L-Proline - a possible source of nitrogen for wine yeast *Saccharomyces cerevisiae* during anaerobic fermentation

by

Christopher Smyl

B.Sc. (Hons). The University of Adelaide

Thesis submitted for the Degree of Doctor of Philosophy

Department of Plant Science
Faculty of Agricultural and Natural Resources Sciences
The University of Adelaide

September, 2000

Abstract

After carbon, in the form of sugar, nitrogen is quantitatively the most important nutritional requirement for growth of *Saccharomyces cerevisiae* wine yeast. An inadequate concentration of assimilable nitrogen in grape musts may lead to incomplete fermentation and/or formation of undesirable flavour compounds in the wine. Musts containing low assimilable nitrogen are common in winemaking and may be corrected by addition of ammonium salts.

L-proline is the most abundant nitrogen-containing compound in the majority of musts. However during the wine fermentation, which is characterized by early onset of anaerobic conditions, yeast can not assimilate proline as its catabolism requires molecular oxygen and its transport is prevented by nitrogen catabolite repression. When provided as the sole source of nitrogen, proline was transported by yeast with the same rate under aerobic and anaerobic conditions.

A laboratory strain of *S. cerevisiae* was genetically modified to utilise proline as a nitrogen source under anaerobic conditions. This was achieved by the introduction of a soybean (*Glicine max*) gene encoding NAD-linked proline oxidoreductase (PDH/P5CR) into yeast. PDH/P5CR, which oxidizes proline to Δ'-pyrroline-5-carboxylate (P5C) with its further conversion to glutamate, is present in some anaerobic bacteria and plants. The reported alkaline pH (10.3) necessary for *in vitro* oxidation of proline by PDH/P5CR was greatly lowered (pH 7.3) by removal of the reaction product, P5C. Growth of the engineered *S. cerevisiae* strain (PU1) on proline as the sole nitrogen source under strict anaerobic conditions confirmed the physiological function of PDH/P5CR.

Transgenic *S. cerevisiae* strain PU1 was able to grow on proline only when the PDH/P5CR gene was overexpressed. Growth was characterised by extended lag

phase and reduced final cell count when compared to growth on $(NH_4)_2SO_4$. This could reflect elevated energetic demands associated with synthesis of the introduced PDH/P5CR protein, proline transport and liberation of all nitrogen required for biosynthesis from proline.

Further work should aim to introduce the NAD-dependent proline degradation pathway into a commercial strain of wine yeast together with the nitrogen-insensitive proline transporter. This would allow for utilization of proline nitrogen as well as open the possibility for controlling the growth of such strains by manipulating the proline content of grape must.

Declaration

The work presented in this thesis is my own unless otherwise acknowledged and has not previously been submitted to any university for the award of any degree or diploma. This thesis may be made available for loan or photocopying provided that an acknowledgment is made in the instance of any reference to this work.

Christopher Smyl

September, 2000

Acknowledgments

I thank my supervisors, Dr Paul Henschke and Dr Peter Langridge, for providing the unique atmosphere of learning and self-analysis during my candidature and constructive critique of the manuscript. I specially thank Dr Langridge for his gratuitousness in providing me with employment during lengthy write-up of this thesis.

I am in debt to my colleague Dr Miguel de Barros Lopes whose intellectual and time input to this work was of "*grandios proporciosos*".

Thank you doctors Ute Baumann, Jeff Eglington, Xinmin Li, Vladimir Jiranek for all your teaching. Thank you Holgar Gockowiak, Angelo Karakousis, Chris Koeller, Jodi Kretchmer, Jan Nield for all your help.

I gratefully acknowledge the financial support for this study through a postgraduate scholarship from the University of Adelaide, Cooperative Research Centre for Viticulture and Australian Wine Research Institute.

Table of contents

Chapter 1

General introduction and project aims

The relationship between nutritional composition of a grape must and the fermentation performance of wine yeast, *Saccharomyces cerevisiae*, is well established (Bisson, 1991; Rapp & Versini, 1991). Although musts contain a complete mixture of nutrients necessary to support yeast growth, it is the concentration and availability of the particular components that determines the rate of growth and the extent of fermentation. Whereas sugars provide yeast with an energy and carbon source, the nitrogen containing compounds are of critical importance to yeast metabolism (Vos *et al.*, 1980; Monk, 1982; Bisson, 1991; Henschke & Jiranek, 1991, 1993; Kunkee, 1991). Deficiency of nitrogen in fermenting must has been linked to slow or stuck fermentations and the production of hydrogen sulfide which detracts from the quality and economic reward of commercially produced wines (Monk, 1982; Stratford & Rose, 1985; Monteiro & Bisson, 1991; Jiranek, 1992).

