), but free diffusion might contribute to some extent to the overall sugar influx at high sugar concentrations (Fuhrmann and Völker, 1993; Walsh *et al*., 1994). This was demonstrated in a variety of cases where the dependence of the rate of transport on the assay sugar concentration yielded a biphasic kinetic plot, indicating the presence of two uptake systems (Spencer-Martins and Van Uden, 1985; Van den Broek *et al*., 1986).

The large majority of yeast strains can either respire sugars or ferment it to ethanol and carbon dioxide. In organisms such as *S. cerevisiae*, alcoholic fermentation is triggered when aerobic sugar-limited cultures are exposed to sugar excess (Boles and Hollenberg, 1997). This instantaneous response is known as the short term Crabtree effect (Petrik *et al*., 1983; Van Urk *et al*., 1988) and is followed by long-term adaptation involving repression of respiratory enzymes (Pronk *et al*., 1996). Crabtree-negative yeasts, such as *C. utilis*, do not exhibit this response (Petrik *et al*., 1983; Rieger *et al*., 1983) and seem to be exemplary of organisms that survive in environments with a low nutrient supply.

A relationship has been observed between the short-term Crabtree-effect and the mode of hexose transport (Boles and Hollenberg, 1997). In general, Crabtree-positive yeasts possess facilitated diffusion systems for the uptake of hexoses, while Crabtree-negative yeasts depend on energy-dependent H+ symport systems (Van Urk *et al*., 1989[b]; Weusthuis *et al*., 1994[a]). This is in agreement with the observation that various Crabtree-negative yeasts are better adapted to grow at low sugar concentrations, which offers an explanation for the competitive advantage of so-called wild yeasts when these contaminate industrial baker's yeast production processes (Postma *et al*., 1989[a]). Yeasts like *C. utilis* possess high-affinity proton/glucose symporters that enable them to effectively scavenge the sugar at low growth rates (Van Urk *et al*., 1989[b]). In contrast, *S. cerevisiae* transports the glucose by low-affinity facilitated transport, leaving relatively high

residual glucose levels in the culture.

The yeasts species presented in Table 4.1 can be divided into two groups with respect to their mechanism for glucose uptake. Those possessing high-affinity uptake systems accumulate glucose via an energy-requiring process, namely H^+-symport, such as the *C. utilis* glucose uptake system. In contrast, *S. cerevisiae* and *S. pombe* exhibit low-affinity uptake systems where glucose uptake probably proceeds by facilitated diffusion only. Some yeasts can transport glucose by an inducible high-affinity (K_m 0.02 – 0.2 mM) H^+-symport mechanism, for example *Kluyveromyces marxianus* (Van den Broek *et al*., 1986; Gasnier, 1987) and some *Candida* species (Spencer-Martins and Van Uden, 1985; Van den Broek *et al*., 1986). Loureiro-Dias (1988) reported that induction of this H+-symport mechanism at low glucose concentrations is particularly common in the genera *Rhodotorula*, *Candida* and *Hansenula*. At high glucose concentrations, this high-affinity transport is repressed and only a low-affinity carrier (K_m 2-3 mM) is detected (Spencer-Martins and Van Uden, 1985; Gasnier, 1987).

Table 4.1. Glucose transport parameters for different yeast species[a]

Species	Affinity (K_m) (mM)[a]		Capacity (V_{max}) (nmol/g/h)[a]	
	High	Low	High	Low
Crabtree-positive yeasts				
Saccharomyces cerevisiae		1.0		12
		20		9
Schizosaccharomyces pombe		1.5		9
Torulopsis glabrata		1.2		31
		18		93
Brettanomyces intermedius	0.03		2.1	
	0.6		2.4	
Crabtree-negative yeasts				
Candida utilis	0.025		8.4	
	0.2		5.4	
Pichia stipitis	0.015		6	
Kluyveromyces marxianus	0.025	1.8	1.2	3
Hansenula nonfermentans	0.02	3.1	3.6	3.3
	0.2		1.5	

[a] Data from Van Urk *et al*. (1989[b])

4.5.1 Sugar Transport in *C. utilis*

Glucose transport. During the growth of yeasts on sugars, the energy requirement for sugar transport can be substantial. Verduyn (1991) calculated that the theoretical energy cost of sugar transport in the yeast *C. utilis* growing on glucose is 8.2 mmol of ATP.g of biomass^{-1}, or 20% of the total ATP requirement. Cells of *C. utilis* display a set of transport systems for glucose in what seems to be a general pattern among several yeasts, including *C. wickerhamii* (Spencer-Martins and Van Uden, 1985), *C. sheatae* (Lucas and Van Uden, 1986), *C. intermedia* (Loureiro-Días, 1987), *Pichia ohmeri* (Verma *et al*., 1987) and *K. marxianus* (Gasnier, 1987). In all these yeasts, two systems were found, namely a H^+-symport when there is no or a very low concentration of glucose in the growth medium, and a facilitated transport system in the presence of higher concentrations of glucose.

Peinado *et al*. (1988) found an H^+ symport in *C. utilis* displaying simple hyperbolic kinetics and a facilitated transport with a complex behaviour. Both systems could be observed simultaneously in chemostat-grown cells at steady-state glucose concentrations below 10 mM, although the affinity of the glucose transport system is related to the residual substrate concentration in the culture. The yeast appears to synthesise carriers with affinity constants that are tuned to the environmental sugar concentration (Postma *et al*., 1989[c]). Moreover, they produce just enough of these carriers to account for the glucose flux required to sustain the growth rate (Postma *et al*., 1988, 1989[b]). The H^+-symport has such a high affinity (K_m about 15 μM) that it is saturated under any conditions where glucose can be detected, so it makes sense that its regulation has to be based on the amount of carrier present in the cells, which is controlled by induction and repression. It is also conceivable that the H^+ symport may be inactivated by glucose or even interconverted into a facilitated transport system, as previously described in other yeasts (Spencer-Martins and Van Uden, 1985; Verma *et al*., 1987). Regulation of transport at high glucose concentrations may also be achieved by changes in the affinity of a single carrier (Peinado *et al*., 1988).

Postma *et al*. (1988) suggested that the kinetics of glucose transport in *C. utilis* are adapted to the residual concentration via a well-balanced synthesis of three different transport systems, which are characterized by their affinity constants. The three transport systems differed by an order of magnitude in their affinity constants, namely a high affinity (K_m of 0.025 mM, V_{max} of 130

µmol/g cells/min), a medium-affinity (K_m of 0.190 mM, V_{max} of 160 µmol/g cells/min) and a low-affinity uptake system (K_m of 2 mM, V_{max} of 300 µmol/g cells/min). It is evident that the residual glucose concentration in the culture is a decisive parameter in the regulation of the synthesis of the three transport systems, with the presence of a transport system with a K_m of 0.025 mM allowing growth of *C. utilis* at very low glucose concentrations.

In order to investigate the relationship between sugar transport and the physiology of the yeasts, the kinetics of glucose oxidation were determined in *S. cerevisiae* and *C. utilis* (Postma *et al*., 1989[c]). The affinity constant for glucose oxidation of *C. utilis* increased when conditions were shifted from glucose excess to glucose limitation from a K_m of 0.68 mM to a K_m of 0.025 mM. In contrast, the affinity constant for glucose oxidation of *S. cerevisiae* did not vary significantly with the conditions applied, with a K_m of 0.34 mM under glucose excess. These finding are in line with the observation that induction of carriers with lower affinity constants occurs during glucose-limited conditions in *C. utilis*. In contrast, the two glucose transport systems of *S. cerevisiae* appear to be present at all growth conditions (Bisson, 1988).

Disaccharide transport. Glucose is by far the most commonly used substrate for fundamental physiological studies on sugar metabolism in yeasts. However, only few industrial applications are based on glucose as a feedstock. Industrial substrates such as molasses, whey, starch hydrolysates and wort all contain disaccharides (sucrose, lactose and maltose) as the major sugar component (Weusthuis *et al*., 1994[a]).

In yeast, the first step in the utilisation of a disaccharide is usually its transport across the plasma membrane from the external medium into the cytosol by means of a stereospecific carrier. The second step is the intracellular hydrolysis of the sugar into its monomers before their subsequent catabolism through the glycolytic pathway. However, in contrast to glucose metabolism, disaccharide metabolism in yeast is not necessarily initiated by the uptake of the sugar molecule. Some yeast species have glycosidases acting outside the plasma membrane, therefore the monosaccharides produced through hydrolysis, rather than the disaccharide itself, is carried into the cell (Rolim *et al*., 2003). For example, in the yeast *K. marxianus*, sucrose is initially hydrolysed to glucose and fructose by the extracellular enzyme inulinase (Rouwenhorst *et al*., 1988; 1991), followed by the transport of the respective hexoses into the cell (Figure 4.4). Conversely, disaccharides can also be transported across the plasma membrane prior to

hydrolysis by an intracellular hydrolase, such as for maltose utilisation by *S. cerevisiae*. In most cases, the uptake of disaccharides by yeasts has been reported to occur via proton symport systems that characteristically have a relatively high affinity constant of 2 to 6 mM (Schulz and Höfer, 1986; Carvelho-Silva and Spencer-Martins, 1990).

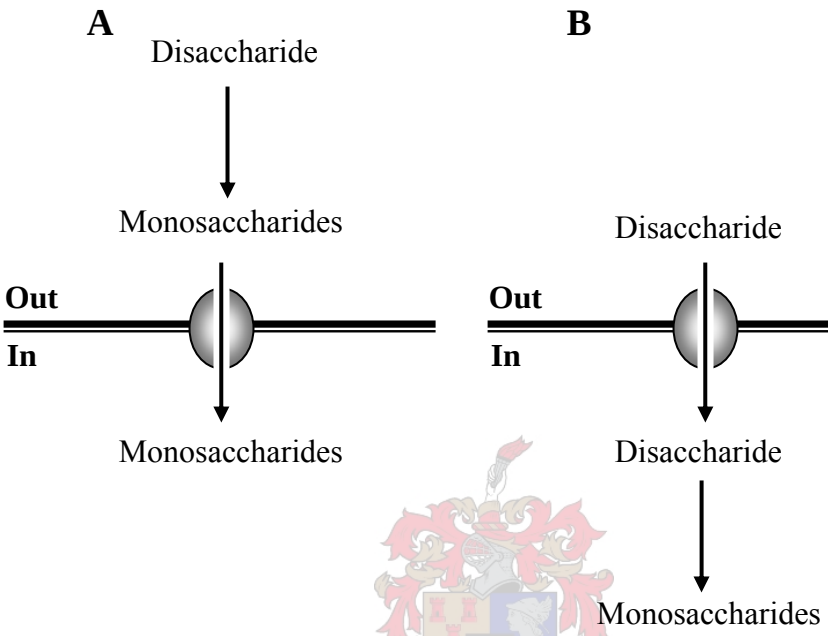

Figure 4.4. Different modes of disaccharide metabolism in yeasts. (A) Extracellular hydrolysis of disaccharides followed by transport of the monosaccharides is the most common method of sucrose metabolism in yeasts. (B) Transport of disaccharides by proton-sugar symport followed by intracellular hydrolysis occurs in lactose and maltose metabolism. (adapted from Weusthuis *et al*., 1994[a]).

The maltose utilisation system of *C. utilis* is affected by glucose through two different mechanisms: catabolite repression and inactivation. The *C. utilis* maltose permease is under the control of both, whereas the α-glucosidase is only repressed by glucose (Peinado *et al*., 1987). In glucose-maltose continuous cultures, both sugars were consumed simultaneously at glucose steady-state concentrations below 100 mg/l. At higher glucose concentrations, repression of the maltose permease increased steeply, being complete when the glucose concentration reached 170 mg/l.

In *C. wickerhamii*, glucose induced inactivation of its glucose transport system has been shown to be triggered by the interaction of the glucose molecule with a target on the cell surface (Spencer-Martins and Van Uden, 1985). This was not the case for the maltose transport system of *C. utilis*, where glucose induced the inactivation of maltose permease (in maltose growing and resting cells) by decreasing V_{max}, without changing the maltose affinity for its transport system. The inactivation process apparently required the entrance of the inactivator into the cell and its subsequent phosphorylation. This was supported by the specific inactivation rate that showed a dependence on glucose similar to that of glucose transport and the observation that only glucose analogues that were rapidly phosphorylated by hexokinases (i.e. 2-deoxyglucose), induced inactivation (Peinado *et al*., 1987).

Maltose uptake by *C. utilis* has been reported to involve an H^+-sugar symport system with a K_m of about 0.4 mM (Peinado *et al*., 1987). Since maltose is taken up as a disaccharide, maltose uptake is an uphill process and is, similar to glucose, PMF-dependent in *C. utilis*. Furthermore, the maltose permease of *C. utilis* is a reversible carrier that acts in an asymmetrical way, leading to slow maltose efflux compared with maltose influx (Van den Broek *et al*., 1997).

The first step in the catabolism of trehalose by *C. utilis* involves its entry into the cell by means of a trehalose membrane transporter. Inside the cell, trehalose is hydrolysed by neutral and/or acidic trehalases, both of which showed increased activities in the presence of external trahalose. The activities of the trehalose transporter, both trehalases as well as α-glucosidase and the maltose transporter were increased when maltose or trehalose, but not glucose, was used as the carbon source (Rolim *et al*., 2003). Thus it appears that maltose and trehalose, or a common metabolite of their catabolism, are able to regulate the activities of the specific enzymes and transporters which are needed for the initial steps of each others catabolism.

Cells of *C. utilis* can transport trehalose by an inducible trehalose transporter with a K_m of 8 mM for trehalose and a V_{max} of 1.8 μmol trehalose min^{-1} mg cell (dry weight)$^{-1}$. The only well-characterised yeast trehalose transporter is the *AGT1*-encoded trehalose transporter of *S. cerevisiae*, which is postulated to have evolved from a duplication of a maltose permease gene. The *AGT1* gene was shown to be transcriptionally repressed by Mig1, a transcriptional factor responsible for glucose repression of several genes (Nehlin and Ronne, 1990), and activated by MalR, the activator of the expression of the *MAL* genes (Hong and Marmur, 1987). This double

regulation causes *AGT1* to be positively regulated by maltose and repressed by glucose (Han *et al*., 1995; Stambuk *et al*., 1998). A similar pattern of activity for the trehalose transporter of *C. utilis* was found by Rolim *et al*. (2003). However, their results did not discriminate between a trehalose/maltose induction or a double regulation involving also glucose repression.

4.5.2 Sugar Transport in *S. pombe*

Unlike the multiple (low- and high-affinity) sugar transport systems described in many yeast species, fission yeast seems to have a much simpler mode of transport. Glucose uptake in *S. pombe* was described to be energy-dependent driven by the plasma membrane ATPase-generated electrochemical gradient (Höfer and Nassar, 1987). Kinetic analysis revealed a specific D-gluconate-H^+-symport activity (Hoever *et al*., 1992; Caspari, 1997) that is responsible for both aerobic and anaerobic glucose transport. It was established that *S. pombe* harbors a multimember family of functional hexose transporters. Of these, Ght5p is the prominently expressed one and represents the high-affinity D-glucose transporter of the *S. pombe* wild-type strain. Ght2p was characterised as a D-glucose transporter, with moderate affinity and transport capacities, while Ght3p encodes a specific D-gluconate transporter. Ght6p exhibits a slightly higher affinity for D-fructose than for D-glucose and is suggested to be the predominant transporter for D-fructose uptake.

A dendrogram of sequence similarities among hexose transporters from humans and different yeast species documents the high similarity between the *S. pombe* transporter proteins and the presence of three clusters of hexose transporters (Heiland *et al*., 2000) (Figure 4.5). Within the yeast monosaccharide transporters, the *S. pombe* proteins are clustered in a group that is distinct from the other groups comprising the *S. cerevisiae* and *Kluyveromyces lactis* transporters (GroupII) and the human glucose transporters (Group III), which are set separately from all the yeast genes. Within the *S. pombe* Ght transporter family, the Ght3p and Ght4p transporters, which are 88% identical to each other, build a subcluster versus Ght1p, Ght2p, Ght5p, and Ght6p. Ght3p and Ght4p are also unusual in that they encode two transporters with significant amino acid exchanges in transmembrane 10, which were suggested to contribute to substrate specificity. In both Ght3p and Ght4p, the most highly conserved phenylalanine residue in the characterized D-glucose transporters is replaced by a tyrosine residue in a way similar to the corresponding residue of GAL2p of *S. cerevisiae*. Furthermore, a tyrosine to tryptophan switch responsible for

discrimination between D-galactose and D-glucose in GAL2p (Kasahara *et al*., 1997), was also found in Ght3p and Ght4p of *S. pombe*. However, since *S. pombe* does not take up or utilise D-galactose, the alteration of these functional amino acids may indicate an alteration of Ght3p and Ght4p substrate specificity to D-gluconate.

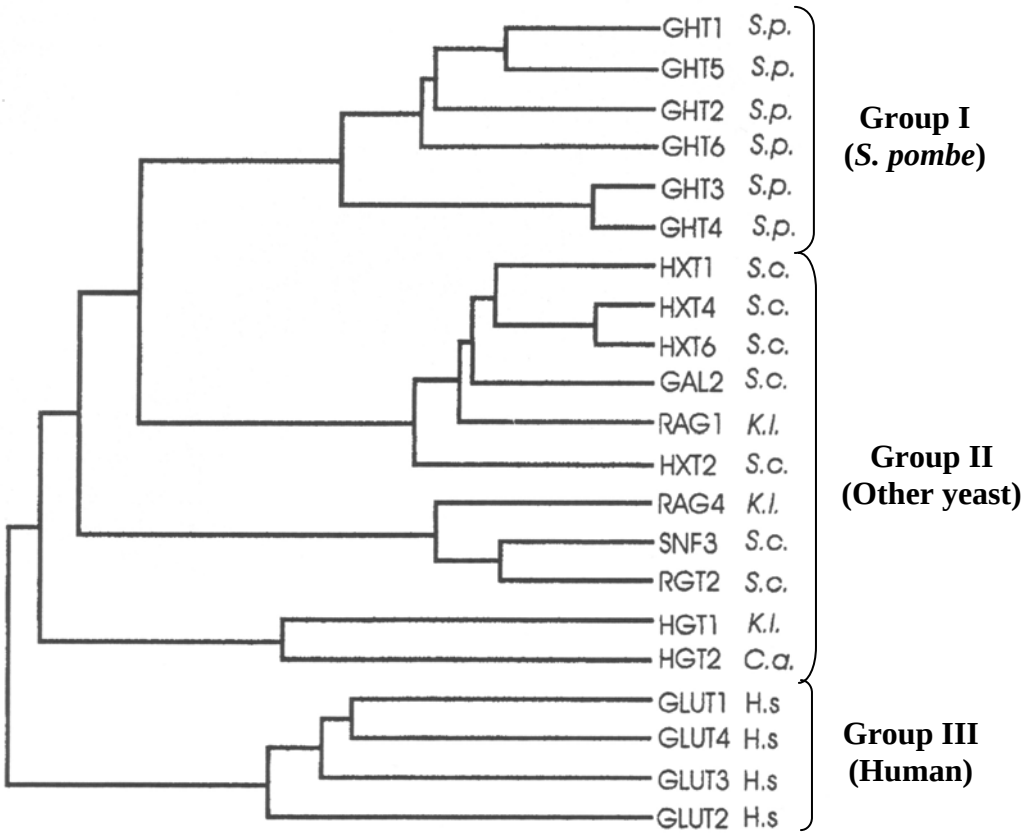

Figure 4.5. Dendrogram of sequence similarities among the human and the yeast hexose transport proteins. The dendrogram was derived from an alighment of some representative amino acid sequences of the hexose transporters of *S. pombe* (S.p.), *S. cerevisiae* (S.c.), *Kluyveromyces lactis* (K.l.), *Candida albicans* (C.a.) and *Homo sapiens* (H.s.) glucose transporters. The dentrogram classifies the relationships of the transport proteins based on their sequence similarities. The lengths of the horizontal branches are inversely proportional to the similarity of the sequences at each branch tip. The *S. pombe* hexose transporters are clustered as a distinct group (Group I) and are less related to the *S. cerevisiae* and *K. lactis* transporters, which comprise Group II. The human glucose transporters GLUT1 and GLUT4 are set separately from the yeast transporters in Group III (taken from Heiland *et al*., 2000).

In *S. pombe* and *C. utilis*, as well as in most prokaryotes, there is a separate transporter for maltose, which subsequently gets hydrolysed intracellularly (Cheng and Michels, 1989; Williamson *et al*., 1993; Liong and Ferrenci, 1994). However, other reports indicated that *S. pombe* might have an extracellular maltase, leading to the extracellular hydrolysis of maltose to glucose (Höfer and Nassar, 1987), resembling results with low glucose concentrations. It is possible that fission yeast has a common transporter for the monosaccharides and no separate transporter for disaccharides.

MALATE METABOLISM IN YEAST

5.1 INTRODUCTION

The study of L-malate metabolism in yeasts is of great biotechnological interest. It is an essential intermediate of cell metabolism, commonly used in a variety of foods and beverages (the D,L-racemate mixture) or in the treatment of liver disfunction and hyperammonemia (the L-isomer). In the wine industry, it may be present during grape must fermentation and/or in the wine, contributing to the "fixed acidity" that defines in an important way the quality of the final product. L-Malate is also used as an acidulant in the food industry and holds about 10% of this market. It is produced commercially by two processes, either by chemical synthesis via hydratation of maleate or fumarate to yield the racemic mixture, or by an enzymatic process using fumarase-containing microbial cells that yield the L-isomer (Chibata *et al*., 1983).

Several yeast species are recognised for their ability to metabolise extracellular L-malate and fall into either the K(-) or K(+) yeast groups, depending on their ability to utilise L-malate and other tricarboxylic acid (TCA) cycle intermediates as sole carbon or energy source (Barnett and Kornberg, 1960; Barnett *et al*., 1990; Rodriquez and Thornton, 1990). The K(+) group includes *Candida sphaerica* (Côrte-Real *et al*., 1989), *C. utilis* (Cássio and Leão, 1993), *Hansenula anomala* (Côrte-Real and Leão, 1990), *Pichia anomala* (Amador *et al*., 1996) and *Kluyveromyces marxianus* (Queiros *et al*., 1998), which have the ability to utilise TCA cycle intermediates as sole carbon sources. The K(-) group of yeasts comprises those yeasts capable of utilising TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources (Barnett and Kornberg, 1960). According to this definition, *Saccharomyces cerevisiae*, *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 malate. The yeast *S. cerevisiae* is regarded as an inefficient metaboliser of extracellular malate, which has been attributed to the lack of a mediated transport system for the acid (Salmon, 1987). Strains of *S. pombe* and *Z. bailii* can degrade high concentrations of L-malate, but only if glucose or another assimilable carbon source is present (Baranowski and Radler, 1984; Rodriquez and Thornton, 1989). In *S. pombe*, the transport of L-malate across the plasma membrane is carrier-mediated (Osothsilp and Subden, 1986[a]; Sousa *et al*., 1992) and the gene encoding the malate permease

(*mae1*) has been cloned by Grobler *et al*. (1995). The cloning of the *C. utilis* dicarboxylic acid transporter will be discussed in Chapter 7.

Genetic and biochemical characterisation of the L-malate utilising pathways in several K(-) and K(+) yeast species indicated that the physiological role and regulation of L-malate metabolism differs significantly between the K(-) and K(+) yeasts. In general, L-malate metabolism in K(-) yeasts is characterised by the absence of glucose repression or substrate induction (Osothsilp and Subden, 1986[a,b]; Rodriquez and Thornton, 1989). In contrast, the regulation of L-malate metabolism in K(+) yeasts typically exhibits strong glucose repression together with substrate induction (Côrte-Real and Leão, 1990; Cássio and Leão, 1993; Amador *et al*., 1996; Queiros *et al*., 1998).

Since very little is known about L-malate metabolism in *C. utilis*, the following discussions are focused on the intracellular degradation of L-malate via the malic enzyme and to a lesser extend, malate dehydrogenase, in the yeasts *S. cerevisiae* and *S. pombe*. This should provide more insight into the mechanisms and regulations involved in L-malate metabolism in general, with some direct correlations with *C. utilis*. In Chapter 8, the cloning of the C. *utilis* malic enzyme gene, *CuME*, as well as some preliminary data on its regulation will be described in more detail.

5.2 METABOLISM OF L-MALATE IN YEAST

L-Malate plays a pivotal role in the metabolism of C_3 and C_4 compounds in different subcellular compartments. Depending on the cellular requirements, malate can be oxidised, dehydrated or decarboxylated (Figure 5.1). The oxidation of malate provides oxaloacetate for the turnover of the TCA cycle or for gluconeogenesis via phosphoenolpyruvate (PEP), while the decarboxylation of malate provides pyruvate for amino acid and other biosynthetic pathways. Synthesis of L-malate via malate synthase in the glyoxalate cycle provides a mechanism for the combination of two C_2 molecules (glyoxalate and acetyl-CoA) to form a C_4 molecule (Fraenkel, 1982).

The ability of a yeast strain to degrade extracellular malate 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[a,b]). The K(-) yeast *S. cerevisiae* only utilises L-malate in the presence of one or more fermentable carbon sources. However, L-malate degradation in *S. cerevisiae* is weak compared to *S. pombe*, which seems to be evolutionarily

optimised for L-malate degradation. The weak degradation of L-malate in *S. cerevisiae* can be linked to the fact that *S. cerevisiae* can import malate and other dicarboxylic acids only via simple diffusion (Salmon, 1987).

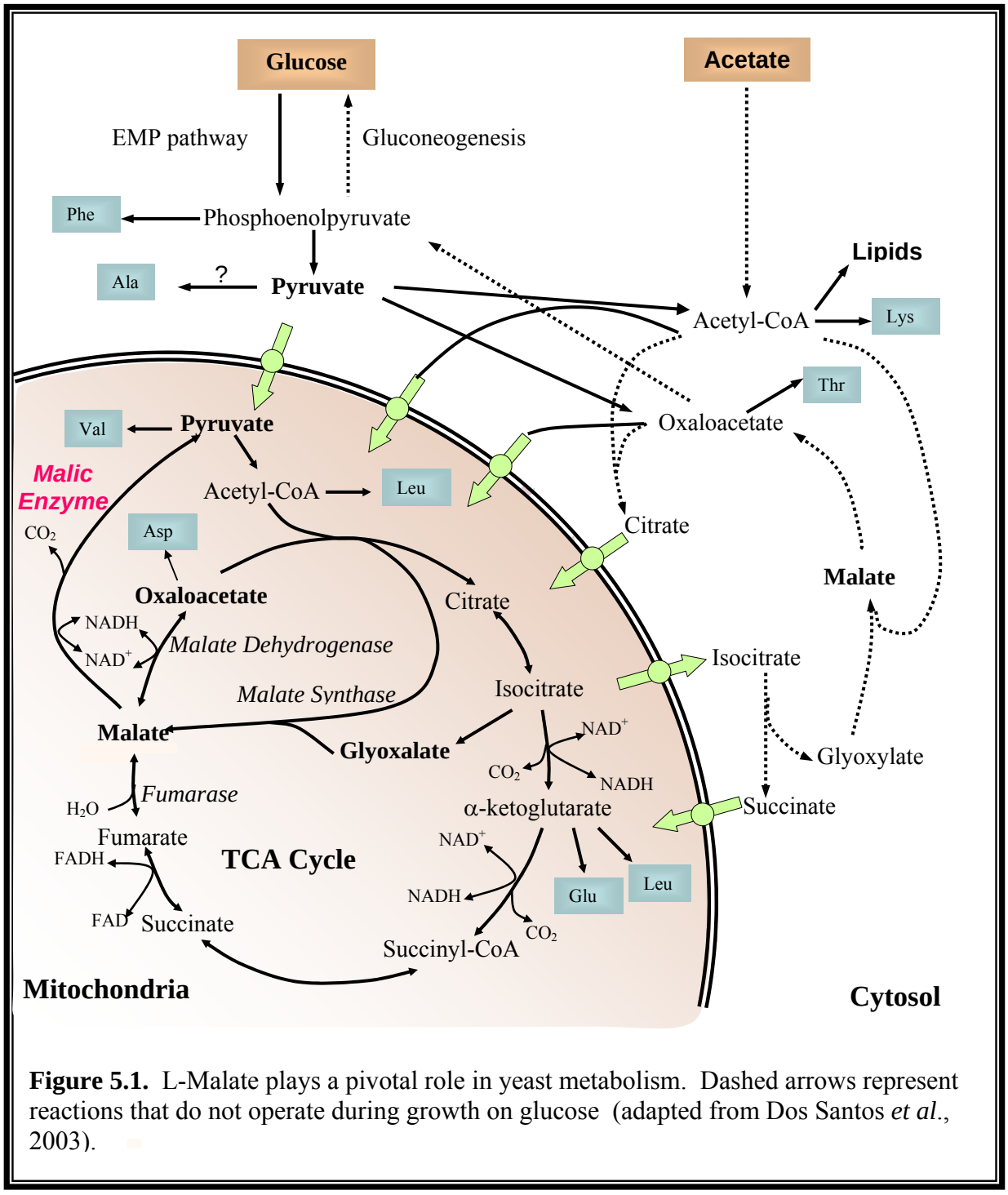

Figure 5.1. L-Malate plays a pivotal role in yeast metabolism. Dashed arrows represent reactions that do not operate during growth on glucose (adapted from Dos Santos *et al.*, 2003).

The yeast *S. pombe* can effectively degrade extracellular L-malate due to an active transport system for malate uptake and a cytosolic NAD-dependent malic enzyme (EC 1.1.1.38) with a high substrate affinity (Maconi *et al*., 1984). Cells of *S. pombe* display an extreme tolerance for high L-malate concentrations, as levels of up to 29 g L-malate/l can be degraded without any negative effect on cell growth, sugar metabolism or ethanol-producing abilities (Temperli *et al*., 1965). Taillandier and Strehaiano (1991) showed that malate is not integrated into biomass after its catabolism by *S. pombe*. It is completely metabolised to ethanol and CO_2 during anaerobiosis, and to CO_2 under aerobiosis (Mayer and Temperli, 1963). As a K(-) yeast, *S. pombe* degrades malate only in the presence of glucose or another assimilable carbon source under both aerobic and anaerobic conditions (Magyar and Panyik, 1989; De Queiros and Pareilleux, 1990), suggesting that the metabolism of malate requires energy, supposedly for transport of malate into the cell (Taillandier and Strehaiano, 1991).

Three enzymes are involved in malate degradation in *S. pombe*, namely the malate transporter, malic enzyme, and a mitochondrial malate dehydrogenase (EC 1.1.1.37) (Osothsilp and Subden, 1986[a]). The transporter encoded by the *mae1* gene (Grobler *et al*., 1995), uses an H^+-symport system for the active transport of L-malate, and the NAD-dependent malic enzyme catalyses the oxidative decarboxylation of L-malate to pyruvate and CO_2. The mitochondrial malate dehydrogenase oxidises L-malate to oxaloacetate in the TCA cycle and is responsible for 10% of the degradation of malate under aerobic conditions, with the remaining L-malate being directly converted to pyruvate and CO_2 via the malic enzyme (Osothsilp and Subden, 1986[a]; Osothsilp, 1987; Subden *et al*., 1998). Therefore, both the malic enzyme and malate dehydrogenase are required for malate utilisation during aerobiosis. 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-malate (Osothsilp, 1987).

5.2.1 Cytosolic vs Mitochondrial Degradation

Malic enzyme. The oxidative decarboxylation of L-malate to pyruvate and CO_2 is catalysed by a mitochondrial or cytosolic malic enzyme that requires divalent cations (Mg^{2+} or Mn^{2+}) and $NAD(P)^+$ as cofactors (Frenkel, 1975). The pyruvate produced in this reaction can be used for amino acid synthesis or further metabolised to the various intermediates shown in Figure 5.1. In *S. pombe*, the pyruvate is immediately decarboxylated to ethanol and CO_2 under anaerobic conditions (Mayer and Temperli, 1963), but the pyruvate can serve to

replenish the TCA cycle under aerobic conditions. The malic enzyme reaction is reversible and carboxylation of pyruvate to malate can provide the necessary precursors for protein biosynthesis for yeast grown on acetate (Fraenkel, 1982). Three types of malic enzymes are found in nature and are classified by their coenzyme specificity and ability to decarboxylate both malate and oxaloacetate. The structure, catalytic mechanism and physiological role of the three isozymes differ remarkably and will be discussed in a later section.

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

5.2.2 L-Malate as Intermediate of the TCA Cycle in Mitochondria

The TCA cycle functions primarily in mitochondria where it allows for the complete degradation of pyruvate produced during glycolysis (Figure 5.1). The TCA cycle is only functional under aerobic conditions and is required for oxidative growth on pyruvate, lactate, acetate and ethanol (Boulton *et al*., 1996). The cycle allows for the metabolic flow of carbon between various metabolic pathways and is a major source of NADH for the production of ATP via oxidative phosphorylation. Some of the enzymes of the TCA cycle are also present in the cytosol and peroxisome where they catalyse similar reactions. Under anaerobic conditions and in the presence of high concentrations of glucose, cells of *S. cerevisiae* do not have functional mitochondria (Fraenkel, 1982), but cytosolic enzymes similar to those in the TCA cycle produce the necessary biosynthetic intermediates.

The enzymatic reactions of the TCA cycle include the hydration of fumarate to L-malate via fumarase and the oxidation of L-malate to oxaloacetate via malate dehydrogenase (Figure 5.1). Both fumarase and malate dehydrogenase catalyse reversible reactions that are regulated by the substrate concentrations and the requirement for either the reductive or oxidative arm of the TCA cycle (Boulton *et al*., 1996). NADH is produced by various enzymes of the TCA cycle during the oxidative arm of the TCA cycle. If the regeneration of NAD^+ from NADH via the conversion of acetaldehyde to ethanol under fermentable conditions is restricted, the

reductive arm of the TCA cycle can be used to regenerate NAD^+ with the production of L-malate and succinate.

5.2.3 Malo-ethanolic Fermentation

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

Fundamental knowledge about the malo-ethanolic pathways from both K(-) and K(+) yeasts is imperative in our understanding of the regulation and physiological role of malate metabolism in yeast and can contribute to the innovative applications of recombinant strains of *S. cerevisiae*. For example, a strong malo-ethanolic phenotype was introduced into a *S. cerevisiae* laboratory strain when the *S. pombe mae1* and *mae2* genes, encoding the malate transporter and malic enzyme respectively, were functionally co-expressed (Volschenk *et al*., 2001). Thereafter, the *S. pombe mae1* and *mae2* genes were integrated into *URA3* locus of an industrial *Saccharomyces bayanus* EC1118 strain. Integration and expression of the malo-ethanolic genes in *S. bayanus* EC1118 had no inverse effect on the fermentation ability of the yeast, while sensory evaluation and chemical analysis of the Chardonnay wines indicated an improvement in wine flavour perception compared to the control wines, without the production of any off-flavours.

5.2.4 Malate Synthesis

Mitochondrial synthesis. L-Malate occurs in mitochondria as an intermediate of the tricarboxylic acid (TCA) cycle. The reversible hydration of fumarate to L-malate (Figure 5.2) is catalysed by the enzyme fumarase (or fumarate hydratase), encoded by *FUM1* in *S. cerevisiae* (Zubay *et al*., 1993). Like most TCA cycle enzymes, fumarase is located in the matrix compartment of mitochondria. The L-malate is directly oxidised to oxaloacetate by malate dehydrogenase (*MDH1*), with NAD^+ serving as the electron acceptor. This reaction is drawn in the direction of malate oxidation by the continued consumption of oxaloacetate in the TCA cycle resulting in no accumulation of L-malate in the mitochondria.

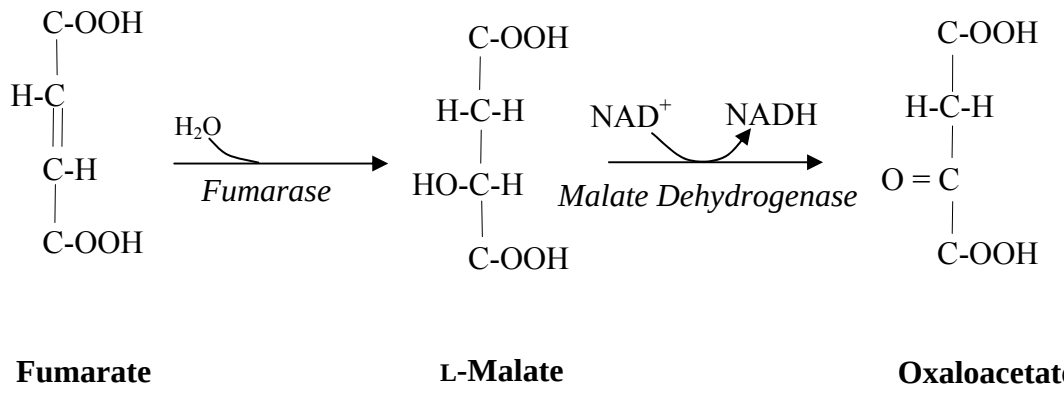

Figure 5.2. Fumarase catalyses the hydration of fumarate to L-malate, followed by oxidation to oxaloacetate.

Cytosolic synthesis. The yeast *S. cerevisiae* contains a cytosolic pathway that involves the enzymes pyruvate carboxylase, malate dehydrogenase (MDH2) and fumarase, and enables the conversion of pyruvate through oxaloacetate to L-malate by the incorporation of CO_2 (Pines *et al*., 1996). This accumulation of L-malate is suggested not to involve the mitochondrial metabolism via the tricarboxylic acid (TCA) cycle (Schwartz and Radler, 1988), but is rather dependent on the cytosolic localisation of pyruvate carboxylase. In this regard, the cytosolic location of pyruvate carboxylase and the mechanism for malate accumulation in *S. cerevisiae* may be similar to that in organic acid producers, such as the fungi *Aspergillus flavus* and *Rhizopus oryzae*, and in contrast to its mitochondrial location in higher eukaryotes (Haarasilta and Taskinen, 1977; Osmani and Scrutton, 1983, 1985; Van Urk *et al*., 1989[c]; Bercovitz *et al*., 1990). However, overexpression of fumarase results in an increase in the levels of malate dehydrogenase (Pines *et al*., 1996), which catalyses conversion of oxaloacetate to L-malate, resulting in L-malate accumulation.

Malate synthesis in peroxisomes. The glyoxalate cycle in yeast peroxisomes is primarily associated with the complete degradation of fatty acids via β-oxidation (Figure 5.3). However, it also plays an important role in the synthesis of C_4 compounds from C_2 carbon substrates by employing some of the TCA cycle enzymes (Fraenkel, 1982). In *S. cerevisiae*, peroxisomal malate synthesis from glyoxalate is catalysed by malate synthase (MLS) as part of the glyoxalate pathway (Van Roermund *et al*., 1995). This reaction combines the two C_2 molecules glyoxalate and acetyl-CoA in the C_4 molecule L-malate. This allows for the synthesis of TCA cycle intermediates in the peroxisome when the mitochondria are non-functional under anaerobic or high glucose conditions (Boulton *et al*., 1996).

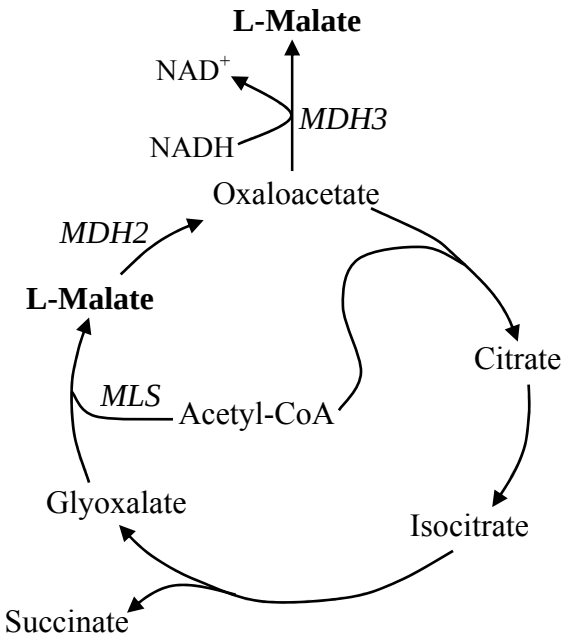

Figure 5.3. Malate synthesis can take place in the peroxisome via malate dehydrogenase (MDH3) and malate synthase (MLS) as part of the glyoxylate cycle (adapted from Solà *et al*., 2004).

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

5.2.5 Comparison of L-Malate Degradation in *S. cerevisiae* and *S. pombe*

Although *S. cerevisiae* and *S. pombe* are both classified as K(-) yeasts, L-malate degradation is significantly weaker in *S. cerevisiae* for the following reasons: (1) *S. cerevisiae* lacks an active transport system for L-malate and extracellular L-malate enters the cells by means of simple diffusion (Figure 5.4); (2) The malic enzyme of *S. cerevisiae* has a significantly lower substrate affinity for L-malate (K_m = 50 mM) than that of *S. pombe* (K_m = 3.2) (Temperli *et*

al., 1965; Fuck *et al*., 1973); and (3) The *S. cerevisiae* malic enzyme is localised in the mitochondria, whereas the *S. pombe* malic enzyme is localised in the cytosol. The mitochondrial location of the *S. cerevisiae* malic enzyme suggests that this enzyme is essentially subjected to the regulatory effect of fermentative glucose metabolism, such as mitochondrial deterioration (Volschenk *et al*., 2003), which is a well-documented phenomenon in Crabtree-positive yeast.

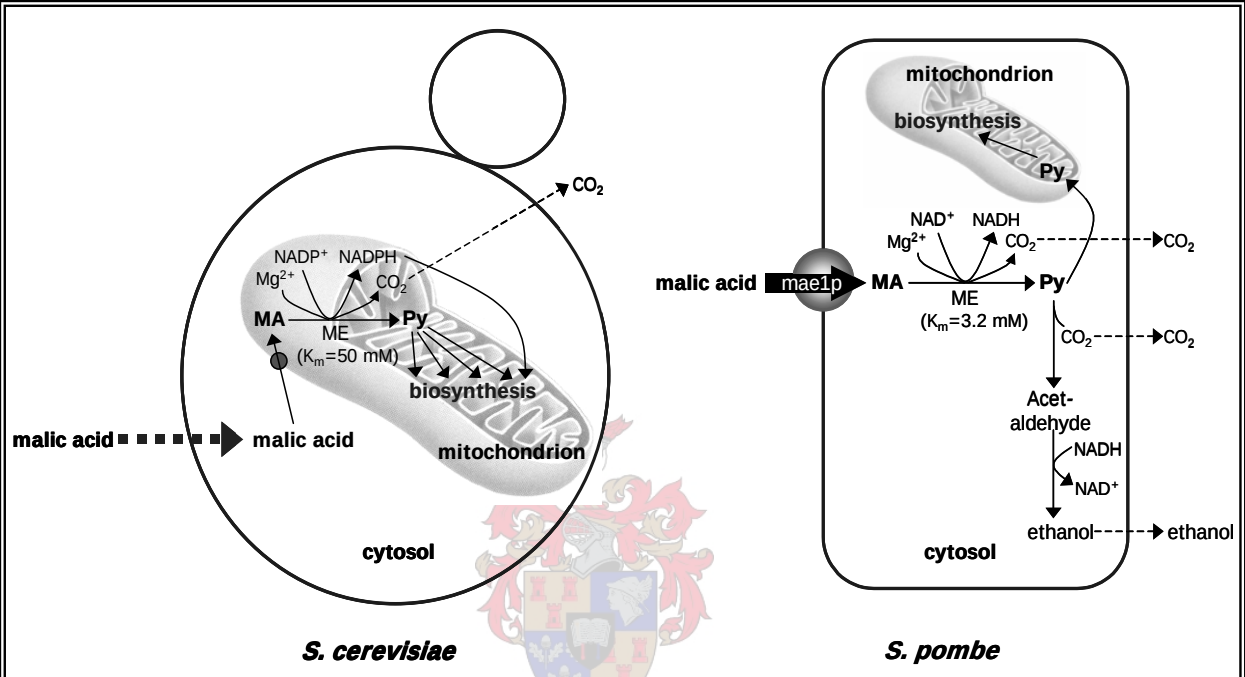

Figure 5.4. The main differences in L-malic acid degradation between *S. cerevisiae* and *S. pombe* involves the transport of malic acid, the substrate affinity of the malic enzyme and the compartmentalisation of the malic enzymes in these two yeast species. MA = malate; Py = pyruvate (taken from Volschenk *et al*., 2003).

Although the contrasting L-malate degradation abilities of *S. cerevisiae* and *S. pombe* suggests that L-malate metabolism should play distinct physiological roles in these two yeast species, biochemical and genetic evaluation of the enzymes and genes involved in this pathway concluded that the malic enzymes from these two yeasts play an almost similar role in the provision of pyruvate for cellular biosynthesis (Volschenk *et al*., 2003). The possibility for the existence of a NADH – NADPH recycling function for the *S. cerevisiae* mitochondrial malic enzyme cannot be ruled out, but additional evidence is required. Furthermore, since the NADP-malic enzyme is implicated in lipogenesis in higher eukaryotes, it is arguable that the *S. cerevisiae* NADP-malic enzyme gene might also be directly involved in the increased synthesis of certain phospholipids, especially in the yeast cell's protective response against

hyper-osmotic stress. Since it has been postulated that the conversion of L-malate to pyruvate to ethanol is a redox-neutral process, the importance of the strong cytosolic malic enzyme of *S. pombe* on maintaining the redox balance and energy production in this yeast remains the topic of further research.

From the above discussions, it is clear that malic enzyme play an important role in L-malate degradation. The nature, regulation and possible functions of malic enzyme will be discussed in more detail in the following sections.

5.3 MALIC ENZYMES

Kornberg (2001) vividly described the way leading to the discovery and characterisation of the pigeon liver malic enzyme by Ochoa *et al*. in 1947. The malic enzyme was later found to be widely distributed in nature, including prokaryotes, fungi, plants and animals. In eukaryotes, the malic enzyme may reside in the cytosol, mitochondria or in plant chloroplasts (Viljoen *et al*., 1994; Schomburg and Stephan, 1995). The presence of a malic enzyme has been demonstrated in the yeasts *S. pombe* (Temperli *et al*., 1965; Viljoen *et al*., 1994), *Rhodotorula glutinis* (Fernández *et al*., 1967), *Z. bailii* (Kuczynski and Radler, 1982) and *S. cerevisiae* (Boles *et al*., 1998).

Malic enzyme catalyses a reversible oxidative decarboxylation of L-malate to yield pyruvate and CO_2, with the concomitant reduction of $NAD(P)^+$ to NAD(P)H (Figure 5.5). In addition to the $NAD(P)^+$ dinucleotide, malic enzymes require divalent cations (Mn^{2+} or Mg^{2+}) as cofactors. The three isozymes, EC 1.1.1.38-40 in general exhibit a high degree of amino acid homology (Viljoen *et al*., 1994; Xu *et al*., 1999; Yang *et al*., 2000), but differ in their intracellular localisation (cytosolic, mitochondrial or hydrogenosomal), substrate affinity and specificity (L-malate and/or oxaloacetate), co-factor specificity [either NAD^+ (EC 1.1.1.38, EC 1.1.1.39) or $NADP^+$ (EC 1.1.1.39, EC 1.1.1.40)] and the degree to which the decarboxylation reaction is reversible (Voegele *et al*., 1999). L-Malate: NAD^+ oxidoreductase (oxaloacetate decarboxylating; EC 1.1.1.38) uses NAD^+ as co-enzyme and has been found in *S. pombe* (Temperli *et al*., 1965). L-Malate: NAD^+ oxidoreductase (decarboxylating; EC 1.1.1.39) is the malic enzyme most commonly found in the mitochondria of plants and animals (Artus and Edward, 1985). This enzyme can use $NADP^+$ as coenzyme in some cases, but prefers NAD^+. L-Malate $NADP^+$-oxidoreductase (oxaloacetate-decarboxylating; EC 1.1.1.40) is $NADP^+$-dependent and although most $NADP^+$-dependent malic enzymes can also

use NAD^+, $NADP^+$ is preferred and acts as a non-competitive inhibitor of NAD^+ activity (Hatch and Mau, 1977).

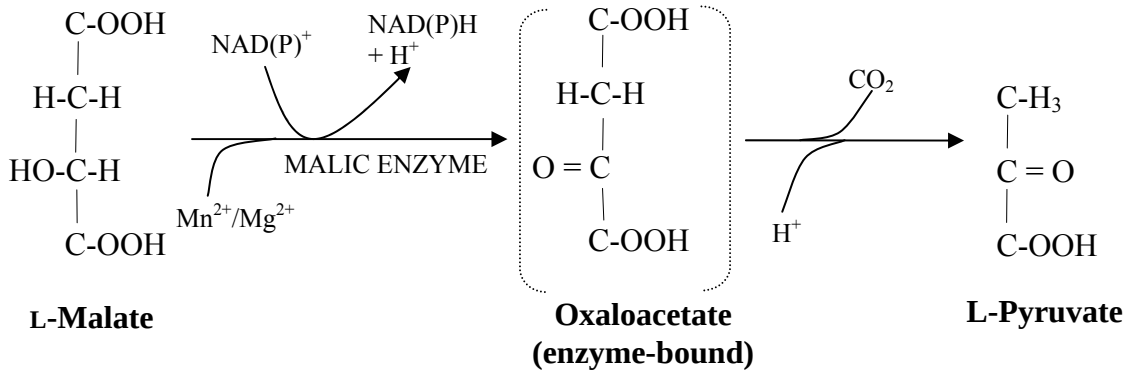

Figure 5.5. The NAD(P)-dependent malic enzyme catalyses the oxidation of L-malic acid to oxaloacetic acid, followed by decarboxylation to pyruvate.

The three malic enzyme isozymes differ with regard to their molecular structure, catalytic properties, and physiological role. In general, the efficacy of a malic enzyme is determined by its substrate affinity and/or the level of expression of the corresponding gene. The *S. pombe* malic enzyme has a very high substrate affinity (K_m = 3.2 mM), whereas the *S. cerevisiae* malic enzyme has a very low substrate affinity (K_m = 50 mM) that contributes to the inefficient degradation of malate by *S. cerevisiae* (Temperli *et al.*, 1965; Osothsilp, 1987). As previously mentioned, the mitochondrial location of the *S. cerevisiae* malic enzyme may contribute to the weak degradation of malate by strains of *S. cerevisiae* under fermentative conditions.

Whereas the *S. pombe* malic enzyme can use only NAD^+ as cofactor (Temperli *et al.*, 1965), the malic enzyme from *S. cerevisiae* has been reported to use both NAD^+ and $NADP^+$ as electron acceptor with NAD^+ being favoured (Kuczynski and Radler, 1982). Both the *S. cerevisiae* and *S. pombe* enzymes are bifunctional (can react with both malate and oxaloacetate), whereas the *Z. bailii* enzyme can only decarboxylate malate, suggesting that a different enzyme does the decarboxylation of oxaloacetate.

The *S. pombe* malic enzyme requires the divalent cations Mn^{2+} or Mg^{2+} for activity, in contrast to the *S. cerevisiae* malic enzyme that prefers Mn^{2+} as a divalent cation (Osothsilp and Subden, 1986[a]; Osothsilp, 1987). The metal ion serves as a bridge between L-malate to properly position the substrate at the active site center and to help polarize the C-2 hydroxyl

group during the initial stage (Chou *et al*., 1995). The metal ion acts as a Lewis acid in the subsequent decarboxylation of oxaloacetate (Figure 5.5) and plays a vital role in chelating the negatively charged enolate-pyruvate intermediate (Chang *et al*., 2002). The *S. pombe* malic enzyme has a pH optimum of 3.5 – 4.0 (Osothsilp and Subden, 1986[a]), which ensures that the substrate is protonated.

The genes encoding the *S. pombe* and *S. cerevisiae* malic enzymes have been cloned and analysed on a molecular level. The *S. pombe* malic enzyme gene, *mae2*, was cloned and characterised as an open reading frame of 1 695 bp (Viljoen *et al*., 1994). It was mapped 29 map units from the *ade6* gene on the right arm of chromosome III near the centromere (Osothsilp, 1987). 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 (Viljoen *et al*., 1994). The *MAE1* gene, identified as the structural gene for the *S. cerevisiae* malic enzyme, encodes a putative protein of 669 amino acids with 47% homology to the *S. pombe* malic enzyme (Boles *et al*., 1998).

5.3.1 Physiological Role of Malic Enzymes

Based on the divergent regulation of malic enzymes in different organisms, tissues and cellular compartments and the evolutionary preservation of malic enzymes throughout a wide spectrum of organisms in nature, it is believed that malic enzymes are responsible for various essential physiological functions in living organisms (Driscol and Finan, 1996; Song *et al*., 2001). The end products of the malic enzyme reaction, i.e. pyruvate, CO_2 and NAD(P)H, feed into numerous biological pathways that can be broadly defined as (1) pathways where NAD^+-dependent malic enzymes are involved in oxidative metabolic processes that yield ATP via the electron transport system, or (2) pathways where the $NADP^+$-dependent enzymes play a role in reductive biosynthesis processes. In line with this broad metabolic view, the NAD^+-dependent malic enzyme isoforms usually play an important role in cellular ATP biosynthesis via the production of NADH and pyruvate. For example, the human NAD^+-dependent malic enzyme is pivotal in energy production via glutamine in rapidly growing tissues, such as the spleen, thymus, mucosal cells of small intestine and tumour cells (Sauer *et al*., 1980; McKeehan, 1982; Bagetto, 1992). In contrast, the reverse reaction of the $NADP^+$-dependent malic enzyme (which involves the carboxylation of pyruvate to malate) plays an important housekeeping role in the anapleurotic reactions of the TCA cycle in both prokaryotes and eukaryotes (Wedding, 1989; Sauer *et al*., 1999). Under fermentative

conditions, the pyruvate generated by the cytosolic NAD-dependent malic enzyme is further metabolised to ethanol, probably to ensure that the redox balance is being maintained.

Lipid biosynthesis. NADP-dependent malic enzyme isoforms found in bacteria, yeast, fungi, birds and mammals play a role in primarily biosynthetic reactions, especially lipid biosynthesis and desaturation through the provision of NADPH (Goodridge and Ball, 1966, 1967; Tanaka *et al*., 1983; Xu *et al*., 1999; Gourdon *et al*., 2000). When ATP supplies are abundant, acetyl-coenzyme A can be converted into fatty acids as an energy reserve. However, mitochondrial acetyl-coenzyme A must be converted to citrate via the tricarboxylate transport system (Figure 5.6) to participate in fatty acid synthesis in the cytosol. Citrate synthase fuses acetyl-coenzyme A with oxaloacetate to produce citrate that is transported from the mitochondria to the cytosol. Once in the cytosol, citrate is converted back to oxaloacetate via the energy-dependent citrate lyase. The oxaloacetate is then reduced to L-malate via malate dehydrogenase; L-malate can be oxidised to pyruvate via the malic enzyme, with the production of NADPH that can feed into the fatty acid biosynthesis pathway. Pyruvate can also be re-imported back into the mitochondria to participate in the TCA cycle yet again. Similarly, L-malate can be transported back into the mitochondria and used to produce NADH via the mitochondrial malate dehydrogenase.

The role of NADP-malic enzyme in lipid biosynthesis in filamentous fungi was studied in depth and strong evidence was obtained that malic enzyme activity is a key factor in ensuring maximal lipid accumulation (Wyn and Ratledge, 1997, 2000). The direct relation between malic enzyme activity and lipid accumulation was until recently still speculative, since maximum lipid accumulation in fungi was not necessarily linked to maximum NADP-malic enzyme activity (Wyn *et al*., 1999; Song *et al*., 2001). However, the identification of multiple isoforms of NADP-malic enzyme in some fungi and the evolution of specific isoforms under specific growth conditions of high lipogenesis, clarified the critical role of NADP-malic enzymes in lipid biosynthesis (Zink, 1972; Savitha *et al*., 1997; Song *et al*., 2001). The current accepted hypothesis suggests that several isoforms of NADP-malic enzyme exist in fungi through the action of post-translational modifications (either partial proteolytic cleavage, phosphorylation or dephosphorylation) and that specific isoforms of the NADP-malic enzyme are directly associated with lipid accumulation, whilst others have other cellular functions (Song *et al*., 2001)

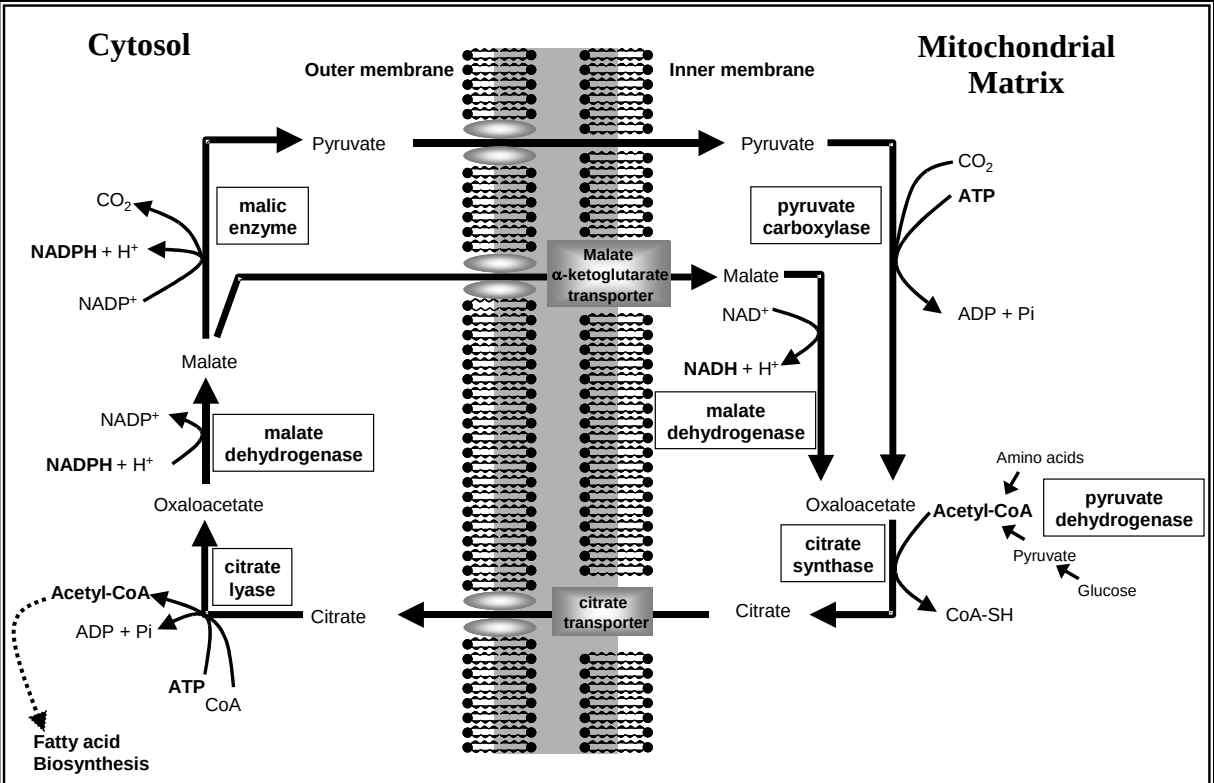

Figure 5.6. The role of the malic enzyme in lipid biosynthesis and desaturation through the provision of cytosolic NADPH in mice and humans. The tricarboxylic transport system is responsible for the export of acetyl-coenzyme A from the mitochondrial matrix into the cytosol where the fatty acid biosynthetic pathway is situated (taken from Volschenk *et al*., 2003)

Generation of NAD(P)H. In *S. cerevisiae*, NADPH is generated only in a few reactions: (1)via the two dehydrogenases of the pentose-phosphate (PP) pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), (2) via the $NADP^+$-dependent isocitrate dehydrogenase, (3) via the $NADP^+$-dependent acetaldehyde dehydrogenase and (4) via the malic enzyme. The *S. cerevisiae* mitochondrial NAD(P)-dependent malic enzyme, ScMae1p, is located in the center of the metabolic network of *S. cerevisiae*, converting malate (an intermediate of the TCA cycle), into pyruvate (a key metabolite for yeast in the split between respiration and fermentation), with the production of one NADPH molecule (Boles *et al*., 1998). In contrast, the NAD^+-dependent malic enzyme from *S. pombe* seems to play a role in the provision of cytosolic NADH under fermentative conditions (Groenewald and Viljoen-Bloom, 2001). The high substrate affinity and cytosolic location of the *S. pombe* malic enzyme enables the yeast to effectively degrade malate to ethanol during alcoholic fermentation (Sousa *et al*., 1995; Taillandier *et al*., 1995).

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

According to this model (Figure 5.7), malic enzyme, pyruvate carboxylase, NAD^+-dependent malate dehydrogenase (MDH2) and the mitochondrial decarboxylic carrier (DIC1) act as a cyclic transhydrogenase shuttle to convert the NADH resulting from biosynthetic metabolism (Van Dijken and Scheffers, 1986), to NADPH in order to sustain the yeast cell's biosynthetic requirements (Bakker *et al*., 2001).

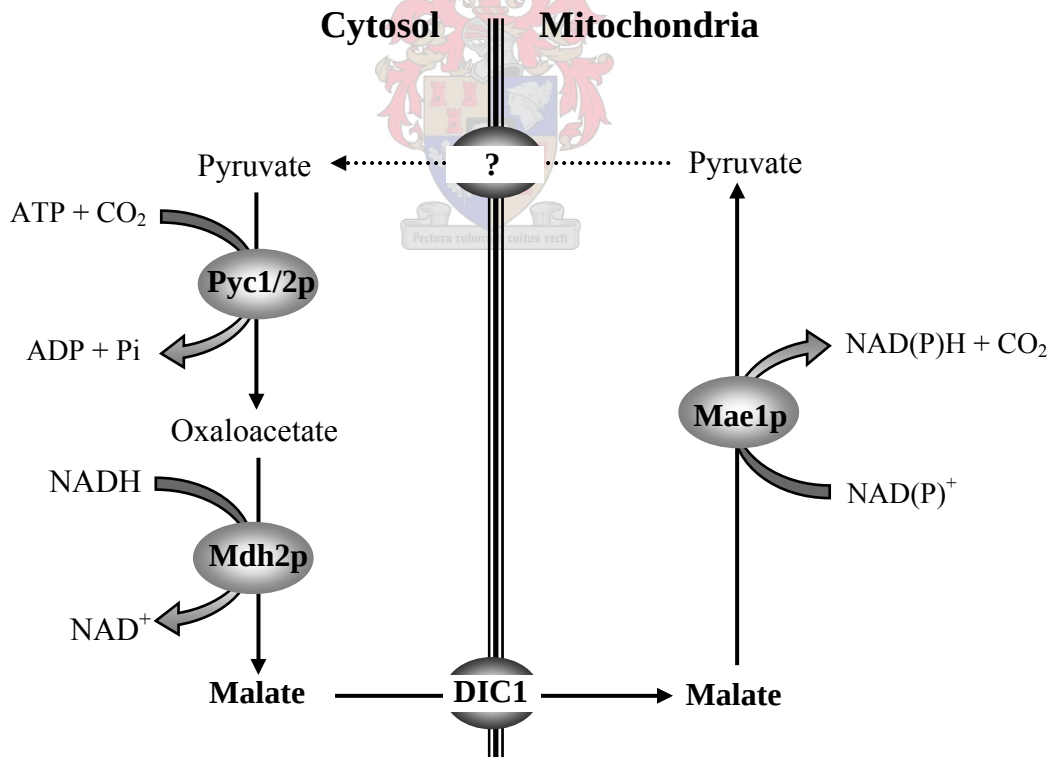

Figure 5.7. A hypothetical malate-pyruvate shuttle as it might work in *S. cerevisiae*, transferring electrons from cytosolic NADH to mitochondrial NAD(P)H. All enzyme activities, except the mitochondrial pyruvate transporter (indicated by the question mark) were identified in *S. cerevisiae* (adapted from Bakker *et al*., 2001).

One of the major shortcomings of this model is that the actual existence of a mitochondrial pyruvate transporter has not yet been established in *S. cerevisiae*. Furthermore, the natural direction of pyruvate flux during respiration is from the cytosol, where glycolysis takes place, into the mitochondria. If a malate-pyruvate shuttle is indeed active in *S. cerevisiae*, it can therefore not work as a full shuttle. It may rather provide an alternative means of pyruvate transport into the mitochondria, with concomitant transport of NAD(P)H into the mitochondria for biosynthetic purposes. In this shunt, NADPH is produced at the expense of one ATP consumed by pyruvate carboxylase and one NADH consumed by malate dehydrogenase: in other words, the operation of the shunt does not have a net effect on the carbon metabolism.

Provision of pyruvate. The induced expression of the cytosolic malic enzyme gene under fermentative conditions when the mitochondria are not fully operational, may also provide pyruvate and NADH for essential anapleurotic reactions (Viljoen *et al*., 1999). Pyruvate plays an important role in the provision of α-ketoglutarate and oxaloacetate for the synthesis of amino acids and nucleotides. Both these precursors are synthesized in the mitochondria and transported to the cytosol for biosynthetic reactions. Alternative pathways must therefore be utilized for the synthesis of these precursors when the mitochondria are not functional. These anapleurotic reactions comprise the carboxylation of pyruvate to oxaloacetate via pyruvate carboxylase, the oxidation of L-malate to pyruvate via the malic enzyme, and the production of succinate via the glyoxylate cycle. Although earlier biochemical studies indicated that the metabolism of L-malate 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 pyruvate for other metabolic requirements (Groenewald and Viljoen-Bloom, 2001).

5.4 MALIC ENZYME STRUCTURE AND CATALYTIC MECHANISM

As mentioned previously, malic enzymes have been isolated from a variety of organisms, including plants, animals, fungi, bacteria and yeast. The malic enzymes isolated and purified thus far show a subunit composition of two to ten identical subunits. The human mitochondrial NAD-malic enzyme (Loeber *et al*., 1991), and the malic enzyme from pigeon liver (Hsu, 1982) are homotetramers, whereas the malic enzyme from *S. pombe* is a homodimer (Osothsilp, 1987). All of these malic enzymes have similar overall tertiary structure albeit with small local differences, that has important structural implications on the

catalytic and regulatory mechanisms. The polypeptide backbone of malic enzymes has a different topology from that of the other oxidative decarboxylases (Xu *et al*., 1999; Yang and Tong, 2000; Coleman *et al*., 2002; Yang *et al*., 2002), establishing malic enzymes as a unique class of oxidative decarboxylases. This section summarises the structural features and the functional implications of this class of oxidative decarboxylases, using the human and pigeon liver malic enzyme as model.

5.4.1 Comparison of Malic Enzyme from Various Sources

Molecular analysis of the *S. pombe mae2* gene and its deduced amino acid sequence revealed the presence of eight highly conserved regions, regions A-H, in malic enzymes from various prokaryotic and eukaryotic organisms (Viljoen *et al*., 1994). The malic enzymes of *Escherichia coli*, *S. pombe* and *S. cerevisiae* showed a closer phylogenetic link with the malolactic enzymes of lactic acid bacteria than with malic enzymes from other organisms (Groisillier and Lonvaud-Funel, 1999). These regions represent clusters of highly conserved residues separated by spacer regions with less homology, but conserved in length. The highly conserved amino acid sequence of malic enzymes indicated a conserved structure and/or catalytic mechanism in spite of differences in their substrate and co-enzyme specificities. Four of the conserved regions identified in malic enzymes, regions A, B, D and E (Loeber *et al*., 1991), were reported to be binding sites for $NAD(P)^+$, L-malate or divalent cations (Rothermel and Nelson, 1989; Börsch and Westhoff, 1990; Hsu *et al*., 1992; Kulkarni *et al*., 1993). Although the physiological role of the other conserved regions is yet unknown, their importance should not be disregarded. For example, a single point mutation in the *S. pombe* malic enzyme gene at nucleotide 1331 (G-to-A) changed amino acid 444 from a glycine to an aspartate residue in the conserved region H and completely abolished the malic enzyme activity (Viljoen *et al*., 1998).

A number of essential amino acid residues in malic enzymes have been identified. The conserved Asp-258 residue shown to be involved in the binding of Mn^{2+} (Wei *et al*., 1995) is contained within the conserved Box C. Arginine residue(s) located at or near the active site of the enzyme may provide a positively charged group that facilitates the binding of the malate anion (Rao *et al*., 1991[b]). This was confirmed by Vernon and Hsu (1983) who showed the involvement of an arginyl residue in the binding of the C_1-carboxyl group of malate. Glycine residues were reported to serve an important function in the folding of the malic enzyme: the conserved glycine residues in regions B and E (G-G- -G) allow the specific folding of the βαβ dinucleotide binding fold for the binding of the coenzyme $NAD(P)^+$ (Wierenga *et al*., 1986).

The active site of malic enzyme constitutes a major conserved region, while some of the subunit contacting regions are also conserved (Chang and Tong, 2003). The enzyme needs an essential divalent metal ion (Mn^{2+} or Mg^{2+}), which plays functional roles in catalysis as well as in structural stability. The amino acid sequences around the metal-binding site are highly conserved, with the direct metal ligands, Glu255, Asp256 and Asp279 (amino acid residues numbered according to human mitochondrial NAD-malic enzyme), being identical among all malic enzymes investigated (Figure 5.8A).

The three metal ligands, Glu255, Asp256 and Asp279, are 2.43, 2.19 and 2.23 Å, respectively, to the manganese ion, forming a reaction core. Many of the other amino acid residues within 7 Å from the metal ion, are hydrophobic residues that form a second sphere for the catalytic metal ion, the other polar groups in the active centre, and ensure an optimal environment for substrate binding and catalytic reactions (Hsu *et al*., 1976). Alteration of these residues, even indirectly, might affect the catalytic efficiency.

5.4.2 Structure of Malic Enzyme

Malic enzymes belong to an amino acid dehydrogenase-like family and a superfamily that contains the NAD(P)-binding Rossmann-fold domain. It has a α/β structure and the core structure includes three layers of α/β/α type, and a parallel β-sheet of six strands. The human malic enzyme monomer was divided into four domains named A, B, C, and D (Figure 5.9A) (Xu *et al*., 1999), which behave mostly as rigid-bodies in the conformational transition between open and closed forms of the enzyme. Domain A is mostly helical (αA1 through αA6) while domain B consists of two segments of the polypeptide chain, with domain C as an inserted cassette. Domain D contains one helix followed by a long extended structure that protrudes away from the rest of the monomer.

Domain B contains a central, parallel five-stranded β-sheet (βB1 through βB5), which is surrounded by helices on both sides (αB1 through αB8) (Chang and Tong, 2003). This β-sheet represents a new backbone-fold for a five-stranded parallel β-sheet. There is a short β-hairpin structure (βB2'-βB3') between strand βB2 and helix αB2 in this domain. Residues in this hairpin structure are highly conserved among malic enzymes.

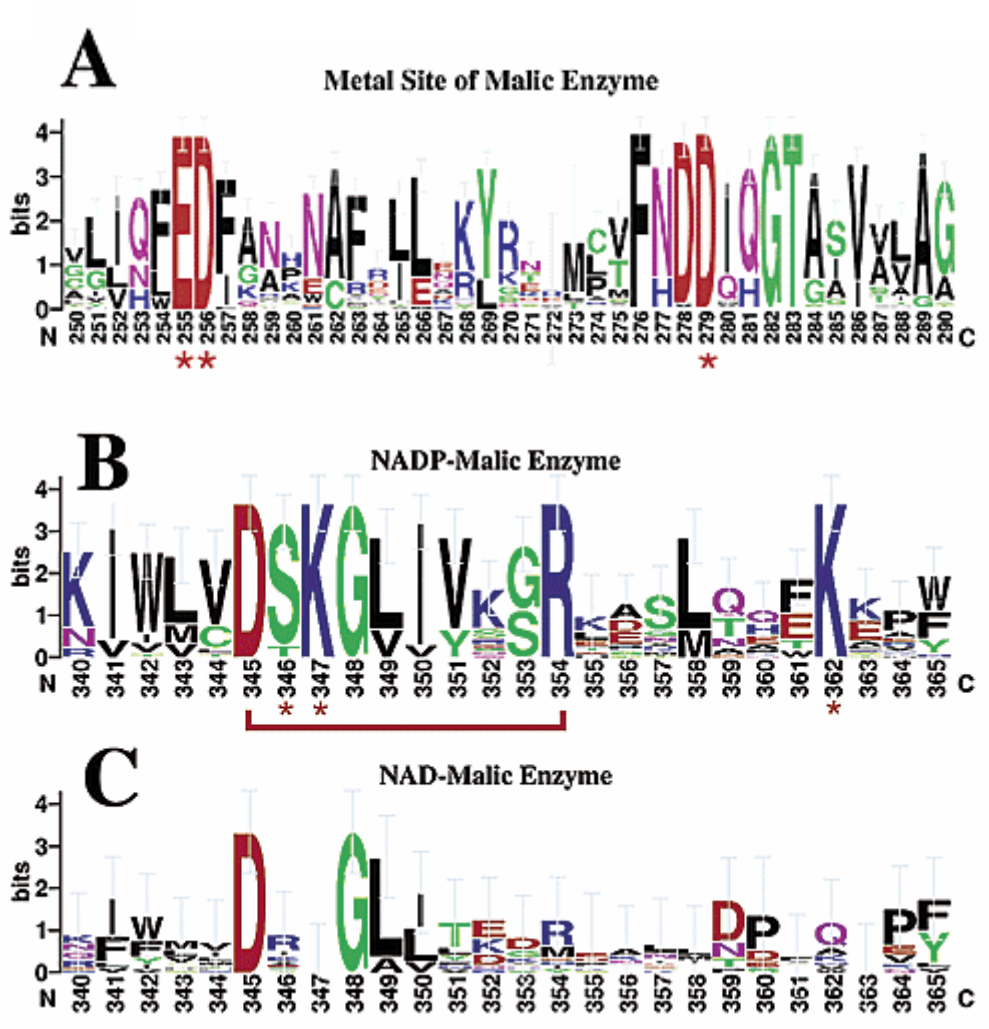

Figure 5.8. (A) Sequence logos of ME around the metal-binding site. The metal-binding ligands, Glu255, Asp256, and Asp279 (red stars), are strictly identical among all ME. Conservation of some putative second sphere hydrophobic amino acid residues is also evident in this figure. (B) Sequence logos of ME around the nucleotide-binding site of NADP-ME. The amino acid residues responsible for the nucleotide specificities are marked with red stars. The Asp345:Arg354 ion pair is highlighted. (C) Sequence logos of ME around the nucleotide-binding site of NAD-ME. Colour codes for the amino acids are as follows: blue for basic residues (Lys, Arg, and His), red for acidic residues (Asp and Glu), violet for amide residues (Asn and Gln), green for other neutral/polar residues, and black for hydrophobic residues (taken from Chang and Tong, 2003).

Domain C has the dinucleotide-binding Rossmann fold, with the exception that strand three is replaced by a short antiparallel strand (βC2) (Chang and Tong, 2003). In addition, there is an extra β-strand (βC7) at the C-terminal end of the domain, together with a β-hairpin insertion between βC6 and βC7. The NAD^+ cofactor in the active site is associated with this domain. The second dinucleotide-binding signature motif, GAGEAA, is located between βC1 and αC1 in this domain and mediates the binding of the phosphates of the cofactor as in other

Rossmann folds. However, the amino acid conservation between this and the Rossmann-fold domains is very low (approx. 15%).

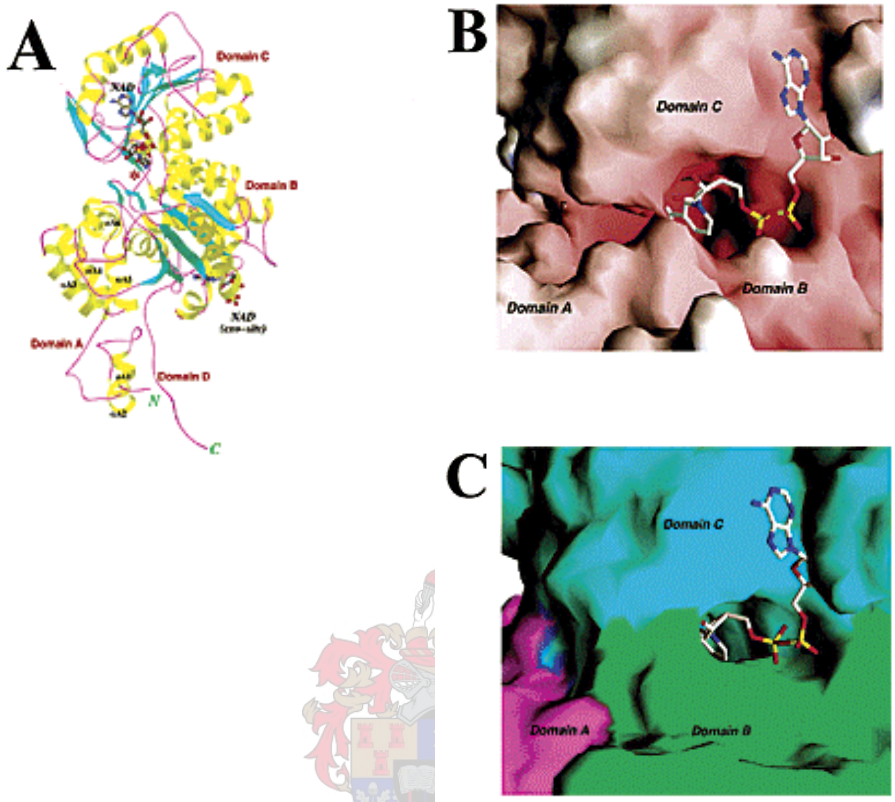

Figure 5.9. Structures of monomers of human m-NAD-ME. (A) The structure of m-NAD-ME in a binary complex with NAD^+, in open form I. The β-strands are shown in cyan, α-helices in yellow, and the connecting loops in purple. The four domains of the structure are labeled. The active site is indicated by the red star. Only the ADP portion of the NAD^+ molecule in the exo site is shown. (B) Molecular surface of the binary complex, in open form I, near the active site, coloured according to electrostatic potential. (C) Molecular surface of the pentary complex, in closed form II, near the active site, coloured by the three domains (taken from Chang and Tong, 2003).

When the human malic enzyme is in the open form, the active site region is fully exposed to the solvent (Figure 5.9B) (Xu *et al.*, 1999). Upon binding the divalent cation and the substrate (malate or pyruvate) or substrate analogue inhibitors (oxaloacetate, tartronate or keto-malonate), the enzyme undergoes a large conformational change (Yang *et al.*, 2000; Yang *et al.*, 2002; Tao *et al.*, 2003). In this closed form of the enzyme, the divalent cation and the substrate or inhibitor is shielded from the solvent (Figure 5.9C). The closed form of the enzyme is likely the catalytic competent conformation, while the open form may be

required for substrate binding and product release. Therefore, it is possible that most malic enzymes can undergo the open-closed transition during catalysis.

The tetramer of malic enzymes obeys a 222 point-group symmetry, with each monomer having essentially the same environment (Figure 5.10) (Chang and Tong, 2003). The four monomers are positioned at the four corners of a square, an arrangement first observed in the electron microscope images of pigeon liver malic enzyme (Nevaldine *et al*., 1974). Most malic enzymes have simple, hyperbolic kinetics with respect to their substrates, suggesting that the four active sites are functioning independently.

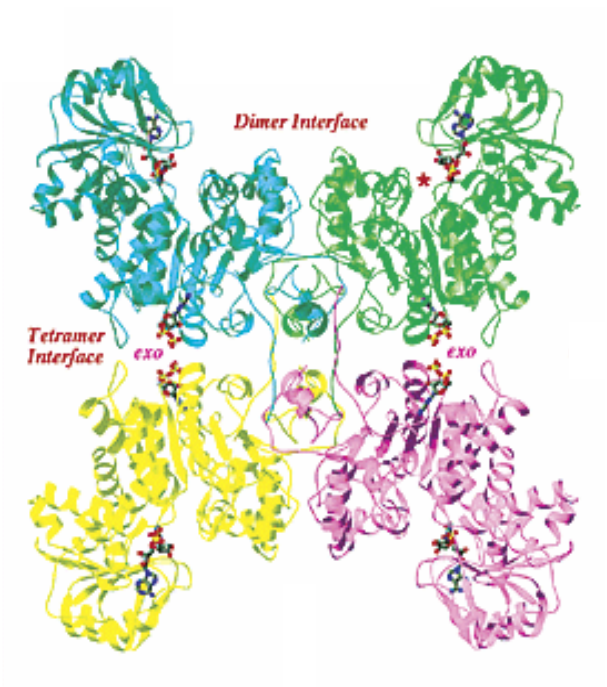

Figure 5.10. Schematic drawing of the tetramer of human m-NAD-ME in open form I (taken from Tao *et al*., 2003).

The tetramer is a dimer of dimers, with intimate contacts at the dimer interface, whereas the association of the two dimers is weaker (Figure 5.10) (Tao *et al*., 2003). This is in agreement with biochemical studies showing that pigeon NADP-malic enzyme exists in a monomer-dimer-tetramer equilibrium in solution (Chang *et al*., 1988). The dimer interface involves residues from domains A and B of the monomer. Helices αA3 and αA4, and their 2-fold symmetry couples, form a four-helical bundle at this interface. Interactions at the tetramer interface are primarily mediated by the long, extended segment at the C-terminus of the malic

enzyme monomer (domain D) (Figure 5.9A) that latches onto the other dimer and interacts with both of its monomers (Figure 5.10).

5.4.3 Acitve Site of the Malic Enzyme and Substrate/Inhibitor Binding Modes

The active site of the malic enzyme is located in a deep cleft at the interface between domains B and C of each molecule (Figure 5.8C) together with several residues from domain A (mostly from helix αA6, Figure 5.11A) (Chang and Tong, 2003). The amino acid residues in the active site region are generally highly conserved among the malic enzymes, supporting their importance in substrate binding and/or catalysis (Figure 5.8A). The active site residues can be roughly divided into four categories: (1) divalent cation-binding residues; (2) substrate-binding residues; (3) $NAD(P)^+$ cofactor binding residues; and (4) catalytic residues. The divalent cation is bound deep in the active site cleft (Figure 5.11A) and is octahedrally coordinated by six oxygens, one each from the side chain carboxylate groups of Glu255, Asp256, and Asp279, two from the substrate or inhibitor, and one from a water molecule (Figure 5.11C).

By studying the dead-end NADH/malate and NAD^+/pyrvate complexes, the bound conformations of the malate and pyruvate substrate molecules have also been determined (Tao *et al*., 2003). Malate is bound in the active site such that the C2 hydroxyl is essentially in the same plane as the C1 carboxylate group (Figure 5.11A) (Chang and Tong, 2003). The C2 hydroxyl and one of the C1 carboxylate oxygen atoms are ligands to the divalent cation (Figure 5.11C). Malate is also involved in a large network of hydrogen-bonding and ionic interactions with the enzyme (Figure 5.11B). The C4 carboxylate group of malate is out of the plane defined by the C1, C2, O2, and C3 atoms (Figure 5.11C).

The bound conformation of the oxaloacetate molecule is consistent with its role as an analogue of the enol-pyruvate transition-state intermediate as well as the pyruvate product (Yang *et al*., 2000; Tao *et al*., 2003). Structural comparison between pyruvate and malate shows that the C2 atoms of the two molecules are separated by about 0.6 Å (Figure 5.11D); partly due to the difference in the hybridization state of this atom (sp^2 vs sp^3) in the two compounds (Tao *et al*., 2003).

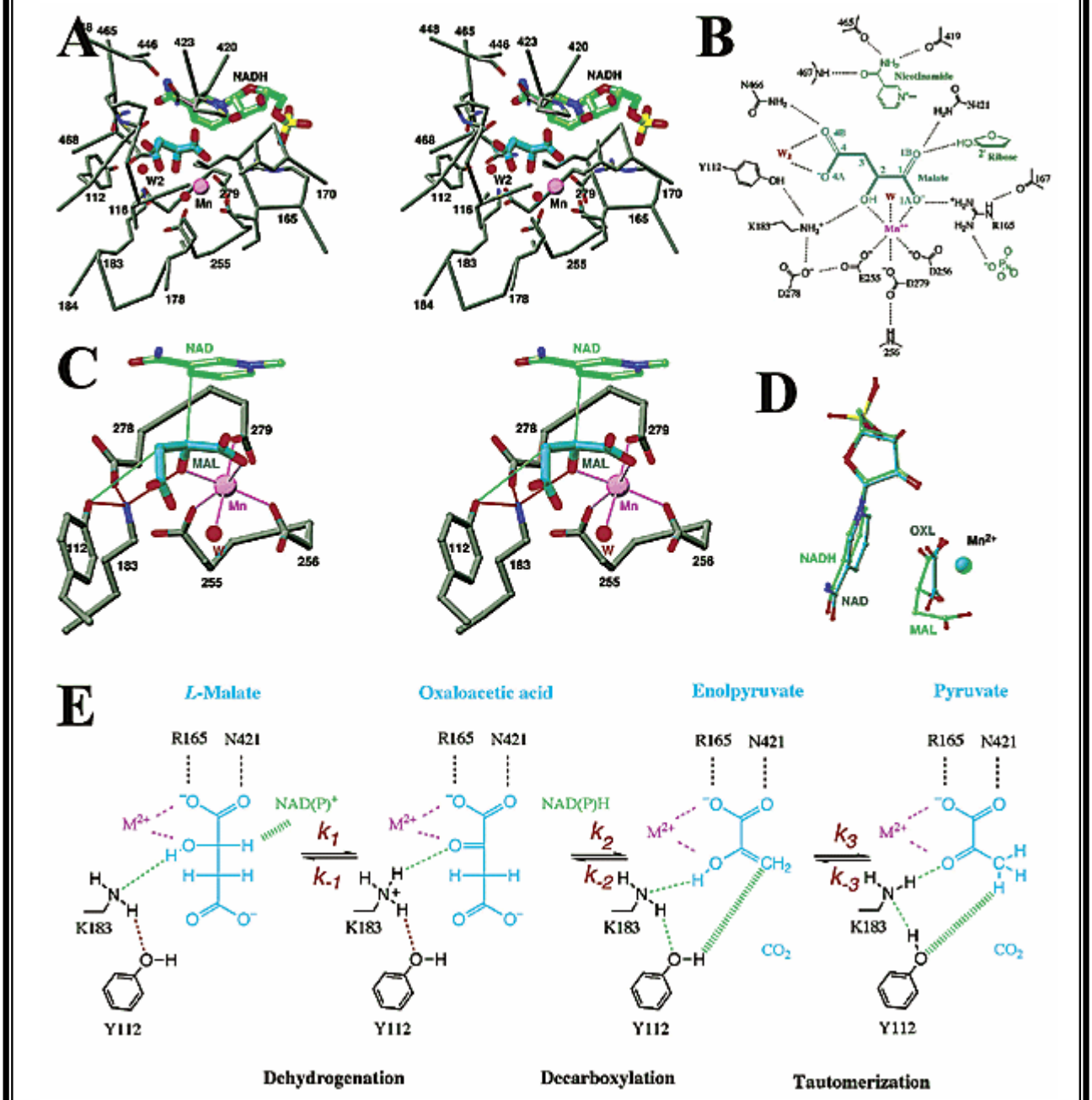

Figure 5.11. Active site of malic enzymes. (A) Residues of human m-NAD-ME near the active site of the enzyme, shown in gray for carbons. The malate molecule is shown with the carbon atoms in cyan and the NAD^+ molecule in green. The Mn^{2+} ion is shown as a purple sphere and the water molecules in red. (B) Schematic drawing of the polar interactions in the active site of human m-NAD-ME. (C) Close-up of the active site of human m-NAD-ME, showing the hydrogen-bonding interactions for the Lys183 side chain. The hydride transfers between the C2 atom of malate and the C4 atom of nicotinamide, and the proton transfer between Tyr112 and C3 atom of the substrate, are indicated in green. (D) Comparison of the binding modes of NAD^+, oxaloacetate, and Mn^{2+} in the quaternary complex with those of NADH, malate, and Mn^{2+} in the pentary complex. (E) A possible catalytic mechanism for malic enzymes. The other proton on the Lys183 side chain is hydrogen bonded to Asp278 throughout the reaction cycle (taken from Chang and Tong, 2003).

The active site also contains several hydrophobic residues and the majority of them do not have direct interactions with the substrate (Figure 5.11A), but instead help shield the active site region from the solvent in the closed form (Chang and Tong, 2003). Interestingly, the two prolines in the active site are both in the *cis* conformation and form a lid over the active site.

5.4.4 NAD(P)+ Binding and Cofactor Specificity

The $NAD(P)^+$ cofactor in the active site is associated with domain C at a position similar to that of the dinucleotide in other Rossmann-fold domains (Chang and Tong, 2003). The adenine ring is on the surface of the protein and the nicotinamide ring is in the anti conformation. Residue Gly444, strictly conserved among malic enzymes, is located close to the amide group in this ring. Mutation of this residue to Asp in the *S. pombe* malic enzyme inactivated the enzyme (Viljoen *et al*., 1994).