The demand of *S. cerevisiae* for nitrogen depends on the strain (Houtman & du Plessis, 1986; Jiranek *et al.*, 1991). It is generally accepted, however, that 140 mg/L of assimilable nitrogen is the minimum required to complete fermentation of a must containing 200 g/L of sugar, while 878 mg N/L is the optimal level for achieving the maximum fermentation rate of the same must (Rapp & Franck, 1971; Reed & Peppler, 1973; Agenbach, 1977; Vos *et al.*, 1980; Monk, 1982; Bely *et al.*, 1990). In contrast to sugars, which are quantitatively the most abundant nutrients, the content of available nitrogen in grape must varies from 40 to 2400 mg N/L and is, therefore, often inadequate for completion of fermentation (Ough, 1968; Ingledew & Kunkee, 1985; Ough & Amerine, 1988; Monteiro & Bisson, 1992). This nitrogen pool consists of a diverse range of compounds including amino acids, ammonium, urea, purines, pyrimidines and small peptides. Of these, the most abundant are amino

acids, comprising between 60 and 90 per cent of total nitrogen in the must (Kliewer, 1970).

The qualitative and quantitative composition of amino acids in musts varies depending on grape variety, climate, cultivation and juice handling practices. Despite these considerable differences, several compounds often predominate, including proline, glutamine, arginine, alanine, glutamate and serine. Amongst these, proline is frequently reported to be the most abundant (Ough, 1968; Ough & Stashak, 1974; Ough & Amerine, 1988). As with other amino acids, the concentration of proline in different musts varies widely, constituting between 24 mg N/L and 563.5 mg N/L of must; with an average of 90.3 mg N/L which amounts to 12% of total must nitrogen (Ough & Stashak, 1974; Ough & Amerine, 1988). There are, however, some grape varieties, for instance Cabernet Sauvignon and Cabernet Merlot, in which the proline nitrogen makes up to 40% of the total must nitrogen (Ough, 1968; Castino *et al.*, 1981). It has been noted for some time that, in contrast to the other amino acids, the amount of proline in the must remains virtually unchanged during or after fermentation (Castor, 1953; Castor & Archer, 1959; Lafon-Lafourcase & Peynaud, 1959; Ough, 1968; Agenbach, 1977), and that, as other nitrogen containing compounds are utilised by yeast, the residual proline accounts for the major proportion of nitrogen present in the finished wine.

The failure of yeast to utilise proline as a nitrogen source is caused by two factors. Firstly, the proline transporters are subject to nitrogen catabolite repression and transinhibition by other nitrogen containing compounds (Lasko & Brandriss, 1981; Horak & Rihova, 1982). Secondly, the first enzyme in the proline degradation pathway, proline oxidase, is linked to the respiratory electron transport chain and thus requires molecular oxygen to function. This nutrient is essentially absent from the must soon after the initial stages of fermentation (Duteurtre *et al.*, 1971).

In commercial winemaking, nitrogen deficiency is commonly encountered (Henschke & Jiranek, 1993). To avert this, musts are routinely supplemented with di-ammonium hydrogen phosphate; a nitrogen source readily assimilated by yeast. Several reports indicate, however, that this treatment can impact on the aroma profile of the wine (Anderson & Kirsop, 1974; Vos *et al.*, 1979; Ough & Lee, 1981; Tromp, 1984) and may be linked to the increased production of urea; a precursor of the suspected carcinogen, ethyl carbamate (Ough *et al.*, 1988).

The development of a wine yeast capable of utilising proline as a nitrogen source under anaerobic conditions will provide the wine industry with an alternative solution to the fermentation of nitrogen deficient musts, especially those in which the relative proline content is high. This project aims to alter the catabolism of proline in wine yeast so that the need for molecular oxygen, presently required for its metabolism, is eliminated. Such a pathway will provide yeast with an abundant, naturally occurring nitrogen source from must. The project also examines L-proline transport and utilisation of proline metabolites by *S. cerevisiae* and selected yeast species.

Chapter 2

Literature review

2.1 General aspects of nitrogen fate during wine fermentation

In wine yeast, *Saccharomyces cerevisia*, the anabolic and catabolic routes of nitrogen metabolism converge at the ammonia/glutamate interconversion through α-ketoglutarate, a feature shared by many organisms. All compounds, which can serve as nitrogen sources, are catabolised to yield glutamate and/or ammonia (Cooper, 1982a; Large, 1986) providing the cell with the ability to synthesise all endogenous nitrogen compounds independent of the nature of the external source, as long as the source is adequate (Jones *et al.*, 1969). Fermenting must contains a range of compounds, which can provide *S. cerevisiae* with nitrogen (Fig. 2.1; Table 2.1). Quantitatively significant nitrogen sources, which influence yeast growth, are ammonium (5-24% of total juice nitrogen, Ough, 1969; Ough & Kriel, 1985), amino acids (60-90%, Kliewer, 1970), peptides (15-20%, Cordonnier, 1966) and proteins (2-13% Amerine *et al.*, 1980; Correa *et al.*, 1988). Amines, nucleic acids derivatives, nitrates and vitamins are present in trace amounts and do not contribute significantly to the utilisable pool of nitrogen (Amerine *et al.*, 1980; Daudt & Ough, 1980; Ough & Crowell, 1980). The content of nitrogen compounds in a must differs greatly depending on the variety, berry maturation state, cultivation practices, region from which grapes where harvested and storage practices (Etievant *et al.*, 1988; Huang & Ough, 1989; Kluba *et al.*, 1978; Houtman & du Plessis, 1981).

Utilisation of a particular nitrogen-containing compound of must by yeast is dictated by an energetic 'effort/reward' balance associated with transport, catabolism, and nutritional value that the compound represents to the cell under particular physiological conditions. This is evidenced by the differing abilities of these compounds to support

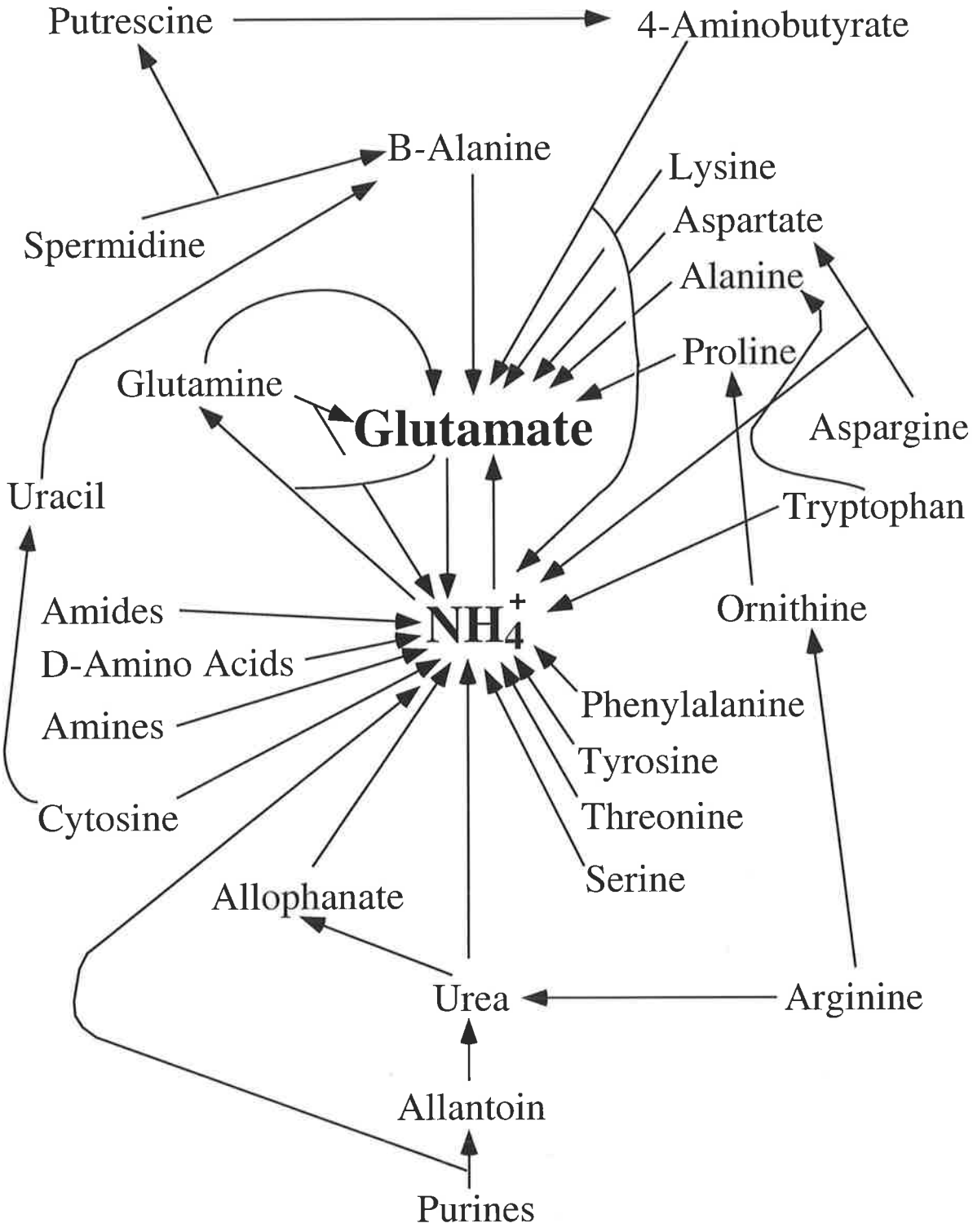


Figure 2.1. Degradation of nitrogenous compounds by yeast (redrawn from Large, 1986).

Nitrogen compound	Grape juice nitrogen content (mg/L)				
Ammonia	10-120	0-146	45-99	45-89	7-127
Amino Acid	170-1120	-	-	704-1070	19-144
Amino	-	15-182	101-168	46-81	14-176
Amide	10-40	-	-	-	-
Humin	5-20	-	-	-	-
Polypeptide	-	38-132	-	-	10-70
Hexosamine	-	18-29	-	-	-
Protein	10-100	28-97	-	-	-
Residual	100-200	-	-	-	-
References	1	2	3	4	5

Table 2.1. Nitrogenius compounds of grape juice (redrawn from Jiranek, 1992. Modified).