Malic enzymes have highly conserved amino acid sequences, but have distinct specificities toward the dinucleotide cofactor. Some malic enzymes can only use NAD^+ as the cofactor, while others can only use $NADP^+$. The molecular basis for cofactor selectivity is still poorly understood. Earlier studies with other enzymes have revealed two major determinants for cofactor specificity (Wierenga *et al*., 1986; Scrutton *et al*., 1990). First of all, an Asp residue near the end of the second strand of the Rossmann fold generally indicates NAD^+ preference, as it recognises the 2'-hydroxyl group in $NADP^+$. Second, enzymes that contain a GXGXXG dinucleotide-binding motif generally prefer NAD^+, whereas those with a GXGXXA motif generally prefer $NADP^+$. However, malic enzymes appear to disobey both of these rules. An Asp residue is conserved among all malic enzymes at the end of the second strand (βC2) in domain C. Moreover, most malic enzymes from animals contain the GXGXXA motif, whereas those from lower organisms contain the GXGXXG motif, irrespective of their cofactor specificity (Chang and Tong, 2003). The structures of the human mitochondrial NAD-malic enzyme show that the Asp345 residue is pointed away from the ribose, forming ion-pair interactions with Arg354 (Figures 5.9B). This may explain why some malic enzymes can use $NADP^+$ as the cofactor even with a conserved Asp at this position. The 2'phosphate group of $NADP^+$ is placed on the surface of the enzyme and interacts with residues Ser346 and the side chain ammonium group of Lys362. These two residues are conserved among the $NADP^+$-dependent malic enzymes (Figure 5.8B). Several other residues near the 2'-phosphate group also have variations between $NADP^+$- and NAD^+-dependent malic enzymes (Figure 5.8B,C).

5.4.5 Possible Catalytic Mechanism

Catalysis by malic enzymes generally proceeds in three steps – dehydrogenation of malate to produce oxaloacetate (k_1), decarboxylation of oxaloacetate to produce enolpyruvate (k_2), and tautomerization of enolpyruvate to produce pyruvate (k_3) (Figure 5.11E) (Cleland, 1999). The divalent cation at the optimal position helps catalyse all the steps of the reaction, which explains its requirement for catalysis by malic enzymes. For the oxidative decarboxylation of malate, a general base is needed to extract the proton from the C2 hydroxyl group to initiate the dehydrogenation reaction (k_1). For the tautomerisation reaction (k_3), a general acid is needed to protonate the enolpyruvate intermediate at the C3 position, and a general base is needed to extract the proton from the C2 hydroxyl of this intermediate.

Based on the structure of human mitochondrial NAD-malic enzyme in complex with malate and pyruvate, Lys183 has been identified as the general base and Tyr112 as the general acid (Tao *et al*., 2003). The Lys183 side chain is hydrogen bonded to the C2 hydroxyl (or carbonyl) of the substrate and the side chains of Tyr112 and Asp278 (Figure 5.11C). The Lys183 side chain, in the neutral form, is perfectly positioned to extract the proton from the C2 hydroxyl of malate (Figure 5.11E). In the decarboxylation reaction (k_2), Lys183 functions as a general acid and donates a proton to the C3 position, while Lys183 extracts the proton from the C2 hydroxyl. During this process, the proton shared between the two residues changes its position to maintain both of them in the neutral state. Therefore, Tyr112-Lys183 functions as a general acid-base pair in this reaction.

In the complex with malate, the proton on the C2 atom is pointed toward the C4 atom of the nicotinamide ring of NAD^+, with a hydride transfer distance of about 2 Å (Chang and Tong, 2003). This explains the stereospecificity of malic enzyme for L-malate, as D-malate cannot adopt the same binding mode (Xu *et al*., 1999).

5.5 REGULATION OF MALIC ENZYMES IN YEAST

Various mechanisms have been described for regulating the levels of activity of malic enzymes. Regulation of the levels of mRNA via transcriptional and post-transcriptional regulation is normally found in higher eukaryotic cells, whereas malic enzymes in fungi and bacteria are usually regulated through competition and activation by other dicarboxylic acids. In higher eukaryotes, the regulation of malic enzymes often involves nutritional or hormonal regulation (Barroso and Santisteban, 1999; Gletsu *et al*., 1999). A good example is the

glucose-induced transcription of the mouse malic enzyme gene via phosphorylation of transcription factors that bind to an insulin response element in the gene promoter (Gletsu *et al*., 1999). However, very little is known about the molecular mechanisms involved in the regulation of malic enzyme genes from yeasts.

5.5.1 The *S. pombe* Malic Enzyme

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 while three negative-acting elements (URSs) are involved in general derepression of *mae2* (Viljoen *et al*., 1999). Both the UAS1 and UAS2 elements have DNA sequences similar to eukaryotic cAMP-responsive regulatory elements: UAS1 has sequence similarity to the binding site for the *S. cerevisiae* cAMP-dependent ADR1 protein (Cherry *et al*., 1989) and mammalian cAMP-inducible AP-2 factor (Xie and Jaiswal, 1996); and UAS2 has similarity to the mammalian activating transcription factor (ATF)/cAMP response element (Hai *et al*., 1989). The three negative *cis*-acting elements, URS1, URS2 and URS3, seem to function co-operatively to repress *mae2* gene expression (Viljoen *et al*., 1999). Only URS3 has sequence similarity to known DNA-binding sites, i.e. that of the mammalian Sp1 and Sp3 transcription factors (Mitchell *et al*., 1987; Li *et al*., 1998). Sequences homologous to that of URS1 and URS2 were also identified in the promoter region of the *S. pombe* malate permease gene (Grobler *et al*., 1995), suggesting the possible co-regulation of pathway-specific enzymes in *S. pombe*.

The sequence similarity of UAS1 and UAS2 with other eukaryotic cAMP-responsive elements raised the possibility of cAMP-regulated expression of the *mae2* gene. In *S. pombe*, the cAMP-dependent (Pka1) and general stress activated (Sty1) pathways operate in parallel to regulate the expression of several genes (Wilkinson *et al*., 1996; Kronstad *et al*., 1998; Wilkinson and Millar, 1998). Transcription of *mae2* in *S. pombe* was induced after growth on 8% or 30% glucose or under non-aerated conditions (Viljoen *et al*., 1999). Furthermore, a two-fold increase in *mae2* transcription was evident within 15 min upon transfer of a culture from 0.5% glucose to fresh medium containing either 30% glucose or 0.5% glucose plus 0.8 M KCl (Groenewald and Viljoen-Bloom, 2001). Both the high glucose concentration and 0.8 M KCl represent osmotic stress conditions that may result in redox imbalances inside the cell. Pap1, as well as Atf1, are partially required for the induced response to 30% glucose, whereas Wis1 may be involved in the response to non-aerated conditions via other transcription factors. Therefore, Groenewald and Viljoen-Bloom (2001) suggested that both

the Pka1 and Sty1 signal transduction pathways are involved in the induced expression of *mae2* under fermentative conditions.

It is possible that there are two levels of regulation for the *mae2* gene in response to glucose. The first level of regulation involves a mild carbon regulated induction in response to high glucose concentrations (e.g. 8% glucose), and the second, a stronger induction in response to osmotic stress conditions (e.g. 30% glucose) (Viljoen *et al*., 1999; Groenewald and Viljoen-Bloom, 2001). Both these conditions can result in redox imbalances, which are rectified by increasing the production of glycerol, with the corresponding oxidation of NADH to NAD^+ (Bakker *et al*., 2001). The additional NAD^+ must be reduced to NADH to maintain the NAD^+/NADH redox balance within the cell.

5.5.2 *Saccharomyces* Malic Enzyme

Preliminary transcriptional regulation studies of the *MAE1* gene in *S. cerevisiae* shed some light on the physiological role of the malic enzyme in this yeast. Expression of the *MAE1* gene was found to be relatively low, but constitutive during continuous cultivation on different carbon sources, i.e. glucose, ethanol and acetate (Boles *et al*., 1998). A clear induction of *MAE1* expression was observed during anaerobic growth of *S. cerevisiae* on glucose in continuous culture, with a 3-fold increase at the transcriptional level and a 4-fold increase in the enzyme activity of cell extracts (Boles *et al*., 1998). However, a database search with the promoter sequence of the *MAE1* gene did not reveal any significant or relevant transcription factor-binding sites (Volschenk *et al*., 2003).

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

These results implicated the native malic enzyme gene as one of the pivotal role players involved in the differential ability of *Saccharomyces* strains to degrade L-malate (Volschenk *et al*., 2003). The results 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 the three *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-malate.

DIFFERENTIAL UPTAKE OF FUMARATE BY *CANDIDA UTILIS* AND *SCHIZOSACCHAROMYCES POMBE*

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ABSTRACT

The dicarboxylic acid fumarate is an important intermediate in cellular processes and also serves as a precursor for the commercial production of fine chemicals such as L-malate. Yeast species differ remarkably in their ability to degrade extracellular dicarboxylic acids and to utilise them as their only source of carbon. In this study we have shown that the yeast *Candida utilis* effectively degraded extracellular fumarate and L-malate, but glucose or other assimilable carbon sources repressed the transport and degradation of these dicarboxylic acids. The transport of both dicarboxylic acids was shown to be strongly inducible by either fumarate or L-malate while kinetic studies suggest that the two dicarboxylic acids are transported by the same transporter protein. In contrast, *Schizosaccharomyces pombe* effectively degraded extracellular L-malate, but not fumarate, in the presence of glucose or other assimilable carbon sources. The *S. pombe* malate transporter was unable to transport fumarate, although fumarate inhibited the uptake of L-malate.

6.1 INTRODUCTION

The C_4-dicarboxylic acid fumarate serves as an intermediate of the tricarboxylic acid (TCA) cycle that allows for the metabolic flow of carbon between various metabolic pathways. Yeast species differ remarkably in their ability to transport and utilise one or more intermediates of the TCA cycle (Barnett and Kornberg, 1960). Previous studies have shown that L-malate can be utilised by *Candida utilis* (Cássio and Leão, 1993), *Candida sphaerica* (Côrte-Real *et al.*, 1989), *Hansenula anomala* (Côrte-Real and Leáo, 1990) and *Kluyveromyces marxianus* (Queiros *et al.*, 1998) as their only source of carbon and energy. In these species, the dissociated form of L-malate is transported across the plasma membrane by

a H^+-symport system that is substrate-inducible and subject to glucose repression. In contrast, *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii* can degrade L-malate only in the presence of an assimilable carbon source (Rodriguez and Thornton, 1990; Osothsilp and Subden, 1986[a]). Other yeasts such as *Saccharomyces cerevisiae* can import L-malate and other dicarboxylic acids only via simple diffusion (Salmon, 1987) and is therefore unable to effectively degrade or utilise extracellular L-malate.

In *S. pombe*, the dissociated form of L-malate is actively transported via a H^+-symport system that operates constitutively, whereas the undissociated acid enters the cell via simple diffusion (Baranowski and Radler, 1984; Osothsilp and Subden, 1986[b]; Sousa *et al*., 1992). The dicarboxylic acids fumarate, D-malate, succinate, oxaloacetate, maleate, malonate and α-ketoglutarate acted as competitive inhibitors for the uptake of L-malate (Sousa *et al.*, 1992), suggesting a common transporter for the uptake of dicarboxylic acids in fission yeast. However, Grobler *et al*. (1995) showed that L-malate, succinate and malonate, but not α-ketoglutarate, were actively transported by *S. pombe* cells.

In addition to its role in metabolic processes, fumarate is also an important precursor for the commercial production of fine chemicals such as L-malate. The D,L-malate racemic mixture is routinely used in a variety of foods and beverages whereas the L-isomer is used for the treatment of conditions such as hyperammonaemia (Rosenberg *et al*., 1999). The racemic mixture is commercially produced via chemical hydratation of maleate or fumarate, and the L-isomer through the enzymatic conversion of fumarate using fumarase-containing microbial cells. The bioconversion of fumarate to L-malate has been obtained by strains of *Brevibacterium* (Takata *et al*., 1980), *Candida rugosa* (Yang *et al*., 1992), *Pichia* (Keruchen'ko *et al*., 1995) and *Dipodascus* (Rosenberg *et al*., 1999) that exhibit high fumarase activities. Over-expression of the *S. cerevisiae* fumarase gene, *FUM1*, also resulted in an increased conversion rate of fumarate to L-malate (Peleg *et al*., 1990). Since *S. cerevisiae* can only import fumarate through diffusion, the introduction of a fumarate transporter gene into *S. cerevisiae* could enable this yeast to actively transport fumarate and consequently improve the bioconversion of fumarate.

Heterologous expression of the *S. pombe* malate transporter gene, *mae1*, in a strain of *S. cerevisiae* resulted in the active transport and efficient degradation of L-malate (Volschenk *et al*., 1997[a,b]). Our first approach was therefore to determine whether expression of the *mae1* gene in *S. cerevisiae* would also enable the recombinant strain to transport fumarate. We

found that neither the recombinant *S. cerevisiae* strain nor the wild type *S. pombe* strain could transport fumarate. In search of an alternative fumarate transporter, several yeast species were evaluated for their ability to degrade extracellular fumarate. Since *C. utilis* proved to be able to degrade both fumarate and L-malate, the transport of these dicarboxylic acids was further investigated in this yeast.

6.2 MATERIALS AND METHODS

6.2.1 Microorganisms and culture media

The yeast strains used in the transport studies included *C. utilis* ATCC 9950 T, *S. pombe* 972 h^- (Osothsilp and Subden 1986[b]) and *S. cerevisiae* YPH259 (MATα *ura*3-52, *lys*2-801_a, *ade*2-101_o, *his*3Δ200, *leu*2-Δ1) (Sikorski and Hieter, 1989). The strains used for the screen on fumarate/malate indicator plates are listed in Table 6.1. Unless otherwise stated, the growth media contained 0.17% YNB (yeast nitrogen base without amino acids and ammonium sulphate [Difco Laboratories, Detroit, Mich.]), 0.5% $(NH_4)_2SO_4$, supplemented with amino acids and buffered at pH 3.3. Different concentrations of fumarate, L-malate and/or a carbon source were added as indicated for the different experiments.

6.2.2 Degradation of extracellular fumarate and L-malate

Indicator agar plates were used to screen different yeast species for the degradation of fumarate and L-malate in the presence of different carbon sources. The yeast strains were streaked onto YNB agar plates containing 0.05% bromocresol-green, 0.3% fumarate or L-malate, together with 2% glucose, fructose, galactose, glycerol, maltose, raffinose or sucrose as carbon source. The plates were incubated at 30°C for 2 days and evaluated for a colour change from yellow at pH 3.3 to blue at pH 5.2.

The utilisation of extracellular fumarate and L-malate by *C. utilis* and *S. pombe* was determined after growth in liquid YNB media containing 2% glucose, raffinose or glycerol/ethanol as carbon source, supplemented with either 0.5% fumarate or L-malate. Cells were harvested at different time intervals and high performance liquid chramatography (HPLC) was used to determine the residual levels of fumarate and L-malate. Glucose concentrations were measured with the glucose oxidase method (Glucose [Trinder], Sigma, St Louis, Mo.) and cell growth was determined spectrophotometrically at OD_{600}. All assays were done in triplicate.

Tabel 6.1. Utilisation of L-malate and fumarate in various yeast species grown on indicator agar with different carbon sources

	Malate							Fumarate						
	Fructose	Galactose	Glucose	Glycerol	Maltose	Raffinose	Sucrose	Fructose	Galactose	Glucose	Glycerol	Maltose	Raffinose	Sucrose
Saccharomyces cerevisiae 228	-	-	-	ng[a]	-	-	-	-	-	-	ng	-	-	-
Schizosaccharomyces pombe 972-	+	ng	+	+	+	+	+	-	ng	-	-	-	-	-
Candida utilis ATCC 9950 T	+	+	-	+	+	+	+	+	+	-	+	+	+	+
Hansenula anomala UOFS YW207 HT	+	-	+	-	-	-	-	+	-	+	-	+	+	-
Rhodosporidium toruloides CBS 0014	-	-	-	-	+	+	-	-	+	-	+	+	+	-
Rhodotorula graminis CBS 2826T	-	-	-	-	+	+	-	-	-	-	+	+	+	-
Tremella fuciformis CBS 6970T	-	-	-	+	+	+	-	-	+	+	+	+	+	-
Yarrowia lypolitica CBS 2073	-	+	-	-	-	+	+	-	+	+	-	+	+	+

[a]No growth

6.2.3 Transport assays

Cells of *S. cerevisiae* YPH259 transformed with plasmid pHV3 containing the *S. pombe mae1* gene (Volschenk *et al*. 1997[b]), and wild type *S. pombe* 972 cells were grown in YNB media containing 2% glucose. For *C. utilis*, cells were cultured in 0.5% fumarate, 0.5% L-malate, 2% glucose, 2% raffinose or 2% glycerol/2% ethanol as the only source of carbon. To further investigate the effect of different carbon sources on the transport of fumarate and L-malate, *C. utilis* cells were cultured to OD_{600} of 0.6 in media containing 0.5% fumarate, 0.5% L-malate, 2% glucose or 2% glycerol/ethanol and divided into two batches. One batch of cultures was assayed immediately while the other was transferred to fresh medium containing either 0.5% L-malate or 2% glucose as carbon source and incubated for another 6 h.

Cells were harvested in the exponential growth phase (OD_{600} of 0.6), washed twice with ice-cold distilled water and resuspended in 0.1M KH_2PO_4 (pH 3.5) to a final concentration of approximately 20 mg dry weight ml^{-1} (adapted from Grobler *et al.* 1995). Cell suspensions were pre-incubated for 5 min at 30 °C in a shaker waterbath at 100 rpm. Assays were initiated by adding 10 µl of an aqueous solution of [1-^{14}C]-fumarate (6.62 µCi/µmol; ICN Pharmaceuticals, CA) or L-[1,4(2,3)-^{14}C]-malate (55 µCi/µmol; Amersham, Bucks, UK). Pre-boiled cells (5 min at 100 °C) were used to determine non-specific binding of ^{14}C-fumarate or ^{14}C-malate to the yeast cells. Samples of 0.5 ml were withdrawn at different time intervals and the reactions were stopped by dilution with 5 ml ice-cold distilled water. The cells were rapidly filtered through 0.45 µm membranes (Millipore Corporation, Bedford, Mass.) and immediately washed with 5 ml ice-cold distilled water. The filters were air dried for 10 min and placed in scintillation vials with 5 ml scintillation reaction mixture (Ecolite, ICN Pharmaceuticals, Calif.). Levels of radioactivity were measured with a Beckman LS 3801 scintillation counter (Beckman Instruments, Calif.).

6.2.4 Cellular fractionation for localisation of dicarboxylic acids

Cultures of *S. pombe* in 10 ml YNB medium containing 2% glucose were harvested at OD_{6oo} of 0.6 and resuspended in 1 ml of 0.1 M KH_2PO_4 (pH 3.5). Cultures were incubated for another hour with 1 µl of either ^{14}C-fumarate (6.62 µCi/µmol) or ^{14}C-malate (55 µCi/µmol). A final concentration of 0.5% non-labeled fumarate or L-malate was added to the ^{14}C-malate or ^{14}C-fumarate cultures, respectively. Cells were harvested and the supernatant was transferred to scintillation vials containing 5 ml scintillation reaction mixture. The cells were resuspended in 300 µl of 0.1 M KH_2PO_4 (pH 3.5) together with 0.3 g glass beads (106 µm diameter). Cells were broken with 10 pulses of 15 s with 1 min on ice between pulses. The supernatant and cell debris were separated through centrifugation and transferred to scintillation vials containing 5 ml scintillation reaction mixture. The levels of radioactivity were determined as described above.

6.2.5 Kinetic parameters for protein-mediated transport

Cells of *C. utilis* were cultured in YNB medium containing 0.5% fumarate as the only carbon source. Cells were harvested in the exponential growth phase (OD_{600} of 0.6), washed twice with ice-cold distilled water and resuspended in 0.1 M KH_2PO_4 (pH 3.5) to a final concentration of 7 mg dry weight ml^{-1}. Transport assays were initiated by adding increasing concentrations of ^{14}C-fumarate (0.015-2 mM) in the presence or absence of 2 mM non-labeled

L-malate. Estimates of kinetic parameters were obtained from Lineweaver-Burk plot of the initial uptake rates of ^{14}C-fumarate. The K_m for total dicarboxylic acids was based on the concentrations of both anionic and undissociated dicarboxylic acids.

6.3 RESULTS

6.3.1 Lack of fumarate transport by recombinant *S. cerevisiae* and wild type *S. pombe*

Strains of *S. cerevisiae* cannot transport extracellular dicarboxylic acids such as L-malate or fumarate (Salmon, 1987). However, transport studies with a recombinant *S. cerevisiae* strain expressing the *mae1* gene of *S. pombe*, showed that active transport of ^{14}C-malate was obtained, whereas fumarate was not transported (Figure 6.1A). The active transport of ^{14}C-malate by the wild type *S. pombe* 972 h$^-$ strain was confirmed (Figure 6.1B), but not in the *S. pombe mae1*$^-$ mutant strain that has a defective malate transporter. No transport of ^{14}C-fumarate was observed in either strain (Figure 6.1B). However, increasing concentrations of fumarate progressively inhibited L-malate uptake by the recombinant *S. pombe* malate transporter (Figure 6.1C). The HPLC analyses confirmed that *S. pombe* cells removed a significant portion (approximately 65%) of the L-malate from the glucose-containing growth media within 28 h (Figure 6.2A), whereas less than 15% of the fumarate was removed (Figure 6.2B). Similar results were obtained for cells grown in media containing raffinose or glycerol/ethanol as carbon source (data not shown).

Since fumarate inhibited the transport of L-malate in the recombinant *S. cerevisiae* strain without being transported itself, the uptake and subsequent cellular localisation of ^{14}C-malate and fumarate was further investigated in wild type *S. pombe* cells (Table 6.2). An hour after the addition of ^{14}C-malate to glucose-grown cells, approximately 30% of the ^{14}C-malate was removed from the extracellular fraction. The majority of this was already further metabolised to pyruvate and CO_2 with only 0.36% and 0.32% retained in the cell debris and intracellular fractions, respectively. The addition of unlabelled fumarate decreased the uptake of ^{14}C-malate by 20% and reduced the localisation of ^{14}C-malate in the cell debris and intracellular fractions by 50% and 28%, respectively.

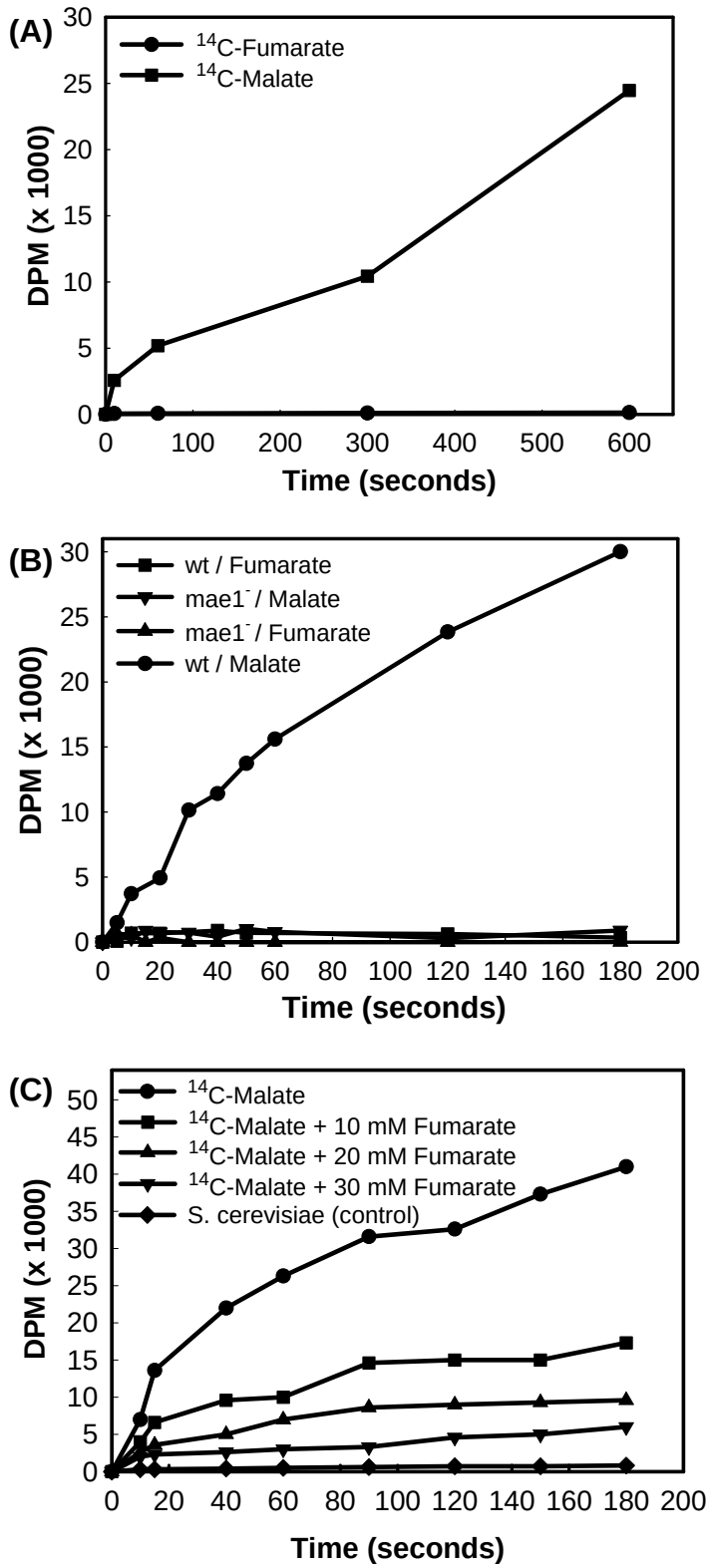

Figure 6.1. Transport studies to determine the uptake of L-malate and fumarate by strains of *S. pombe* and *S. cerevisiae*. (A) Uptake of ^{14}C-malate and ^{14}C-fumarate in *S. cerevisiae* cells transformed with the *S. pombe mae1* gene. (B) Uptake of ^{14}C-malate and ^{14}C-fumarate by *S. pombe* 972 h^{-} (wt) and *S. pombe mae1*$^{-}$ grown in 2% glucose. (C) Competition by fumarate for the transport of 4 mM ^{14}C-malate at pH 3.5 by the *S. cerevisiae* YPH259 host strain (control) or transformed with the *S. pombe mae1* gene. The yeast strains were grown in 2% glucose without fumarate, or with 10 mM, 20 mM or 30 mM non-labelled fumarate added simultaneously with the ^{14}C-malate.

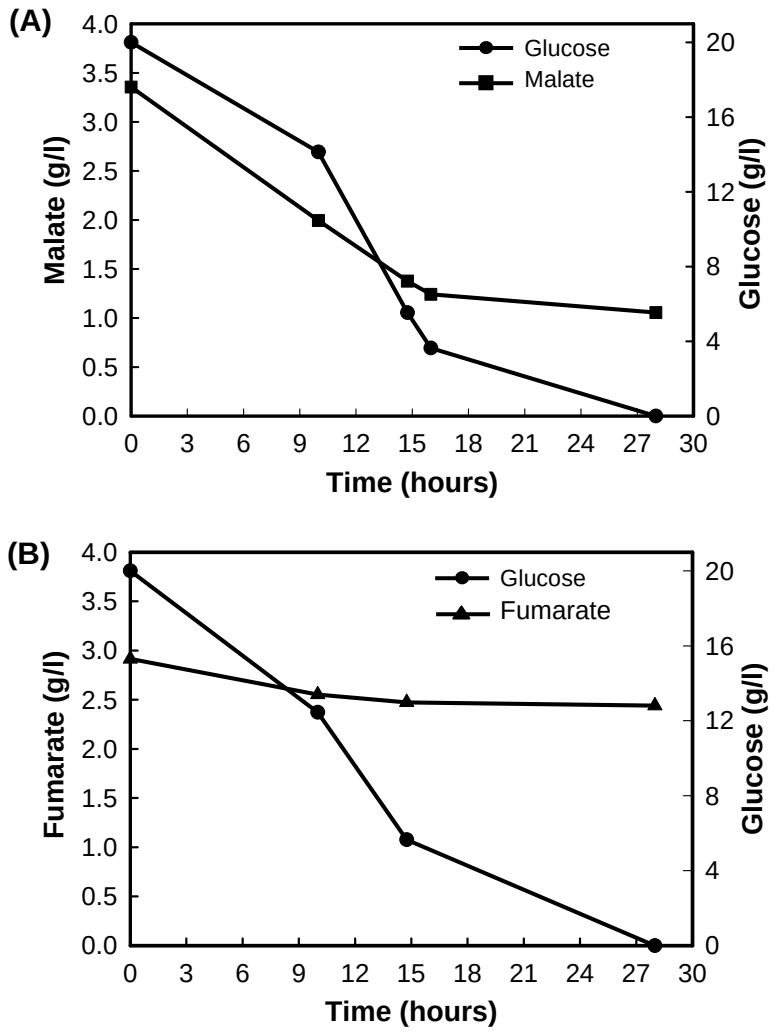

Figure 6.2. HPLC analysis of extracellular concentrations of (A) L-malate and (B) fumarate during growth of *S. pombe* 972 h^- on medium containing 2% glucose

When ^{14}C-fumarate was added to the *S. pombe* cells, only 1.9% was removed from the extracellular fraction after 1 h, but almost 10% of this was retained in the cell debris (Table 6.2). Although the addition of unlabelled L-malate did not significantly influence the uptake of ^{14}C-fumarate, it decreased its localisation in the cell debris by more than 50%. These results suggested that fumarate competes for the uptake of L-malate by inhibiting its binding to the malate transporter, although only L-malate is actively transported by the protein.

Table 6.2 Distribution of ^{14}C-labelled fumarate and L-malate in different cellular fractions in *S. pombe* cells after incubation for 60 min with or without unlabelled L-malate or fumarate

Culture conditions	Cellular distribution (% of total ^{14}C added)		
	Cell debris	Intracellular	Extracellular
Grown in 2% glucose, add ^{14}C-malate	0.36	0.32	69.36
Grown in 2% glucose, add fumarate and ^{14}C-malate	0.18	0.09	83.19
Grown in 2% glucose, add ^{14}C-fumarate	0.18	0.14	98.10
Grown in 2% glucose, add L-malate and ^{14}C-fumarate	0.06	0.07	98.41

6.3.2 Screening of yeast species for degradation of extracellular fumarate and L-malate

In a screen for yeasts capable of transporting fumarate and L-malate, several species were screened for their ability to degrade extracellular fumarate or L-malate incorporated into fumarate/malate indicator agar plates (Table 6.1). The yeasts *S. pombe* and *S. cerevisiae* are not able to utilise intermediates of the TCA cycle as their only source of carbon (Barnett and Kornberg, 1960), whereas the other species that were investigated are known for their ability to utilise TCA cycle intermediates. No degradation of either fumarate or L-malate was found for *S. cerevisiae*, since the yeast is unable to transport either of the dicarboxylic acids. In *S. pombe*, L-malate was effectively degraded in the presence of all the carbon sources that sustained growth, but no degradation of fumarate was observed. For *C. utilis*, degradation of both fumarate and L-malate were found in all the carbon sources investigated, except for glucose (Table 6.1). The other yeast species showed varying abilities to utilise fumarate or L-malate that seemed to be dependent on the available carbon source. Since the indicator plates only provided limited information, further investigation was required to better understand the regulatory mechanisms involved in the degradation and transport of fumarate and L-malate by *C. utilis*.

6.3.3 Degradation and transport of fumarate and L-malate by *C. utilis*

Cells of *C. utilis* effectively degraded extracellular fumarate when grown in YNB medium containing fumarate as the only carbon source (Figure 6.3A). However, the degradation of fumarate was less efficient when grown in the presence of either raffinose or glycerol/ethanol as carbon source, suggesting that other assimilable carbon sources may result in catabolite

repression of fumarate transport. In support of this, the degradation of fumarate by cells grown in glucose/fumarate media only commenced once the glucose had been depleted (Figure 6.3B). Similar results were obtained for L-malate (data not shown), indicating that the degradation of both fumarate and L-malate is subject to catabolite repression.

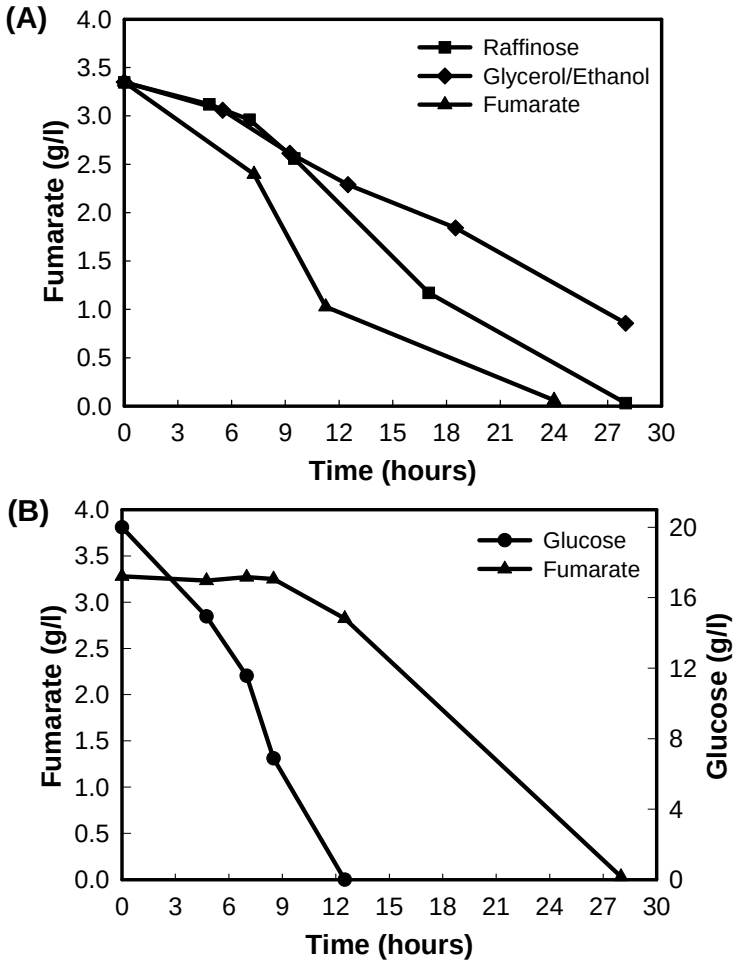

Figure 6.3. HPLC analyses showing the residual levels of fumarate after growth of *C. utilis* on (A) 0.5% fumarate, 2% raffinose or 2% glycerol/ethanol or (B) 2% glucose as carbon source (residual concentration of glucose is also indicated).

When *C. utilis* cells were pre-cultured in either fumarate or L-malate as the only carbon source, most of the ^{14}C-fumarate was taken up within 10 s of addition (Figure 6.4A). However, the uptake of ^{14}C-fumarate by cells grown on either glucose or raffinose as the only carbon source was almost non-detectable, with only a small amount transported by cells grown on glycerol/ethanol. Similar results were obtained for the transport of L-malate in *C. utilis* (date not shown), indicating that active transport of both fumarate and L-malate was subject to substrate induction by either dicarboxylic acid.

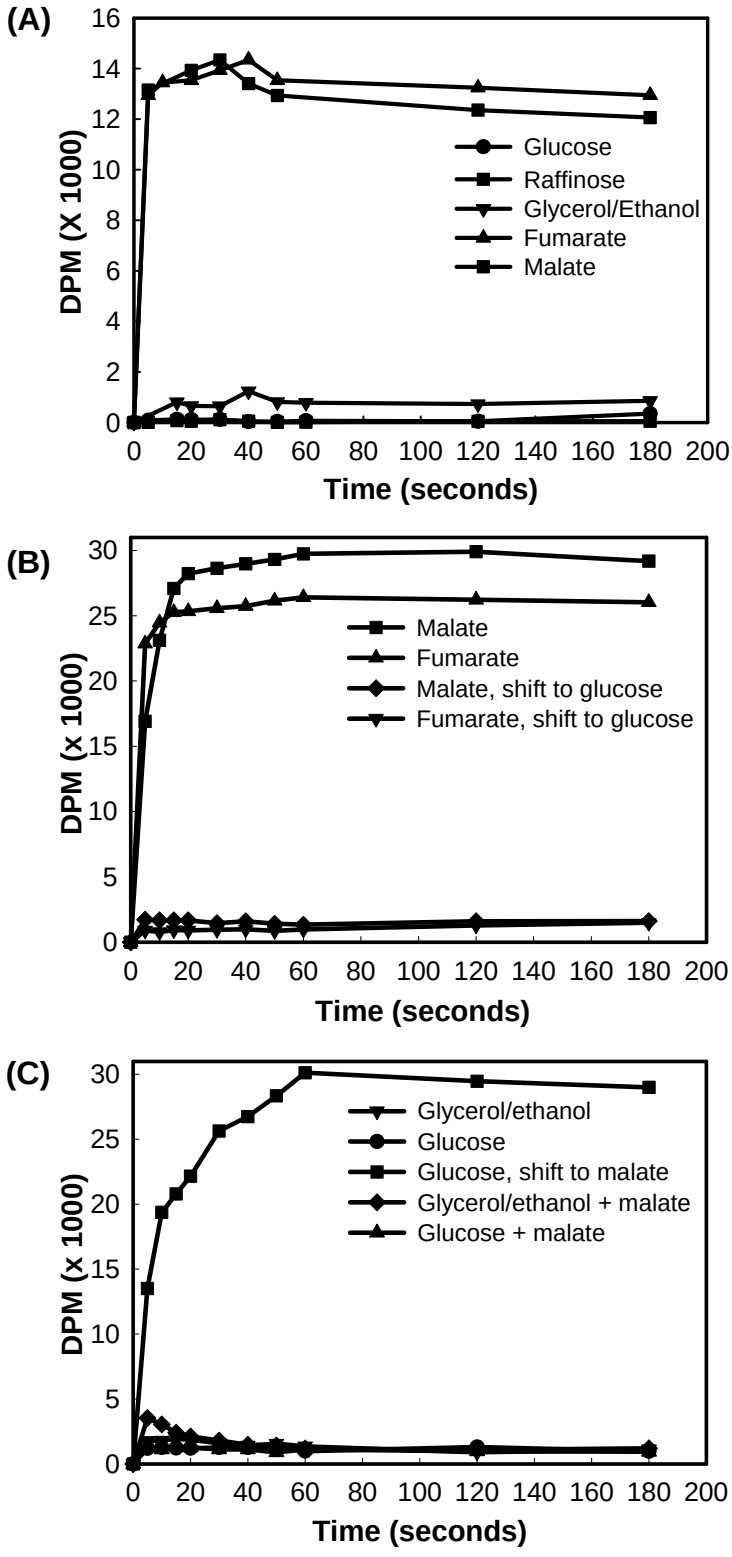

Figure 6.4. Transport studies to determine the uptake of ^{14}C-labelled L-malate and fumarate by *C. utilis*. (A) Uptake of ^{14}C-fumarate after growth on 2% glucose, 2% raffinose, 2% glycerol/ethanol, 0.5% fumarate or 0.5% L-malate as only carbon source. (B) Uptake of ^{14}C- malate after growth on 0.5% fumarate or 0.5% L-malate as only carbon source, and shifted to fresh medium containing 2% glucose. (C) Uptake of ^{14}C-malate after growth on 2% glucose or 2% glycerol/ethanol with or without 0.5% L-malate. Glucose-grown cells were also shifted to fresh medium containing 0.5% L-malate.

The transport of ^{14}C-malate by *C. utilis* was further investigated by shifting cultures grown on different carbon sources to fresh medium containing either 0.5% L-malate or 2% glucose (Figure 6.4B,C). ^{14}C-malate was quickly transported by cells grown on either fumarate or L-malate, but transport ceased when cells were transferred to glucose-containing medium (Figure 6.4B). Cells were unable to transport ^{14}C-malate when grown on glucose or glycerol/ethanol medium, not even when 0.5% L-malate was included in the glucose medium (Figure 6.4C). However, cells grown on glucose medium regained their ability to transport ^{14}C-malate when transferred to medium containing L-malate as the only carbon source (Figure 6.4C).

Preliminary kinetic studies were done to determine whether *C. utilis* uses the same transporter protein for the uptake of fumarate and L-malate. Lineweaver-Burk plots of the initial rates of uptake of ^{14}C-labelled fumarate at pH 3.5 were linear over the concentration range of 0.08 – 2 mM (Figure 6.5). The following kinetic parameters were calculated: $V_{max(fumarate)}$(pH 3.5) = 1.058 nmol s^{-1} mg (dry weight) $cells^{-1}$; K_m (pH 3.5) = 0.11 mM. These results indicated that fumarate and L-malate were mutually competitive inhibitors, suggesting that they might share the same carrier protein in *C. utilis*.

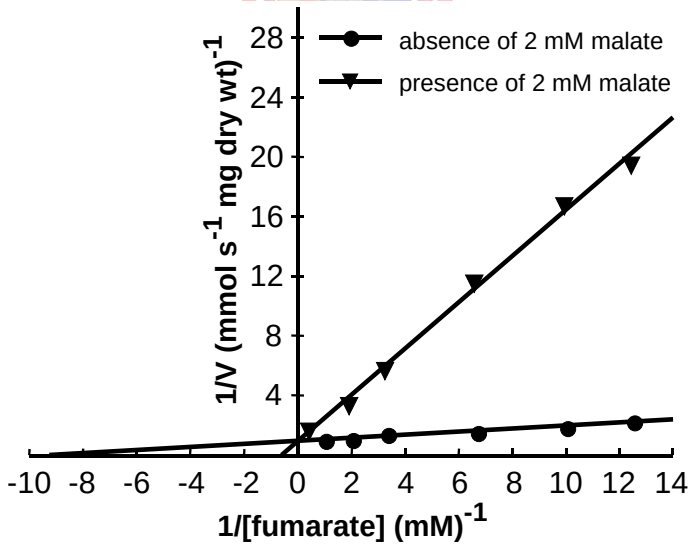

Figure 6.5. Lineweaver-Burk plots of the initial uptake rates of 4 mM ^{14}C-fumarate by fumarate grown cells as a function of the fumarate concentration in the media. Assays were done in the presence or absence of 2 mM L-malate.

6.4 DISCUSSION

The dicarboxylic acid L-malate is widely employed in both the pharmaceutical and food industries. Due to its industrial importance, several groups have investigated the bioconversion of fumarate to L-malate using microbial cells (Takata *et al*., 1980; Yang *et al*., 1992; Keruchen'ko *et al*., 1995; Rosenberg *et al*., 1999). Increased bioconversion of fumarate to L-malate (80.4 mmol fumarate/h per g of cel wet weight) was obtained by over-expression of the *S. cerevisiae* fumarase gene, *FUM1* (Peleg *et al*., 1990). This efficiency may be further improved if the *S. cerevisiae* cells were able to actively transport fumarate and not have to rely only on diffusion of the substrate. This could be realised through heterologous expression of a suitable fumarate transporter from another yeast in *S. cerevisiae*.

A screen for yeast strains that could degrade extracellular fumarate showed significant differences in the regulation and specificity for the uptake of fumarate and L-malate between yeast species. A common dicarboxylic acid transporter was suggested for *S. pombe* strain ICV.M (Sousa *et al*., 1992), but results presented here showed that neither the wild type *S. pombe* 972 h^- strain nor a recombinant strain of *S. cerevisiae* containing the *S. pombe* malate transporter gene was able to transport fumarate (Figure 6.1). However, increasing concentrations of fumarate were able to progressively inhibit the uptake of L-malate by the recombinant strain. Cellular fractionation of glucose-grown cells (Table 6.2) showed that the addition of unlabelled fumarate decreased both the uptake and membrane localisation of ^{14}C-malate. The data suggested that fumarate could also bind to the malate transporter and therefore inhibit the uptake of L-malate. The binding of both fumarate and L-malate to the *S. pombe* malate transporter can be ascribed to the structural relatedness of the two dicarboxylic acids. Similarly, Grobler *et al*. (1995) reported that α-ketoglutarate was not transported by *S. pombe*, although it competed for the uptake of L-malate (Sousa *et al*., 1992).

The results presented here indicate a significant difference in the transport of fumarate and L-malate by *C. utilis* and *S. pombe*. Cells of *S. pombe* 972 effectively transported L-malate, but not fumarate, and no evidence for substrate induction or glucose repression for the uptake of L-malate was found. In contrast, the *C. utilis* ATCC 9950 T strain effectively transported both fumarate and L-malate and the uptake of both dicarboxylic acids was induced by either of the substrates. The kinetic data suggest the fumarate and L-malate are transported by the same carrier protein in *C. utilis*, which explains the similar regulatory mechanisms observed for the transport of fumarate and L-malate.

The degradation of either fumarate or L-malate by *C. utilis* was sensitive to the presence of glucose (Figure 6.3). This supports previous reports that the utilisation of L-malate in *C. utilis* strain IGC 3092 was subject to glucose repression (Cássio and Leão, 1993). In addition, we observed that the transport of either fumarate or L-malate was also insignificant in the presence of other carbon sources such as raffinose and glycerol/ethanol (Figure 6.4). This confirmed that *C. utilis* employs a double regulatory mechanism for the transport of L-malate and fumarate with the dicarboxylic acids only being transported in the presence of either of the inducers and when no alternative carbon source is available.