References:
(1) Hening, 1945 cited by Koch & Sajak, 1959
(2) Cordonnier, 1966
(3) Lafon-lafourcade & Peynaud, 1959
(4) Lafon-Lafourcade & Guimberteau, 1962
(5) Bizeau, 1963

growth when supplied as sole nitrogen sources as well as the selective accumulation of these compounds when present as a mixture (Monk *et al.*, 1987; Jiranek, 1992). Ammonium, arginine, asparagine, aspartate, glutamate, isoleucine, leucine, lysine, methionine, serine and threonine support the highest growth rates, measured as culture doubling time, when provided as the single source of nitrogen. These compounds are also rapidly utilised during the initial stages of growth (Watson, 1976; Cooper, 1982a; Jiranek, 1992). A significant delay precedes the uptake of alanine, histidine, phenylalanine and valine, whereas glycine, tryptophan and tyrosine are transported after depletion of the preferred group of compounds (Jiranek, 1992). Proline is virtually unutilised during wine fermentation. The selective uptake of nitrogenous compounds allowed for their segregation into four groups depending on the timing and extend of their removal from the fermenting medium (Table 2.2). The above observations may indicate the ability of the yeast cell to differentiate between good and poor nitrogen source. Compounds from Group A represent an efficient nitrogen source by either playing the central role in nitrogen metabolism (ammonium, glutamate), providing more than one nitrogen atom per molecule transported (arginine, asparagine, lysine), or their transport requires less energy expenditure than the two remaining groups (Seaston *et al.*, 1973; Eddy, 1982a). Cysteine and lysine, although utilised by *S. cerevisiae* when present in a mixture of compounds, fail to sustain cell growth when provided as the sole source of nitrogen. High intracellular concentrations of cysteine are toxic to the cell in a manner similar to other sulfhydryl compounds (Maw, 1965), whereas lysine catabolism results in the production of toxic α-amino adipate semialdehyde, which accumulates in the absence of a better nitrogen source (Cooper, 1982b). Proline is characterised as a Group D amino acid since liberation of its nitrogen requires the oxygen-dependent, electron transport chain-linked, proline oxidase. As already stated, molecular oxygen is practically absent from must during fermentation, and thus proline nitrogen can not be utilised (Duteurtre *et al.*, 1971).

Group A	Group B	Group C	Group D
Ammonium	Alanine	Glycine	Proline
Arginine	Histidine	Tryptophan	
Asparagine	Phenylalanine	Tyrosine	
Aspartate	Valine		
Glutamate			
Isoleucine			
Leucine			
Lysine			
Methionine			
Serine			
Threonine			

Table 2.2. Groupings for the ordered absorption of amino acids and ammonium ions by yeast from growth medium. Adopted from Jones *et al.*, 1969 and Jiranek, 1992.

All nitrogenous compounds enter yeast cell through energy dependent (obtained through ATP hydrolysis), membrane transport proteins (permeases). Transport proceeds down the gradient of electrochemical potential of hydrogen protons, which are co-transported with the nitrogen compound into the cell. Preferred nitrogen sources enter the cell by at least two transport systems differing in substrate affinity (K_m) and maximal transport rate (V_{max}). Entry of transported solutes through a specific transport system with high affinity for the given substrate is generally more energetically efficient (requiring less co-transported protons), and proceeds at a significantly higher rate. Compounds from Groups A and B are transported by high affinity permeases during the initial stages of fermentation (Rose & Keenan, 1981; Eddy, 1982a; Knatchbull & Slaughter, 1987). Once these are exhausted, less favourable nitrogen sources (Group C) are transported through the general amino acid permease (GAP) which serves as a nitrogen scavenging system. GAP can transport D- and L- isomers of neutral and basic amino acids and operates as the major amino acid permease towards the end of fermentation (Grenson *et al.*, 1970; Wiame *et al.*, 1985). Oligopeptides (Calderbank *et al.*, 1984; Moneton *et al.*, 1986; Naider *et al.*, 1974), nucleotides, nucleic acid derivatives (Lee, 1987) and vitamins (Rogers & Lichstein, 1969; Perl *et al.*, 1976) can be accumulated and utilised by *S. cerevisiae*. Precise mechanisms of transport of these nitrogen sources however, is not fully understood. A summary of *S. cerevisiae* nitrogen compound transport systems is presented in Table 2.3.

Control and regulation of nitrogen utilisation in *S. cerevisiae* is an interactive process involving transport and catabolism and is mediated at the level of gene transcription, translation; enzyme structure - precursor/active enzyme (permease) and physical enzyme/substrate interaction – compartmentation. In the ever-changing environment of a fermenting must, solute transport is constantly controlled to ensure a steady and sufficient supply of nitrogen at minimal energy cost to the cell. Excess accumulation of single compounds, when a mixture of good nitrogen sources is available, is prevented by feedback inhibition (accumulated substrate inhibits further accumulation

Table 2.3. Amino acid transport mechanism of *Saccharomyces* yeast. Adopted from Jiranek, 1992.

References: (1) Grenson *et al.*, 1970; (2) Grenson *et al.*, 1987; (3) McKelvey *et al.*, 1990; (4) Grenson *et al.*, 1966; (5) Chan & Cossins, 1976; (6) Gregory *et al.*, 1982; (7) Darte & Grenson, 1975; (8) Joiris & Grenson, 1969; (9) Grenson & Dubois, 1982; (10) Ballarin-Denti *et al.*, 1984; (11) Crabeel & Grenson, 1970; (12) Bussey & Umbarger, 1970; (13) Gits & Grenson, 1969; (14) Ramos *et al.*, 1980; (15) Grenson, 1966; (16) Gits & Grenson, 1967; (17) Horak & Rihova, 1982; (18) Magana-Schwencke & Schwencke, 1969; (19) Magana-Schwencke *et al.*, 1973; (20) Lasko & Brandriss, 1981; (21) Shukla *et al.*, 1982; (22) Verma *et al.*, 1984; (23) Wiame *et al.*, 1985; (24) Greasham & Moat, 1973; (25) Kotyk & Dvorakova, 1990.

[a] Activity in ammonium grown cells

[b] nmol/mg/protein/min

[c] nmol/g/dry weight/min

[d] nmol/g/dry weight/s

Amino Acid	Transport System[e]	Ammonium sensitive[a]	K_m (mM)	V_{max}[b]	Reference
L-Alanine	GAP	Yes			1
γ-Amino butyric acid	Proline permease	Yes	12		
	GABA permease	Yes	100		2,3
	GAP	Yes			2
L-Arginine	Arginine permease	No	10	12	4,5
	GAP	Yes	7.6	23	1,5
L-Asparagine	Glutamine permease	No	350	33[c]	6,7
	GAP	Yes			6,7
L-Citrulline	GAP	Yes	80		1
L-Glutamic acid	Dicarboxylic amino-acid permease 1	No	17		7,8
	Dicarboxylic amino-acid permease 2	Yes			7,8
	GAP	Yes	1000	20[c]	7
L-Glutamine	Glutamine permease 1	No			9
	Glutamine permease 2	No			9
	GAP	Yes	40		1,9
L-Glycine	Glycine permease	No	400	2	10
	GAP	Yes	14 000	2	1,10
L-Histidine	Histidine permease 1	No	17	11	11
	Histidine permease 2	No	4 000	28	11
	GAP	Yes	25	16	1
L-Leucine	Leucine permease	No	1 000		12,13,14
	GAP	Yes			12,14
L-Lysine	Lysine permease	No	25	8.1	15
	Arginine permease	No	200	17	15
	GAP	Yes	3.1		1
L-Methionine	Methionine permease 1	No	12	13	16
	Methionine permease 2	No	770	13	16
	GAP	Yes			1
L-Proline	Proline permease	Yes	31	40[d]	17,18,19
	GAP	Yes	>2 500	160[d]	17,20
L-Serine	Serine specific permease	Partial	250		21,22
	GAP	Yes	500		1,21,22
L-Threonine	Threonine specific permease	No			13
	GAP	Yes			23
L-Tryptophan	Aromatic amino acids permease	No	410	1.2[c]	23,24,25
	GAP	Yes	10		1,23
L-Valine	GAP	Yes			1

by the same permease due to an increase in intracellular concentration) and transinhibition (inhibition between permeases for unrelated substrate through competition for a common energy source). Delay of transport and catabolism of poor nitrogen sources is achieved by inactivation/reactivation of particular permeases and nitrogen catabolite repression (NCR) of their catabolic enzymes. Preferred nitrogen compounds, mostly ammonium and glutamate, their metabolites and/or enzymes associated with their catabolism are involved in these processes (Courchesne & Magasanik, 1983; Wiame *et al.*, 1985; Vandenbol *et al.*, 1987).

The key reaction in *S. cerevisiae* nitrogen metabolism, namely interconversion of glutamate and ammonium through α-ketoglutarate, is catalysed by enzymes which are responsive to ammonium concentration. Ammonium increases the activity of NADP-dependent glutamate dehydrogenase (NADP-GDH), catalysing the synthesis of glutamate from ammonium and α-ketoglutarate, and represses the NAD-dependent GDH, which catalyses the reverse reaction (Heirholzer & Holzer, 1963). A number of permeases, including GAP, are subject to this control (refer to Table 2.3). Examples of regulation through inactivation/reactivation and nitrogen catabolite repression of the specific proline permease and GAP are discussed further in detail.

Certain pathways that share common intermediates can be regulated by separation of the anabolic and catabolic reactions into different compartments. Proline anabolism and catabolism involves a common intermediate, Δ'-pyrroline-5-carboxylate. To avoid futile recycling, proline catabolism occurs entirely in the mitochondria, whereas its synthesis is cytosolic (Brandriss & Magasanik, 1980). Compartmentation also serves as a means for storage of excess nitrogen. A portion of nitrogen influx into the cell during the initial stages of fermentation is protected from degradation by accumulation in the vacuole. In response to an increasing cytosolic concentration of nitrogen compounds, vacuolar transport proteins are induced which mediate transfer of excess nitrogen into the vacuole (Sato *et al.*, 1984). These vacuolar reserves, mostly amino

acids (Weimken & Durr, 1974), may constitute up to 90% of a particular nitrogen source transported into the cell (Messenguy *et al.*, 1980; Kitamoto *et al.*, 1988) and are utilised when the cytosolic and extracellular sources are exhausted.