The carbon sensitivity and substrate induction observed for the uptake of fumarate and L-malate by *C. utilis* could be interpreted in the context of its ability to utilise intermediates of the TCA cycle as the only source of carbon and energy. The yeast *C. utilis* is Crabtree-negative and can therefore ferment sugars only under oxygen-limited conditions (Van Dijken *et al*., 1993). Under aerobic growth conditions, the yeast tended to channel most of its pyruvate into the TCA cycle, resulting in an adequate supply of intracellular TCA cycle intermediates such as fumarate and L-malate. Since the degradation of glucose, raffinose or glycerol/ethanol can provide pyruvate for the TCA cycle, the dicarboxylic acids will most likely only be utilised if a more efficient carbon source is not available. The results presented here support the notion that *C. utilis* cells allow the transport of fumarate and L-malate only in the presence of the inducers and when an alternative carbon source is not available. Furthermore, the results presented in Figure 6.3 indicate that the catabolite repression is stronger when cells are grown on glucose than on the less favourable carbon source glycerol/ethanol.

Although the transport of dicarboxylic acids has been described for a number of yeast species, the *S. pombe mae1* gene is the only malate transporter gene cloned and sequenced thus far (Grobler *et al*., 1995). In this study, we demonstrated significant differences between *S. pombe* and *C. utilis* concerning the uptake of fumarate and L-malate and the regulation thereof. However, a proper investigation into the molecular basis for the transport of fumarate and L-malate by *C. utilis* can only be done once the fumarate/malate transporter gene from *C. utilis* is cloned.

6.5 ACKNOWLEDGEMENTS

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CLONING OF THE DICARBOXYLIC ACID TRANSPORTER FROM *CANDIDA UTILIS*

7.1 INTRODUCTION

Significant differences between *Schizosaccharomyces pombe* and *Candida utilis* regarding the uptake of fumarate and L-malate and the regulation thereof have been discussed in Chapter 6. The *C. utilis* transporter has a much higher substrate affinity than the *S. pombe* transporter with a K_m of 0.11 mM (Saayman *et al*., 2000) as apposed to 3.7 mM for *S. pombe* (Osothsilp and Suben, 1986[b]). Further characterisation of the dicarboxylic acid transporter from *C. utilis* and comparison with the *S. pombe* malate transporter, showed that these two transporters have different substrate specificities, i.e. *S. pombe* cells only transport L-malate whereas *C. utilis* cells transport both L-malate and fumarate. Furthermore, the kinetic data indicated that L-malate and fumarate are transported by the same carrier protein in *C. utilis*. It therefore seems likely that the *C. utilis* transporter may be able to also recognise and transport other dicarboxylic acids.

These findings raised the possibility of industrial applications of the *C. utilis* dicarboxylic acid transporter. The *C. utilis* dicarboxylic acid transporter may be considered as an alternative to the *S. pombe* L-malate transporter gene, *mae1*, to introduce L-malate degradation in *Saccharomyces cerevisiae* via heterologous expression. Another possible application would be the bioconversion of fumarate, a highly insoluble compound present in high concentrations in the effluents of certain chemical industries. In general, fumarate is removed from these effluents by incinerating of the fumarate precipitate, a procedure that does not only pose an environmental hazard, but is also costly. A biological alternative to remove the excess fumarate and convert it into L-malate, an important commercial chemical used in the food and wine industry, would be more cost-effective and environmentally friendly.

Fumarate is a C_4-dicarboxylic acid that serves as an intermediate of the tricarboxylic acid (TCA) cycle, which is completed with the conversion of succinate to oxaloacetate. Three reactions are involved in this conversion:

1. Conversion of succinate to fumarate catalysed by succinate dehydrogenase. This enzyme is stereospecific with only the *trans*-isomer fumarate produced, and not the *cis*-isomer maleate.

2. Hydration of fumarate to malate catalysed by fumarate hydratase, also known as fumarase.
3. Reduction of L-malate to oxaloacetate via malate dehydrogenase.

In order to achieve the biological conversion of fumarate to L-malate in yeast, two important proteins are required: a fumarate transporter for the uptake of fumarate, and the fumarase enzyme for the enzymatic conversion. The yeast *S. cerevisiae*, well known as a host for the expression of heterologous proteins, contains a fumarase gene, but lacks a transport system for the uptake of fumarate. Effective degradation of high concentrations of extracellular fumarate will therefore require heterologous expression of a fumarate transporter protein in *S. cerevisiae*. Together with the overexpression of the native *S. cerevisiae* fumarase gene (*FUM1*), this could provide the yeast cell with a strong influx of fumarate, as well as a high intracellular concentration of the enzyme required to convert the fumarate into L-malate. When introduced into industrial effluents containing high concentrations of fumarate, the yeast should therefore be able to effectively convert fumarate to L-malate.

The following sections will describe a number of strategies employed for the cloning of the *C. utilis* dicarboxylic acid transporter, as well as those used for overexpression of the *S. cerevisiae FUM1* gene. Please note that this is presented as a compilation of successive strategies and not written according to a style suitable for publication.

7.2 CLONING OF THE *C. UTILIS* DICARBOXYLIC ACID TRANSPORTER

Only a few transporter proteins have been cloned from yeast and the DNA sequences of these show very little homology. Although the dicarboxylic acid transporters of *C. utilis* and *S. pombe* are responsible for the transport of L-malate, DNA sequence analyses of the respective genes may explain the differences in substrate specificities. To avoid problems that *S. cerevisiae* may encounter with expression and post-transcriptional regulation of *C. utilis* genes, it was decided to construct a cDNA library of *C. utilis* under regulation of *S. cerevisiae PGK1* promoter and terminator sequences that will allow the constitutive expression of the *C. utilis* dicarboxylic acid transporter in *S. cerevisiae*.

7.2.1 Construction of *C. utilis* cDNA Library

Construction of Expression Vector, pMH107. Standard molecular techniques were performed essentially as described by Ausubel *et al*. (1995). The expression vector pHVX2

(Volschenk *et al*., 1997[a]) was modified by removing the *Sal*I and *Xba*I sites and subcloning of a synthetic oligonucleotide, containing various restriction sites, as a *Eco*RI - *Xho*I fragment, resulting in pMH107 (Figure 7.1). A *C. utilis* cDNA library was directionally cloned into the *Sal*I - *Not*I sites of pMH107 under control of the constitutive *PGK1* promoter and terminator sequences of *S. cerevisiae.*

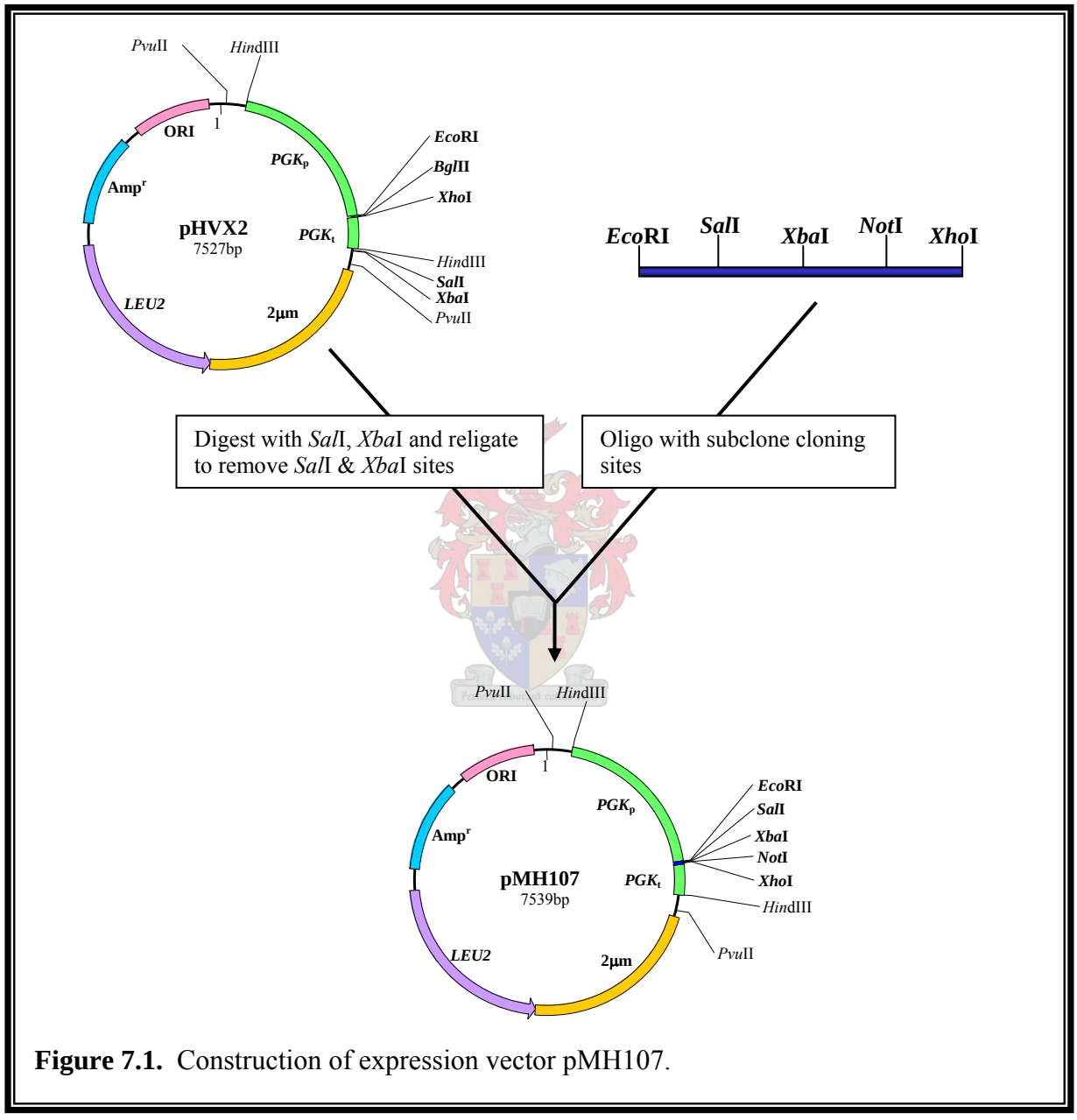

Figure 7.1. Construction of expression vector pMH107.

Isolation of mRNA. Since the *C. utilis* dicarboxylic acid transporter is subject to glucose repression and substrate induction (Cássio and Leão, 1993; Saayman *et al*., 2000), cells of *C. utilis* ATCC 9950 T were cultured at 30ºC to an optical density of 0.6 at 600nm in 100 ml selective YNB media containing 0.5% L-malate as sole carbon source, to ensure that the genes

necessary for L-malate metabolism will be transcribed. Total RNA was isolated with the FastRNA Pro Red Kit (Bio 101, Carlsbad, CA) and mRNA was prepared from 2 mg total RNA with the Gibco BRL MessageMaker mRNA Isolation System (Life Technologies, Inchinnan, UK). The mRNA was eluted with 2 ml DEPC-treated water at 65ºC and purified with oligo(dT)-cellulose.

Synthesis of cDNA. First strand cDNA synthesis was done for 1 hr at 42 ˚C with a reaction mixture containing 5 µg mRNA, 50 µg/ml *Not*I primer-adapter, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 µM each dATP, dCTP, dGTP and dTTP, 10 µCi [α-^{32}P]-dATP and 50 000 U/ml SUPERSCRIPT RT (Life Technologies, Inchinnan, UK). The mRNA and *Not*I primer-adapter (blunt-end) was first heated to 65 ˚C for 10 min and immediately chilled on ice. Before addition of the enzyme, the rest of the mixture was heated to 42 ˚C for 2 minutes to equilibrate. Second strand synthesis proceeded for 2 hours at 16 ˚C (reaction mixture containing 25 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM β-NAD^+, 250 µM each dATP, dCTP, dGTP and dTTP, 1.2 mM DTT, 65 U/ml DNA ligase, 250 U/ml DNA polymerase I and 13 U/ml RNase H). The cDNA overhangs were filled to blunt ends (2 min, 16˚C with 10 U T_4 DNA polymerase) and the reaction stopped with the addition of 30 mM EDTA. The cDNA was now ready for *Sal*I adaptor (sticky end) ligation, which was done for 16 hours at 16 ˚C (reaction mixture containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 5 % (w/v) PEG 8000, 1 mM DTT, 200 µg/ml *Sal*I adapters and 100 U/ml T_4 DNA ligase).

To enable the cloning of the corresponding cDNA fragments, the cDNA was digested with *Not*I (2 hours, 37 ˚C) and size fractionated by SEPHACRYL S-500 HR gel-exclusion chromatography (Life Technologies, Inchinnan, UK). Fractions containing cDNA fragments between 1.0 and 4.0 kb were ligated into the *Not*I – *Sal*I sites of vector pMH107 and transformed into LIBRARY EFFICIENCY DH5α Competent Cells (F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44n λ^- *thi*-1 *gyr*A96 *rel*A1) using standard heat shock transformation procedures (Ausubel *et al*., 1995).

This procedure was repeated nine times, producing 1.3 x 10^5 to 1.5 x 10^6 clones per µg DNA, but only had 60% inserts ≥ 200 bp (Table 7.1).

Table 7.1. Summary of best results for construction of *C. utilis* cDNA library

	Specification	Actual Results
Number of clones / ml	$\geq 3 \times 10^6$	1.5×10^6
Average insert size	$\geq$ 1kb	1.5 kb
% Inserts $\geq$ 200 bp	87 %	60 %

7.2.2 Commercial Production of a cDNA Library

Life Technologies (www.invitrogen.com) was approached for the commercial production of a *C. utilis* cDNA library. To ensure that the genes necessary for L-malate metabolism will be transcribed, cells of *C. utilis* were cultured to an optical density of 0.6 at 600nm in 200 ml selective YNB media containing 0.5% L-malate at 30˚C. Cells were harvested by centrifugation, washed three times with 30 ml 0.85% NaCl and flash-frozen in liquid nitrogen. It was shipped on dry ice to Life Technologies for cDNA preparation and directional cloning into the *Not*I-*Sal*I sites of plasmid pEXP-AD502 (Figure 7.2). The library contained 100% inserts of more than 200 bp with an average insert size of 1.844 bp (Table 7.2). Transformation of the cDNA into *E. coli* yielded 1.07×10^7 cfu/ml.

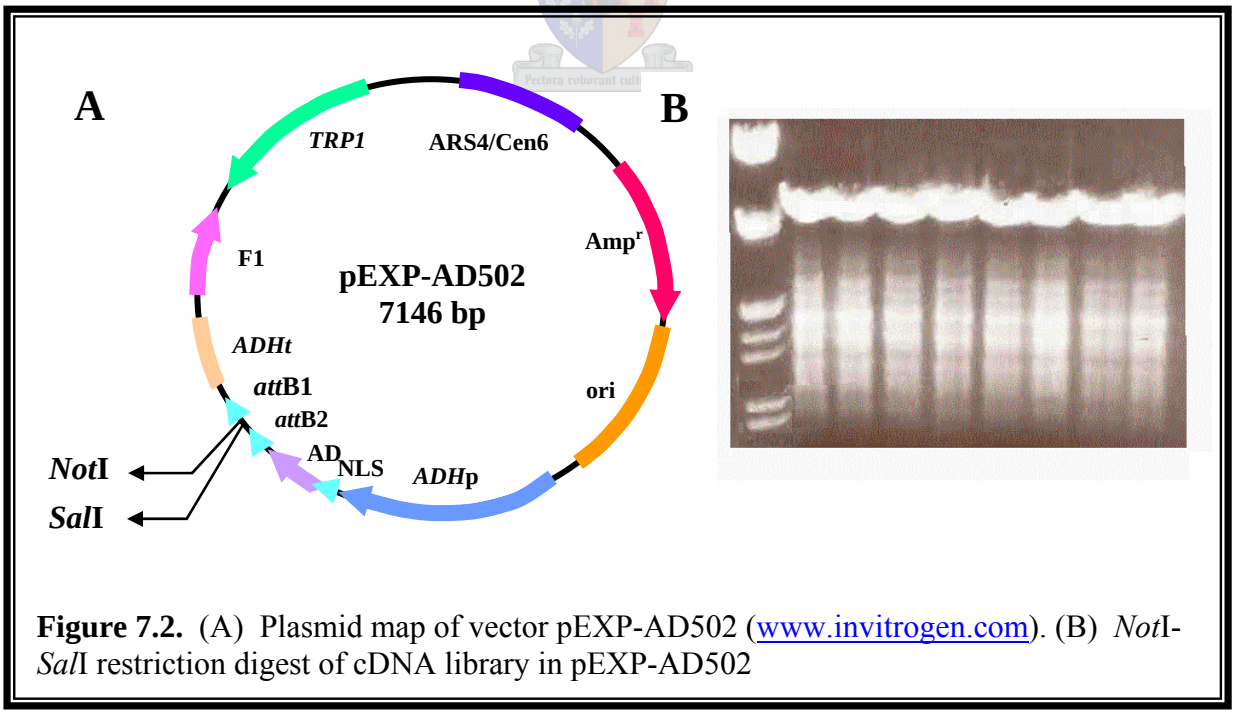

Figure 7.2. (A) Plasmid map of vector pEXP-AD502 (www.invitrogen.com). (B) *Not*I-*Sal*I restriction digest of cDNA library in pEXP-AD502

Table 7.2. Results of commercial cDNA library

	Specification	Commercial Library
Number of clones / ml	$\geq 3 \times 10^6$	1.07×10^7
Average insert size	≥ 1kb	1.844 kb
% Inserts ≥ 200 bp	87 %	100 %

To amplify the library, 4×10^5 primary cDNA transformants were inoculated into 450 ml 2x LB medium (37˚C) containing 1.35 g SeaPrep (FMC BioProducts, Rockland, Maine, USA) agarose and 200 µg Ampicillin. It was incubated in an ice water bath for 1 hr, followed by 30˚C for 45 hours. The cells were collected by centrifugation at 8000 rpm for 20 min at room temperature and resuspended in 25 ml 2x LB medium containing 12.5% glycerol. The amplified library yielded 1.15×10^9 cfu/ml.

Plasmid DNA was isolated from the cDNA library (Ausubel *et al*., 1995) and transformed into LiOAc-competent cells of *S. cerevisiae* YPH259 (*MAT*α, *ura*3-52, *lys*2-801^{amber}, *ade*2-101^{ochre}, *his*3Δ200, *leu*2-Δ1) carrying plasmid pHV4, which contains the *S. pombe* malic enzyme gene (*mae2*) (Volschenk *et al*., 1997[a]). Transformants were screened on pH-sensitive modified Glucose-Malate- Indicator Agar (GMIA) plates (Volschenk *et al*., 2004) containing 2% (w/v) of various carbon sources (Table 7.3) together with 0.5% malate as inducer.

Approximately 4.95×10^5 colonies were screened, but no positive colonies were obtained (i.e. discolouring of pH-sensitive GMIA medium that would indicate a shift in pH). The cloning plasmid (pEXP-AD502) contains a nuclear localisation signal that could target the expressed protein to the nucleus instead of the cell membrane. To overcome this problem, the cDNA library was subcloned into the *Sal*I – *Not*I sites of plasmid pMH107 (Figure 7.1). This yielded very low transformation frequencies (Table 7.3) and although approximately 9.9×10^4 colonies were screened, no positive clones were observed.

Table 7.3. Transformation frequencies of the *C. utilis* cDNA library into *S. cervisiae*.

	Colony Forming Units (cfu) / µg DNA				
Plasmid	**Glucose**	**Raffinose**	**Glycerol/Ethanol**	**Galactose**	**Sucrose**
pEXP-AD502	5 210	1628	6773	3047	4062
pMH107	208	120	94	130	105

7.2.3 Gateway Cloning Technoloy

The low transformation frequencies obtained with *S. cerevisiae* and the absence of any positive clones in this study led us to investigate alternative strategies. Conventional cDNA cloning protocols typically include the use of a restriction enzyme cleavage step for directional cloning, which may result in overlooking cDNA clones that happen to have an internal restriction site that is also cleaved by the same restriction enzyme. Recently, the GATEWAY Cloning Technologywas developed (Hartley *et al*., 2000; Walhout *et al*., 2000) for cloning and subcloning of DNA sequences, facilitating gene functional analysis and protein expression. DNA segments are transferred between vectors using phage lambda-based site-specific recombination instead of restriction endonucleases and ligase. This recombination system is used by phage λ during the switch between the lytic and lysogenic pathways. This powerful system can easily transfer one or more DNA sequences into multiple vectors in parallel reactions, while maintaining orientation and reading frame.

The key DNA recombination sequences (*att* sites) and proteins that mediate the recombination reactions are the foundation of GATEWAY Cloning Technology. Two reactions constitute the GATEWAY System: (1) The BP reaction uses reaction sites *att*B and *att*P to generate a product flanked by *att*L1 and *att*L2, and (2) the LR reaction uses reaction sites *att*L and *att*R to generate a product flanked by *att*B1 and *att*B2 (Table 7.4). The reactions are conservative, i.e., there is no net synthesis or loss of nucleotides - the DNA segments that flank the recombination sites are merely switched. The recombination sites of each vector comprise a hybrid sequence, donated by the sites on the parental vectors. The recombination can occur between DNA's of any topology (supercoiled, linear or relaxed), although efficiency varies.

Table 7.4. Summary of GATEWAY reactions and nomenclature.

Reaction	Reaction Sites	Catalysed by	Product	Structure of Product
BP Reaction	*att*B x *att*P	BP Clonase Enzyme Mix	Entry Clone	*att* L1-gene-*att*L2
LR Reaction	*att*L x *att*R	LR Clonase Enzyme Mix	Expression Clone	*att*B1-gene-*att*B2

The BP reaction is a recombination reaction between an Expression Clone, in this case pEXP-AD502, and a Donor Vector (pDONR201; Life Technologies, Inchinnan, UK) (Figure 7.3A). Homologous recombination then occurs between the B1, B2 and P1, P2 sites, by means of the BP Clonase Enzyme. This system has a dual selection system. The expression clone contains an ampicillin resistance gene whereas the donor vector has a kanamycin resistance gene. Furthermore, the Donor Vector carries the *ccdB* gene that interferes with *E. coli* DNA gyrase and thereby inhibits growth of most *E. coli* strains. The end result is a byproduct containing the *ccdB* gene on the ampicillin resistant vector, and an Entry Clone carrying the cDNA products on the kanamycin resistant vector. Positive colonies are selected on kanamycin-containing LB-plates.

The next step is the LR reaction, a recombination reaction between the Entry Clone generated in the previous step and a Destination Vector, to create an Expression Clone (Figure 7.3B). The Destination Vector also contains the *ccdB* gene. After homologous recombination by means of the LR Clonase enzyme gene, the end result is the cDNA genes on the ampicillin resistance vector and the *ccdB* gene on the kanamycin resistance vector. Positive clones can then be selected for on ampicillin-containing LB-plates.

Constructing a new Destination Vector for cDNA-library. For any vector to serve as a Destination Vector, it must have *att*R sites flanking the *ccd*B gene. For the *C. utilis* cDNA library, vector pHVX2 (Volschenk *et al*., 1997[a]) was converted to a GATEWAY Destination Vector by ligating a blunt-ended cassette, containing *att*R sites, the *ccd*B gene and a chloramphenicol resistance marker, into the *Eco*RI - *Xho*I sites of pHVX2 (Figure 7.4). After linearisation of pHVX2 with *Eco*RI and *Xho*I, the ends were blunted by a Klenow fill-in reaction (Ausubel *et al*., 1995), after which the blunt-end cassette (*att*R1-Cm^r-*ccdB*-*att*R2) was ligated into the vector, resulting in Destination Vector pMH108.

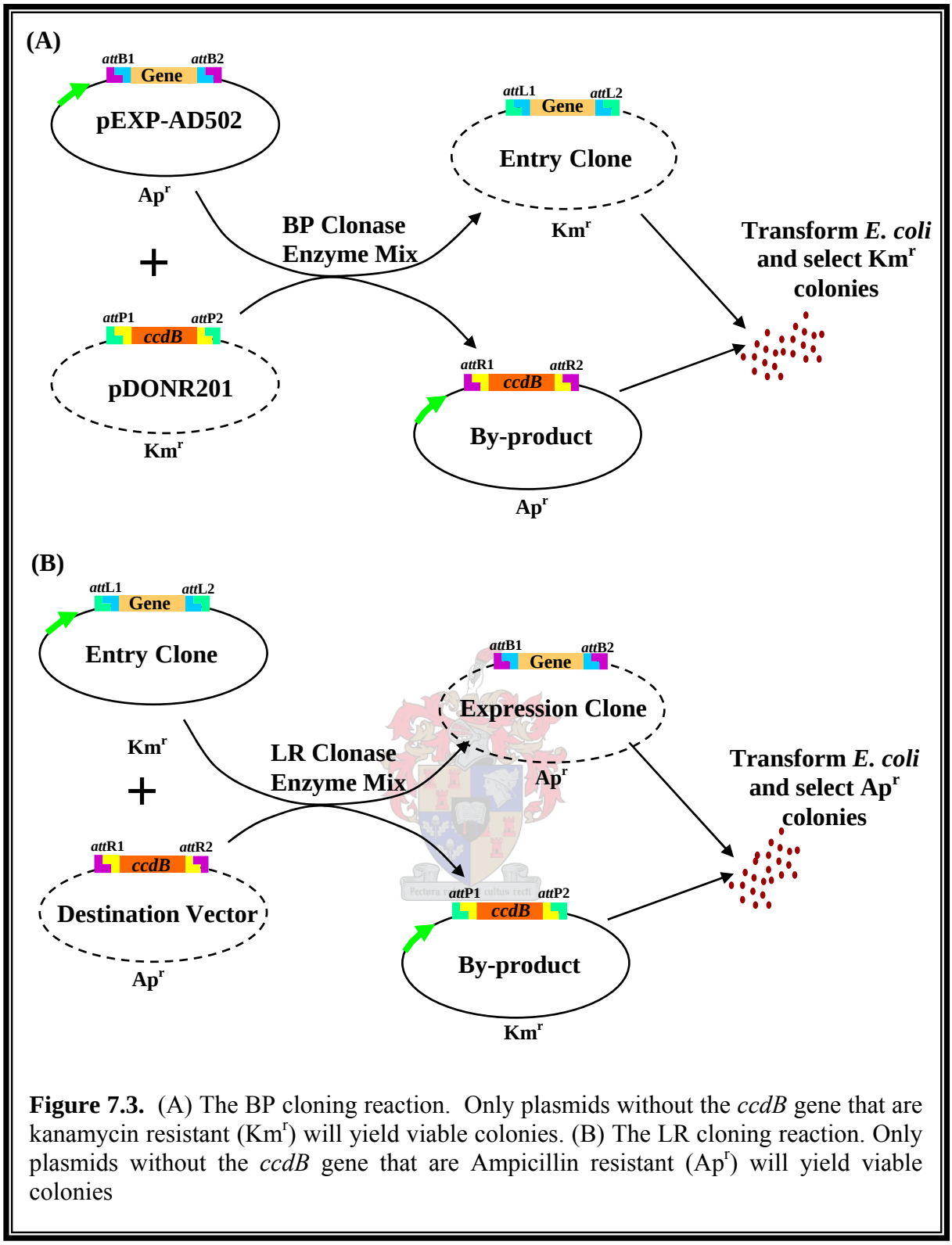

Figure 7.3. (A) The BP cloning reaction. Only plasmids without the *ccdB* gene that are kanamycin resistant (Km^r) will yield viable colonies. (B) The LR cloning reaction. Only plasmids without the *ccdB* gene that are Ampicillin resistant (Ap^r) will yield viable colonies

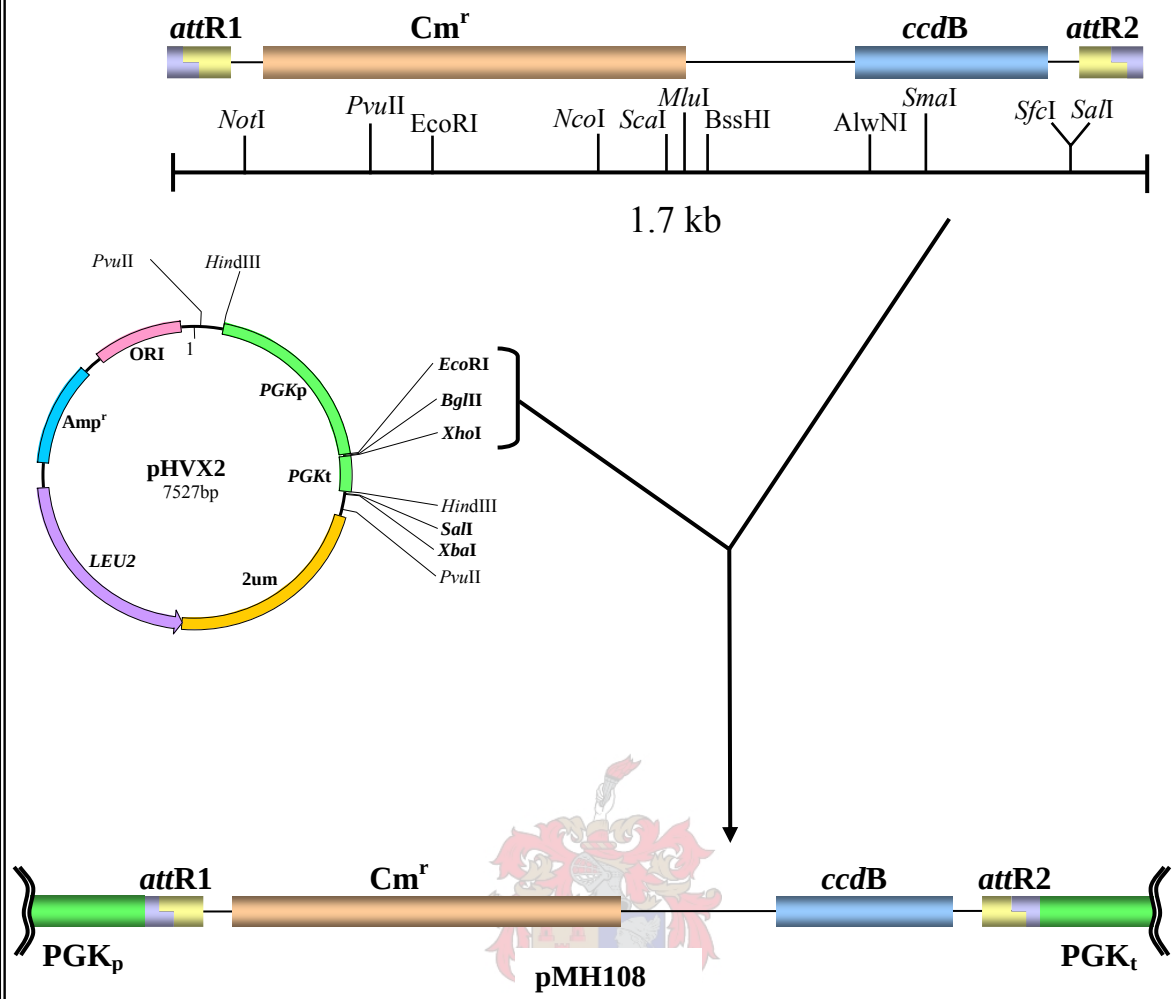

Figure 7.4. Construction of Destination Vector pMH108.

Plasmid pMH108 was propagated in LIBRARY EFFICIENCY DB3.1 competent cells (F^- *gyr*A462 *end*A1 D(*sr*1 - *rec*A) *mcr*B *mrr hsd*S20 (r_B^-, m_B^-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*-5 λ^- *leu mtl*-1) (Life Technologies, Inchinnan, UK), containing a gyrase mutation (*gyr*A462). The ccdB protein interferes with *E. coli* DNA gyrase and thereby inhibits growth of most *E. coli* strains. Strains of *E. coli* that contain an F' episome also carry the *ccd*A gene that encodes an antidote to ccdB protein toxicity. Therefore, strains with F episomes must not be used for selection following BP or LR reactions.

The cDNA library of *C. Utilis* was subcloned into the new Destination Vector, pMH108, by means of GATEWAY Cloning Technology and transformed into *S. cerevisiae* Y294 carrying plasmid pHV4 (containing the *S. pombe* malic enzyme gene, *mae2*). Good transformations frequencies were obtained (Table 7.5) and 6.0 x 10^5 *S. cerevisiae* colonies were screened on

pH-sensitive GMIA plates containing 0.5% malate together with 2% (w/v) glucose, rafffinose, glycerol/ethanol, galactose or sucrose, but no positive colonies were obtained.

Table 7.5. Summary of transformation frequencies for plasmid pMH108

	Colony Forming Units (cfu) / µg DNA				
Plasmid	**Glucose**	**Raffinose**	**Glycerol/Ethanol**	**Galactose**	**Sucrose**
pMH108	12 250	8 750	6 550	9 800	11 760

7.2.4 Expression of cDNA Library under Inducible GAL1-Expression

pYES-DEST52 - Destination Vector. The *C. utilis* cDNA library was also subcloned into the commercial plasmid pYES-DEST52 (Figure 7.5) by means of GATEWAY Cloning Technology. The Destination Vector pYES-DEST52 is a GATEWAY-adapted vector for cloning and inducible *GAL1*-expression in *S. cerevisiae*. The pYES-DEST cDNA library was again transformed into *S. cerevisiae* Y294 containing the *S. pombe* malic enzyme gene, *mae2*. Approximately 5.0 x 10^5 colonies were screened on plates containing 2% raffinose and 0.5% galactose, or 0.5% raffinose and 2% galactose, with transformation frequencies of 12 355 and 12 950 cfu/µg DNA, respectively, with no positive colonies obtained.

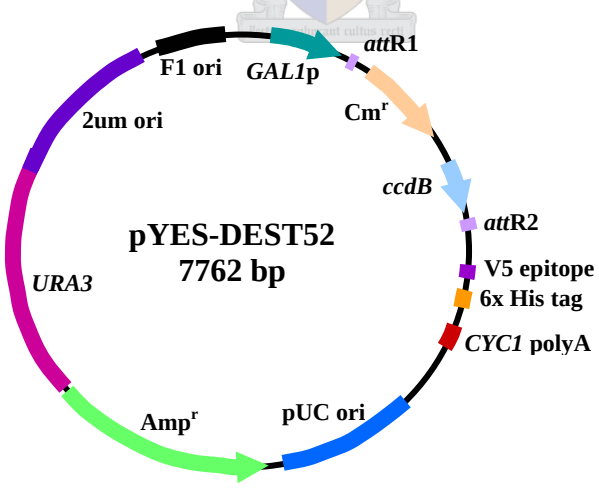

Figure 7.5. Plasmid map of vector pYES-DEST52 (www.invitrogen.com).

7.3 BIO-CONVERSION OF FUMARATE TO L-MALATE

7.3.1 Over-expression of *FUM1*

As mentioned previously, fumarase catalyses the interconversion of fumarate and L-malate as part of the TCA cycle. Like most TCA cycle enzymes, fumarase is located in the matrix compartment of mitochondria in *S. cerevisiae*. However, two compartmentally distinct forms of fumarase have been suggested, namely mitochondrial and cytosolic fumarase isoenzymes, encoded by a single nuclear gene, *FUM1* (Wu and Tzagoloff, 1987). The mitochondrial isoenzyme is probably processed by removal of a signal peptide during translocation of the protein from the cytosol through the mitochondrial membranes. Very little research has been done to elucidate the mechanism and function of fumarase distribution between subcellular compartments in *S. cerevisiae*. We therefore cloned the *FUM1* gene with and without the signal peptide to over-express both fumarase isoenzymes.

Experimental strategy. DNA manipulations were performed in the expression vector pHVX2 (Volschenk *et al*., 1997[a]), a derivative of YEplac181, containing the *PGK1* promoter and terminator sequences. The *FUM1* open reading frame (ORF), with or without the signal peptide, was isolated from *S. cerevisiae* genomic DNA as a *Xho*I fragment by means of PCR, and sub-cloned into the *Xho*I site of pHVX2 (Figure 7.6). pMH102 contains the complete *FUM1* ORF of 1.5 kb (mitochondrial isoenzyme), and pMH103 contains the *FUM1* gene without the proposed mitochondrial import signal of 69 bp (cytosolic isoenzyme). *E. coli* strain DH5α was transformed by electroporation and transformants were selected on LB medium supplemented with ampicillin.

7.3.2 Secretion of Fumarase Enzymes

When it became apparent that the cloning of a *C. utilis* dicarboxylic acid transporter was more difficult than anticipated, alternative strategies were investigated for the expression of *FUM1*. One of these was the possible secretion of the fumarase enzyme by means of the *MFα1* secretion signal, which would allow the extracellular degradation of fumarate to L-malate. Since little is known about the extracellular activity of fumarase, both the mitochondrial and cytosolic open reading frames were subcloned and constitutively expressed under regulation of the *PGK1* promoter and terminator sequences in pHVX2.

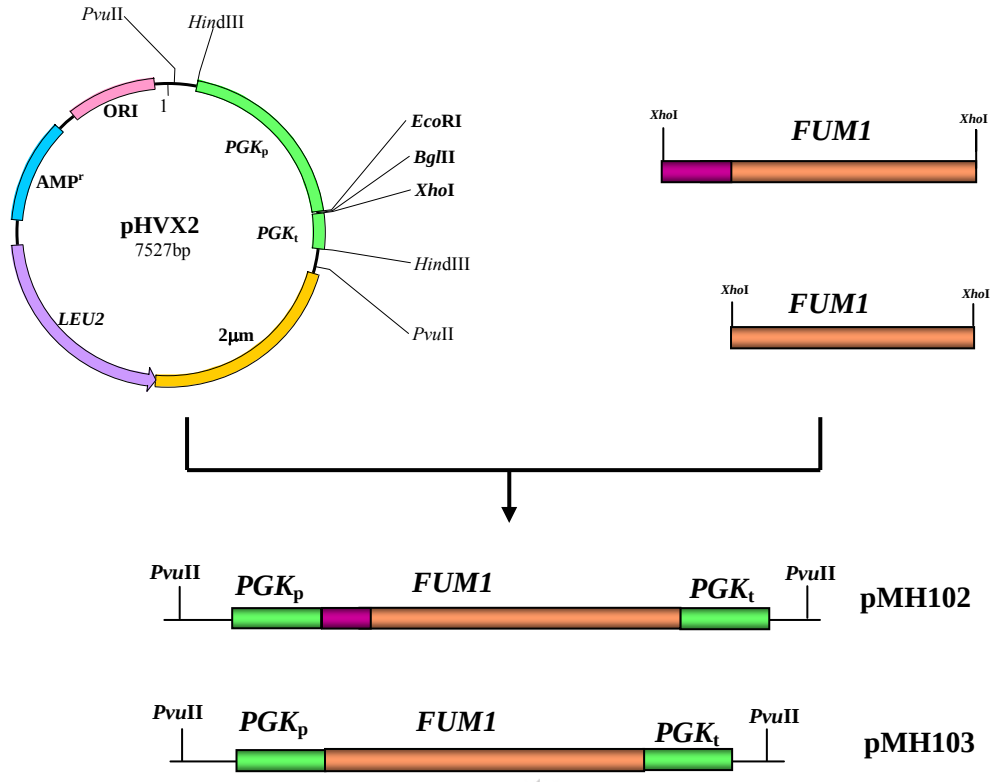

Figure 7.6. Strategy for cloning of the mitochondrial and cytosolic *FUM1* isozymes under control of the *PGK1* promoter and terminator sequences in pHVX2.
■, mitochondrial leader peptide, ■, *FUM1* ORF.

Experimental strategy. The *MFα1* secretion signal from plasmid pPRL1 (Van Rensburg *et al*., 1995) was sub-cloned into the *Eco*RI - *Xho*I site of vector pHVX2, resulting in plasmid pAM100 (Figure 7.7). The *FUM1* gene was subcloned into the *Xho*I site of pAM100, resulting in plasmids pMH105 and pMH106 containing the mitochondrial and cytosolic fumarase genes, respectively, linked to the *MFα1* secretion signal under control of the *PGK1* promoter and terminator sequences. Plasmids pMH105 and pMH106 were transformed into *S. cerevisiae* Y294 (*α leu2-3, 112 ura3-52, his3, trp1-289*) and transformants were selected on selectice YNB agar plates (0.17% Yeast Nitrogen Base without amino acids and ammonium sulphate [Difco Laboratories, Detroit, MI]) supplemented with amino acids as required. Cells cultured in media containing 1% glucose, 2% bactopeptone, 1% yeast extract and 0.3% fumarate were harvested at different time intervals during growth and the supernatant analysed by means of HPLC to determine the extracellular conversion of fumarate to L-malate.

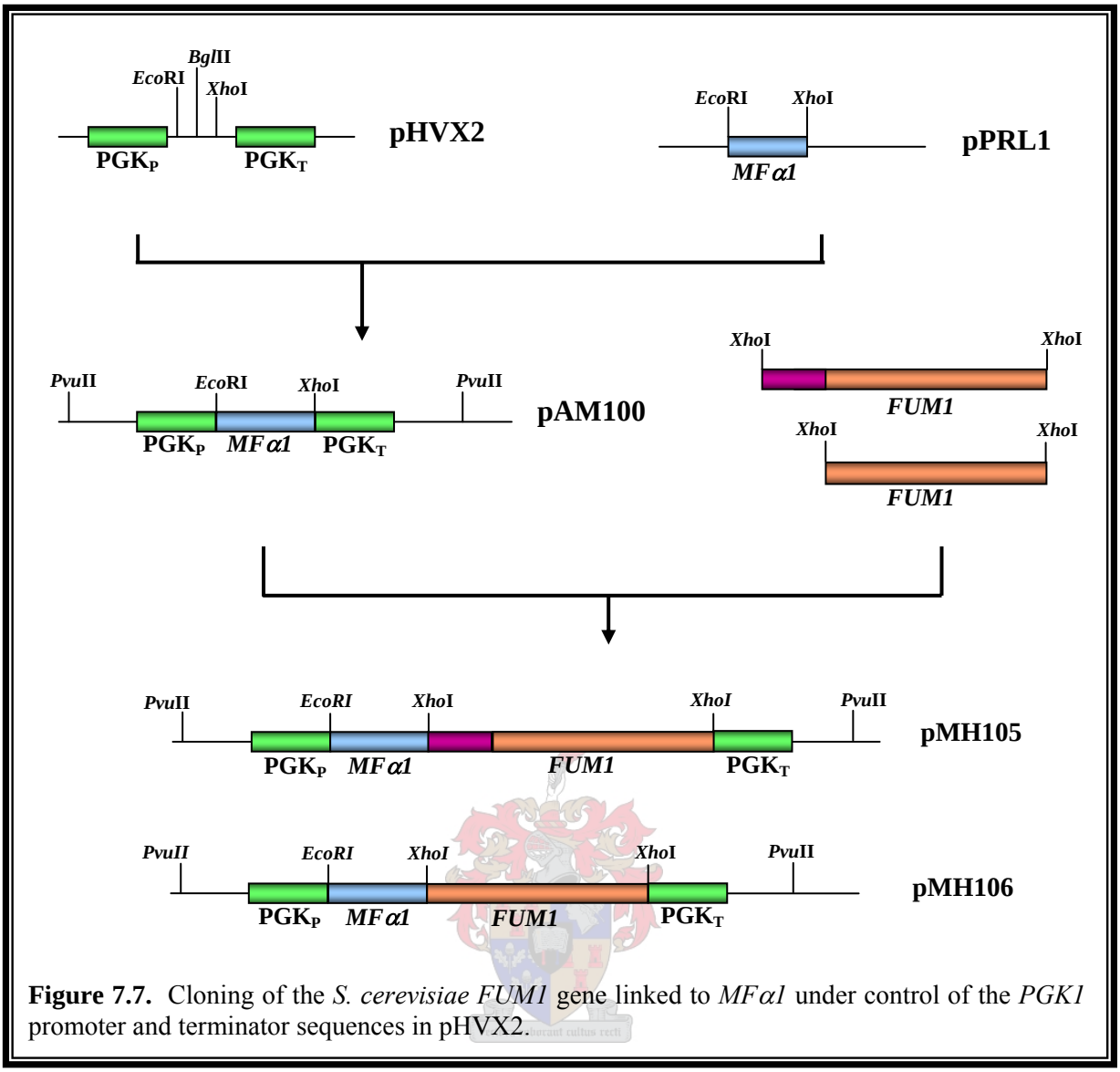

Figure 7.7. Cloning of the *S. cerevisiae FUM1* gene linked to *MFα1* under control of the *PGK1* promoter and terminator sequences in pHVX2.

Results. As shown in Figure 7.8, small amounts of L-malate were produced by all the recombinant strains (less than 0.1 g/l) after 72 hrs. The *S. cerevisiae* host strain transformed with the pAM100 vector only and pMH105 (mitochondrial fumarase enzyme) degraded similar amounts of fumarate (26% and 25% after 72 hrs, respectively). It is possible that the fumarase enzyme was still targeted to the mitochondria, despite the presence of the *MFα1* secretion signal. Although the cytoplasmic enzyme (pMH106 in Figure 7.8C) was able to provide a more effective degradation of fumarate (1.7 g/l after 72 hrs), the amount of L-malate produced was similar to the other two strains (less than 0.5 g/l).

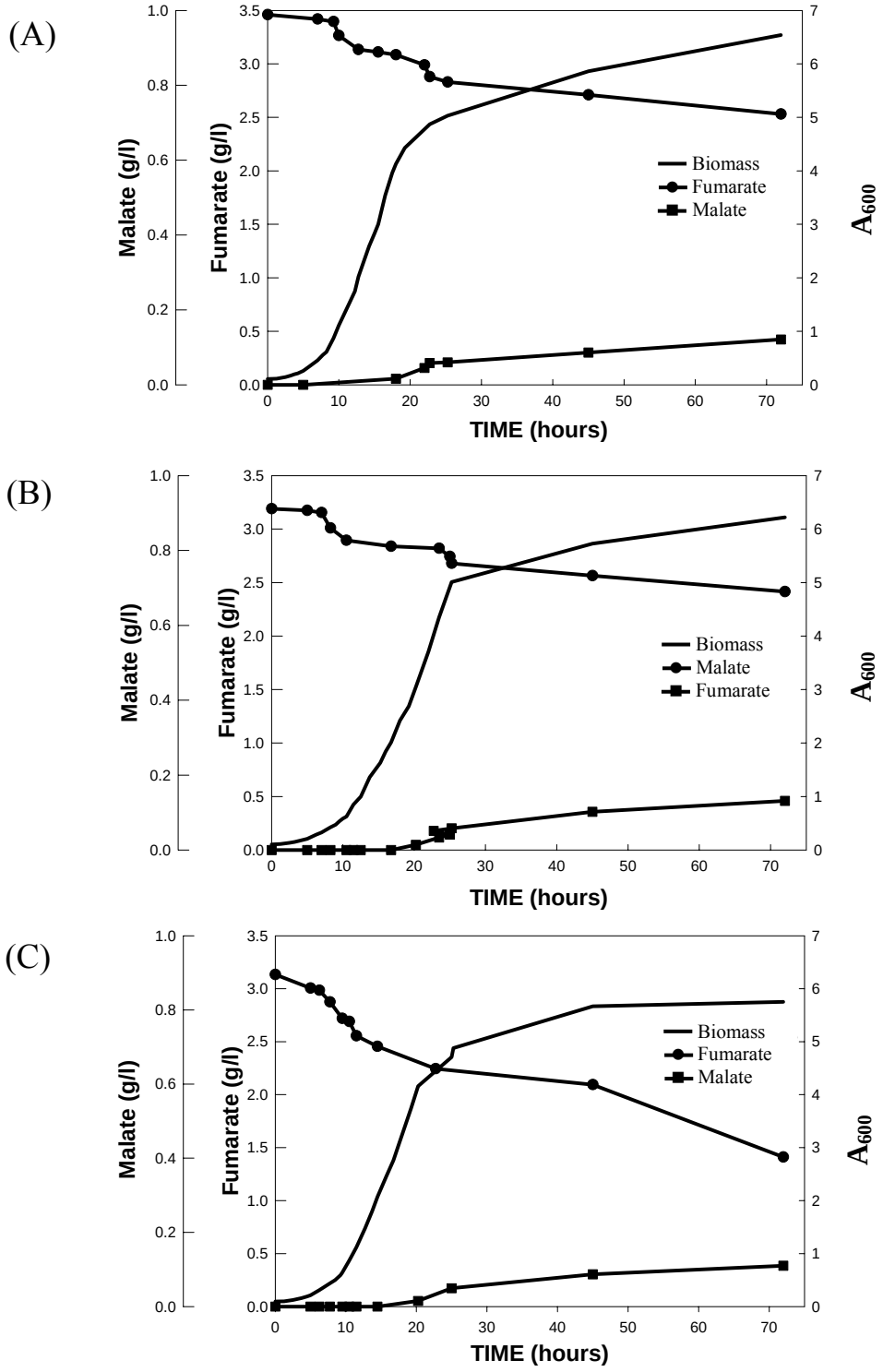

Figure 7.8. Degradation of extracellular fumarate, and the formation of malate by *S. cerevisiae* containing (A) pAM100 (no *FUM1* insert), (B) the *MFα1* secretion signal linked to the mitochondrial fumarase enzyme (pMH105), and (C) the *MFα1* secretion signal linked to the cytosolic fumarase enzyme (pMH106).

7.4 CONCLUDING REMARKS

Only a few dicarboxylic acid transporters have been cloned from yeast thus far. This includes the *S. pombe* malate transporter (Grobler *et al*., 1995) and the *Kluyveromyces lactis* dicarboxylic acid transporter, encoded by *KlJEN2* (Lodi *et al*., 2004). The *C. utilis* malate/fumarate transporter is highly effective (K_m of 0.11 mM for fumarate), but the protein is subject to glucose repression and substrate induction (Saayman *et al*. 2000). If the *C. utilis* malate/fumarate transporter gene could be cloned, it would provide the possibility of heterologous expression of the gene under constitutive or induced regulation for various commercial applications.

In this study, several strategies were employed to clone the *C. utilis* malate/fumarate transporter gene. The low level of homology observed for those transporters already known did not favour the selection of conserved regions for the design of homologous or even degenerate primers. This strategy was, however, successful for the cloning of the *C. utilis* malic enzyme gene (see Chapter 8) due to the high level of homology between malic enzymes in general (Viljoen *et al*., 1994). It was therefore decided to attempt cloning of the *C. utilis* malate/fumarte transporter gene from a cDNA library constructed from cells grown in medium containing L-malate as sole carbon source, to increase the copy number of the relevant gene. Two host strains were considered, namely the *S. pombe mae1*$^-$ strain (deficient in malate transport) or the recombinant *S. cerevisiae* strain carrying the *S. pombe* malic enzyme gene, *mae2*. The best candidate was the recombinant *S. cerevisiae-mae2* strain, since the low transformation frequencies normally associated with *S. pombe*, made it an unlikely candidate for screening of a library.

A cDNA library from *C. utilis* was constructed employing a number of strategies to ensure representativeness and high transformation frequencies. Several expression vectors containing *S. cerevisiae* promoter and terminator sequences were constructed to allow the expression of the *C. utilis* malate/fumarate transporter in either a constitutive or regulated fashion. The cDNA library was transformed into an *S. cerevisiae* strain carrying a plasmid containing the *S. pombe* malic enzyme gene (*mae2*) to allow screening for a malate-degrading *S. cerevisiae* clone. Although a good representative library was constructed using the GATEWAY Cloning Technology, combined with good transformation frequencies for the *S. cerevisiae-mae2* strain, no positive clones (i.e. malate-degrading) were detected during screening on various modified GMIA-plates.

The failure to clone the *C. utilis* malate/fumarate transporter gene from the cDNA libary could be due to intrinsic problems with the GMIA plates used for the library screen. The GMIA plates were originally developed for screening for *S. pombe* mutants (Subden *et al*., 1986[b]) and subsequently modified by Volschenk *et al*. (2004) to allow for screening of malate-degrading *S. cerevisiae* strains expressing the *S. pombe* malate transporter (mae1p) and malic enzyme (mae2p). It is, however, possible that the recombinant *S. cerevisiae* strain expressing the *C. utilis* malate/fumarate transporter may not be able to degrade the malate in the GMIA plates with the same efficacy as the *S. cerevisiae mae1-mae2* strain, which will therefore result in a deminished pH change in the GMIA plates and hence no detectable colour change.

Effective expression of the *S. pombe* malate transporter in *S. cerevisiae* suggested that the *C. utilis* transporter may also be effective when expressed in *S. cerevisiae*. As was clearly showed in this study, these assumptions don't always take significant differences between the two relevant genes/proteins into consideration. Only a few dicarboxylic acid transporters have been studied in yeast, but it is evident that they don't share the high level of homology that was observed for malic enzymes. It is therefore possible that sequence differences between the *S. pombe* and *C. utilis* transporters may impact on the translation efficiency of the two genes in *S. cerevisiae*, the folding and chanelling of the proteins towards the outer membrane as well as the actual embedding of the protein inside the membrane. Transporter proteins are membrane bound and contains various hydrophilic regions on both sides of the membrane lipid bilayer, connected by hydrophobic membrane-spanning α-helices (Solomon *et al*., 2005). In order for the protein to be functional, it needs to be folded correctly to associated with the membrane and to transport the required substrate. It is also possible that this specific folding pattern may require specific chaperone proteins not present in the *S. cerevisiae* host strain.

One of the possible industrial application for a recombinant *C. utilis* transporter would have been to provide a biological means to remove excess fumarate from effluents of certain chemical industries, and convert it (via fumarase) to L-malate, an important commercial chemical used in the food and wine industry. This could be achieved by expressing the *C. utilis* dicarboxylic acid transporter gene in *S. cerevisiae* together with over-expression of the *S. cerevisiae* fumarase gene (*FUM1*). In anticipation of the successful cloning the *C. utilis* malate/fumarate transporter gene, vectors for overexpression of the *S. cerevisiae FUM1* genes (mitochondrial and cytosolic) isozymes were constructed. However, when it became apparant that the *C. utilis* transporter could not be cloned in the course of this study, an alternative

strategy was investigated, namely to target the *FUM1* gene for secretion under control of the *S. cerevisiae MFα1* secretion signal.

The degradation of extracellular fumarate was improved with the overexpression and secretion of the cytosolic fumarase, but this was not quantitatively converted to L-malate. The low efficiency of the over-expressed *FUM1* gene could be ascribed to a number of reasons, including a lower than expected expression level or the ineffective secretion thereof due to structural problems associated with the fumarase protein that may prohibit it from being transported across the membrane. Since the fumarase is not a protein usually targeted for secretion, it may require specific membrane-associated proteins for translocation across the plasmamembrane.

CLONING, CHARACTERISATION AND HETEROLOGOUS EXPRESSION OF THE *CANDIDA UTILIS* MALIC ENZYME GENE

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ABSTRACT

The *Candida utilis* malic enzyme gene, *CuME*, was isolated from a cDNA library and characterised on a molecular and biochemical level. Sequence analysis revealed an open reading frame of 1926 bp, encoding a 641 amino acid polypeptide with a predicted molecular weight of approximately 70.2 kDa. The optimum temperature for the *C. utilis* malic enzyme activity was 52°C and the enzyme was stable at 50°C for 2 hours. The inferred amino acid sequence suggested a cytosolic localisation for the malic enzyme, as well as 37% and 68% homology with the malic enzymes of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively. Expression of the *CuME* gene is subject to glucose repression and substrate induction, suggesting similar regulatory pathways than for the *C. utilis* malate/fumarate transporter. The *CuME* gene was successfully expressed in *S. cerevisiae* under control of the *S. cerevisiae PGK1* promoter and terminator. When co-expressed with the *S. pombe* malate permease gene (*mae1*), it resulted in a recombinant strain of *S. cerevisiae* able to degrade 90% of the extracellular L-malate within 24 hours.

Nucleotide sequence data reported are available in the DDBJ/EMBL/Genbank databases under the accession number DQ173437.

8.1 INTRODUCTION

L-Malate is a α-hydroxyl C_4-dicarboxylic acid that serves as an intermediate in the tricarboxylic acid (TCA) cycle in mitochondria of living cells. It is commercially used as a food additive, having greater tartness and taste retention than most other food acids (Neufeld *et al*., 1991). L-Malate is metabolised in various subcellular compartments of the eukaryotic cell and serves a pivotal role in the metabolism of C_3- and C_4-metabolites. The oxidative decarboxylation of L-malate to pyruvate and carbon dioxide (CO_2) is catalysed by a

mitochondrial or cytosolic malic enzyme that requires divalent cations (Mg^{2+} or Mn^{2+}) and $NAD(P)^+$ as cofactors. Three types of malic enzymes are found in nature and are classified by their coenzyme specificity and ability to decarboxylate both L-malate and oxaloacetate (Viljoen *et al*., 1994). The NAD^+-specific L-Malate: NAD^+ oxidoreductase (oxaloacetate-decarboxylating) [EC 1.1.1.38] is usually associated with bacterial cells; L-malate: $NAD(P)^+$ oxidoreductase (decarboxylating) [EC1.1.1.39] prefers NAD^+ to $NADP^+$ as coenzyme and is found in mitochondria, hydrogenosomes and bacteria, while the $NADP^+$-dependent L-malate: $NADP^+$ oxidoreductase (oxaloacetate-decarboxylating) [EC 1.1.1.40] is found in bacteria and the cytosol and plastids of eukaryotic cells (Doležal *et al*., 2004).

Yeast species differ remarkably in their ability to utilise and degrade extracellular L-malate. Cells of *Schizosaccharomyces pombe* and *Candida utilis* are able to effectively transport and degrade extracellular L-malate, whereas *Saccharomyces cerevisiae* cells have to rely on simple diffusion due to the lack of a transport system for L-malate (Volschenk *et al*., 2003). There are also significant differences between the malic enzymes of *S. pombe* and *S. cerevisiae*: the *S. cerevisiae* malic enzyme is localised in the mitochondrion (Boles *et al*., 1998) and has a low substrate affinity for L-malate (K_m = 50 mM) (Fuck *et al*., 1973). In contrast, a cytoplasmic localisation was proposed for the *S. pombe* malic enzyme (Viljoen *et al*., 1999) which also has a much stronger substrate affinity for L-malate (K_m = 3.2 mM) (Temperli *et al*., 1965). Despite the different L-malate degradation abilities of *S. cerevisiae* and *S. pombe*, biochemical and genetic analyses of the relevant enzymes and genes suggested that the *S. cerevisiae* and *S. pombe* malic enzymes both play a role in the provision of pyruvate for cellular biosynthesis (Volschenk *et al*., 2003).

Although cells of *S. pombe* and *C. utilis* can effectively degrade L-malate, the proteins involved are subject to different regulatory mechanisms. In *S. pombe*, L-malate can not serve as sole carbon or energy source and is only metabolised in the presence of glucose or another assimilable carbon source (Baranowski and Radler, 1984; Taillandier and Strehaiano, 1991). In contrast, *C. utilis* can utilise intermediates of the TCA cycle as the only source of carbon and energy, but the degradation and transport of L-malate in *C. utilis* is subject to carbon catabolite repression repression and substrate induction (Cássio and Leão, 1993; Saayman *et al*., 2000). In *C. utilis*, the major pathway for carbon metabolism is via the mitochondrial TCA cycle to generate energy as well as for the biosynthesis of cell material (especially amino acids and lipids). The TCA cycle isozyme families are therefore often critical points for control of metabolic flux (McAlister-Henn and Small, 1997).

The yeast *C. utilis* is an industrially important microorganism that is widely used for the production of biologically useful materials, such as glutathione, certain amino acids and enzymes. It has been utilised in the large-scale production of single-cell protein from biomass-derived sugars, such as sugar molasses and spent sulfite liquor (Boze *et al*., 1994). Furthermore, *C. utilis* has been approved for use as a foodstuff by the US Food and Drug Administration and may therefore be considered for other biotechnological applications in the food and beverage industry.

Effective degradation of extracellular L-malate in *C. utilis* has been ascribed to the active transport of L-malate by the malate/fumarate transporter (Saayman *et al*., 2000), as well as the presence of an active intracellular malic enzyme. The malate/fumarate transporter was reported to be sensitive to catabolite repression and substrate induction by both L-malate and fumarate (Saayman *et al*., 2000), but information on the nature and regulation of the *C. utilis* malic enzyme has been limited. The aim of this study was therefore to clone and characterise the *C. utilis* malic enzyme gene, *CuME*, and determine its relatedness to other malic enzyme genes, especially those of *S. pombe* and *S. cerevisiae*. The regulation of the *C. utilis* malic enzyme was studied on a transcriptional level and the commercial potential of the *CuME* gene was evaluated in a heterologous *S. cerevisiae* strain together with the *S. pombe* malate transporter gene.

8.2 MATERIALS AND METHODS

8.2.1 Strains and Culture Conditions

The bacterial and yeast strains and plasmids used in this study are listed in Table 8.1. Cells of *Escherichia coli* DH5α were transformed by heat shock (Ausubel *et al*., 1995) with selection on LB plates (0.5% yeast extract, 1% NaCl, 1% tryptone) supplemented with 200 mg/l ampicillin. Cells of *S. cerevisiae* were cultured in liquid YPD media (1% yeast extract, 2% bactopeptone, 2% glucose) at 30°C and competent cells (Moreno *et al*., 1991) were transformed or co-transformed with plasmids pHVX2, pHV4, pMH109 and pMH110 (Table 8.1). Transformants were isolated on selective YNB agar plates (0.17% Yeast Nitrogen Base without amino acids and ammonium sulphate [Difco Laboratories, Detroit, MI], 0.5% $(NH_4)_2SO_4$, 2% glucose, 1.7% bacto-agar [Difco Laboratories, Detroit, MI]) supplemented with amino acids as required. Cells of *S. pombe* and *C. utilis* were grown in YNB media, supplemented with amino acids as required, or in YPD media.

Table 8.1. Strains and plasmids used for the isolation and characterisation of the malic enzyme gene of *C. utilis*

Strains	Description	Reference
E. coli DH5α	F^- Φ80d*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	www.invitrogen.com
C. utilis ATCC 9950 T	Wild type strain	Saayman *et al.*, 2000
S. cerevisiae Y294	α *leu*2-3, 112 *ura*3-52 *his*3 *trp*1-289	Crous *et al.*, 1995
S. pombe 972^-	*leu*1-32 h^-	Osothsilp, 1987
S. pombe mae2$^-$	972 h^- *leu*1-32 *mae2*$^-$ LH67	Osothsilp and Subden, 1986[a]
Plasmids	**Description**	**Reference**
pEXP-AD502	Shuttle vector (*TRP1* marker gene), containing the $ADH1_p$-$ADH1_t$ expression cassette	www.invitrogen.com
pYES-DEST52	Shuttle vector (*URA3* marker gene) containing the *GAL1* promoter and *CYC1* terminator	www.invitrogen.com
pJC1	Shuttle vector Yep352 (*URA3* marker gene), containing the $PGK1_p$-$PGK1_t$ expression cassette	Crous *et al.*, 1995
pHVX2	Shuttle vector Yeplac181 (*LEU2* marker gene), containing the $PGK1_p$-$PGK1_t$ expression cassette	Volschenk *et al.*, 1997[a]
pHV3	pHVX2 with *S. pombe mae1* ORF ($PGK1_p$-*mae1*-$PGK1_t$)	Volschenk *et al.*, 1997[a,b]
pHV4	pHVX2 with *S. pombe mae2* ORF ($PGK1_p$-*mae2*-$PGK1_t$)	Volschenk *et al.*, 1997[a]
pMH109	pHVX2 with *CuME* ORF ($PGK1_p$-*CuME*-$PGK1_t$)	This study
pMH110	pJC1 with *mae1* ORF ($PGK1_p$-*mae1*-$PGK1_t$)	This study

Modified GMIA plates containing 0.17% Yeast Nitrogen Base (Difco Laboratories, Detroit, MI), 0.5% $(NH_4)_2SO_4$, 10% glucose, 10% L-malic acid, 0.01% bromocresol green, 2% Noble agar (Difco Laboratories, Detroit, MI) with the pH adjusted to 3.3 with KOH (Volschenk *et al.*, 2004), was used to screen for *S. cerevisiae* transformants with a malo-ethanolic

phenotype. These plates (supplemented with amino acids as required) produce blue colonies with a surrounding blue zone due to a shift in pH from 3.3 to 5.2 units when L-malate is converted to pyruvate.

8.2.2 DNA Manipulations

Standard recombinant DNA techniques were performed 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).

8.2.3 Cloning of *CuME* via PCR

PCR amplification of an internal *CuME* fragment from the *C. utilis* cDNA library constructed in Chapter 7 (Figure 8.1) was done with primers 5'-MH3 and 3'-MH4 (Table 8.2), which were designed based on perceived homology with the *S. pombe* malic enzyme (*mae2*) sequence. The DNA sequence of the PCR fragment was used to design primers 5'-pEXP-AD502F and 3'-CuMERint (5' region of *CuME*) as well as 5'-CuMEFint and 3'-pEXP-AD502R (3' region of *CuME*) to amplify and sequence the remaining *CuME* fragments. The entire *CuME* ORF was then amplified by PCR using primers 5'-CuMalicF and 3'-CuMalicR and subcloned into the pGEM®-T Easy Vector System (Promega Corporation, Madison, USA). Plasmids were transformed into *E. coli* DH5α cells by electroporation and selected on LB medium supplemented with 200 mg/l ampicillin (Ausubel *et al*., 1995).

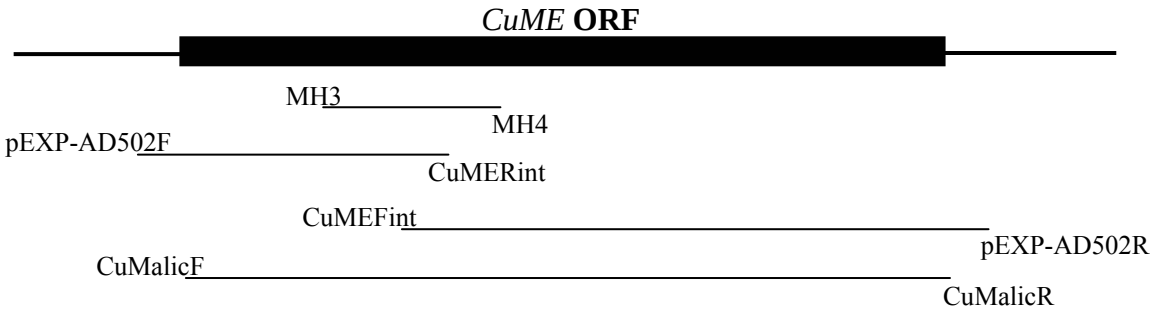

Figure 8.1. Strategy for cloning of the *C. utilis* malic enzyme gene, *CuME*.

Table 8.2. Primers used to isolate and sequence *CuME* ORF and other gene fragments

Primer	Sequence
5'-MH3	5'AAGGAACTAGTTCCTATCATCTATACACCTACC3'
3'-MH4	5'GGCCATCGATACGGCACCGGTTCCCTG GAT3'
5'-pEXP-AD502F	5'TATAACGCGTTTGGAATCACT3'
3'-CuMERint	5'ACACTGGGCTGACTCTACCTGGGT3'
5'-CuMEFint	5'ACCCAGGTAGAGTCATCCCAGTGT3'
3'-pEXP-AD502R	5'GTAAATTTCTGGCAAGGTAGAC3'
5'-CuMalicF	5'GTTTCAACGATGATATCCAGGGAACCGGTGCCGT3'
3'-CuMalicR	5'TCACCTTCGGTAGGTGTATAGATGATAGGA3'
5'-mae1ORF-F	5'GATCGAATTCATGGGTGAACTCAAGGAAATC3'
3'-mae1ORF-R	5'GATCAGATCTTTAAACGCTTTCATGTTCACT3'
5'-MalicF1	5'CCTACTCCCACAGATTTAGA3'
3'-MalicR1	5'GTCTTGTTGGGTTGGACAAT3'
5'-MalicF2	5'TCTCAAGTCCATGAAGGAGA3'
3'-MalicR2	5'GACGATTTGATCAGCAATAC3'
5'-5SF	5'GGTTGCGGCCATATCTAGCAGAAA3'
3'-5SR	5'AGATTGCAGCACCTGAGTTTCGCG3'

Primers 5'-CuMalicF and 3'-CuMalicR were also used for PCR amplification of the genomic copy of the *CuME* ORF from 1 µg genomic DNA isolated from *C. utilis* ATCC 9950 T using the glass bead-phenol extraction (Hoffmann and Winston, 1987). The PCR program consisted of an initial denaturation step at 94ºC for 5 minutes, followed by 30 cycles of 94ºC for 45 seconds, 45ºC for 1 minute and 72ºC for 1.5 minutes. The PCR product was purified and transformed into *E. coli* DH5α cells as described for the cDNA fragment.

8.2.4 DNA Sequencing and Analysis

Plasmid DNA was isolated from three subclones with the High Pure Plasmid Purification Kit (Roche Biochemicals, Germany) and submitted for automated sequencing using various primers as indicated in Figure 8.2. Cycle sequencing reactions were performed using the BigDye 3 V1 Terminator kit (Applied Biosystems) on a GeneAmp PCR system 9700

(Applied Biosystems). Unincorporated terminators were removed and electrophoresis was performed on an ABI PRISM (R) 3100 Genetic Analyser (Applied Biosystems).

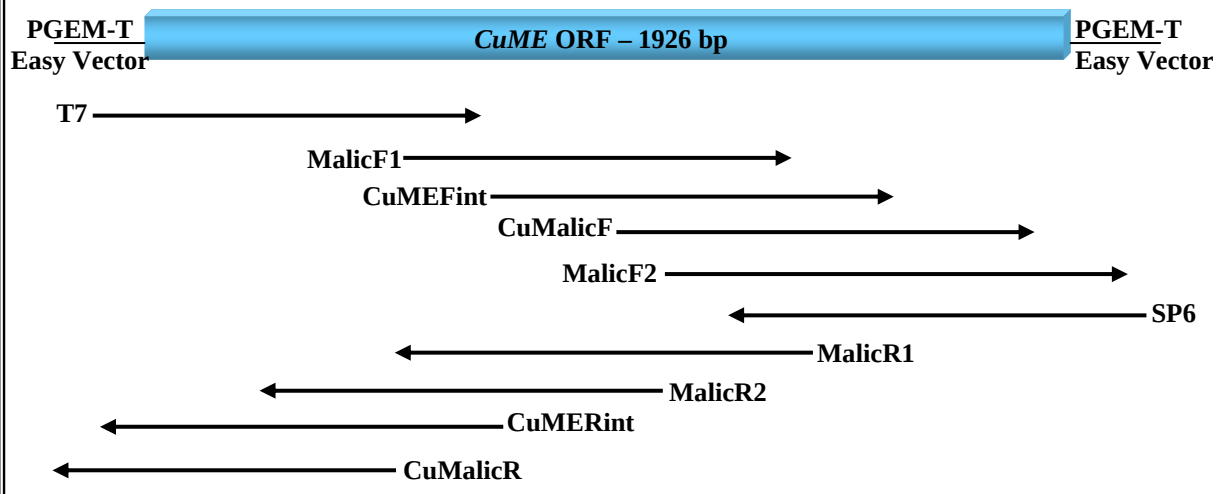

Figure 8.2. DNA sequencing strategy for the coding region of *CuME* (cDNA copy). Arrows indicate the regions that were sequenced from each primer. Both strands were

The nucleotide sequence for the *CuME* gene was submitted to the DDBJ/EMBL/Genbank databases under the accession number DQ173437. The DNA sequence of the *C. utilis* malic enzyme gene (cDNA and genomic copies) was compared with that of *S. pombe* and *S. cerevisiae* using the PC-based DNAMAN software (version 4.13) from Lynnon Biosoft. Comparison searches were performed with the NCBI nucleotide BLAST program (http://www.ncbi.nih.gov/) and consensus multiple alignment and phylogenetic tree calculations with the ClustalW program (Thompson *et al.*, 1994). Prediction of cellular localisation of the *C. utilis* malic enzyme was done using Predotar version 1.03 (http://genoplante-info.infobiogen.fr/predotar/predotar-html).

8.2.5 Malic Enzyme Activity Assays

Strains were cultured overnight in 10 ml YNB medium containing various carbon sources as indicated for the respective experiments. Total cellular proteins were isolated and used for malic enzyme assays as described by Osothsilp and Subden (1986[a]). The assay mixture of 3 ml included of 0.05 ml 0.2 M L-malate, 0.05 ml 4 mM NAD^+ or $NADP^+$, 0.05 ml $MgCl_2$ or $MnCl_2$, 1.3 ml 0.1 M phosphate buffer (pH 7.5), and 0.05 ml total cellular protein extract. Unless stated otherwise, the assays were performed at 25°C. The reaction was started with the addition of the protein extract and the increase in absorbance at 340 nm was measured at

various time intervals. Enzyme activities are given as µmoles of NADH produced per mg of protein as determined by Bradford assays (Bio-Rad Laboratories, Hercules, CA).

To determine the optimum temperature for the *C. utilis* malic enzyme, enzyme activity reactions were done at temperatures between 10°C and 100°C at pH 7.5 for 15 min. The thermal stability of the enzyme was determined by exposing the crude protein extracts to 30°C, 40°C, 50°C, 60°C or 70°C for up to 120 min prior to quantification of the residual enzyme activity.

8.2.6 RNA Isolation and Slot Blot Analyses

Cultures of *C. utilis* ATCC 9950 T were prepared in duplicate in 100 ml Minimal Medium containing 0.17% YNB without amino acids and ammonium sulphate (Difco Laboratories, Detroit, MI), 0.5% $(NH_4)_2SO_4$, and either 2% D-glucose (with or without 0.5% L-malate), 2% raffinose (with or without 0.5% L-malate), 2% glycerol/ethanol, 0.5% L-malate or 0.5% fumarate. The cultures were grown to an optical density of 0.8 (A_{600}) at 30ºC under aerated conditions unless stated otherwise. The cells were harvested, 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*., 1995). Hyridisation was done with PCR-generated DIG-labelled fragments of *CuME* and the *C. utilis* 5S genes using primers MH3 + MH4 and 5SF + 5SR, respectively (Table 2). Transcripts were visualised with the Chemiluminescent Detection Kit (Roche Biochemicals, Germany) and relative concentrations of the transcripts were quantified by means of densitometry and expressed as percentage relative to the *C. utilis* 5S transcripts.

For expression studies with shift assays, YNB media with 2% D-glucose or 0.5% L-malate was inoculated in duplicate with *C. utilis* ATCC 9950 T and grown under aerated conditions to an optical density of 0.8 at 600 nm at 30ºC. The cells were harvested and the pellets resuspended in fresh Minimal Medium containing 0.5% L-malate (for glucose-grown cells) or 2% D-glucose (for malate-grown cells). Cells were cultured under aerated conditions for an additional 90 min with 10 ml samples taken at 0, 15, 30, 60 and 90 min for RNA isolation and slot blot analysis as described above.

8.2.7 Heterologous Expression of *CuME*

The *CuME* ORF was subcloned as an *Eco*RI – *Xho*I fragment into pHVX2 under regulation of the *S. cerevisiae PGK1* promoter and terminator regions (Volschenk *et al*., 1997[a,b]), resulting

in plasmid pMH109 (Figure 8.3). The *S. pombe* malate transporter gene, *mae1*, was isolated from pHV3 using primer set 5'-mae1ORF-F and 3'-mae1ORF-R (Table 8.2), and subcloned as an *Eco*RI – *Bgl*II fragment into pJC1 to yield plasmid pMH110. Competent cells of *S. cerevisiae* Y294 were prepared (Moreno *et al*., 1991) and transformed with pMH110 together with pHVX2, pHV4 or pMH109.

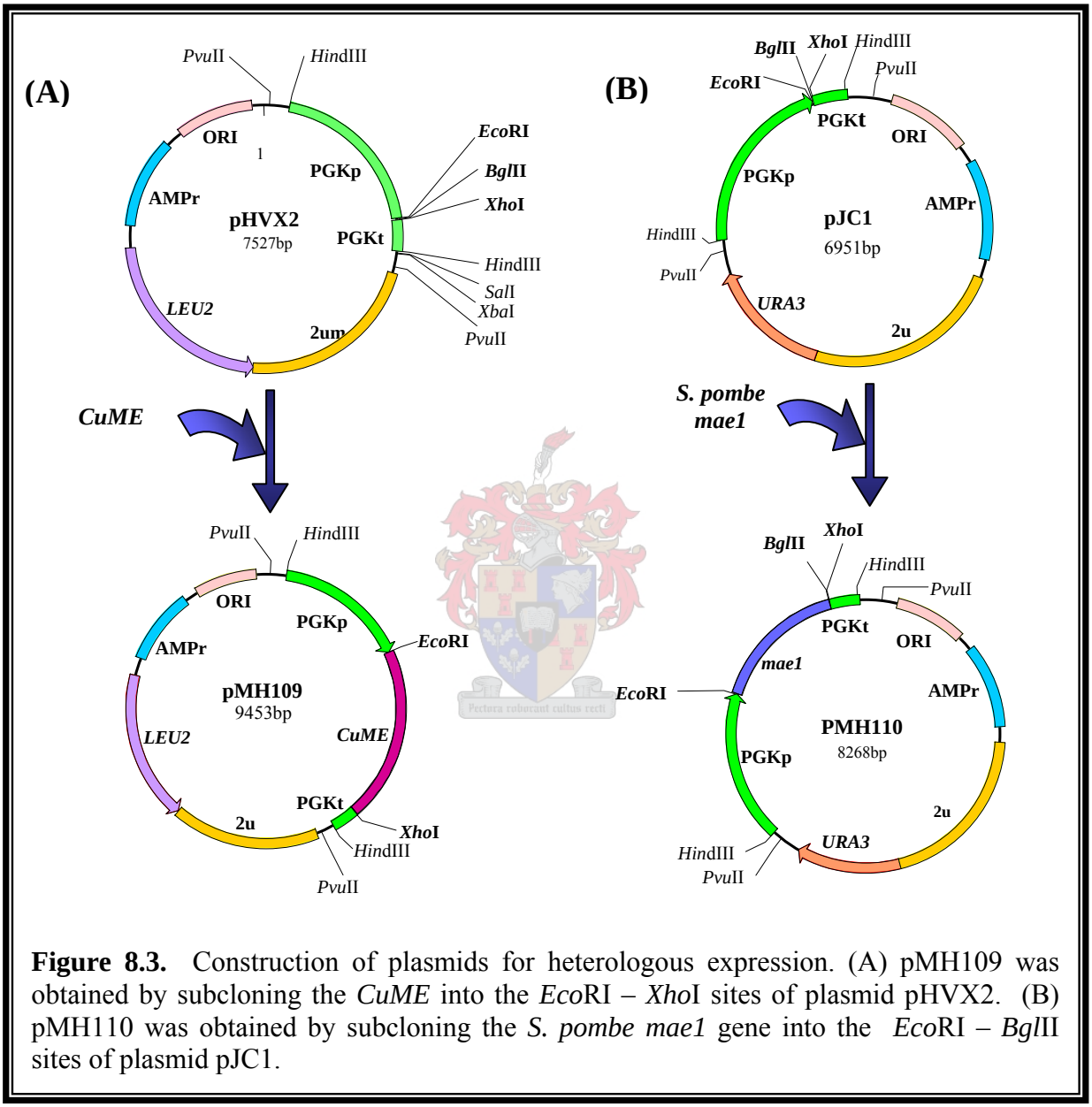

Figure 8.3. Construction of plasmids for heterologous expression. (A) pMH109 was obtained by subcloning the *CuME* into the *Eco*RI – *Xho*I sites of plasmid pHVX2. (B) pMH110 was obtained by subcloning the *S. pombe mae1* gene into the *Eco*RI – *Bgl*II sites of plasmid pJC1.

Strains of *C. utilis* ATCC 9950 T, *S. pombe* 972h$^-$, *S. cerevisiae* Y294 and *S. cerevisiae* Y294 transformants were inoculated in 10 ml YNB containing 0.5% L-malate at an optical density of 0.01 at 600 nm and incubated on a gyrotory shaker at 30˚C for 4 days. The cells were harvested by centrifugation and the supernatant filter-sterilised with Millex-GS 0.22 µm filter

units (Millipore, Bedford, MA). The L-malate content of the supernatant was determined with the L-malate test kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Transformants were also plated on modified GMIA plates (described above) to evaluate their ability to degrade L-malate.

8.3 RESULTS

8.3.1 Cloning and Sequence Analyses of *CuME*

The *C. utilis* cDNA library contained 100% inserts of more than 200 bp with an average insert size of 1.844 bp (Chapter 7). Transformation of the cDNA clones into *E. coli* yielded 1.07×10^7 cfu/ml and the amplified library yielded 1.15×10^9 cfu/ml.

The cDNA clone of the *C. utilis* malic enzyme gene comprised 2112 bp, including an ORF of 1926 bp (Figure 8.4). The deduced amino acid sequence suggests it encodes a 641 amino-acid protein with a predicted molecular weight of approximately 70 kDa. The size and sequence of the genomic DNA copy correlated with the ORF of the cDNA fragment (data not shown), suggesting the absence of introns.

Alignment of the amino acid sequences of the *C. utilis*, *S. cerevisiae* and *S. pombe* malic enzymes (Figure 8.5) showed that the putative CuMEp shared 37% and 68% homology with the *S. pombe* mae2p and *S. cerevisiae* mae1p respectively, and mae1p shares 47% homology with mae2p. Furthermore, eight highly conserved regions previously identified in malic enzymes (Viljoen *et al*., 1994, 1998) are also conserved in the *C. utilis* malic enzyme (Figure 8.5). The protein is predicted to be localised in the cytosol, similar to that of *S. pombe* (Viljoen *et al*., 1994).

A phylogenetic tree constructed with the deduced amino acid sequences of 44 other malic enzymes indicated six groups of malic enzymes (Figure 8.6). The malic enzyme from *C. utilis* clusters together with *S. pombe* and *S. cerevisiae* in Group V, with a closer relationship with *S. cerevisiae*. Group VI comprises isoenzymes from eubacteria, which seem to be more closely related to the yeast malic enzymes (Group V) than to those of higher eukaryotes, such as plants (Group II and IV), animals and humans (Group I) and human parasites (Group III). Within Groups I and II, both cytosolic and mitochondrial isoenzymes are found that can be either NAD^+ or $NADP^+$ dependent.

```
1      ATGACAATCTCAAACACCCAAGCTGCTGATGGCAGACGTTACTCCACAACTGTGGGTGCCAGTGGAGCTTCGACG
1       M  T  I  S  N  T  Q  A  A  D  G  R  R  Y  S  T  T  V  G  A  S  G  A  S  T
76     ACAAGAGACACGACGATAGGTTCCGTTAACGAGTACCCAGATGAGTGCATTTCGAAGGAGCCTATTGGTGAGGCT
26      T  R  D  T  T  I  G  S  V  N  E  Y  P  D  E  C  I  S  K  E  P  I  G  E  A
151    GCTCGTGCAGCCATCAATGTTGCAAAGGCCACAAGACTCTCTGCTGTTGGCCCAATTGAGTGTTCTCTCAACGGT
51      A  R  A  A  I  N  V  A  K  A  T  R  L  S  A  V  G  P  I  E  C  S  L  N  G
226    TTCCAGCTGCTGAACTCTCCATTGTTCAACAAAGGTTCCGCTTTCACACTCGAGGAGCGTGCTGCGTTTGGCCTC
76      F  Q  L  L  N  S  P  L  F  N  K  G  S  A  F  T  L  E  E  R  A  A  F  G  L
301    GAAGGTTTGTTACCTGCCCAGGTCAACGACCTTAATGCCCAAGTTGAGAGGGCTTACAAGCAATTGTGCTACTTG
101     E  G  L  L  P  A  Q  V  N  D  L  N  A  Q  V  E  R  A  Y  K  Q  L  C  Y  L
376    AAGACACCATTGGCCAAGAACGACTTCTGTTCGTCAATGAGAGTGCAGAACAAGGTTCTGTTTTACGAATTGGTG
126     K  T  P  L  A  K  N  D  F  C  S  S  M  R  V  Q  N  K  V  L  F  Y  E  L  V
451    AGAAGACACATTAGAGAGCTGGTTCCAATCATCTACACACCAACTGAAGGTGACGCCATTGCTGCCTACTCCCAC
151     R  R  H  I  R  E  L  V  P  I  I  Y  T  P  T  E  G  D  A  I  A  A  Y  S  H
526    AGATTTAGAAAACCAGAAGGTTGTTTCCTCGATATCACAGATCCAGACTCCATCGACCGCAGACTGGCCAACTTT
176     R  F  R  K  P  E  G  C  F  L  D  I  T  D  P  D  S  I  D  R  R  L  A  N  F
601    GGTGAAGATAAGGATGTTGACTACATTGTTGTCTCTGACGGTGAAGGTATCTTGGGTATTGGTGACCAAGGTGTT
201     G  E  D  K  D  V  D  Y  I  V  V  S  D  G  E  G  I  L  G  I  G  D  Q  G  V
676    GGTGGTGTCCGTATTGCCATCTCTAAGCTTGCCCTTATGACCCTTTGTGGTGGTATCCACCCAGGTAGAGTCATC
226     G  G  V  R  I  A  I  S  K  L  A  L  M  T  L  C  G  G  I  H  P  G  R  V  I
751    CCAGTGTGTCTTGATGTTGGTACCAACAACAAGAAGCTGATCACCGATGATTTGTACATGGGTAACAGATTCCCA
251     P  V  C  L  D  V  G  T  N  N  K  K  L  I  T  D  D  L  Y  M  G  N  R  F  P
826    AGAGTCCGTGGTAAGGAGTACGACGACTTTGTCGACAAGTTTATCCAGGTTTTGAAGAGAAGATTCCCATCTGCA
276     R  V  R  G  K  E  Y  D  D  F  V  D  K  F  I  Q  V  L  K  R  R  F  P  S  A
901    ACTCTTCATTTTGAGGATTTCGGTGTCACCACTGGTCGTCCACTCTTGCAAAGATACAGAAACGAGTTGGCTTGT
301     T  L  H  F  E  D  F  G  V  T  T  G  R  P  L  L  Q  R  Y  R  N  E  L  A  C
976    TTCAACGATGATATCCAAGGTACCGGTGCTGTCGTGATGGCTTCCCTCTCTGCAGCCTTGAAGCACACCAACCGT
326     F  N  D  D  I  Q  G  T  G  A  V  V  M  A  S  L  S  A  A  L  K  H  T  N  R
1051   AACCTTTTGGATTCCCAAATTGTCATCTACGGTGCTGGTTCCGCCGGTTTGGGTATTGCTGATCAAATTGTCAAC
351     N  L  L  D  S  Q  I  V  I  Y  G  A  G  S  A  G  L  G  I  A  D  Q  I  V  N
1126   CACATGGTTACACACGGTGCCACTGTCGAGGAGGCCAAGTCTAAGATCCACGCTCTAGATATCAGAGGGTTGATT
376     H  M  V  T  H  G  A  T  V  E  E  A  K  S  K  I  H  A  L  D  I  R  G  L  I
1201   CTCAAGTCCATGAAGGAGACCTCCACTCCAGATCAACACTCTTATGCCGACGAAGATGCTGATTGGGAGGGAATC
401     L  K  S  M  K  E  T  S  T  P  D  Q  H  S  Y  A  D  E  D  A  D  W  E  G  I
1276   GACACGAAGTCATTGTACGAAGTTGTCAAGAAGATCAAACCAACTTGTTTGATTGGATGTTCCACACAAGCTGGT
426     D  T  K  S  L  Y  E  V  V  K  K  I  K  P  T  C  L  I  G  C  S  T  Q  A  G
1351   GCATTCACACAGCAGATTGTGCAAGAGATGCACAAGCACAACCCACGTCCAATCATCTTCCCATTGTCCAACCCA
451     A  F  T  Q  Q  I  V  Q  E  M  H  K  H  N  P  R  P  I  I  F  P  L  S  N  P
1426   ACAAGACTCCACGAGGCTGTTCCAGAGGACTTGATGGCCTGGACAAACAACGACGCCATGGTTGCCACCGGTTCT
476     T  R  L  H  E  A  V  P  E  D  L  M  A  W  T  N  N  D  A  M  V  A  T  G  S
1501   CCATTCAAACCTGTCGATGGATGGGTCATCTCGGAGAACAACAACTGTTTTGCATTCCCAGGTATCGGCCTGGGC
501     P  F  K  P  V  D  G  W  V  I  S  E  N  N  N  C  F  A  F  P  G  I  G  L  G
1576   TCTGTTCTCTCAAGAGCCAAGATCATCTCGGACAAGATGATCTCTGCTGCCGTGGACCAGCTGGCCTCTCTGTCC
526     S  V  L  S  R  A  K  I  I  S  D  K  M  I  S  A  A  V  D  Q  L  A  S  L  S
1651   CCATTGTCCAAGGACCCTAAGGCTGGTTTGCTTCCACCATTGGAGGTCATCAACGACACATCTGCCAAGGTTGCG
551     P  L  S  K  D  P  K  A  G  L  L  P  P  L  E  V  I  N  D  T  S  A  K  V  A
1726   GCTGCTGTCATCCTACAAGCACTGGACGAGGGCCTTGCCCGTATCGAGGACGAGGTGCAGCCAGGCAAGGATGAG
576     A  A  V  I  L  Q  A  L  D  E  G  L  A  R  I  E  D  E  V  Q  P  G  K  D  E
1801   TACGTTACTGTCCCAGGGGACTTTGATGCCTGTGTGGAGTGGGTGAAACAGCAGATGTGGAAGCCAGAGTACAGA
601     Y  V  T  V  P  G  D  F  D  A  C  V  E  W  V  K  Q  Q  M  W  K  P  E  Y  R
1876   CCAATGGTTAAGGTGGAACACAGACACGATATCCATACGCATCAGTTCTGA
626     P  M  V  K  V  E  H  R  H  D  I  H  T  H  Q  F  *
```

Figure 8.4. DNA and deduced amino acid sequence of the coding region of *CuME*. The deduced amino acid sequence of the protein is written below the nucleotide sequence.

```
C. utilis       -------------------------MTISNTQAADGRRYSTTVGASGASTTRDTTIGSVN
S. cerevisiae   MLRTRLSVSVAARSQLTRSLTASRTAPLRRWPIQQSRLYSSNTRSHKATTTRENTFQKP-
S. pombe        ------------------------------------------------------------

C. utilis       EYPDECISKEPIGEAAR--AAINVAKATRLSAVGPIECSLNGFQLLNSPLFNKGSAFTLE
S. cerevisiae   -YSDEEVTKTPVGSRARKIFEAPHPHATRLTVEGAIECPLESFQLLNSPLFNKGSAFTQE
S. pombe        -------------------------MPAGTKEQIECPLKGVTLLNSPRYNKDTAFTPE
                                         *** *    *****  **  *** *

C. utilis       ERAAFGLEGLLPAQVNDLNAQVERAYKQLCYLKT-PLAKNDFCSSMRVQNKVLFYELVRR
S. cerevisiae   EREAFNLEALLPPQVNTLDEQLERSYKQLCYLKT-PLAKNDFMTSLRVQNKVLYFALIRR
S. pombe        ERQKFEISSRLPPIVETLQQQVDRCYDQYKAIGDEPLQKNLYLSQLSVTNQTLFYALISQ
                **  *    **  *  *  * * *       ** **       * *  *   *

                        Box A
C. utilis       HIRELVPIIYTPTEGDAIAAYSHRFRKPEGCFLDITDPDS--IDRRLANFGEDKDVDYIV
S. cerevisiae   HIKELVPIIYTPTEGDAIAAYSHRFRKPEGVFLDITEPDS--IECRLATYGGDKDVDYIV
S. pombe        HLIEMIPIIYTPTEGDAIKQFSDIYRYPEGCYLDIDHNDLSYIKQQLSEFGKSDSVEYII
                *  *  ***********   *   * ***  ***   *   *   *    *    * **

                        Box B                                   Box C
C. utilis       VSDGEGILGIGDQGVGGVRIAISKLALMTLCGGIHPGRVIPVCLDVGTNNKKLITDDLYM
S. cerevisiae   VSDSEGILGIGDQGIGGVRIAISKLALMTLCGGIHPGRVLPVCLDVGTNNKKLARDELYM
S. pombe        ITDSEGILGIGDQGVGGVLISVAKGHLMTLCAGLDPNRFLPIVLDVGTNNETHRKNHQYM
                   * ********** *** *   *  ***** *  * *  *  *******         **

                                                                        Box D
C. utilis       GNRFPRVRGKEYDDFVDKFIQVLKRRFPSATLHFEDFGVTTGRPLLQRYRNELACFNDDI
S. cerevisiae   GNKFSRIRGKQYDDFLEKFIKAVKKVYPSAVLHFEDFGVKNARRLLEKYRYELPSFNDDI
S. pombe        GLRKDRVRGEQYDSFLDNVIKAIREVFPEAFIHFEDFGLANAKRILDHYRPDIACFNDDI
                *    * **  ** *    *        * *  ******       *  **     *****

                                        Box E
C. utilis       QGTGAVVMASLSAALKHTNRNLLDSQIVIYGAGSAGLGIADQIVNHMVTHGATVEEAKSK
S. cerevisiae   QGTGAVVMASLIAALKHTNRDLKDTRVLIYGAGSAGLGIADQIVNHMVTHGVDKEEARKK
S. pombe        QGTGAVALAAIIGALHVTKSPLTEQRIMIFGAGTAGVGIANQIVAGMVTDGLSLDKARGN
                ******  *    **  *  *      * *** ** *** ***  *** *     *

                                                                    Box F
C. utilis       IHALDIRGLILKSMKETSTPDQHSYADEDADWEGID--TKSLYEVVKKIKPTCLIGCSTQ
S. cerevisiae   IFLMDRRGLILQSYEANSTPAQHVYAKSDAEWAGIN--TRSLHDVVENVKPTCLVGCSTQ
S. pombe        LFMIDRCGLLLERHAKIATDGQKPFLKKDSDFKEVPSGDINLESAIALVKPTILLGCSGQ
                    *  ** *        *  *      *            *       *** * *** *

                                Box G                           Box H
C. utilis       AGAFTQQIVQEMHKHNPRPIIFPLSNPTRLHEAVPEDLMAWTNNDAMVATGSPFKPVDG-
S. cerevisiae   AGAFTQDVVEEMHKHNPRPIIFPLSNPTRLHEAVPADLMKWTNNNALVATGSPFPPVDG-
S. pombe        PGKFTEKAIREMSKHVERPIIFPISNPTTLMEAKPDQIDKWSDGKALIATGSPLPPLNRN
                 * **     ** **  ****** **** * ** *     *    *  *****  *

C. utilis       ---WVISENNNCFAFPGIGLGSVLSRAKIISDKMISAAVDQLASLSPLSK-DPKAGLLPP
S. cerevisiae   ---YRISENNNCYSFPGIGLGAVLSRATTITDKMISAAVDQLAELSPLREGDSRPGLLPG
S. pombe        GKKYVISQCNNALLYPALGVACVLSRCKLLSDGMLKAASDALATV-PRSLFAADEALLPD
                      **  **    *  *   ****     * *  ** *  *   *         ***

C. utilis       LEVINDTSAKVAAAVILQALDEGLARIEDEVQPGKD--EYVTVPGDFDACVEWVKQQMWK
S. cerevisiae   LDTITNTSARLATAVILQALEEGTARIEQEQVPGGAPGETVKVPRDFDECLQWVKAQMWE
S. pombe        LNNAREISRHIVFAVLKQAVSEGMSTVDLPKDDAKLK-EWIIEREWNPEYKPFV------
                *      *     **  **  **              *              *

C. utilis       PEYRPMVKVEHRHDIHTHQ------------------------------------------
S. cerevisiae   PVYRPMIKVQHDPSVHTNQ------------------------------------------
S. pombe        ------------------------------------------------------------
```

Figure 8.5. Amino acid sequence alignment of the malic enzymes from *C. utilis*, *S. cerevisiae* and *S. pombe*. Homologous regions A-H are indicated by the yellow boxes, while amino acid changes are indicated in blue for *S. pombe* and in green for *S. cerevisiae*.