It has been established that the optimal concentration of utilisable must nitrogen for *S. cerevisiae* to complete fermentation of sugars is 878 mg N/L (Agenbach, 1977; Vos *et al.*, 1980; Bely *et al.*, 1990). In this respect, musts are often deficient in nitrogen. However, the intracellular nitrogen deficiency may arise not only as a result of low must concentration of utilisable nitrogen but also through impaired cellular transport. Several factors associated with the process of must fermentation may interfere with the accumulation of sufficient nitrogen to satisfy the metabolic demands of *S. cerevisiae*. Relatively early depletion of molecular oxygen during fermentation prevents *S. cerevisiae* from utilising proline. Anaerobic conditions also negatively affect synthesis of sterols and unsaturated fatty acids; lipids essential for membrane function (Andreasen & Stier, 1954; David & Kirsop, 1973; Lafon-Lafourcade, 1986). Progressive increase of carbon dioxide pressure (Pekur *et al.*, 1981; Knatchbull & Slaughter, 1987), ethanol (Larue & Lafon-Lafourcade, 1989; van Uden, 1989) and pH (Greasham & Moat, 1973; Ballarin-Denti *et al.*, 1984) during fermentation leads to marked reduction in transport rate.

2.2 Transport of L-proline

2.2.1 Transport proteins

To sustain life a cell must maintain its internal homeostasis. The key role in this process is played by a cytoplasmic membrane, a structure separating a cell from its environment. The phospholipid bilayer composition of this membrane renders it hydrophobic and makes it a highly selective barrier for the transport of solutes into and out of the cell. Only small, hydrophobic (nonpolar), uncharged molecules like CO_2, O_2, NH_3 and fat-soluble substances, such as fatty acids and alcohols can penetrate the

cytoplasmic membrane easily by passive diffusion. Ions, large inorganic and organic compounds, which are crucial to the cell physiology, can not enter the cell freely due to their polarity. These substances pass through the membrane with the assistance of membrane transport proteins (Oxender, 1972; Wilson, 1978; Eddy, 1982b; Stein, 1986). These proteins float freely while suspended in the cytoplasmic membranes. One end of the protein is therefore exposed to the exterior while the other end is within the enclosed by the membrane space.

The transport proteins are classified on the basis of their mechanism of action. Primarily they can be divided into channels and carriers (the latter are also named permeases or transporters). Channels enable the solute to penetrate the cell membrane while remaining inert during the process. Differentiation between transported solutes is based here mainly on the solute size. Transport is passive and occurs only down the concentration gradient. Carrier proteins actively partake in the transport. Because they undergo conformational changes and bind to the transported solute, they can be considered as membrane-bound enzymes. Instead of catalysing the conversion of a substrate to a product, carriers mediate the vectorial reaction of solute transfer from one side of the membrane to the other. Their binding sites alternatively face external and internal membrane surface which influence K_m (substrate affinity) and J_a (transport rate) of the transported substrate.

Carriers can be categorised as those catalysing (a) facilitated diffusion and (b) active transport. The first process, like channel mediated transport, does not utilise energy. Transport proceeds down the concentration gradient and stops when concentrations of the transported solute are equal on both sides of the membrane. In contrast to channels, the recognition of substrate by the carriers is strict, allowing for selective and accelerated accumulation of the solute. Carriers responsible for the active transport utilise energy. This enables substances to be transported against the concentration gradient and creates substantial difference in their concentrations across the membrane.

Two types of active transport carriers exist; primary carriers, also known as chemiporters, and secondary carriers. Primary carriers utilise the phosphate bond energy of ATP to pump ions across the membrane. Hydrogen ions are employed by procaryotes and plants for this process, whereas sodium and potassium ions are used in the animal kingdom. The created electrochemical ion potential is then utilised by primary carriers for translocation of required nutrients. When a primary carrier protein functions only as an ion transporter it is often referred to as a pump. The secondary carriers are unable to generate ion gradients. They are energised by the electrochemical potential created by the primary carriers or proton gradient generated by the respiratory chain or light reactions.

During transport, ions and solutes can move through the carrier in the same direction - symport or co-transport, or in opposite directions - antiport or countertransport. Sugars, amino acids and other large molecules are predominantly transported by co-transport whereas antiport systems are generally used for the excretion of metabolic by-products from the cytoplasm (Hama *et al.*, 1987; Reizer *et al.*, 1990; Kalman *et al.*, 1991). The previously described process of facilitated diffusion, which transports solutes independently of any coupling ions, is also known as uniport. Pumps also transport ions in the uniport fashion (Higgins *et al.*, 1990; Ames *et al.*, 1992; Konings *et al.*, 1992).

Carriers mediating active transport can also be classified by their specificity towards the transported solute. Carriers transporting only a single compound or a group of structurally related compounds are known as specific carriers. Affinity of these transport systems for a target solute is usually very high enabling efficient scavenging of solutes which are present in the medium at very low concentration. For this reason, specific carriers are particularly prevalent amongst unicellular organisms living in environments that demand a high competition for nutrients. The second group consists of the non-specific or general carriers. These are capable of transporting a large number of structurally unrelated compounds, they usually have low substrate affinity

and are therefore only able to transport solutes present at high concentration in the external medium. Mammalian and plant cells are equipped mostly with these carriers but they are also present in procaryotic and eucaryotic microorganisms.