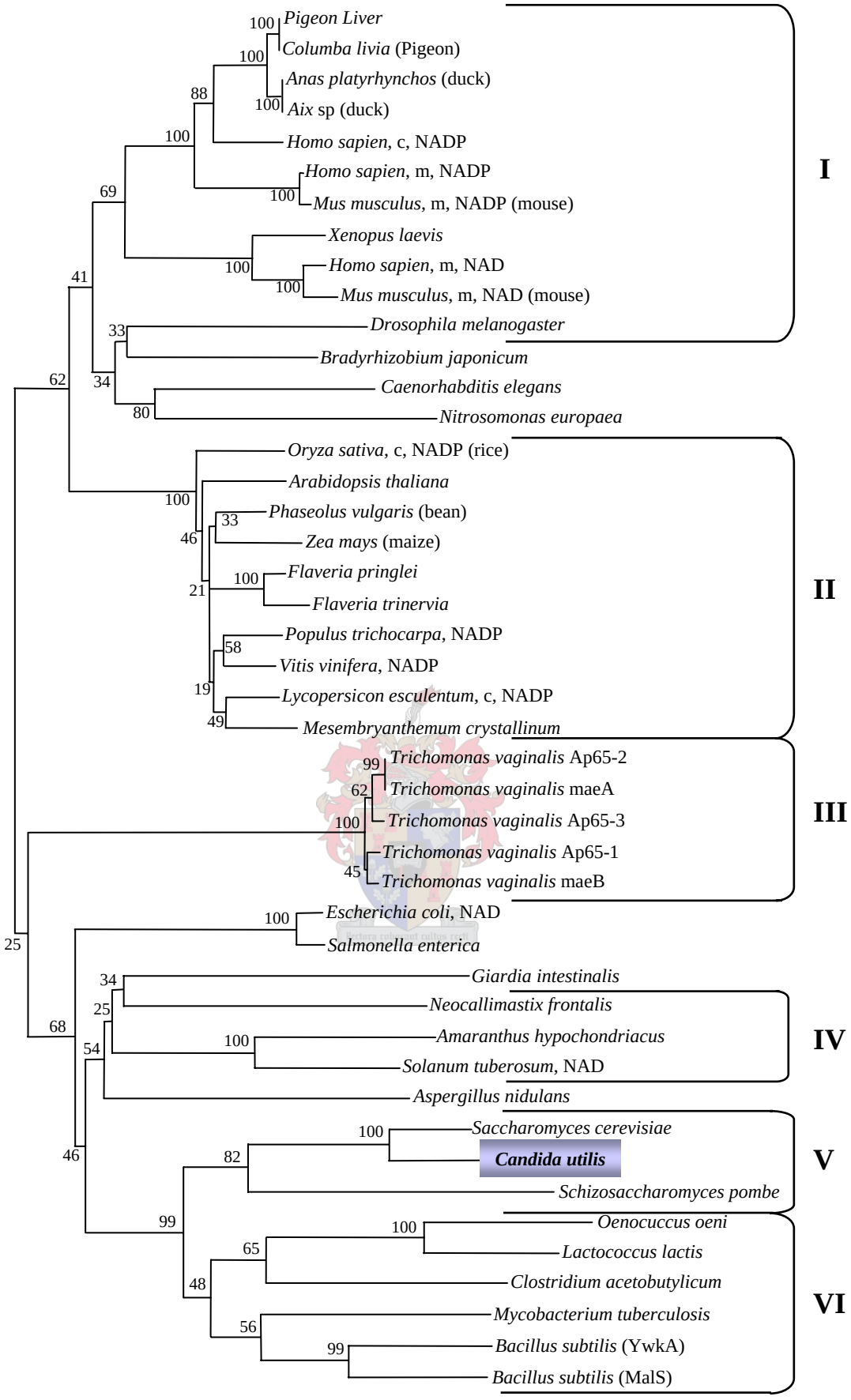

Figure 8.6. Phylogenetic relationship of malic enzymes from different organisms. Blocks of homology are numbered I to VI. If known, the corresponding intracellular localization is indicated: c, cytoplasm; m, mitochondria.

8.3.2 Characterisation of the *C. utilis* Malic Enzyme

Malic enzyme activity assays on total protein extracts of *C. utilis* cells showed that the malic enzyme is able to decarboxylate both L-malate and oxaloacetate, while utilising either Mg^{2+} or Mn^{2+} as divalent cations (Table 8.3). It can utilise either NAD^+ or $NADP^+$ for the decarboxylation of oxaloacetate, but prefers NAD^+ as coenzyme for the decarboxylation of L-malate.

Table 8.3. Substrate and co-factor specificity for the *C. utilis* malic enzyme

	L-Malate		Oxaloacetate	
	NAD+	NADP+	NAD+	NADP+
$MnCl_2$	1943.79[a]	330.88	1084.57	1037.97
$MgCl_2$	1793.80	14.62	800.90	865.41

[a]Malic enzyme activity (μmoles NADH/mg prot.)

The optimum temperature for the malic enzyme activity was 52°C (Figure 8.7A), which is comparable to that reported for the malic enzymes from *Bacillus stearothermophilus* (55ºC: Kobayashi *et al*., 1989) and *Pseudomonas diminuta* (50ºC: Suye *et al*., 1992). Enzyme activity was stable at 30°C for up to 120 min (Figure 8.7B), with circa 75% and 53% of the activity retained after 120 min at 50°C and 70°C, respectively.

Carbon catabolite repression and substrate induction of the *C. utilis* malic enzyme was confirmed by the degradation of extracellular L-malate as well as malic enzyme activity assays. When cultured in 0.5% L-malate as sole carbon source, *C. utilis* cells degraded approximately 90% of the L-malate 30 hours (Figure 8.8). However, degradation of L-malate in medium containing 2% glucose/0.5% L-malate only started when all the glucose was depleted. The malic enzyme activity was induced more than 35-fold (relative to growth in 2% glucose) in cells grown in 0.5% L-malate as sole carbon source (Figure 8.9A), but repressed by glucose as well as 2% raffinose and 2% glycerol/2% ethanol. Closer inspection of the data in Figure 8.9B revealed at least 3-fold higher levels of activity when grown in the presence of 2% raffinose/0.5% L-malate and 2% glycerol/2% ethanol relative to cells grown in 2% glucose.

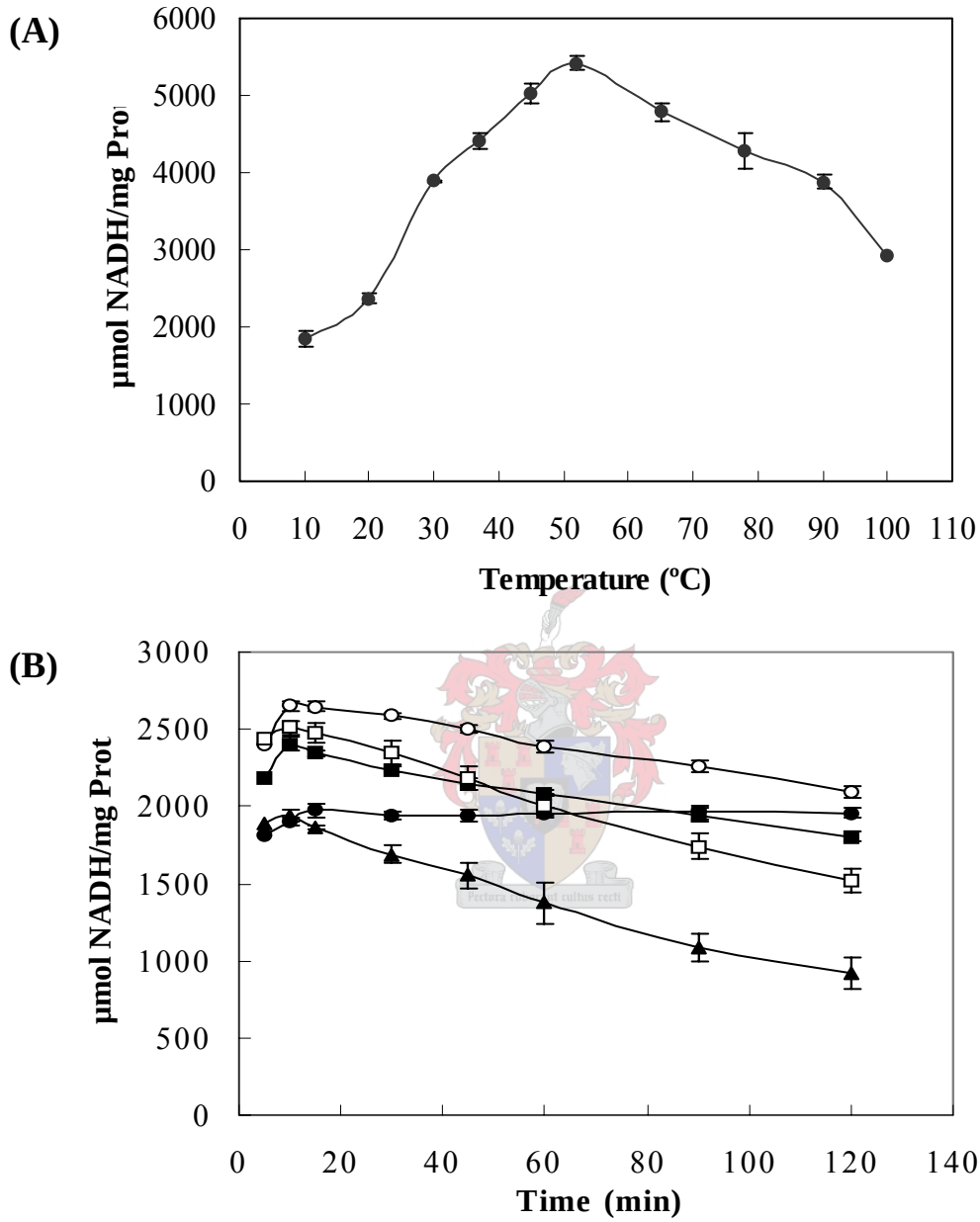

Figure 8.7. (A) The effect of temperature on the activity of the *C. utilis* malic enzyme as determined by malic enzyme activity assays (B) Thermo-stability of the *C. utilis* malic enzyme with exposure to different temperatures and time periods [● = 30°C; ■ = 40°C; ○ = 50°C; □ = 60°C; ▲ = 70°C] (error bars indicate standard deviation of three repeats).

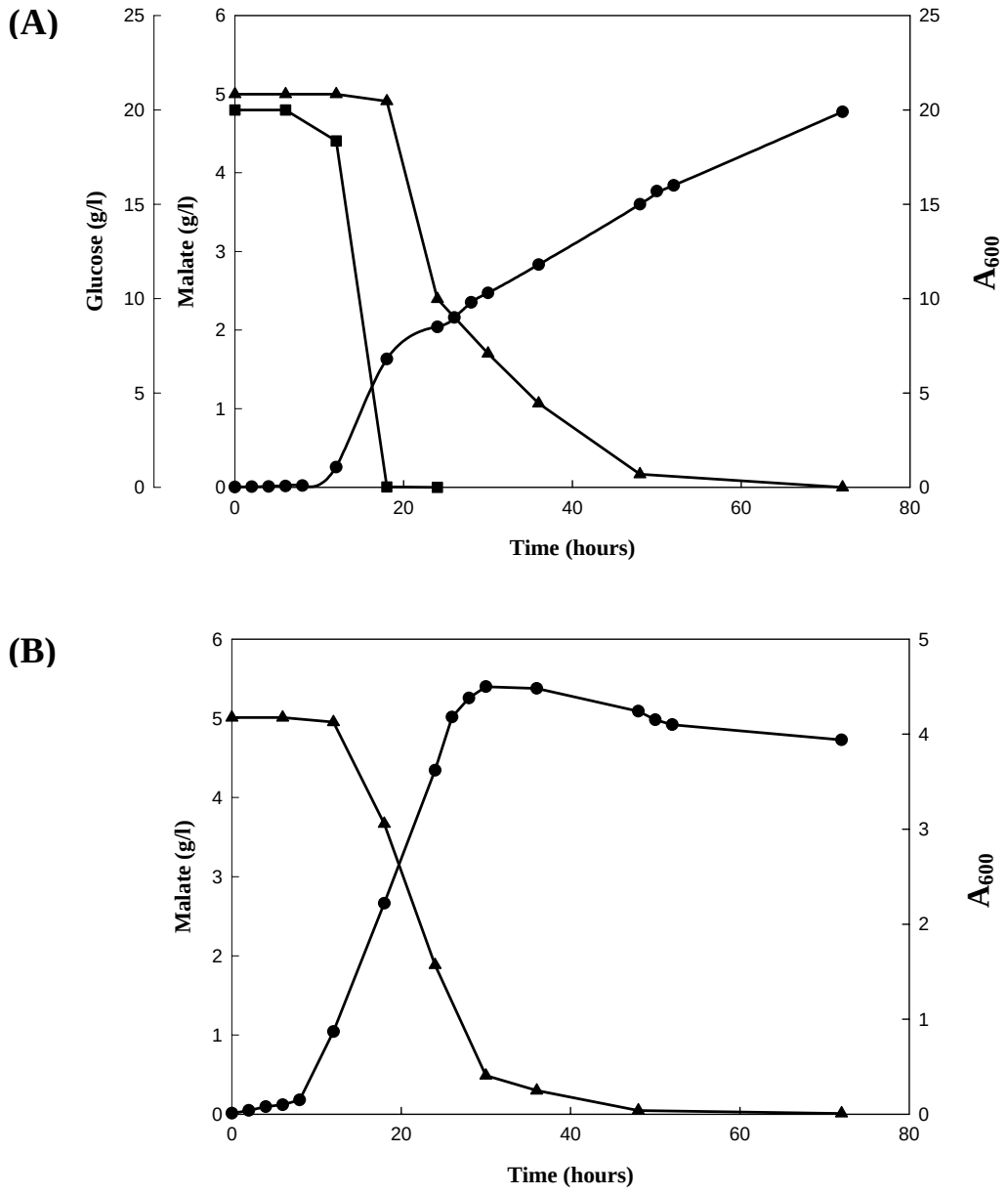

Figure 8.8. L-Malate (▲) and glucose (■) degradation by *C. utilis* in media containing (A) 2% glucose and 0.5% L-malate compared to (B) *C. utilis* cells cultured in 0.5% L-malate as sole carbon source. (●) Growth curve of *C. utilis* in the respective growth media as measured by cell density at 600 nm (note different scales for (A) and (B)).

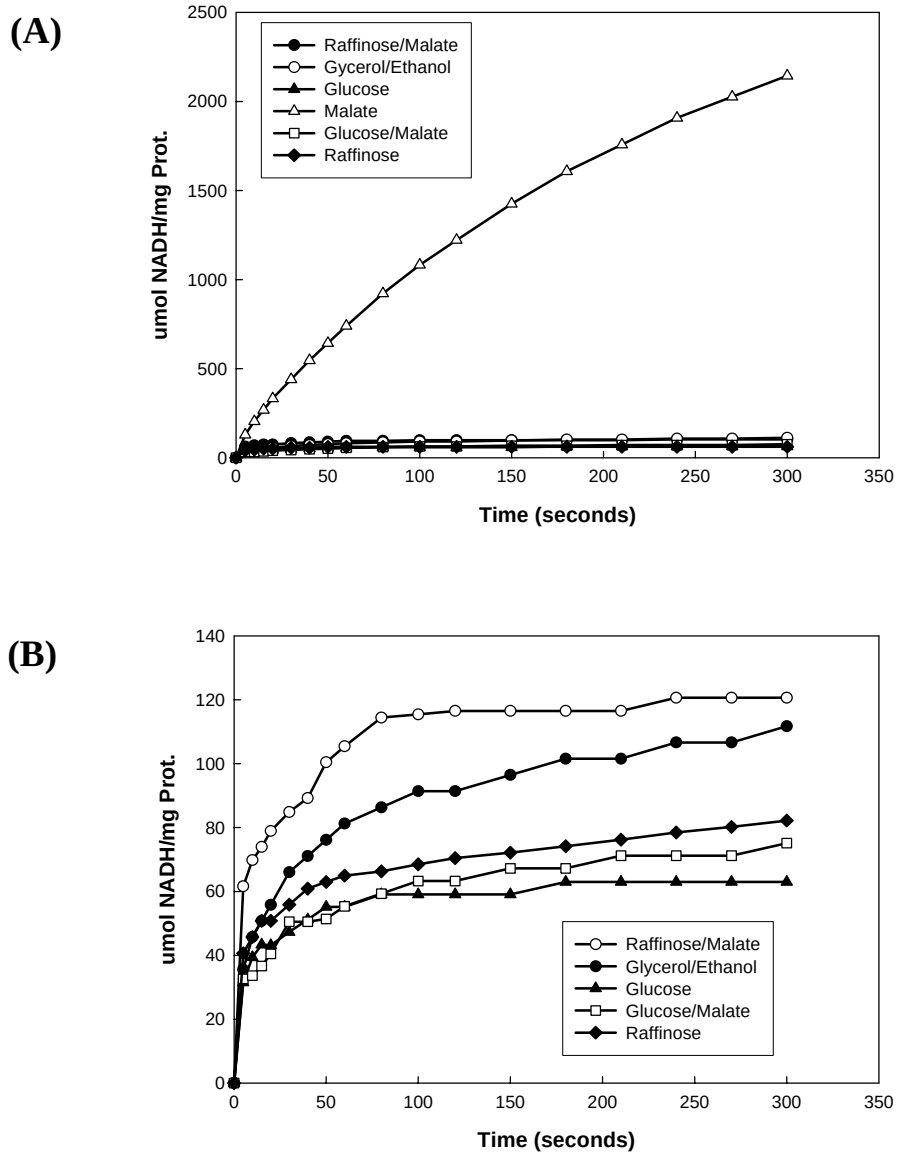

Figure 8.9. (A) Malic enzyme activity assays on *C. utilis* cell extracts after growth in various carbon sources. (B) Smaller scale to show minor differences in enzyme activities (excluding data for malate).

When cultured in different concentrations of glucose and L-malate, the highest level of *C. utilis* malic enzyme activity was obtained after growth in 0.5% L-malate, with very little enzyme activity in any of the glucose-containing cultures under aerobic conditions (Figure 8.10). However, the enzyme activity was at least 2-fold higher when cells were grown in 2% glucose under non-aerated conditions than under aerobic conditions.

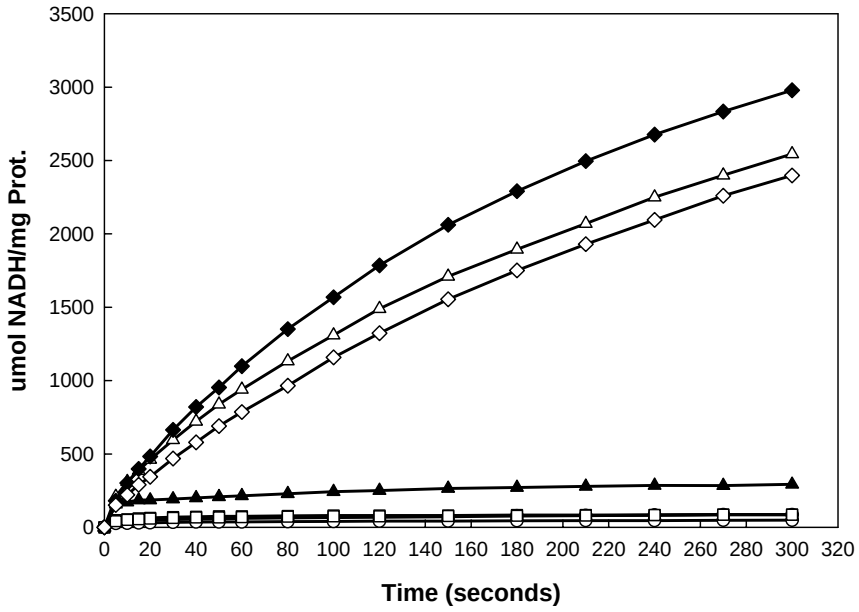

Figure 8.10. Malic enzyme activity of *C. utilis* grown in different consentrations of glucose and L-malate; (●) 0.2% glucose, (○) 0.5% glucose, (■) 2% glucose, (□) 8% glucose, (▲) 2% glucose (anaerobic), (Δ) 0.1% L-malate, (♦) 0.5% L-malate, (◊) 1% L-malate.

The induction/repression patterns observed with the malic enzyme activity assays correlated with transcription levels of the *CuME* gene. When compared to cells grown in 2% glucose as sole carbon source (Figure 8.11A), induced levels of *CuME* were observed for cells grown in 0.5% L-malate (10 fold induction), 2% raffinose/0.5% L-malate (3.7-fold), 0.5% fumarate (3-fold), 2% glycerol/2% ethanol (2-fold) or 2% raffinose (1.6-fold). When cells grown overnight in 2% glucose were shifted to fresh medium containing 0.5% L-malate as sole carbon source, the transcription levels of *CuME* experienced a lag during the first 30 minutes (Figure 8.11B), followed by a 2.2-fold induction at 60 min.

8.3.3 Recombinant Expression of *CuME* in *S. cerevisiae* Y294

The ability of *S. pombe* 972h$^-$ (wild type), *S. pombe mae2*$^-$, *C. utilis* ATCC9950 T and recombinant strains of *S. cerevisiae* Y294 to degrade L-malate was evaluated on modified GMIA-plates as well as in liquid cultures. The *S. pombe* wt strain successfully degraded L-malate incorporated into the GMIA plates (indicated by the blue colonies in Figure 8.12A), but not the *S. pombe mae2*$^-$ mutant (deficient in malic enzyme activity). The *C. utilis* strain

grew in the presence of both 0.5% L-malate and 2% glucose/0.5% L-malate, but degradation of L-malate only occurred in the absence of glucose. The parental *S. cerevisiae* Y294 and recombinant *S. cerevisiae* strain carrying the *S. pombe* L-malate transporter gene (*mae1*, pHVX2), was unable to degrade L-malate. However, the recombinant *S. cerevisiae* strains carrying the *S. pombe* L-malate transporter gene (*mae1*, pMH110), together with the malic enzyme gene of either *S. pombe* (*mae2*, pHV4) or *C. utilis* (*CuME*, pMH109) effectively degrade the L-malate in the GMIA plates.

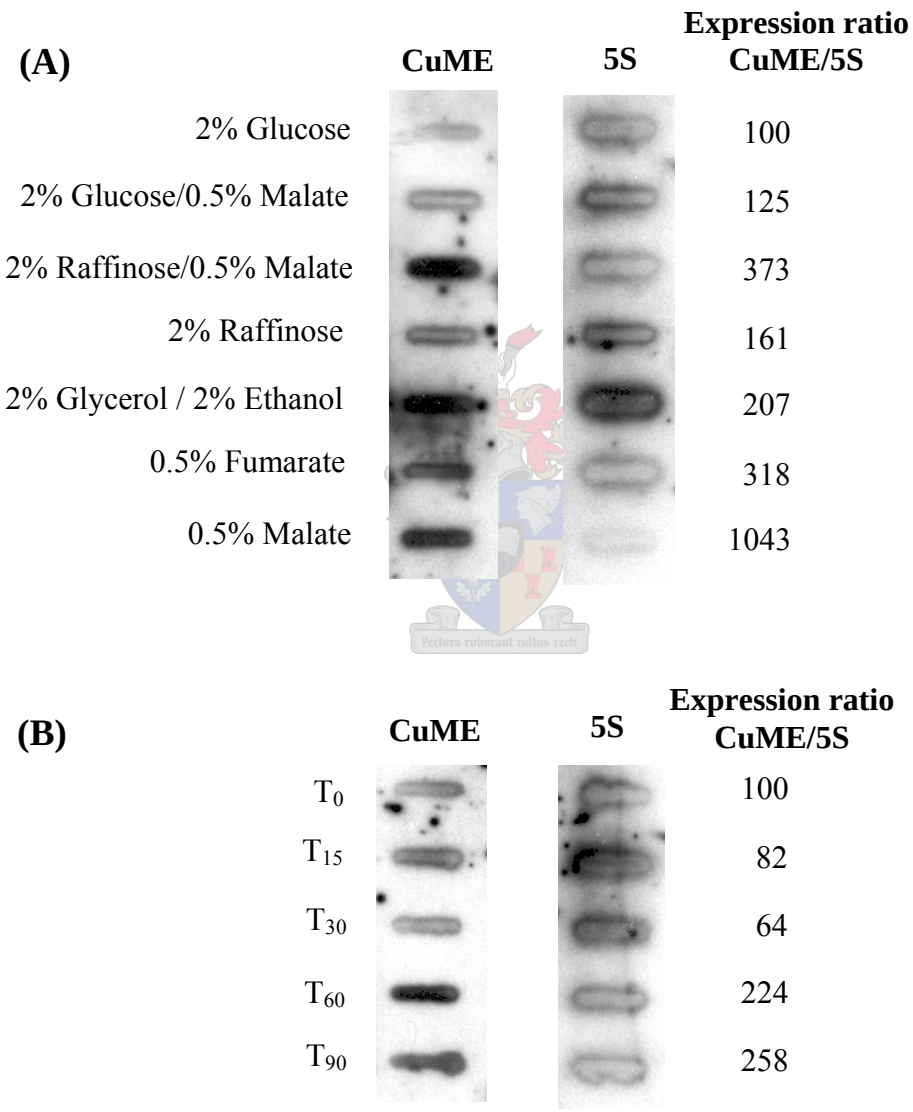

Figure 8.11. (A) Quantification of transcription levels of the malic enzyme gene, *CuME*, in different carbon sources by means of northern slot blot analyses. Values are indicated relative to expression after growth in 2% glucose (taken as 100). (B) Quantification of transcription levels of the malic enzyme gene, *CuME*, after a shift from 2% glucose to 0.5% L-malate.

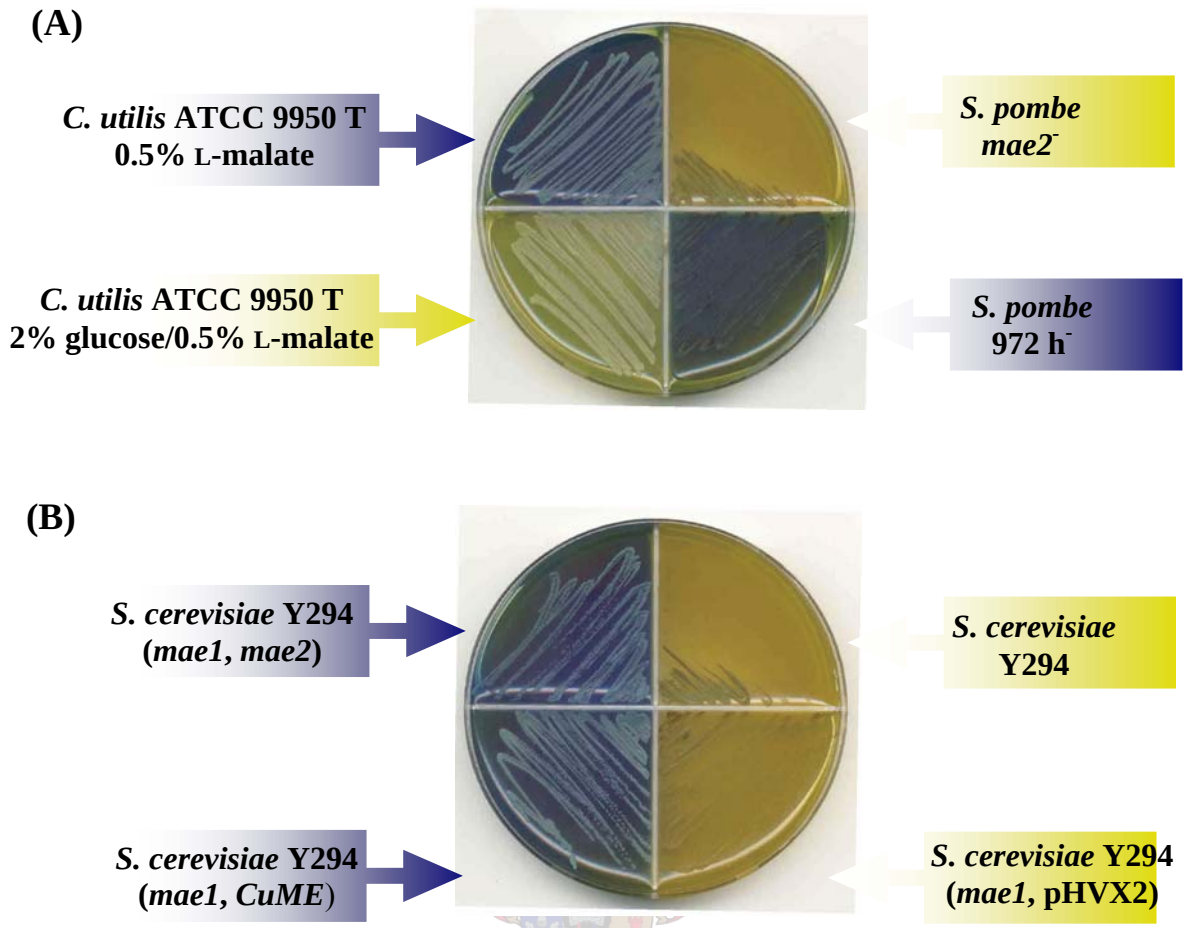

Figure 8.12. (A) Cells of *S. pombe* 972 h$^-$ showed a clear malo-ethanolic phenotype as apposed to the *S. pombe mae2*$^-$ mutant (yellow/brown colonies). Cells of *C. utilis* displayed degradation of L-malate in the presence of L-malate as sole carbon source, but not when glucose was present. (B) Transformants of *S. cerevisiae* Y294 containing the *PGK*p-*mae1*-*PGK*$_t$ together with either the *PGK*$_p$-*CuME*-*PGK*$_t$ or the *PGK*$_p$-*mae2*-*PGK*$_t$ expression cassettes showed a clear degradation of L-malate on GMIA plates (blue colonies), as apposed to transformants containing only the *PGK*$_p$-*mae1*-*PGK*$_t$ expression cassette and the control plasmid, pHVX2.

When cultured in liquid YNB media containing 2% glucose and 0.5% L-malate, *S. cerevisiae* Y294 showed no significant degradation of L-malate, while the *S. pombe* and *C. utilis* strains showed rapid degradation within 2 days (Figure 8.13). The efficacy of *S. cerevisiae* improved with the introduction of the *S. pombe* malate transporter gene (*mae1*), with a more profound effect when the *S. pombe* malate transporter was combined with either the *S. pombe* or the *C. utilis* malic enzymes: the recombinant *S. cerevisiae mae1-mae2* and *mae1-CuME* strains

degraded ca. 5 g/l L-malate within 2 days. The rate of degradation by the recombinant *S. cerevisiae mae1-CuME* strain was significantly better than the wild type *C. utilis* strain since the recombinant proteins were not subject to the glucose repression experienced by the wt *C. utilis* strain. Furthermore, the *S. cerevisiae mae1-CuME* strain was more effective than the malo-ethanolic *S. cerevisiae mae1-mae2* strain previously reported by Volschenk *et al*. (1997[a]).

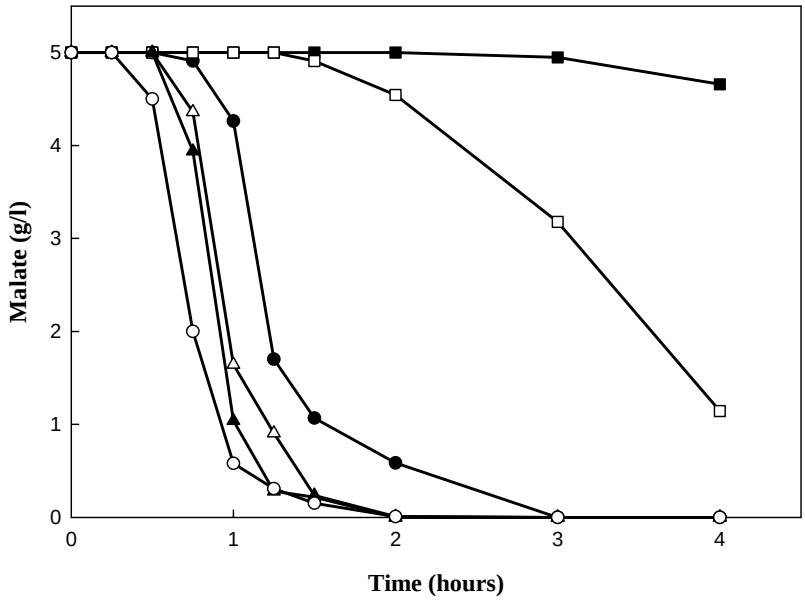

Figure 8.13. L-Malate degradation by different yeast strains. ○ = *S. cerevisiae* Y294 (*mae1*, *CuME*); Δ = *S. cerevisiae* Y294 (*mae1*, *mae2*); □ = *S. cerevisiae* Y294 (*mae1*, pHVX2); ■ = *S. cerevisiae* Y294; ▲ = *S. pombe* 972h$^-$, wt; ● = *C. utilis* ATCC 9950 T

8.4 DISCUSSION

The ability of a yeast strain to degrade extracellular L-malate is dependent on the efficient transport of the dicarboxylic acid, as well as the efficacy of the intracellular malic enzyme for the decarboxylation of L-malate to yield pyruvate and CO_2. Based on its substrate specificity and cofactor requirements, the *C. utilis* malic enzyme can be classified as L-malate: NAD^+ oxidoreductase (oxaloacetate decarboxylating: EC 1.1.1.38). It demonstrated good temperature stability with an optimum temperature of 52ºC, which is comparable to that reported for the malic enzymes from *Bacillus stearothermophilus* (55ºC; Kobayashi *et al*., 1989) and *Pseudomonas diminuta* (50ºC; Suye *et al*., 1992).

DNA and amino acid sequence analyses showed that the highly conserved regions in malic enzymes previously identified by Viljoen *et al*. (1994, 1998), are also conserved in the *C. utilis* malic enzyme. The conserved P—YTPTVG-AC motif in Region A is the proposed binding site for L-malate (Kulkarni *et al*., 1993) and NAD^+ (Rothermel and Nelson, 1989). The ILGLGD-G—G and GAG-A—GIA motifs in regions B and E, respectively, represent $NAD(P)^+$ binding sites (Rothermel and Nelson, 1989; Börsch and Westhoff, 1990; Hsu *et al*., 1992). The direct metal ligands Glu^{305}, Asp^{306} and Asp^{329} in region C (numbers corresponding to position in *C. utilis* malic enzyme) are identical among all malic enzymes investigated, suggesting that the catalytic mechanism should essentially be the same for all malic enzymes.

The DNA sequence of the *CuME* gene showed strong homology with the malic enzyme genes from *S. cerevisiae* and *S. pombe*, although these three malic enzymes differ in their substrate specificity (L-malate and/or oxaloacetate), cofactors (NAD^+ and/or $NADP^+$) and intracellular localisation (cytosolic or mitochondrial) (Voegele *et al*., 1999). The cytosolic malic enzymes from *C. utilis* and *S. pombe* are bifunctional, reacting with either L-malate or oxaloacetate, and requires NAD^+ and the divalent cations Mn^{2+} or Mg^{2+} for activity. In contrast, the mitochondrial *S. cerevisiae* malic enzyme prefers Mn^{2+} and can utilise both NAD^+ and $NADP^+$ as electron acceptor with NAD^+ being favoured (Kuczynski and Radler, 1982).

Phylogenetic analyses of the evolutionary relationship between *C. utilis* CuMEp and 44 known malic enzymes revealed that the *C. utilis* malic enzyme clusters together with *S. pombe* and *S. cerevisiae* in Group V, with a closer relationship with *S. cerevisiae*. Group VI comprises isoenzymes from eubacteria, which seem to be more closely related to the yeast malic enzymes (Group V) than to those of higher eukaryotes, such as plants (Group II and IV), animals and humans (Group I) and human parasites (Group III). Within Groups I and II, both cytosolic and mitochondrial isoenzymes are found that can be either NAD^+ or $NADP^+$ dependent. These findings suggest that sequence homologies among malic enzymes from various organisms are primarily determined by phylogenetic relationships between the organisms, rather than being the result of functional constraints related to catalytic properties and intracellular localisation.

Results presented in this study confirmed that the glucose repression/substrate induction observed for malate degradation and malate transport in *C. utilis* also applies to the *C. utilis* malic enzyme. Malic enzyme activity was induced more than 35-fold (relative to growth in 2% glucose) in cells grown in 0.5% L-malate as sole carbon source, while carbon sources that

can provide pyruvate for the TCA cycle, repressed the malic enzyme activity. The transport and degradation of L-malate will therefore be delayed until these carbon sources (e.g. glucose) are depleted (Van Dijken *et al*., 1993).

An interesting observation was that growth in 2% glucose under non-aerated conditions (exclusion of oxygen) increased the enzyme activity by 2-fold. Under aerobic growth conditions, the yeast tends to channel most of its pyruvate into the TCA cycle, resulting in an adequate supply of intracellular TCA cycle intermediates. Under non-aerated conditions, the mitochondrial TCA enzymes may be repressed (McCammon *et al*., 2003) which leaves the cytosolic malic enzyme with the task of replenishing TCA intermediates for other cellular requirements. Since the *C. utilis* malic enzyme is predicted to be cytosolic, induction of the malic enzyme activity will therefore contribute to replenishing of the TCA cycle intermediates.

The induction/repression patterns observed with the malic enzyme activity can be ascribed to regulation of the *CuME* gene on a transcriptional level. A 10-fold induction was observed for cells grown in the presence of 0.5% L-malate (relative to growth in 2% glucose). The 3-fold induction by 0.5% fumarate was not surprising given the fact that fumarate is a precursor for L-malate in the TCA cycle. High levels of extracellular fumarate (transported by the inducible malate/fumarate transporter) will lead to an increase in the levels of intracellular L-malate, therefore providing an induction signal for transcription of the *CuME* gene.

The results presented here confirm that transcription of the *C. utilis* malic enzyme was induced in the presence of L-malate, but only when a better carbon source was not available. When cells grown overnight in 2% glucose were shifted to fresh medium containing 0.5% L-malate, the transcription levels of the *CuME* gene showed a lag phase due to adaptation to the new growth medium, followed by a 2-fold induction in *CuME* transcription at 60 min. Instrumental in the metabolism of extracellular L-malate is the uptake thereof by means of an active malate/fumarate transporter that is also regulated by substrate induction and catabolite repression (Cássio and Leão, 1993; Saayman *et al*., 2000). This strong carbon sensitivity displayed for both the malate transporter and malic enzyme can be linked to the ability of *C. utilis* to utilise intermediates of the TCA cycle as the only source of carbon and energy. When no better assimilable carbon source is available, the cell will rapidly adjust its metabolism to utilise L-malate for the provision of pyruvate for biogenesis as well as energy production via the TCA cycle. Since the activity of the *C. utilis* malate/fumarate transporter

and malic enzyme exhibit similar regulatory patterns, it suggests concerted regulatory mechanisms that govern the degradation of extracellular malate in this organism.