As the metabolic pathway, for a given compound, starts with its transport or transport of necessary intermediates, facilitated diffusion and active transport carriers are integrated into the enzymatic machinery of that pathway and are strictly regulated by a number of mechanisms. These operate on several levels associated with a synthesis of transport protein itself or its function. Being biologically active compounds, carriers are also influenced by a number of physiological factors such as temperature, pH and concentration of certain substances such as ethanol and carbon dioxide. This in turn determines the transport rate for the particular substance (Stein, 1990).

2.2.2 Mechanisms of L-proline transport and its regulation

Transport of L-proline has been the subject of extensive studies in both unicellular organisms and tissues of higher plants and animals. This transport can proceed through L-proline specific carriers or non-specific carriers. Similar to the transport of other amino acids, L-proline transport is always energy dependent.

a) Kinetics of proline transport in Saccharomyces cerevisiae

In the yeast *Saccharomyces cerevisiae* L-proline enters the cell through two distinctive transport systems, the low and the high affinity carries. The low affinity transport system, with a K_m of 13 mM and a maximum rate of transport V_{max} of 150-165 nmol/s/(g dry wt.), is capable of transporting L-proline only when its concentration in the medium is greater than 0.1%. Experimental data indicate that this low affinity L-proline transport is mediated by the general amino acid permease (GAP) (Lasko & Brandriss, 1981; Horak & Rihova, 1982). Besides proline, this carrier catalyses the uptake of a wide range of L- and D-amino acids, with basic and neutral amino acids being preferred substrates. In fact, GAP is the only transport system in yeast capable of transporting D-amino acids, non-proteinogenic L-amino acids and several metabolic

intermediates such as citrulline and α-aminoadipic acid (Grenson *et al.*, 1970; Darte & Grenson, 1975; Rytka, 1975).

GAP is a membrane-bound multiprotein complex consisting of three different polypeptides of molecular weight of 53 000, 45 000 and 30 000 Da. It also contains a loosely associated periplasmic protein, MW 14 000, that is capable of binding to the transported solute (Woodward & Kornberg, 1980). The translocation of amino acids by GAP is driven by the proton motive force and involves a proton-symport mechanism (Seaston *et al.*, 1973). The affinity of the carrier for solutes is affected by pH in a charge-related fashion. For the acidic amino acids a lower *Km* was observed at low pH, whereas high pH renders lower *Km* for basic amino acids (Eddy, 1982b; Olivera *et al.*, 1993).

The second proline carrier, the specific or the high affinity transport system, is able to transport L-proline when its external concentration is below 0.02%. Its K_m is 0.025mM and V_{max} 40 nmol/s/(g dry wt.) (Lasko & Brandriss, 1981; Horak & Rihova, 1982). This carrier, named Put4 for proline utilisation, is a single protein with hydrophobic regions forming membrane-spanning α-helical segments separated by very short hydrophilic, electrically charged stretches. Its composition is similar to four other *S. cerevisiae* transporters which include GAP, histidine permease (Hip1), arginine permease (Can1) and GABA permease (Uga4) (Hoffmann, 1985; Tanaka & Fink, 1985; Jauniaux *et al.*, 1989; Vandenbol *et al.*, 1989; Jauniaux & Grenson, 1990). The presence of a histidine residue in the active site of the Put4 protein and the spatial arrangement of the imino and carboxyl groups of proline are essential for the successful binding of the proline to the carrier molecule and its subsequent transport (Horak, 1986). Besides the close structural analogues of proline, such as *N*-methylglycine, L-thiazolidin carboxylic acid, 3,4-dehydro-DL-proline, D- and L-azetidin carboxylic acid; L-alanine and GABA are the only other natural substrates for the specific proline carrier and are transported only in the absence of proline in the

external medium (Magana-Schwencke & Schwencke, 1969; Horak & Rihova, 1982; Grenson, 1992). The functioning of Put4 is pH and temperature dependent, with an optimal pH of 5.9 and optimal temperature of 30^0C (Horak & Rihova, 1982). Although these characteristics indicate energy involvement, as confirmed by changes in ATP levels associated with proline transport, no conclusive evidence exists to what energises the proline uptake in *S. cerevisiae*. No exchange of ions, alkalisation or changes in conductivity were observed, indicating that factors other than/or additional to the proton motive force, which provides energy for transport of large number of amino acids, are involved in proline transport (Eddy, 1982b; Horak & Rihova, 1982; Horak & Kotyk, 1986; Calahorra *et al.*, 1989; Kotyk *et al.*, 1990).

Both GAP and Put4 proteins lack an ATP-binding site indicating that they are proton-gradient driven secondary carriers. Together, they are capable of accumulating proline intracellularly against the concentration gradient in the ratio of 800:1. (Magana-Schwencke & Schwencke, 1969; Lasko & Brandriss, 1981; Horak & Rihova, 1982; Horak & Kotyk, 1986).

b) Regulation of proline transport in <u>Saccharomyces cerevisiae</u>

Regulatory mechanisms of L-proline transport mediated by the general amino acid permease and Put4 display similar characteristics (Grenson, 1983a, 1983b, Jauniaux *et al.*, 1987). Both carriers are trans-inhibited by all protein building L-amino acids; exceptions here are alanine and GABA which are, besides L-proline, the only natural substrates for Put4 and thus show competitive inhibition of the carrier.