L-Malate is commercially used as a food additive, having greater tartness and taste retention than most other food acids (Neufeld *et al.*, 1991). It is, however, also one of the most important determinants of wine quality as it contributes to total acidity and tartness in wines if present at high levels. Winemakers routinely use bacterial malolactic fermentation (MLF) alter alcoholic fermentation to deacidify and stabilise their wines, but this is often regarded as problematic and undesirable. Alternative methods for reducing the amounts of L-malate in wine have been investigated, which led to the heterologous expression of the *S. pombe* malate permease and malic enzyme genes via genomic integration in a commercial wine yeast strain (Volschenk *et al*., 2004). This newly introduced malo-ethanolic strain was able to effectively degrade L-malate during synthetic and grape must fermentation without any negative effect on fermentation kinetics and wine quality.

In this study, the *CuME* gene was successfully sub-cloned and co-expressed with the *S. pombe mae1* gene under control of the *PGK1* promoter and terminator sequences in *S. cerevisiae*. The efficacy of the recombinant strain to degrade extracellular L-malate was better than that of a wild type *S. pombe*, as well as the recombinant *S. cerevisiae mae1-mae2* strain (Volschenk *et al*., 2004). This suggests that the *C. utilis* malic enzyme may have significant potential for industrial applications that should be further investigated.

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this study was to gain insight into the regulatory mechanisms that govern the degradation of extracellular L-malate in *Candida utilis* and the physiological relevance thereof. The genes encoding the *C. utilis* malate transporter and malic enzyme have not yet been cloned and there is little information on other genes from *C. utilis*. However, the genes encoding the *Schizosaccharomyces pombe* L-malate transporter (*mae1*) and malic enzyme (*mae2*) have been cloned and characterised, as well as the malic enzyme gene (*MAE1*) from *Saccharomyces cerevisiae*. These three genes could therefore be used as a reference for the isolation and characterisation of the corresponding *C. utilis* genes. Cloning of the *C. utilis* transporter and/or malic enzyme genes could also provide us with an alternative dicarboxylic acid transporter and/or malic enzyme for heterologous expression of the genes for commercial applications.

We have demonstrated significant differences between *S. pombe* and *C. utilis* concerning the uptake of L-malate and fumarate and the regulation thereof (Chapter 6, Saayman *et al*., 2000). Cells of *C. utilis* effectively degraded extracellular L-malate and fumarate, but glucose or other assimilable carbon sources repressed the transport and degradation of these dicarboxylic acids. The transport of both dicarboxylic acids was shown to be strongly inducible by either fumarate or L-malate, while kinetic studies suggest that the two dicarboxylic acids are transported by the same protein. In contrast, *S. pombe* effectively degraded extracellular L-malate only in the presence of glucose or other assimilable carbon sources. The *S. pombe* malate transporter was unable to transport fumarate, although fumarate inhibited the uptake of L-malate.

Understanding the mechanism and regulation of dicarboxylic acid transport in *C. utilis* required the cloning and molecular analysis of the corresponding genes. Due to the generally low level of homology between known dicarboxylic acid transporters, it was impossible to design homologous or degenerate primers for direct cloning of the *C. utilis* dicarboxylic acid transporter via PCR or homologous recombination. The only alternative was to construct a cDNA library from *C. utilis* that could be used to complement an *S. cerevisiae* strain (malate transporter absent) or the *S. pombe mae1* mutant strain (mutated malate transporter). Due to the low transformation frequencies usually obtained with *S. pombe*, it was decided to transform the cDNA library into an *S. cerevisiae* strain carrying a plasmid containing the

S. pombe malic enzyme gene (*mae2*) to allow screening for a malate-degrading *S. cerevisiae* clone.

Constitutive and regulated expression vectors containing *S. cerevisiae* promoter and terminator sequences were constructed to overcome potential problems with heterologous expression of a *C. utilis* gene under regulation of its own promoter sequences in *S. cerevisiae* (Chapter 7). A number of strategies were employed to ensure representativeness and high transformation frequencies for the cDNA library, but despite numerous screens with different vector systems, we were unable to clone the *C. utilis* dicarboxylic acid transporter gene. This could be due to a number of reasons, including weak translation of the gene due to codon bias, ineffective channelling of the protein via the endoplasmatic reticulum and Golgi complex, misfolding of the protein due to the absence of specific chaperone proteins, poor association with the plasma membrane, etc. It should be kept in mind that the *S. cerevisiae* host strain does not have a malate transporter and may therefore not be able to process the foreign *C. utilis* gene correctly. Effective expression of the *S. pomb*e malate transporter in *S. cerevisiae* raised the possibility that this could also be the done with the *C. utilis* transporter, but there may be significant sequence differences between the two genes or proteins that could account for the poor or lack of expression of the *C. utilis* gene.

Future research would involve alternative strategies to isolate the *C. utilis* dicarboxylic acid transporter. One option is to transform the cDNA library into the *S. pombe mae1* mutant strain and screen for complementation of its ability to degrade extracellular malate. However, even if high transformation frequencies could be obtained with *S. pombe*, there is still the possibility that the *C. utilis* gene may also not be functionally expressed in *S. pombe*. An alternative would be to follow the strategy described by Gerós *et al*. (2000) for the isolation of the lactate transporter from *C. utilis*: plasma membranes from cells grown either on malate (induce dicarboxylate transporter) or glucose (repress dicarboxylate transporter) are incubated with ^{14}C-malate, followed by SDS-PAGE to visualise the ^{14}C-labelled band only in the malate-grown cells, which would indicate the presence of the dicarboxylate transporter. Cloning of the *C. utilis* transporter gene will allow DNA sequence analyses and comparison with the *S. pombe* malate transporter to identify conserved areas that may be involved in the membrane localisation, substrate specificity and/or mode of action.

The ability of a yeast strain to degrade extracellular L-malate is dependent on the efficient transport of the dicarboxylic acid, as well as the efficacy of the intracellular malic enzyme for the decarboxylation of L-malate to yield pyruvate and CO_2. Cloning and molecular analysis

of the *C. utilis* malic enzyme gene, *CuME* revealed a high degree of homology with the malic enzyme genes of *S. pombe* and *S. cerevisiae* (Chapter 8). The *C. utilis* malic enzyme had a temperature optimum of 52˚C with a loss of only 40% of its activity after exposure to 60˚C for 120 min. Furthermore, the *C. utilis* malic enzyme uses either NAD^+ or $NADP^+$ for decarboxylation of oxaloacetate, but is unable to utilise $NADP^+$ as coenzyme for the decarboxylation of L-malate. Based on its substrate specificity and cofactor requirements, the *C. utilis* malic enzyme can therefore be classified as L-Malate: NAD^+ oxidoreductase (oxaloacetate decarboxylating; EC 1.1.1.38).

The regulation of the *C. utilis* malic enzyme was determined on the transcriptional and translation level by means of northern analysis and enzyme activity assays, respectively. Results presented in Chapter 8 demonstrated a 10-fold increase in transcription of *CuME* when grown in media containing 0.5% malate as sole carbon source, compared to transcription when grown in media containing 2% glucose. When cells were shifted from media containing 2% glucose to media containing 0.5% malate, a 2-fold increase in transcription levels was observed after 60 min. The malic enzyme activity assays corresponded well with the transcriptional data, indicating that expression of the *C. utilis* malic enzyme is indeed subject to glucose repression and substrate induction.

The strong carbon sensitivity displayed for the malate transporter and malic enzyme can be linked to the ability of *C. utilis* to utilise intermediates of the TCA cycle as the only source of carbon and energy. When no better assimilable carbon source is available, the cell will adjust its metabolism to utilise L-malate for the provision of pyruvate for biogenesis as well as energy production via the TCA cycle. Since the activity of the *C. utilis* malate/fumarate transporter and malic enzyme exhibit similar regulatory patterns, it suggests concerted regulatory mechanisms that govern the degradation of extracellular malate in this organism.

The yeast *C. utilis* is an industrially important microorganism that is widely used for the production of biologically useful materials, single-cell protein, etc. (Boze *et al*., 1994) and has been approved for use as a foodstuff by the US Food and Drug Administration. A possible industrial application for a recombinant *C. utilis* transporter would be to provide a biological means to remove excess fumarate from effluents of certain chemical industries and convert it (via fumarase) to L-malate, an important commercial chemical used in the food and wine industry. As discussed in the second half of Chapter 7, this could be achieved by expressing the *C. utilis* dicarboxylic acid transporter gene in *S. cerevisiae* together with the over-expression of the *S. cerevisiae* fumarase gene (*FUM1*). When cloning of the *C. utilis*

transporter gene proved to be more difficult than anticipated, an alternative strategy was investigated, namely to target the *FUM1* gene for secretion under control of the *S. cerevisiae MFα1* secretion signal. Degradation of extracellular fumarate was improved by over-expression of the cytosolic fumarase, but at a slower rate than expected. This could be due to the lack of auxillary proteins required for translocation across the membrane. However, it was also noted that the fumarate was not quantitatively converted to L-malate, which is difficult to explain without further investigation as to the effect of product inhibition, etc.

The *CuME* gene was also subcloned and introduced into a *S. cerevisiae* laboratory strain, together with the *S. pombe* malate transporter gene (*mae1*), both under the constitutive regulation of the *S. cerevisiae* 3-phosphoglycerate kinase (*PGK1*) promoter and terminator elements. The results (Chapter 8) clearly showed that the malic enzyme was active in *S. cerevisiae* and the degradation ability of the recombinant strain compares well with results obtained for the co-expression of the *S. pombe mae1* and *mae2* genes in *S. cerevisiae.* This suggests that the *C. utilis* malic enzyme may have significant potential for industrial applications that should be further investigated.

During this study, we only started to elucidate the mechanisms involved in L-malate metabolism in *C. utilis*. It is clear that the regulation of both the transporter and intracellular malic enzyme is quite complex and the expression of the respective genes are sensitive to the specific substrates as well as other available carbon sources. Further analysis of the CuMEp and other proteins involved in the degradation of malic acid in *C. utilis* will provide more information on the physiological importance of this substrate, as well as the various routes for its metabolism inside the cell.

LITERATURE CITED

Alsina, A., Mason, M., Uphoff, R.A., Riggsby, W.S., Becker, J.M. and D. Murphy. 1988. Catheter-associated *Candida utilis* fungemia in a patient with acquired immunodeficiency syndrome: species verification with a molecular probe. J. Clin. Microbiology. **26**: 621-624.

Amador, P., Borges, F. and M. Côrte-Real. 1996. Biochemical characterization of a mutant of the yeast *Pichia anomala* derepressed for malic acid utilization in the presence of glucose. FEMS Micriobiol. Lett. **141**: 227-231.

Ansanay, V., Dequin, S., Camarasa, C., Schaeffer, V., Grivet, J., Blondin, B., Salmon, J. and P. Barre. 1996. Malolactic fermentation by engineered *Saccharomyces cerevisiae* as compared with engineered *Schizosaccharomyces pombe*. Yeast **12**: 215-225.

Aquila, H., Link, T.A. and M. Klingenberg. 1985. The uncoupling protein from brown fat mitochondria is related to the mitochondrial ADP/ATP carrier. Analysis of sequence homologies and of folding of the protein in the membrane. EMBO J. **4**: 2369-2376.

Aquila, H., Misra, D., Eulitz, M. and M. Klingenberg. 1982. Complete amino acid sequence of the ADP/ATP carrier from beef heart mitochondria. Hoppe-Seyler's Z. Physiol. Chem. **363**: 345-349.

Araujo, A. and J. D'Souza. 1986. Enzymic saccharification of pretreated rice straw and biomass production. Biotechnol. Bioeng. **28**: 1503-1509.

Armstrong, D.A., Martin, S.M. and H. Yamazaki. 1984. Production of ethyl acetate from dilute ethanol solutions by *Candida utilis*. Biotechnol. Bioeng. **26**: 1038.

Armstrong, M. and H. Yamazaki. 1984. Production of acetaldehyde from ethanol by *Candida utilis*. Biotechnol. Lett. **6**: 183-188.

Arnold, J.L., Knapp, J.S. and C.L. Johnson. 2000. The use of yeasts to reduce the polluting potential of silage effluent. **34**: 3699-3708.

Artus, N.N. and G.E. Edwards. 1985. NAD-malic enzyme from plants. FEBS Lett. **182**: 225-233.

Asakura, K., Iwaguchi, S-I., Homma, M., Sukai, T., Higashide, K. and K. Tanaka. 1991. Electrophoretic karyotypes of clinically isolated yeasts of *Candida albicans* and *Candida glabrata*. J. Gen. Microbiol. **137**: 2531-2538.

Ausubel, F.M., Brent, R., Kinston, R., Moore, D.D., Seidman, J.G., Smith, J.A. and K. Struhl. 1995. Current protocols in Molecular Biology. John Wiley and Sons, New York.

Azzam, A.M. and Y.A. Heikel. 1989. Aerobic treatment of molasses distillery waste water and biomass production. J. Environ. Sci. Health Part A **A24**: 967-978.

Bagetto, L.G. 1992. Deviant energetic metabolism of glycolytic cancel cells. Biochim. **74**: 959-974.

Bakker, B.M., Overkamp, K.M., Van Maris, A.J.A., Kötter, P., Luttik, M.A.H., Van Dijken, J.P. and J.T. Pronk. 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **25**: 15-37.

Baranowski, K., and F. Radler. 1984. The glucose-dependent transport of L-malic acid in *Zygosaccharomyces bailii*. Antonie van Leeuwenhoek **50**: 329-340.

Barnett, J.A. and H.L. Kornberg. 1960. The utilisation by yeast of acids of the tricarboxylic acid cycle. J. Gen. Microbiol. **23**: 65-82.

Barnett, J.A., R.W. Payne, and D. Yarrow. 1990. Yeasts: Characteristics and identification, 2nd ed. University Press, Cambridge.

Barroso, I. and P. Santisteban. 1999. Insulin-induced early growth response gene (*Egr-1*) mediates a short term repression of rat malic enzyme gene transcription. J. Biol. Chem. **274**: 17997-18004.

Basabe, L., Domínguez, I. and F.P. Chávez. 2004. Cloning and sequence analysis of the *Candida utilis HIS3* Gene. Biomol. Eng. **21**: 15-20.

Beck, L. 1989. A review of farm waste pollution. L. Inst. Water and Environ. Manag. **3**: 467-477.

Belcarz, A., Ginalska, G., Lobarzewski, J., and C. Penel. 2002. The novel non-glycosylated invertase from *Candida utilis* (the properties and the conditions of production and purification). Biochim. Biophys. Acta **1594**: 40-53.

Beneman, J.R. 1992. Microalgae aquaculture feeds. J. Appl. Phycol. **4**: 233-245.

Bercovitz, A., Peleg, Y., Battat, E., Rokem, J.S. and I. Goldberg. 1990. Localozatio of pyruvate carboxylase in organic acid producing *Aspergillus* strains. Appl. Environ. Microbiol. **56**: 1594-1597.

Bisson, L.F. 1988. High-affinity glucose transport in *Saccharomyces cerevisiae* is under general glucose repression control. J. Bacteriol. **170**: 4838-4845.

Bojunga, N. and K.D. Entian. 1999. Cat8p, the activator of gluconeogenic genes in *Saccharomyces cerevisiae*, regulates carbon source-dependent expression of NADP-dependent cytosolic isocitrate dehydrogenase (Idp2p) and lactate permease (Jen1p). Mol. Gen. Genet. **262**: 869-875.

Bojunga, N., Kotter, P. and K.D. Entian. 1998. The succinate/fumarate transporter Acr1p of *Saccharomyces cerevisiae* is part of the gluconeogenic pathway and its expression is regulated by Cat8p. Mol. Gen. Genet. **5**: 453-461.

Boles, E. and C.P. Hollenberg. 1997. The molecular genetics of hexose transport in yeasts. FEMS Microbiol. Rev. **21**: 85-111.

Boles, E., de Jong-Gubbels, P. and J.T. Pronk. 1998. Identification and characterization of *MAE1*, the *Saccharomyces cerevisiae* structural gene encoding mitochondrial malic enzyme. J. Bacteriol. **180**: 2875-2882.

Börsch, D. and P. Westhoff. 1990. Primary structure of NADP-dependent malic enzyme in the dicotyledonous C_4 plant *Flaveria trinervia*. FEBS Lett. **273**: 111-115.

Boulton, R.B., Singleton, V.L., Bisson, L.F. and R.E. Kunkee. 1996. In *Principles and practices of winemaking*, Chapman & Hall, New York.

Boze, H., Moulin, G. and P. Galzy. 1994. Production of food and fodder yeast. Crit. Rev. Biotechnol. **12**: 65-86.

Boze, H., Moulin, G., and P. Galzy. 1992. Production of food and fodder yeast. Crit. Rev. Biotechnol. **12**: 65-86.

Bruinenberg, P.M., Van Dijken, J.P. and I. Schaaff-Gerstenschläger. 1983[a]. An enzymic analysis of NADPH production and consumption in *Candida utilis*. J. Gen. Microbiol. **129**: 965-971.

Bruinenberg, P.M., Van Dijken, J.P. and W.A. Scheffers. 1983[b]. A theoretical analysis of NADPH production and consumption in yeasts. J. Gen. Microbiol. **129**: 965-971.

Bruinenberg, P.M., Van Dijken, J.P., Kuenen, J.G. and W.A. Scheffers. 1985. Oxidation of NADH and NADPH by mitochondria from the yeast *Candida utilis*. J. Gen. Microbiol. **131**: 1043-1051.

Burford, J.R. 1976. Effect of the application of cow slurry to grassland on the composition of the soil atmosphere. J. Sci. Food Agr. **27**: 115-126.

Byrne, S.M. and C.S. Hoffman. 1993. Six *git* genes encode a glucose-induced adenylate cyclase activation pathway in the fission yeast *Schizosaccharomyces pombe*. J. Cell. Sci. **105**: 1095-1100.

Camarasa, C., Bidard, F., Bony, M., Barre, P. and S. Dequin. 2001. Characterization of *Schizosaccharomyces pombe* malate permease by expression in *Saccharomyces cerevisiae*. Appl. Environ. Microb. **67**: 4144-4151.

Carvalho-Silva, M. and I. Spencer-Martins. 1990. Modes of lactose uptake in the yeast species *Kluyveromyces marxianus*. Antonie van Leeuwenhoek. **57**: 77-81.

Casal, M., Blázquez, M., Gamo, F-J., Gancedo, C. and C. Leão. 1995. Lack of lactate-proton symport activity in *pck1* mutants of *Saccharomyces cerevisiae*. FEMS Microb. Lett. **128**: 279-282.

Casal, M., Cardoso, H. and C. Leão. 1996. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. Microbiology **142**: 1385-1390.

Casal, M., Paiva, S., Andrade, R.P., Gancedo, C. and C. Leão. 1999. The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1*. J. Bacteriol. **181**: 2620-2623.

Caspari, T. 1997. Onset of gluconate-H^+ symport in *Schizosaccharomyces pombe* is regulated by the kinases Wis1 and Pka1, and requires the *gti1*$^+$ gene product. J. Cell. Sci. **110**: 2599-2608.

Cássio, F., and C. Leão. 1991. Low- and high-affinity transport systems for citric acid in the yeast *Candida utilis*. Appl. Environ. Microbiol. **57**: 3623-3628.

Cássio, F., and C. Leão. 1993. A comparative study on the transport of L(-)malic acid and other short-chain carboxylic acids in the yeast *Candida utilis*: Evidence for a general organic acid permease. Yeast **9**: 743-752.

Cássio, F., Leão, C. and N. van Uden. 1987. Transport of lactate and other short-chain monocarboxylates in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **53**: 509-513.

Castrillo, J.I., Kaliterna, J., Weusthuis, R.A., van Dijken, J.P. and J.T. Pronk. 1996. High-cell-density cultivation of yeasts on disaccharides in oxygen-limited batch cultures. Biotech. Bioeng. **49**: 621-628.

Chakravorty, M., Veiga, L.A., Bacila, M. and B.L. Horecker. 1962. Pentose Metabolism in Candida. II. The Dephosphopyridine Nucleotide-Specific Polyol Dehydrogenase of *Candida utilis*. J. Biol. Chem. **237**: 1014-1020.

Chang, G.G., Huang, T.M. and T.C. Chang. 1988. Reversible dissociation of the catalytically active subunits of pigeon liver malic enzyme. Biochem. J. **254**:123-130.

Chang, G-G. and L. Tong. 2003. Structure and function of malic enzymes, a new class of oxidative decarboxylases. Biochemistry **42**: 12721-12733.

Chang, H., Chou, W. and G. Chang. 2002. Effect of metal binding on the structural stability of pigeon liver malic enzyme. J. Biol. Chem. **277**: 4663-4671.

Chávez, F.P., Pons, T, Delgado, J.M. and L. Rodriquez. 1998. Cloning and Sequence analysis of the gene encoding invertase (*INV1*) from the yeast *Candida utilis*. Yeast **14**: 1223-1232.

Chávez, F.P., Rodriquez, L., Díaz, J., Delgado, J.M. and J.A. Cremata. 1997. Purification and characterization of an invertase from *Candida utilis*: Comparison with natural and recombinant yeast invertases. J. Biotechnol. **53**: 67-74.

Cheng, C. and J-H. Ma. 1997. Glucose metabolism and bioreduction of 2-butanone by *Candida utilis* studied by means of ion-exchange chromatography. J. Chromatogr. **A763**: 205-211.

Cheng, Q., and C.A. Michels. 1989. The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. Genetics **123**: 477-484

Cherry, J.R., Johnson, T.R., Dollard, C., Shuster, J.R. and C.L. Denis. 1989. Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. Cell **56**: 409-419.

Chibata, I., Tosa, T. and I. Takata. 1983. Continuous production of L-malic acid by immobilized cells. Trends Biotechnol. **1**: 9-11.

Choi, M.H., Ji, G.J., Koh, K.H., Ryu, Y.W., Jo, D.H., and Y.H. Park. 2002. Use of waste chinese cabbage as a substrate for yeast biomass production. Bioresource. Technol. **83**: 251-253.

Chou, W., Tsai, W., Lini, C. and G. Chang. 1995. Selective oxidative modification and affinity cleavage of pigeon liver malic enzyme by the Cu^{2+}-ascorbate system. J. Biol. Chem. **270**: 25935-25941.

Christen, P., Domenech, F., Michelena, G., Auria, R. and S. Revah. 2002. Biofiltration of volatile ethanol using sugar cane bagasse inoculated with *Candida utilis*. J. Hazard Mater. **B89**: 253-265.

Cleland, W.W. 1999. Mechanisms of enzymatic oxidative decarboxylation. Acc. Chem. Res. **32**: 862-868.

Clifton, D., Walsh, R.B. and D.G. Fraenkel. 1993. Functional studies of yeast glucokinase. J. Bacteriol. **175**: 3289-3294.

Coleman, D.E., Rao, G.S., Goldsmith, E.J., Cook, P.F. and B.G. Harris. 2002. Crystal structure of the malic enzyme from *Ascaris suum* complexed with nicotinamide adenine dinucleotide at 2.3 Å resolution. Biochemistry **41**: 6928-6938.

Conover, T.E. 1987. Does citrate transport supply both acetyl groups and NADPH for cytoplasmic fatty acid synthesis? Trend Biochem. Sci. **12**: 88-89.

Cooper, T.G. 1982. Transport in *Saccharomyces cerevisiae*. In *The Molecular Biology of the Yeast* Saccharomyces*; Metabolism and Gene Expression* Strathern, J.N. and E.W. Jones (Ed.)

Côrte-Real, M., and C. Leão. 1990. Transport of malic acid and other dicarboxylic acids in the yeast *Hansenula anomala*. Appl. Environ. Microbiol. **56**: 1109-1113.

Côrte-Real, M., Leão, C. and N. van Uden. 1989. Transport of L-malic acid and other dicarboxylic acids in the yeast *Candida sphaerica*. Appl. Microbiol. Biotechnol. **31**: 551-555.

Corzo, F., Revah, S. and P. Christen. 1995. Effect of oxygen on the ethyl acetate production from continuous ethanol stream by *Candida utilis* in submerged cultures. In *Food Flavours: generation analysis and process influence*. pp 1141-1154. G Charalambous (Ed.) Amsterdam, Elsevier.

Cozens, A.L., Runswick, M.J. and J.E. Walker. 1989. DNA sequences of two expressed nuclear genes for human mitochondrial ADP/ATP translocase. J. Mol. Biol. **206**: 261-280.

Crabtree, H.G. 1929. Observations on the carbohydrate metabolism of tumours. Biochem. J. **23** 536-545.

Crous, J.M., Pretorius, I.S. and W.H. Van Zyl. 1995. Cloning and expression of an *Aspergillus kawachii* endo-1,4-b-xylanase gene in *Saccharomyces cerevisiae*. Curr. Genet. **28**: 467-473.

Daniel, H-M., Sorrell, T.C, and W. Meyer. 2001. Partial sequence analysis of the actin gene and its potential for studying the phylogeny of *Candida* species and their teleomorphs. Int. J. Syst. Evol. Micr. **51**: 1593-1606.

Davids, S.J., Simard, R.E. and B-H. Lee. 1986. Aerobic yeast growth on hydrolyzed potato Eextracts. Entropic. **22**: 49-52.

De Jong-Gubbels, P., Bauer, J., Niederberger, P., Stuckrath, I., Kotter, P., van Dijken, J.P. and J.T. Pronk. 1998. Physiological characterization of a pyruvate-carboxylase-negative *Saccharomyces cerevisiae* mutant in batch and chemostat cultures. Antonie van Leeuwenhoek. **74**: 253-263.

De Queiroz, H. and A. Pareilleux. 1990. Growth kinetics of *Schizosaccharomyces pombe* under various culture conditions: influence of pH, malate, ethanol and oxygenation. Appl. Microbiol. Biotechnol. **33**: 578-581.

Delcourt, F., Taillandier, P., Vidal, F. and P. Strehaiano. 1995. Influence of pH, malic acid and glucose concentrations on malic acid consumption by Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol. **43**: 321-324.

Delfin, J., Perdomo, W., García, B. and J. Menendez. 2001. Isolation and sequence of the MIG1 homologue from the yeast *Candida utilis*. Yeast **18**: 597-603.

DeVit, M.J., Waddle, J.A. and M. Johnston. 1997. Regulated nuclear translocation of the Mig1 glucose repressor. Mol. Biol. Cell. **8**: 1603-1618.

Dinoupoulou, G., Rudd, T. and J.N. Lester. 1988. Anaerobic acidogenesis of a complex wastewater. Biotechnol. Bioeng. **31**: 958-968.

Divjak, S. and J.R. Mor. 1973. On the activity of carbon dioxide fixation in growing yeasts. Arch. Microbiol. **94**: 1991-1999.

Doležal, P., Vaňáčová, S., Tacheza, J. and I. Hrdý. 2004. Malic enzymes of *Trichomonas vaginalis*: two enzyme families, two distinct origins. Gene **329**: 81-92.

Domagk, G.F. and K.M. Doering. 1975. D-Ribose-5-phosphate isomerase from *Candida utilis*. Meth. Enzymol. **41**: 427-429.

Domagk, G.F. and R. Chilla. 1975. Glucose-6-phosphate dehydrogenase from *Candida utilis*. Meth. Enzymol. **41**: 205-208.

Domagk, G.F., Doering, K.M. and R. Chilla. 1973. Purification and properties of ribose-phosphate isomerase from *Candida utilis*. Eur. J. Biochem. **38**: 259-264.

Dos Santos, M.M., Gombert, A.K., Christensen, B., Olsson, L. and J. Nielsen. 2003. Identification of *in vivo* enzyme activities in the co-metabolism of glucose and acetate by *Saccharomyces cerevisiae* by using ^{13}C-labeled substrates. Eukariot. Cell. **2**: 599-608.

Driscoll, B.T. and T.M. Finan. 1996. $NADP^+$-dependent malic enzyme of *Rhizobium meliloti*. J. Bacteriol. **178**: 2224-2231.

Elmaleh, S., Defrance, M.B, and C. Ghommidh. 1999. Organic acids oxidation by *Candida utilis*: application to industrial waste water treatment. Process. Biochem. **35**: 441-449.

Elmaleh, S., Defrance, M.B. and C. Ghommidh. 1996. Acidogenic effluents treatment in a yeast reactor. Water Res. **30**: 2526-2529.

Endemann, G., Goetz, P.G., Edmond, J. and H. Brunengraber. 1982. Lipogenesis from ketone bodies in the isolated perfused rat liver. Evidence for the cytosolic activation of acetoacetate. J. Biol. Chem. **257**: 3434-3440.

Eraso, P. and J.M. Gancedo. 1984. Catabolite repression in yeasts is not associated with low levels of cAMP. Eur. J. Biochem. **141**:195-198.

Espinel, A.E. and J.M. Peinado. 1994. Hexokinases and catabolite repression in *Candida utilis*. Folia Microbiol. **39**: 515.

Espinel, A.E., Gómez-Toribio, V. and J.M. Peinado. 1996. The inactivation of hexokinase activity does not prevent glucose repression in *Candida utilis*. FEMS Microbiol. Lett. **135**: 327-332.

Fellows, P.J. and J.T. Worgan. 1986. Studies on the growth of *Candida utilis* on D-galacturonic acid and the products of pectin hydrolysis. Enzyme. Microb. Technol. **8**: 537-540.

Fellows, P.J. and J.T. Worgan. 1987[a]. Growth of *Saccharomyces fibuliger* and *Candida utilis* in mixed culture on apple processing wastes. Enzyme. Microb. Technol. **9**: 434-437.

Fellows, P.J. and J.T. Worgan. 1987[b]. Growth of *Saccharomyces fibuliger* and *Candida utilis* in mixed culture on pectic materials. Enzyme. Microb. Technol. **9**: 430-433.

Fernández, M., Fernández, E. and R. Rodicio. 1994. *ACR1*, a gene encoding a protein related to mitochondrial carriers, is essential for acetyl-CoA synthetase activity in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **242**: 727-735.

Fernández, M.J., Medrano, L., Ruiz-Amil, M. and M. Losada. 1967. Regulation and function of pyruvate kinase and malate enzyme in yeast. Eur. J. Biochem. **3**: 11-18.

Fiechter, A., Fuhrmann, G.F. and O. Käppeli. 1981. Regulation of glucose metabolism in growing yeast cells. Adv. Microb. Physiol. **22**: 123-183.

Fields, M.L., Tantratian, S. and R.E. Baldwin. 1991. Production of bacterial and yeast biomass in ground corn cob and ground corn stalk media. J. Food Prot. **54**: 117-120.

Fletcher, T.S., Kwee, I.K., Nakada, T., Largman, C. and B.M. Martin. 1992. DNA sequence of the yeast transketolase gene. Biochemistry **31**: 1892-1896.

Flores, C-L., Rodriquez, C., Petit, T. and C. Gancedo. 2000. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. FEMS Microbiol. Rev. **24**: 507-529.

Fraenkel, D.G. 1982. Carbohydrate metabolism. In *The molecular biology of the yeast* Saccharomyces*: Metabolism and gene expression.* pp. 1-38. Strathern, J.N., Jones, E.W. and J.R. Broach (Ed.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Frenkel, R. 1975. Regulation and physiological functions of malic enzymes. Curr. Top. Cell. Regul. **9**: 157-181.

Fuck, E., Stark, G. and F. Radler, 1973. Äpfelsäurestoffwechsel bei *Saccharomyces* II. Anreicherung und Eigenschaften eines Malatenzymes. Arch. Mikrobiol. **89**: 223-231.

Fuhrmann, G.F. and B. Völker. 1993. Misuse of graphical analysis in nonlinear transport kinetics by Eadie-Hofstee plots. Biochim. Biophys. Acta. **1145**: 180-182.

Fukuhara, H. 2003. The Kluyver effect revisited. FEMS Yeast Res. **3**: 327-331.

Gamal, R.F., El-Sawy, M., Ramadan, E.M. and H.M. Abd El-Hady. 1985. Protein production from *Candida utilis* in continuous culture. Egypt. J. Microbiol. Spec. Issue 65-80.

Gancedo, C. and R. Serrano. 1989. Energy yielding metabolism in yeast. In *The yeasts* vol. 3. 2nd ed, p. 205-259. J.S. Harrison and A.H. Rose (Ed.). Academic Press, Inc., New York.

Gancedo, J.M. 1998. Yeast carbon catabolite repression. Microbiol. Mol. Biol. Rev. **62**: 334-361.

Gancedo, J.M. and R. Lagunas. 1973. Contribution of the pentose phosphate pathway to glucose metabolism in *Saccharomyces cerevisiae*: a critical analysis on the use of labelled glucose. Plant Sci. Lett. **1**: 193-200.

Gasnier, B. 1987. Characterization of low- and high-affinity glucose transports in the yeast *Kluyveromyces marxianus*. Biochim. Biophys. Acta. **903**: 425-433.

Gennis, R.B. 1989. In: *Biomembranes: Molecular structure and function*. pp1-350. Springer-Verlag. New York Inc.

Gerós, H., Baltazar, F., Cássio, F. and C. Leão. 2000. L-[U-14C] lactate binding to a 43 kDa protein in plasma membranes of *Candida utilis*. Microbiology. **146**: 695-699.

Giovannucci, E., Ascherio, A., Rimm, E.B., Stampfer, M.J., Colditz, G.A. and W.C. Willet. 1995. Intake of carotenoids and retinol in relation to risk of prostate cancer. J. Natl. Cancer Inst. **87**: 1767-1776.

Gletsu, N., Dixon, W. and M.T. Clandinin. 1999. Insulin receptor at the mouse hepatocyte nucleus after a glucose meal induces dephosphorylation of a 30-kDa transcription factor and a concomitant increase in malic enzyme gene expression. J. Nutr. **129**: 2154-2161.

Gonzalés-Siso, M.I., Freire Picos, M.A. and M.E. Cerdan. 1996. Reoxidation of the NADPH produced by the pentose phosphate pathway is necessary for the utilization of glucose by *Kluyveromyces lactis* rag2 mutants. FEBS Lett. **387**: 7-10.

Goodridge, A.G. and E.G. Ball. 1966. Lipogenesis in the pigeon: *in vitro* studies. Am. J. Physiol. **211**: 803-808.

Goodridge, A.G. and E.G. Ball. 1967. Lipogenesis in the pigeon: *in vivo* studies. Am. J. Physiol. **213**: 245-249.

Gourdon, P., Baucher, M., Lindley, N.D. and A. Guyonvarch. 2000. Cloning of the malic enzyme gene from *Corynebacterium glutaminm* and role of the enzyme in lactate metabolism Appl. Environ. Microbiol. **66**: 2981-2987.

Grobler, J., Bauer, F., Subden, R.E. and H.J.J. van Vuuren. 1995. The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C_4 dicarboxylic acids. Yeast **11**: 1485-1491.

Groenewald, M. and M. Viljoen-Bloom. 2001. Factors involved in the regulation of the *Schizosaccharomyces pombe* malic enzyme. Curr. Genet. **39**: 222-230.

Groisillier, A. and A. Lonvaud-Funel. 1999. Comparison of partial malolactic enzyme gene sequences for phylogenetic analysis of some lactic acid bacteria species and relationships with the malic enzyme. Int. J. Syst. Bacteriol. **49**: 1417-1428.

Gross, F. 1972. The formation of fermentation liquor during ensilage and its elimination. Bayer. Landw. Jb. **49**: 964-970.

Gupta, L.K., Pathak, G. and R.P. Tiwari. 1990. Effect of nutrition variables on solid state alcoholic fermentation of apple pomace by yeasts. J. Sci. Food Agric. **50**: 55-62.

Haarasilta, S. and L. Taskinen. 1977. Location of three key enzymes of gluconeogenesis in baker's yeast. Arch. Microbiol. **113**: 159-161.

Hai, T.W., Lui, F., Coukos, W.J. and M.R. Green. 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. **3**: 2083-2090.

Han, E., Cotty, F., Sottas, H.J. and C.A. Micheles. 1995. Characterization of *AGT1* encoding a general α-glucoside transporter from *Saccharomyces cerevisiae*. Mol. Microbiol. **17**: 1093-1107.

Haron, E., Feld, R., Tuffnell, P., Patterson, B., Hasselback, R. and A. Mallow. 1987. Hepatic Candiasis: an increasing problem in immunocompromised patients. Am. J. Med. **83**: 17-26.

Hartley, J.L., Temple, G.F. and M.A. Brasch. 2000. DNA cloning using *in vitro* site-specific recombination. Genome Res. **10**: 1788-1795.

Hatanaka, T., Kihira, Y., Shinohara, Y., Majima, E. and H. Terada. 2001. Characterization of loops of the yeast mitochondrial ADP/ATP carrier facing the cytosol by site-directed mutagenesis. Biochem. Biophys. Res. Comm. **286**: 936-942.

Hatch, M.D. and S.L. Mau. 1977. Association of NADP- and NAD-linked malic enzyme activities in *Zea mays*: relation to C_4 pathway photosynthesis. Arch. Biochem. Biophys. **179**: 361-369.

Heiland, S., Radovanovic, N., Höfer, M., Winderickx, J. and H. Lichtenberg. 2000. Multiple hexose transporters of *Schizosaccharomyces pombe*. J. Bacteriol. **182**: 2153-2162.

Hendriks, L., Goris, A., van der Peer, Y., Neefs, J.M., Vancanneyt, M., Kersters, K., Berny, J.F., Hennebert, G.L. and R. de Wachter. 1992. Phylogenetic relationships among Ascomycetes and Ascomycete-like yeasts as deduced from small ribosomal subunit RNA sequences. Syst. Appl. Microbiol. **15**: 98-104.

Hoever, M., Milbradt, B. and M. Höfer. 1992. D-Gluconate is an alternative growth substrate for cultivation of *Schizosaccharomyces pombe* mutants. Arch. Microbiol. **157**: 191-193.

Höfer, M. and F.R. Nassar. 1987. Aerobic and anaerobic uptake of sugars in *Schizosaccharomyces pombe*. J. Gen. Microbiol. **133**: 2163-2172.

Hoffmann, C.S. and F. Winston. 1987. A ten minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57**: 267-272.

Holzer, H. 1961. Regulation of carbohydrate metabolism by enzyme competition. Cold Spring Harb.Sym.Quant.Biol. **26**: 277-288.

Hong, S.H., and J. Marmur. 1987. Upstream regulatory regions controlling the expression of the yeast maltase gene. Mol. Cell. Biol. **7**: 2477-2483.

Horitsu, H., Sasaki, I., Kikuchi, T., Suzuki, H., Sumida, M. and M. Tomoyeda. 1976. Purification, properties and structure of ribose-5-phosphate ketol isomerase from *Candida utilis*. Agric. Biol. Chem. **40**: 257-264.

Hsu, R.Y. 1982. Pigeon liver malic enzyme. Mol. Cell. Biochem. **43**: 3-26.

Hsu, R.Y., Glynias, M.J., Satterlee, J., Feeney, R., Clarke, A.R., Emery, D.C., Roe, B.A., Wilson, R.K., Goodridge, A.G. and J.J. Holbrooks. 1992. Duck liver 'malic' enzyme.

Expression in *Escherichia coli* and characterization of the wild-type enzyme and site-directed mutants. Biochem. J. **284**: 869-876.

Hsu, R.Y., Mildvan, A.S., Chan, G.G. and C. Fung. 1976. Mechanism of malic enzyme from pigeon liver. Magnetic resonance and kinetic studies of the role of Mn^{2+}. J. Biol. Chem. **251**: 6574-6583.

Imshenetskii, A.A., Kondrat'eva, T.F. and N.A. Lobacheva. 1987. Biomass synthesis by diploid *Candida utilis* strains in a medium with ethanol. Mikrobiologiya **56**: 347-349.

Indiveri, C., Iacobazzi, V., Giangregorio, N. and F. Palmieri. 1997. The mitochondrial carnitine carrier protein: cDNA cloning, primary structure and comparison with other mitochondril transport proteins. Biochem J. **3**: 713-719.

Kaliterna, J., Weusthuis, R.A., Castrillo, J.I., van Dijken, J.P. and J.T. Pronk. 1995. Transient responses of *Candida utilis* to oxygen limitation. Regulation of the Kluyver effect for maltose. Yeast **11**: 317-325.

Kaplan, R.S. 2001. Structure and function of mitochondrial anion transport proteins. J. Membr. Biol. **179**: 165-183.

Kaplan, R.S. Mayor, J.A. and D.O. Wood. 1993. The mitochondrial tricarboxylate transport protein. cDNA cloning, primary structure, and comparison with other mitochondrial transport protein. J. Biol. Chem. **268**: 13682-13690.

Kaplan, R.S., Mayor, J.A., Gremse, D.A. and D.O. Wood. 1995. High level expression and characterization of the mitochondrial citrate transport protein from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **270**: 4108-4114.

Kasahara, M., Shimoda, El and M. Maeda. 1997. Amino acid residues responsible for galactose recognition in yeast Gal2 transporter. J. Biol. Chem. **272**: 16721-16724.

Kawai, S., Murao, S., Mochizuki, I., Shibuya, K.Y. and M. Takagi. 1992. Drastic alteration of cycloheximide sensitivity by substitution of one amino acid in the L41 ribosomal protein of yeast. J. Bacteriol. **174**: 254-262.

Keruchen'ko, Y.S., Keruchen'ko, I.D. and K.L. Gladilin. 1995. Formation of malate by yeast of the genus *Pichia*. Prikl. Biochim. Mikrobiol. **31**: 143-144.

Keys, D.A. and L. McAlister-Henn. 1990. Subunit structure, expression and function of NAD(H)-specific isocitrate dehydrogenase in *Saccharomyces cerevisiae*. J. Bacteriol. **172**: 4280-4287.

Klein, R.D. and Favreau, M.A. 1995. The Candida Species: Biochemistry, Molecular Biology, and Industrial Applications. In *Food Biotechnology Microorganisms*, pp 297-371. Hui, Y.H. and G.G. Khachatourians (Ed.) VCH Publishers. New York.

Kobayashi, H., Mitobe, H., Takahashi, K., Yamamoto, T., Shibata, N. and S. Suzuki. 1992. Structural study of a cell wall mannan-protein complex of the pathogenic yeast *Candida glabrata* IFO 0622 strain. Arch. Biochem. Biophys. **294**: 662-669.

Kobayashi, K., Doi, S., Negoro, S., Urabe, I. and H. Okada. 1989. Structure and properties of malic enzyme from *Bacillus stearothermophilus*. J. Biol. Chem. **264**: 3200-3205.

Kockova-Kratochvilova, A. 1990. In *Yeast and Yeast-like Organisms*. pp.77-130. K. Sigler (Ed.). VCR Verlagsgesslschaft. Weinheim.

Kolarov, J., Kolarov, N. and N. Nelson. 1990. A third ADP/ATP translocator gene in yeast. J. Biol. Chem. **265**: 12711-12716.

Kondo, K., Miura, Y., Sone, H., Kobayashi, K. and H. Iijima. 1997. High-level expression of a sweet protein monellin, in the food yeast *Candida utilis*. **15**: 453-487.

Kondo, K., Saito, T., Kajiwara, S., Takagi, M. and N. Misawa. 1995. A transformation system for the yeast *Candida utilis*: Use of a modified endogenous ribosomal protein gene as a drug-resistant marker and ribosomal DNA as an integration target for vector DNA. J. Bacteriol. **177**: 7171-7177.

Kornberg, A. 2001. Remembering our teachers. J. Biol. Chem. **276**: 3-11.

Kreger-van Rij, N.J.W. 1984. The Yeasts, a Taxonomic Study. Amsterdam. Elsevier.

Kronstad, J., De Maria, A., Funnell, D. Laidlaw, R.D. Lee, N., De Sa, M.M. and M. Ramesh. 1998. Signaling via cAMP in fungi: interconnections with mitogen-activated protein kinase pathways. Arch. Microbiol. **170**: 395-404.

Kruckeberg, A.L. and J.R. Dickinson. 2004. Carbon metabolism. In *The metabolism and molecular physiology of Saccharomyces cerevisiae*. J.R. Dickinson and M. Schweizer (Ed.), pp. 42 – 103. CRC Press, London.

Kuczynski, J.T. and F. Radler. 1982. The anaerobic metabolism of malate of *Saccharomyces bailii* and the partial purification and characterization of malic enzyme. Arch. Microbiol. **131**: 266-270.

Kulkarni, G., Cook, P.F. and B.G. Harris. 1993. Cloning and nucleotide-sequence of a full-length cDNA encoding *Ascaris suum* malic enzyme. Arch. Biochem. Biophys. **300**: 231-237.

Kurtzman, C.P. 1984. Synonymy of the yeast genera *Hansenula* and *Pichia* demonstrated through comparisons of deoxyribonucleic acid relatedness. Antonie van Leeuwenhoek **50**: 209-217.

Kurtzman, C.P., Johnson, C.J. and M.J. Smiley. 1979. Determination of conspecificity of *Candida utilis* and *Hansenula jadinii* through DNA reassociation. Mycologia **71**: 844-847.

Kusano, M., Sakai, Y., Kato, N. and H.A. Yoshimoto. 1999. Novel hemiacetal dehydrogenase activity involved in ethyl acetate synthesis in *Candida utilis*. J. Biosci. Bioeng. **87**: 690-692.

Kwon-Chung, K.J., Hicks, J.B. and P.N. Lipke. 1990. Evidence that *Candida stellatoidea* type II is a mutant of *Candida albicans* that does not express sucrose-inhibitable α-glucosidase. Infect. Immun. **58**: 2804-2808.

Lagunas, R. 1993. Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **104**: 229-242.

Lang, J.M. and V.P. Cirillo. 1987. Glucose transport in a kinaseless *Saccharomyces cerevisiae* mutant. J. Bacteriol. **169**: 2932-2937.

Larson, C., Pahlman, I., Ansell, R., Rigoulet, M., Adler, L. and L. Gustafsson. 1998. The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. Yeast **14**: 347-357.

Lawford, G.R., Kligeman, A., and T. Williams. 1979. Production of high-quality edible protein from *Candida* yeast grown in continous culture. Biotechnol. Bioeng. **21**: 1163-1173.

Lawson, J.E. and M.G. Douglas. 1988. Separate genes encode functionally equivalent ADP/ATP carrier proteins in *Saccharomyces cerevisiae*. Isolation and analysis of *AAC2*. J. Biol. Chem. **263**: 14812-14818.

Leão, C. and N. van Uden. 1984. Effects of ethanol and other alkanols on passive proton influx in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. **774**: 43-48.

Leão, C. and N. van Uden. 1986. Transport of lactate and other short-chain monocarboxylates in the yeast *Candida utilis*. Appl. Microbiol. Biotechnol. **23**: 389-393.

Lengeler, J.W. 1993. Carbohydrate transport in bacteria under environmental conditions, a black box? Antonie van Leeuwenhoek. **63**: 275-288.

Li, N., Seetharam, S. and B. Seetharam. 1998. Characterization of the human transcobalamin II promoter. A proximal $G^{C}/_{G}T$ box is a dominant negative element. J. Biol. Chem. **273**: 16104-16111.

Liong, E.C. and T. Ferrenci. 1994. Molecular cloning of a maltose transporter gene from Bacillus *stearothermophilus* and its expression in *Escherichia coli* K-12. Mol. Gen. Genet. **243**: 343-352.

Litchfield, J.H. 1979. Production of single-cell protein for use in food or feed. In *Microbial Technology: Microbial Processes* Vol 1., 2nd edn, pp 93-155. Peppler, H.J. and D. Perlman. (Eds.) New Yor. Academic Press.

Litchfield, J.H. 1983. Single-cell proteins. Science **219**: 740-746.

Liti, G., Wardrop, F.R., Cardinali, G., Martini, A. and G.M. Walker. 2001. Differential responses to antimycin A and expressions of the Crabtree effect in selected *Kluyveromyces* spp. Ann. Microbiol. **51**: 235-243.

Liu, Y.J., Whelen, S. and B.D. Hall. 1999. Phylogenetic relationships among Ascomycetes: evidence from an RNA polymerase II subunit. Mol. Biol. Evol. **16**: 1799-1808.

Lodi, T., Fontanesi, F. and B. Guiard. 2002. Co-ordinate regulation of lactate metabolism genes in yeast: the role of the lactate permease gene *JEN1*. Mol. Genet. Genomics. **266**: 838-847.

Lodi, T., Fontanesi, F., Ferrero, I. and C. Donnini. 2004. Carboxylic acids permeases in yeast: two genes in *Kluyveromyces lactis.* Gene **339**: 111-119.

Loeber, G., Infante, A.A., Maurer-Fogy, I., Krystek, E. and M.B. Dworkin. 1991. Human NAD^{+}-dependent mitochondrial malic enzyme. cDNA Cloning, primary structure, and expression in *Escherichia coli*. J. Biol. Chem. **266**: 3016-3021.

Loureiro-Dias, M.C. 1987. Glucose and polyol transport systems in *Candida intermedia* and their regulation. J. Gen. Microbiol. **133**: 2737-2742.

Loureiro-Dias, M.C. 1988. Movements of protons coupled to glucose transport in yeasts. A comparative study among 248 yeast strains. Antonie van Leeuwenhoek. **54**: 331-343

Lucas, C. and N. van Uden. 1986. Transport of hemicellulose monomers in the xylose fermenting yeast *Candida sheatae*. Appl. Microbiol. Biotechnol. **23**: 491-495.

Lundin, M., Nehlin, J.O. and H. Ronne. 1994. Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. Mol. Cell. Biol. **14**: 1979-1985.

Mackler, B., Haynes, B., Person, R. and G. Palmer. 1980. Electron transport systems of *Candida utilis*. Purification and properties of the respiratory chain-linked external NADH dehydrogenase. Biochim. Biophys. Acta **591**: 289-297.

Maconi, E., Manachini, P., Aragozzini, F., Gennari, C. and G. Ricca. 1984. A study on the maloalcoholic fermentation pathway in *Schizosaccharomyces pombe*. Biochem. J. **217**: 585-588.

Magyar, I. and I. Panyak. 1989. Biological deacidification of wine with Schizosaccharomyces *pombe* entrapped in Ca-alginate gel. Am. J. Enol. Vitic. **40**: 233-240

Makuc, J., Paiva, S., Schauen, M., Kramer, R., Andre, B., Casal, M., Leão, C. and E. Boles. 2001. The putative monocarboxylate permeases of the yeast *Saccharomyces cerevisiae* do not transport monocarboxylic acids across the plasma membrane. Yeast **18**: 1131-1143.

Malathi, S. and G.S. Laddha. 1989. Single-cell protein from defatted mango (*Mangifera indica*) kernels. Indian. J. Exp. Biol. **27**: 792-794.

Malluta, E.F., Decker, P. and B.U. Stambuk. 2000. The Kluyver effect for trehalose in *Saccharomyces cerevisiae*. J. Basic. Microbiol. **40**: 199-205.

Martin, A.M., Goddard, S. and P. Bemister. 1993. Production of *Candida utilis* biomass as aquaculture feed. J. Sci. Food. Agric. **61**: 363-370.

Maugeri-Filho, F. and G. Goma. 1988. SCP production from organic acids with *Candida utilis*. Rev. Microbiol. **19**: 446-452.

Maugeri-Filho, F. and G. Goma. 1980. SCP production from organic acids with *Candida utilis*. Rev.Microbiol. 19 446-454.

Mayer, K. and A. Temperli. 1963. The metabolism of L-malate and other compounds by *Schizosaccharomyces pombe*. Arch. Mikrobiol. **46**: 321-328.

McAlister-Henn, L. and W.C. Small. 1997. Molecular Genetics of Yeast TCA cycle isozymes. Prog. Nucleic Acid Res. **57**: 317-339.

McCammon, M.T., Epstein, C.B., Przybyla-Zawislak, B., McAlister-Henn, L. and R.A. Butow. 2003. Global transcription analyses of Krebs tricarboxylic acid cycle mutants reveals an alternating pattern of gene expression and effects on Hypoxic and oxidative genes. Mol. Biol. Cell. **14**: 958-972.

McGuire, N., Hutson, J. and H. Heubl. 1992. Gangrenous cholecystitis secondary to *Candida tropicalis* Infection in a Patient with Leukemia. Clin. Infect. Dis. **14** 367-368.

McKeehan, W.L. 1982. Glycolysis, glutaminolysis and cell proliferation. Cell. Biol. Int. Rep. **6**: 635-650.

McQuillen, D.P., Zingman, B.S., Meunier, F. and S.M. Levitz. 1992. Inasive infections due to *Candida krusei*: report of ten cases of fungemia that include three cases of endophtalmitis. Clin. Infect. Dis. **14**: 472-478.

Meijer, A.J. and K. van Dam. 1974. The metabolic significance of anion transport in mitochondria. Biochim Biophys Acta. **346**: 213-244.

Meyer, S.A., Ahearn, D.G. and D. Yarrow. 1984. Genus H. *Candida* berkhout. In *The Yeasts - A Taxonomic Study.*, pp 585-840. N.J.W. Kreger-van Rij (Ed.) Elsevier Science Publishers. B.V. Amsterdam.

Mian, F.A., Fencl, Z., Prokop, A., Mohagheghi, A. and A. Fazeli. 1974. Effect of growth rate on the glucose metabolism of yeast grown in continuous culture. Radiorespirometric studies. Folia. Microbiol. **19**: 191-198.

Miki, W. 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem. **63**: 141-146.

Minard, K.I. and L. McAlister-Henn. 1991. Isolation, nucleotide sequence analysis and disruption of MDH2 gene from *Saccharomyces cerevisiae*: evidence for three isozymes of yeast malate dehydrogenase. Mol. Cell. Biol. **11**: 370-380.

Minard, K.I. and L. McAlister-Henn. 2001. Antioxidant function of cytosolic sources of NADPH in yeast. Free Radic. Biol. Med. **31**: 832-843.

Minard, K.I., Jennings, G.T., Loftus, T.M. Xuan, D. and L. McAlister-Henn. 1998. Sources of NADPH and expression of mammalian $NADP^+$-specific isocitrate dehydrogenases in *Saccharomyces cerevisiae*. J. Biol. Chem. **273**: 31486-31493.

Miosga, T. and F.K. Zimmerman. 1996. Cloning and characterization of the first two genes of the non-oxidative part of the *Saccharomyces cerevisiae* pentose-phosphate pathway. Curr. Genet. **30**: 404-409.

Mitchell, P.J., Wang, C. and R. Tjian. 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**: 847-861.

Miura, Y., Kondo, K., Saito, T., Shimada, H., Fraser, P.D. and N. Misawa. 1998. Production of the carotenoids lycopene, β-carotene, and astaxanthin in the food yeast *Candida utilis*. Appl. Environ. Microb. **64**: 1226-1229.

Moreno, S., Klar, A. and P. Nurse. 1991. Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194**: 795-823.

Mueller, R.H. and W. Babel. 1989. Continuous non-fermentative synthesis of acetone. Wiss. Z. **38**: 296-302.

Neal, W.K., Hoffmann, H-P. and C.A. Price. 1971. Sedimentation behavior and ultrastructure of mitochondria from repressed and derepressed yeast, *Saccharomyces cerevisiae*. Plant Cell. Physiol. **12**: 181-192.

Nehlin, J.O. and H. Ronne. 1990. Yeast MIG1 repressor is related to the mammalian early growth response and Wilm's tumour finger proteins. EMBO J. **9**: 2891-2898.

Neufeld, R.J., Rokem, J.S., Pines, O. and I. Goldberg. 1991. l-malic acid formation by immobilized *Saccharomyces cerevisiae* amplified for fumarase. Enz. Microbiol. Technol. **13**: 991-996.

Nevaldine, B.H., Bassel, A.R. and R.Y. Hsu. 1974. Mechanism of pigeon liver malic enzyme subunit structure. Biochim. Biophys. Acta. **336**: 283-293.

Nogae, I. and M. Johnston. 1990. Isolation and characterization of the *ZWF1* gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase. Gene **96**: 161-169.

Ochoa, S., Mehler, A.H. and A. Kornberg. 1947. Reversible oxidative decarboxylation of malic acid. J. Biol. Chem. **167**: 871-872.

Odds, F.C. 1988. *Candida* and Candidosis: A Review and Bibliography. London. Bailliere-Tindale.

Olesen, P.T. and Stahnke, L.H. 2000. The influence of *Debaromyces hansenii* and *Candida utilis* on the aroma formation in garlic spiced fermented sausages and model minces. Meat Sci. **56**: 357-368.

Ortiz, C.P, Steyer, J-P, and A. Bories. 1997. Carbon and nitrogen removal from wastewater by *Candida utilis*: kinetics aspects and mathematical modelling. Process. Biochem. **32**: 179-189.

Osmani, S.A. and M.C. Scrutton. 1983. The subcellular localization of pyruvate carboxylase and of some other enzymes in *Aspergillus nidulans*. Eur. J. Biochem. **133**: 551-560.

Osmani, S.A. and M.C. Scrutton. 1985. The subcellular localization and regulatory properties of pyruvate carboxylase from *Rhizopus arrhizus*. Eur. J. Biochem. **147**: 119-128.

Osothsilp, C. 1987. Genetic and biochemical studies of malic acid metabolism in *Schizosaccharomyces pombe*. Ph.D. thesis, University of Guelph, Ontario, Canada.

Osothsilp, C. and R.E. Subden. 1986[a]. Isolation and characterization of *Schizosaccharomyces pombe* mutants with defective NAD-dependent malic enzyme. Can. J. Microbiol. **32**: 481-486.

Osothsilp, C., and R.E. Subden. 1986[b]. Malate transport in *Schizosaccharomyces pombe*. J. Bacteriol. **168**: 1439-1443.

Östling, J., Carlberg, M. and H. Ronne. 1996. Functional domains in the Mig1 repressor. Mol. Cell. Biol. **16**: 753-761.

Páca, J. and J. Votruba. 1990. Effect of external pH on the respiration actitivy of *Candida utilis* induced by ethanol. Appl. Microbiol. Biot. **33**: 438-441.

Pallotta, M.L., Fratianni, A. and S. Passarella. 1999. Metabolite transport in isolated yeast mitochondria: fumarate/malate and succinate/malate antiports. FEBS Lett. **462**: 313-316.

Palmer, J.M. and I.M. Møller. 1982. Regulation of NAD(P)H dehydrogenases in plant mitochondria. Trends. Biochem. Sci. **7**: 258-261.

Palmieri, F. 1994. Mitochondrial carrier protein. FEBS Lett. **346**: 48-54.

Palmieri, F. 2004. The mitochondrial transporter family (SLC25): physiological and pathological implications. Eur. J. Physiol. **447**: 689-709.

Palmieri, L., Lasorsa, F.M., De Palma, A., Palmieri, F., Runswick, M.J. and J.E. Walker. 1997. Identification of the yeast *ACR1* gene product as a succinate-fumarate transporter essential for growth on ethanol or acetate. **417**: 114-118.

Palmieri, L., Lasorsa, F.M., Vozza, A., Agrimi, G., Fiermonte, G., Runswick, M.J., Walker, J.E. and F. Palmieri. 2000[a]. Identification and functions of new transporters in yeast mitochondria. Biochim. Biophys. Acta. **1459**: 363-369.

Palmieri, L., Palmieri, F., Runswick, M.J. and J.E. Walker. 1996. Identification by bacterial expression and functional reconstitution of the yeast genomic sequence encoding the mitochondrial dicarbocylate carrier protein. FEBS Lett. **399**: 299-302.

Palmieri, L., Runswick, M.J., Fiermonte, G., Walker, J.E. and F. Palmieri. 2000[b]. Yeast mitochondrial carriers: bacterial expression, biochemical identification and metabolic significance. J. Bioenerg. Biomembr. **32**: 67-77.

Palmieri, L., Vozza, A., Agrimi, G., De Marco, V., Runswick, M.J., Palmieri, F. and J.E. Walker. 1999[a]. Identification of the yeast mitochondrial transporter for oxaloacetate and sulfate. J. Biol. Chem. **274**: 22184-22190.

Palmieri, L., Vozza, A., Hönlinger, A., Dietmeier, K., Palmisano, A., Vincenzo, Z. and F. Palmieri. 1999[b]. The mitochondrial dicarboxylate carrier is essential for the growth of *Saccharomyces cerevisiae* on ethanol or acetate as the sole carbon source. Mol. Microbiol. **31**: 569-577.

Parulekar, S.J., Semones, G.B., Rolf, M.J., Lievense, J.C. and H.C. Lim. 1986. Induction and elimination of oscillations in continuous cultures in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **28**: 700-710.

Patterson, D.C. and D.J. Kilpatrick. 1991. Effluent from grass silage for finishing pigs. J. Agr. Sci. **116**: 119-124.

Peinado, J.M., Barbero, A. and N. van Uden. 1987. Repression and inactivation by glucose of the maltose transport system of *Candida utilis*. Appl. Microbiol. Biotechnol. **26**: 154-157.

Peinado, J.M., Cameira-dos-Santos, P.J. and M.C. Loureiro-Días. 1988. Regulation of glucose transport in *Candida utilis*. J. Gen. Microbiol. **134**: 195-201.

Peinado, J.M., Cameira-Dos-Santos, P.J. and M.C. Loureiro-Días. 1989. Regulation of glucose transport in *Candida utilis*. J.Gen.Microbiol. **135**: 195-201.

Peleg, Y., Rokem, J.S., Goldberg, I., and O. Pines. 1990. Inducible overexpression of the *FUM1* gene in *Saccharomyces cerevisiae*: Localization of fumarase and efficient fumaric acid bioconversion to L-malic acid. Appl. Environ. Microbiol. **56**: 2777-2783.

Petrik, M., Käppeli, O. and A. Fiechter. 1983. An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: involvement of short- and long-term regulation. J. Gen. Microbiol. **129**: 43-49.

Pines, O., Even-Ram, S., Elnathan, N., Battat, E., Aharonov, O., Gibson, D. and I. Goldberg. 1996. The cytosolic pathway of L-malic acid synthesis in *Saccharomyces cerevisiae*: the role of fumarase. Appl. Microbiol. Biotechnol. **46**: 393-399.

Poncet, S., Jacob, F.H., Berton, M.C. and A. Couble. 1985. Ethanol fermentation of inulin in some yeasts. Ann. Inst. Pasteur/Microbiol. **136B**: 99-109.

Postma, E., Kuiper, A., Tomasouw, W.F., Scheffers, W.A. and J.P. van Dijken. 1989[a]. Competition for glucose between the yeasts *Saccharomyces cerevisiae* and *Candida utilis*. Appl. Environ. Microb. **55**: 3214-3220.

Postma, E., Scheffers, W.A. and J.P. Van Dijken. 1988. Adaptation of the kinetics of glucose transport to environmental conditions in the yeast *Candida utilis* CBS 621: a continuous-culture study. J. Gen. Microbiol. **134**: 1109-1116.

Postma, E., Scheffers, W.A. and J.P. van Dijken. 1989[b]. Kinetics of growth and glucose transport in glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066. Yeast **5**: 159-165.

Postma, E., Verduyn, C., Scheffers, W.A. and J.P. Van Dijken. 1989[c]. Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl. Environ. Microb. **55**: 468-477.

Pronk, J.T., Steensma, H.Y. and J.P. Van Dijken. 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast **12**: 1607-1633.

Queiros, O., Casal, M., Althoff, S., Morades-Ferreira, P. and C. Leão. 1998. Isolation and characterization of *Kluyveromyces marxianus* mutants deficient in malate transport. Yeast **14**: 401-407.

Raa, J. 1990. Biotechnology in aquaculture and the fish processing industry: A success story in Norway. In *Advances in fisheries technology and biotechnology for increased profitability*. Voigt, M.N. and J.R. Botta (Eds.) Technomic Publishing. Lancastor, PA, USA.

Rao, N.A., Nerenberg, A.V., and D.J. Forster. 1991[a]. *Torulopsis candida* (*Candida famata*) endophthalmitis simulating propionibacterium acnes syndrome. Arcg. Opthalmol. **109**: 1718-1721.

Rao, S.R., Kamath, B.G. and A.S. Bhagwat. 1991[b] . Chemical modification of the functional residue(s) of malic enzyme from *Zea mays*. Biochemistry **30**: 431-435.

Redruello, B., Valdes, E., Lopez, M.L. and R. Rodicio. 1999. Multiple regulatory elements control the expression of the yeast *ACR1* gene. FEBS Lett. **445**: 246-250.

Redzepovic, S., Orlic, S., Majdak, A., Kozina, B., Volschenk, H. and M. Viljoen-Bloom. 2003. Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. Int. J. Food. Microbiol. **83**: 49-61.

Richard, G.-F., Fairhead, C. and B. Dujon. 1997. Complete transcriptional map of yeast chromosome XI in different life conditions. J. Mol. Biol. **268**: 303-321.

Rieger, M., Käppeli, O. and A. Fiechter. 1983. The role of a limited respiration in the complete oxidation of glucose by *Saccharomyces cerevisiae*. J. Gen. Microbiol. **129**: 653-661.

Rippa, M. and M. Signorini. 1975. 6-Phosphogluconate dehydrogenase from *Candida utilis*. Methods Enzymol. **41**: 237-240.

Rodriquez, L., Chávez, F.P., González, M.E., Basabe, L., and T. Rivero. 1998. Isolation and sequence analysis of the orotidine-5'-phosphate decarboxylase gene (*URA3*) of *Candida utilis*. Comparison with the OMP decarboxylase gene family. Yeast **14**: 1399-1406.

Rodriquez, S.B. and R.J. Thornton. 1989. A malic acid dependent mutant of *Schizosaccharomyces malidevorans*. Arch. Microbiol. **152**: 564-566.

Rodriquez, S.B. and R.J. Thornton. 1990. Factors influencing the utilisation of L-malate by yeast. FEMS Lett. **72**: 17-22.

Rolim, M.F., de Araujo, P.S., Panek, A.D., Paschoalin, V.M.F. and J.T. Silva. 2003. Shared control of maltose and trehalose utilization in *Candida utilis*. Brazilian J. Med. Biol. Res. **36**: 829-837.

Romano, A.H. 1982. Facilitated diffusion of 6-deoxy-D-glucose in bakers' yeast: evidence against phosphorylation-associated transport of glucose. J. Bacteriol. **152**: 1295-1297.

Romanos, M.A., Scorer, C.A. and J.J. Clare. 1992. Foreign gene expression in yeast: A review. Yeast **8**: 423-488.

Rose, M., Albig, W. and K.-D. Entian. 1991. Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexokinase phosphorylation by hexokinases PI and PII. Eur. J. Biochem. **199**: 511-518.

Rosenberg, M., Miková,H. and L. Krištofiková. 1999. Formation of l-malic acid by yeasts of the genus *Dipodascus*. Lett. Appl. Microbiol. **29**: 221-223.

Rothermel, B.A. and T. Nelson. 1989. Primary structure of the maize NADP-dependent malic enzyme. J. Biol. Chem. **264**: 19587-19592.

Roukas, T. 1998. Pretreatment of beet molasses to increase pullulan production. Proc. Biochem. **33**: 805-810.

Rouwenhorst, R.J., van der Baan, A.A., Scheffers, W.A. and J.P. van Dijken. 1991. Production and localization of β-fructosidase in asynchronous and synchronous chemostat cultures of yeasts. Appl. Environ. Microbiol. **57**: 557-562.

Rouwenhorst, R.J., Visser, L.E., van der Baan, A.A., Scheffers, W.A. and J.P. van Dijken. 1988. Production, distribution and kinetic properties of inulase in continuous cultures of yeasts. Appl. Environ. Microbiol. **54**: 1131-1137.

Rumsey, G.L. 1978. Recent advance in nutrition of salmonids. Salmonoid **2**: 14-17.

Runswick, M.J., Powell S.J., Nyren, P. and J.E. Walker. 1987. Sequence of the bovine mitochondrial phosphate carrier protein: structural relationship to ADR/ATP translocase and the brown fat mitochondria uncoupling protein. EMBO J. **6**: 1367-1373.

Runswick, M.J., Walker, J.E., Bisaccia, F., Iacobazzi, V. and F. Palmieri. 1990. Sequence of the bovine 2-oxoglutarate/malate carrier protein: structural relationship to other mitochondrial transport proteins. Biochemistry **29**: 11033-11040.

Saayman, M., Van Vuuren, H.J.J., Van Zyl, W.H. and M. Viljoen-Bloom. 2000. Differential uptake of fumarate by *Candida utilis* and *Schizosaccharomyces pombe*. Appl. Microbiol. Biotechnol. **54**: 792-798.

Salmon, J.M. 1987. L-Malic acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. **901**: 30-34.

Sandor, A., Johnson, J.H. and P.A. Srere. 1994. Cooperation between enzyme and transporter in the inner mitochondrial membrane of yeast. J. Biol. Chem. **269**: 29609-29612.

Sauer, L.A., Dauchy, R.T., Nagel, W.O. and H.P. Morris. 1980. Mitochondrial malic enzymes: mitochondrial NAD(P)'-dependent malic enzyme activity and malate-dependent pyruvate formation are progression-linked in Morris hepatomass. J. Biol. Chem. **255**: 3844-3848.

Sauer, U.W.E., Lasko, D.R., Fiaux, J., Hochuli, M. Glaser, R., Szyperski, T., Wüthrich, K. and J.E. Bailey. 1999. Metabolic flux ratio analysis of genetic and environmental modulations of *Escherichia coli* central carbon metabolism. J. Bacteriol. **181**: 6679-6688.

Savitha, J., Wynn, J.P. and C. Ratledge. 1997. Malic enzyme: its purification and characterization from *Mucor circineloides* and occurrence in other oleaginous fungi. World J. Microbiol. Biotechnol. **13**: 7-9.

Schaaff-Gerstenschläger, I. and T. Miosga. 1997. The Pentose Phosphate Pathway. In *Yeast Sugar Metabolism. Biochemistry, Genetics, Biotechnology and Applications*, Zimmermann, F.K. and K.-D. Entian (Eds.). Technomic. Lancaster, P.

Schaaff-Gerstenschläger, I., Mannhaupt, G., Vetter, I., Zimmermann, F.K. and H. Feldmann. 1993. *TKL2*, a second transketolase gene of *Saccharomyces cerevisiae*. Cloning, sequence and deletion analysis of the gene. Eur. J. Biochem. **217**: 487-492.

Schomburg, D. and D. Stephan. 1995. In *Enzyme Handbook*, Vol. 9. Class 1.1 oxidoreductases EC 1.1.1.1 – EC 1.1.1.149. Springer Verlag, Berlin, Germany.

Schulz, B. and M. Höfer. 1986. Utilization of lactose in non-respiring cells of the yeast *Debaryomyces polymorphus*. Arch. Microbiol. **145**: 367-371.

Schwartz, H. and F. Radler. 1988. Formation of L(-)malate by *Saccharomyces cerevisiae* during fermentation. Appl. Microbiol. Biotechnol. **27**: 553-560.

Scrutton, N.S., Berry, A. and R.N. Perham. 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature **343**: 38-43.

Shay, L.K. and G.H. Wegner. 1985. Improved fermentation process for producing *Torula* yeast. Food Technol. **39**: 61-66.

Shay, L.K., Hunt, H.R. and G.H. Wegner. 1987. High-productivity fermentation process for cultivating industrial microorganisms. J. Ind. Microbiol. **2**: 79-85.

Shimada, H., Kondo, K., Fraser, P.D., Miura, Y., Saito, T. and N. Misawa. 1998. Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the Isoprenoid pathway. Appl. Environ. Microb. **64**: 2676-2680.

Sikorski, R.S. and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**: 19-27.

Sims, A.P. and J.A. Barnett. 1978. The requirement of oxygen for the utilization of maltose, cellobiose and D-galactose by certain anaerobically fermenting yeasts (Kluyer effect). Gen. Microbiol. **106**: 288.

SivaRama, H., Pundle, A.V. and A.A. Prabhune. 1984. Growth of *Candida utilis* on distillery effluent. Biotechnol. Lett. **6**: 759-762.

Solomon, E.P., L.R. Berg, and D.W. Martin. 2005. In *Biology*, Seventh Ed. N. Rose (Ed.). Brooks/Cole-Thomson Learning, USA.

Song, Y., Wynn, J.P., Li, Y., Grantham, D. and C. Ratledge. 2001. A pre-genetic study of the isoforms of malic enzyme associated with lipid accumulation in *Mucor circinelloides*. Microbiol. **147**: 1507-1515.

Sousa, M.J., M. Mota, and C. Leão. 1992. Transport of malic acid in the yeast *Schizosaccharomyces pombe*: evidence for a proton-dicarboxylate symport. Yeast. **8**: 1025-1031.

Sousa, M.J., M. Mota, and C. Leão. 1995. Effects of ethanol and acetic acid on the transport of L-malic acid and glucose in the yeast *Schizosaccharomyces pombe*: implications in wine deacidification. FEMS Microbiol. Lett. **126**: 197-202.

Spencer-Martins, I. and N. van Uden. 1985. Catabolite interconversion of glucose transport systems in the yeast *Candida wickerhamii*. Biochim. Biophys. Acta. **812**: 168-172.

Stambuk, B.U., Panek, A.D., Crowe, J.H., Crowe, L.M. and P.S. de Araujo. 1998. Expression of high-affinity trehalose-H^+ symport in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. **1374**: 118-128.

Steffan, J.S. and L. McAlister-Henn. 1992. Isolation and characterization of the yeast gene encoding the *MDH3* isoenzyme of malate dehydrogenase. J. Biol. Chem. **267**: 24708-24715.

Stein, W.D. 1986. Transport and diffusion across cell membranes, pp. 114 – 209. Academic Press, New York. NY.

Stoltenburg, R., Klinner, U., Ritzerfeld, P., Zimmerman, M. and C.C. Emeis. 1992. Genetic diversity of the yeast *Candida utilis*. Curr. Genet. **22**: 441-446.

Streit, J.D., Prior, B.A. and S.G. Kilian. 1987. Substrate utilization by yeast in a spent sulfite liquor permeate. Water SA. **13**: 145-150.

Stucka, R., Dequin, S., Salmon, J.M. and C. Gancedo. 1991. DNA sequences in chromosomes II and VII code for pyruvate carboxylase isoenzymes in *Saccharomyces cerevisiae*: analysis of pyruvate carboxylase-deficient strains. Mol.Gen.Genet. **229**: 307-315.

Subden, R.E., Krizus, A., Osothsilp, C., Viljoen, M. and H.J.J. van Vuuren. 1998. Mutational analysis of malate pathways in *Schizosaccharomyces pombe*. Food Res. Int. **31**: 37-42.

Sundström, M., Lindqvist, Y., Schneider, G., Hellman U. and H. Ronne. 1993. Yeast *TKL1* gene encodes a transketolase that is required for efficient glycolysis and biosynthesis of aromatic amino acids. J. Biol. Chem. **268**: 24346-24352.

Suye, S., Okada, Y., Funada, A., Kawagoe, M. and S. Inuta. 1992. Purification and properties of malic enzyme from *Pseudomonas diminuta* IFO 13182. J. Ferment..Bioeng. **73**: 343-347.

Taillandier, P. and P. Strehaiano. 1991. The role of L-malic acid in the metabolism of *Schizosaccharomyces pombe*: substrate consumption and cell growth. Appl. Microbiol. Biotechnol. **35**: 541-543.

Taillandier, P., Gilis, M. and P. Strehaiano. 1995. Deacidification by *Schizosaccharomces*: interactions with *Saccharomyces*. J. Biotechnol. **40**: 199-205.

Takata, I., Yamamoto, K., Tosa, T. and I. Chibata. 1980. Immobilization of *Brevibacterium flavum* with carrageenen and its application for continuous production of L-malic acid. Microb. Technol. **2**: 30-36.

Tam, J.Y., Blume, K.G. and C.G. Prober. 1992. Prophylactic fluconazole and *Candida krusei* Infections. N. Engl. J. Med. **326**: 891.

Tanaka, K., Ohtani, S. and K. Shigeno. 1983. Effect of increasing dietary energy on hepatic lipogenesis in growing chicks I. Inreasing energy by carbohydrate supplementation. Poultry Sci. **62**: 445-451.

Tao, X., Yang, Z. and L. Tong. 2003. Chrystal structures of substrate complexes of malic enzyme and insights into the catalytic mechanism. Structure **11**:1141-1150.

Temperli, A., Künsch, V., Mayer, K. and I. Busch. 1965. Reinigung und Eigenschaften der Malatdehydrogenase (decarboxylierent) aus Hefe. Biochem. Biophys. Acta. **110**: 630-632.

Thompson, J.D., Higgins, D.G. and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research **22**: 4673-4680.

Treitel, M.A. and M. Carlson. 1995. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc. Natl. Acad. Sci. USA **92**: 3132-3136.

Tub, R.S. 1986. Amylolytic yeasts for commercial application. Trends Biotechnol. **4**: 98-105.

Van den Broek, P.J.A., Schuddemat, J., Van Leeuwen, C.C.M. and J. Van Steveninck. 1986. Characterization of 2-deoxyglucose and 6-deoxyglucose transport in *Kluyveromyces marxianus*: evidence for two different transport mechanisms. Biochim. Biophys. Acta. **860**: 626-631.

Van den Broek, P.J.A., van Gompel, A.E., Luttik, M.A.H. and J.T. Pronk. 1997. Mechanism of glucose and maltose transport in plasma-membrane vesicles from the yeast *Candida utilis*. **321**: 487-495.

Van Dijken, J.P. and W.A. Scheffers. 1986. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Rev. **32**: 199-224.

Van Dijken, J.P., Weusthuis, R.A, and J.T. Pronk. 1993. Kinetics of growth and sugar consumption in yeasts. Antonie van Leeuwenhoek **63**: 343-352.

Van Rensburg, P., van Zyl, W.H. and I.S. Pretorius. 1995. Expression of the *Ruminococcus flavefaciens* cellodextrinase gene in *Saccharomyces cerevisiae*. Biotech. Lett. **17**: 481-486.

Van Roermund, C.W.T., Elgersma, Y., Singh, N., Wanders, R.J.A. and H.F. Tabak. 1995. the membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. EMBO J. **14**: 3480-3486.

Van Rooijen, R.J., Chrispijn, H. and R. Baankreis. 1994. The Kluyver effect in *Saccharomyces cerevisiae* for trehalose and the isolation of putative trehalose uptake mutants from *Saccharomyces exiguus*. Folia Microbiol. (Praha) **39**: 553-554.

Van Urk, H., Bruinenberg, P.M., Veenhuis, M., Scheffers.W.A. and J.P. Van Dijken. 1989[a]. Respiratory capacities of mitochondria of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 grown under glucose limitation. Antonie van Leeuwenhoek **56**: 211-220.

Van Urk, H., Mak, P.R., Scheffers, W.A. and J.P. Van Dijken. 1988. Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. Yeast **4**: 283-291.

Van Urk, H., Postma, E., Scheffers, W.A. and J.P. van Dijken. 1989[b]. Glucose transport in Crabtree-positive and Crabtree-negative yeasts. J. Gen. Microbiol. **135**: 2399-2406.

Van Urk, H., Schipper, D., Breedveld, G.J. Mak, P.R., Scheffers, W.A. and J.P. van Dijken. 1989[c]. Localization and kinetics of pyruvate metabolizing enzymes in relation to aerobic alcoholic fermentation in *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621. Biochim. Biophys. Acta. **991**: 78-86.

Van Winkel, L.J. 1999. Thermodynamics and Transport. In *Biomembrane Transport*. Academic Press, USA.

Verduyn, C., Stouthamer, A.H., Scheffers, W.A. and J.P. Van Dijken. 1991. A theoretical evaluation of growth yields of yeasts. Antonie van Leeuwenhoek **59:** 49-63.

Verduyn, C. 1991. Physiology of yeasts in relation to biomass yields. Antonie van Leeuwenhoek Int. J. Gen. Mol. Biol. **60**: 325-353.

Verma, R.S., Spencer-Martins, I. and N. van Uden. 1987. Role of de novo protein synthesis in the interconversion of glucose transport systems in the yeast *Pichia ohmeri*. Biochim. Biophys. Acta. **900**: 139-144.

Vernon, C.M. and R.Y. Hsu. 1983. Pigeon liver malic enzyme: involvement of an arginyl residue at the binding site for malate and its analogs. Arch. Biochem. Biophys. **225**: 296-305.

Viljoen, M., Subden, R.E., Krizus, A. and H.J.J. van Vuuren. 1994. Molecular analysis of the malic enzyme gene (*mae2*) of *Schizosaccharomyces pombe*. Yeast **10**: 613-624.

Viljoen, M., van der Merwe, M., Subden, R.E. and H.J.J. van Vuuren. 1998. Mutation of Gly-444 inactivates the *S. pombe* malic enzyme. FEMS Microbiol. Lett. **167**: 157-162.

Viljoen, M., Volschenk, H., Young, R.A. and H.J.J. van Vuuren. 1999. Transcriptional regulation of the *Schizosaccharomyces pombe* malic enzyme gene, *mae2*. J. Biol. Chem. **274**: 9969-9975.

Visser, W., Scheffers.W.A., Batenburg-VanDerVegte, W.H. and J.P. Van Dijken. 1990. Oxygen requirements of yeasts. Appl. Environ. Microb. **56**: 3785-3792.

Voegele, R.T., Mitsch, M.J. and T.M. Finan. 1999. Characterization of two members of a novel malic enzyme class. Biochim. Biophys. Acta. **1432**: 275-285.

Volschenk, H., van Vuuren, H.J.J. and M. Viljoen-Bloom. 2003. Malo-ethanolic fermentation in *Saccharomyces* and *Schizosaccharomyces*. Curr. Genet. **43**: 379-391.

Volschenk, H., Viljoen, M., Grobler, J., Bauer, F., Lonvaud-Funel, A., Denayrolles, M., Subden, R.E. and H.J.J. van Vuuren. 1997[a]. Malolactic fermentation in grape musts by a genetically engineered strain of *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. **48**: 193-196.

Volschenk, H., Viljoen,M., Grobler, J., Petzold, B., Bauer, F., Subden, R.E., Young, R.A., Lonvaud, A., Denayrolles, M. and H.J.J. van Vuuren. 1997[b]. Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nature Biotech. **15**: 253-257.

Volschenk, H., Viljoen-Bloom, M., Subden, R.E. and van H.J.J. Vuuren. 2001. Malo-ethanolic fermentation in grape must by recombinant strains of *Saccharomyces cerevisiae*. Yeast **18**: 963-970.

Volschenk, H., Viljoen-Bloom, M., van Staden, J., Husnik, J. and H.J.J. van Vuuren. 2004. Genetic engineering of an industrial strain of *Saccharomyces cerevisiae* for L-malic acid degradation via an efficient malo-ethanolic pathway. S. Afr. J. Enol. Vitic. **25**: 63-73.

Von Jagow, G. and M. Klingenberg. 1970. Pathways of hydrogen in mitochondria of *Saccharomyces cerevisiae*. Eur. J. Biochem. **12**: 583-592.

VonArx, J.A. 1980. A mycologists view of yeasts. In *Biology and Activities of Yeast*. pp 53-61. Skinner, F.A., Passmore, S.M. and R.R. Davenport. (Eds.) The Society for Applied Bacteriology, Symp. Ser. No. Academic Press. New York.

Walhout, A.J., Sordella, R., Lu, X., Hartley, J.L., Temple, G.F., Brasch, M.A., Thierry-Mieg, N. and M. Vidal. 2000. Protein interaction mapping in *C. elegans* using proteins involved in vulval development. Science **287**: 116-122.

Walker, M.E., Val, D.L., Rohde, M., Devenish, R.J. and J.C. Walace. 1991. Yeast pyruvate carboxylase: identification of two genes encoding isoenzymes. Biochem. Biophys. Res. Commun. **176**: 1210-1217.

Walsh, M.C., Smits, H.P., Scholte, M. and K. van Dam. 1994. Affinity of glucose transport in *Saccharomyces cerevisiae* is modulated during growth on glucose. J. Bacteriol. **176**: 93-958.

Weaver, T., Lees, M., Zaitsev, V., Zaitseva, I., Duke, E., Lindley, P., McSweeny, S., Svensson, A., Keruchenko, J., Keruchenko, I., Gladilin, K. and L. Banaszak. 1998. Crystal structures of native and recombinant yeast fumarase. J. Mol. Biol. **280**: 431-442.

Watteeuw, C.M., Armiger, W.B., Ristoph, D.L. and A.E. Humphrey. 1979. Production of single cell protein from ethanol by fed-batch process. Biotechnol. Bioeng. **21**: 1221-1237.

Wedding, R.T. 1989. Malic enzymes of higher plants. Characteristics, regulation, and physiological function. Plant Physiol. **90**: 367-371.

Weems, J.J.J. 1992. *Candida parapsilosis* epidemiology, pathogenicity, clinical manifestations, and antimicrobial susceptibility. Clin. Infect. Dis. 756-766.

Wei, C.H., Chou, W.Y. and G.G. Chan. 1995. Identification of Asp258 as the metal coordinate of pigeon liver malic enzyme by site-specific mutagenesis. Biochemistry **34**: 7949-7954.

Weusthuis, R.A., Luttik, M.A.H., Scheffers, W.A., Van Dijken, J.P. and J.T. Pronk. 1994[a]. Is the Kluyver effect in yeast caused by product inhibition? Microbiol. **140**: 1723-1729.

Weusthuis, R.A., Visser, W., Pronk, J.T., Scheffers, W.A. and J.P. van Dijken. 1994[b]. Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect. Microbiol. **140**: 703-715.

Wickerham, L.J. and K.A. Burton. 1948. Carbon assimilation tests for the classification of yeasts. J. Bacteriol. **56**: 363-371.

Wierenga, R.K., Terpstra, P. and W.G.J. Hol. 1986. Prediction of the occurrence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. **187**: 101-107.

Wigler, M., Field, J., Powers, S., Broek, D., Toda, T., Cameron, S., Nikawa, J., Michaeli, T., Colicelli, J. and K. Ferguson. 1988. Studies of RAS function in the yeast *Saccharomyces cerevisiae*. Cold Spring Harbor Symp. Quant. Biol. **53**: 649-655.

Wilkinson, M.G. and J.B.A. Millar. 1998. SAPKs and transcription factors do the nucleocytoplasmic tango. Genes Dev. **12**: 1391-1397.

Wilkinson, M.G. Samuels, M., Takeda, T., Toone, W.M. Shieh, J.-C, Toda, T., Millar, J.B.A. and N. Jones. 1996. The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. Genes Dev. **10**: 2289-2301.

Williamson, P.R., Huber, M.A. and J.E. Bennett. 1993. Role of maltase in the utilisation of sucrose in *Candida albicans*. Biochem. J. **291**: 765-771.

Wohlrab, H. and C. Briggs. 1994. Yeast mitochondrial phosphate transport protein expressed in *Escherichia coli*. Site-directed mutations at threonine-43 and at a similar location in the second tandem repeat (isoleucine-141). Biochemistry **33**: 9371-9375.

Wood, T. 1981. The preparation of transketolase free from D-ribulose-5-phosphate-3-epimerase. Biochim. Biophys. Acta. **659**: 233-243.

Wu, M. and Tzagoloff, A. 1987. Mitochondrial and cytoplasmic fumarases in *Saccharomyces cerevisiae* are encoded by a single nuclear gene *FUM1*. J. Biol. Chem. **262**: 12275-12282.

Wu, N. and B. Ye. 1989. One-step fermentative production of single cell protein from sweet potato starch. Gongye. Weisheng. Wu. **19**: 18-22.

Wynn, J.P. and C. Ratledge. 1997. Malic enzyme is a major source of NADPH for lipid accumulation by *Aspergillus nidulans*. Microbiol. **143**: 253-257.

Wynn, J.P. and C. Ratledge. 2000. Evidence that the rate-limiting step for the biosynhesis of arachionic acid I *Mortierella alpina* is at the level of the 18:3 to 20:3 elongase. Microbiology **146**: 2325-2331.

Wynn, J.P., Hamid, A.A. and C. Ratledge. 1999. The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. Microbiology **145**: 1911-1917.

Xie, T. and A.K. Jaiswal. 1996. AP-2 mediated regulation of human NAD(P)H:quinone oxidoreductase 1 (NQO1) gene expression. Biochem. Pharmacol. **51**: 771-778.

Xu, Y., Bhargava, G., Wu, H., Loeber, G. and L. Tong. 1999. Crystal structure of human mitochondrial $NAD(P)^+$-dependent malic enzyme: a new class of oxidative decarboxylases. Structure **7**: 877-889.

Yagupsky, P., Dagan, R., Chipman, M., Goldschmied-Reouven, A., Zmora, E. and M. Karplus. 1991. Pseudo-outbreak of *Candida guilliermondii* fungemia in a neonatal intensive care unit. Pediatr. Infect. Dis. J. **10**: 928-932.

Yang, L.W., Wang, X.Y. and S. Wei. 1992. Immobilization of *Candida rugosa* having high fumarase activity with polyvinyl alcohol. Ann. NY Acad. Sci. **672**: 563.

Yang, Z. and L. Tong. 2000. Structural studies of a human malic enzyme. Protein Pept. Lett. **7**: 287-296.

Yang, Z., Floyd, D.L., Loeber, G., and L. Tong. 2000. Structure of a closed form of human malic enzyme and implications for catalytic mechanism. Nat. Struct. Biol. **7**: 251-257.

Yang, Z., Zhang, H., Hung, H.C., Kuo, C.C., Tsai, L.C., Yuan, H.S., Chou, W.Y., Chang, G.G. and L. Tong. 2002. Structural studies of the pigeon cytosolic $NADP^+$-dependent malic enzyme. Protein Sci. **11**:332-341.

Ziino, M., Lo Curto, R.B., Salvo, F., Signorino, D., Chiofalo, B. and D. Giuffrida. 1999. Lipid composition of *Geotrichum cendidum* single cell protein grown in continuous submerged culture. Bioresource. Technol. **67**: 7-11.

Zink, M.W. 1972. Regulation of the two malic enzymes in *Neurospora crassa*. Can. J. Microbiology **18**: 611-617.

Zubay, G. 1993. Biochemistry, Vol. 2: Catabolism and biosynthesis (3rd ed.). Wm.C. Brown Publishers, Dubuque, USA.