When presented with a preferred nitrogen source, for example ammonia, cells of *S. cerevisiae* halt transport of L-proline through GAP and Put4 in the same manner. This is accomplished by a double control mechanism involving inhibition of carrier activity, a process known as nitrogen catabolite inhibition (NCI); and repression of carrier protein synthesis, a system known as nitrogen catabolite repression (NCR). The inhibition of GAP and Put4 rests on the balance between two proteins which assist in

the function of both carriers. These are the NPR (nitrogen permease reactivtor) and Npi1 and Npi2 (nitrogen permease inactivator, formerly Mut2 and Mut4) proteins (Jauniaux *et al.*, 1987). When yeast are grown on proline, or other poor nitrogen sources, the *NPR* gene is fully expressed and the presence of its product renders GAP and Put4 fully functional by eliminating the inhibitive action of Npi1/Npi2 proteins (Vandenbol *et al.*, 1987). Although the mechanism of this interaction is not fully understood it is postulated that Npi1/Npi2, which are thought to be expressed constitutively, bind to GAP and Put4 and render them inactive. Npr protein prevents this binding from occurring by blocking Npi1/Npi2 binding site (Grenson and Acheroy, 1982). Addition of ammonium ions prevents the synthesis of the *NPR* gene product, allowing unrestricted binding of Npi proteins to GAP and Put4 and halting proline transport. $NADP^+$-linked glutamate dehydrogenase (GDH), an enzyme partaking in the synthesis of glutamate, plays a crucial role in the inactivation of Npr synthesis by ammonia. This occurs through the regulation of the expression of *NPR* gene by Gdh protein or Gdh-ammonia complex (Vandenbol *et al.*, 1987). This is illustrated by (a) the GAP and Put4 of *gdh* mutants are insensitive to ammonia inhibition (Grenson and Hou, 1972; Dubois *et al.*, 1974; Roon *et al.*, 1974) and (b) the rate of the $NADP^+$-GDH synthesis is related to the intracellular concentration of ammonium ions (Bogonez *et al.*, 1985).

The second level of L-proline transport regulation, the repression of synthesis of GAP and PUT4 carriers, appears to be associated with the catabolic, NAD^+-linked glutamate dehydrogenase (GDH-CR). It is thought that this protein forms a complex with ammonium ions when it enters the cell and/or glutamine, which is synthesised in the presence of ammonia (Dubois *et al.*, 1977; Grenson, 1983b; Jauniaux *et al.*, 1987; Soussi-Boudekou & Andre, 1999). It is not known whether this complex acts at the level of transcription or translation of the *GAP* and *PUT4* genes, or regulates post-translation modifications necessary for the formation of the fully functional GAP and Put4 proteins (Roon & Even, 1973; Roon *et al.*, 1974; Grenson, 1983a; Wiame *et al.*, 1985).

This NCI/NCR regulation system of both proline carriers is rare amongst the amino acids transport systems in *S. cerevisiae*, as most high affinity transporters of the amino acids are unaffected by either ammonia or other nitrogen sources in the NCI/NCR fashion (Wiame *et al.*, 1985; Horak, 1986; Grenson, 1992; Ter Schure *et al.*, 1998). As a result of the trans-inhibition and NCI/NCR repression in *S. cerevisiae*, L-proline is transported only when it serves as the sole nitrogen source.

c) Proline transport in microorganisms and filamentous fungi

In most microorganisms studied, proline enters the cell by specific and/or non-specific proline carriers. In number of these organisms, these proline specific carriers are induced by proline and are not subjected to repression by ammonia or other nitrogen source. They are energised by energy derived from glycolysis, which enables them to function under anaerobic conditions. A summary of proline carriers is presented in Table 2.4.

d) Proline transport in animals and higher plants

In contrast to free-living microbes, cells of multicellular organisms are surrounded either by neighbouring cells or a rich nutrient milieu which includes amino acids. Therefore, there is no need for a specific, high affinity transport systems capable of scavenging amino acids. Generally, the cells of vascular plants and animals possess three amino acid transport systems; one for neutral, one for acidic and one for basic amino acids. Such a mode of transport has been described in various organisms including soybean (King & Hirji, 1975), oat (Kinraide & Etherton, 1980), castor bean (Robinson & Beevers, 1981), broadbean (Despeghel & Delrot, 1983), sugarcane (Wyse & Komor, 1984) and in investigated animal tissues including kidney (Fox *et al.*, 1964), intestinal mucosa (Schultz & Zalusky, 1965), muscle (Christensen & Liang, 1966) and brain (Margolis & Lajtha, 1968). Thus L-proline enters cells of higher plants (Shtarkshall *et al.*, 1970; Jung & Luttge, 1980; Kinraide & Etherton, 1980; Robinson & Beevers, 1981) and animal tissues (Finch & Hird, 1960a, 1960b;

Table 2.4. Characteristics of proline transporters in microorganisms and filamentous fungi.

References: (1) van Veen *et al.*, 1994; (2) Behki, 1967; (3) Arst & Cove, 1973; (4) Arst *et al.*, 1980; (5) Dabrowa & Howard, 1981; (6) Jayakumar & Prasad, 1978; (7) Jayakumar *et al.*, 1979; (8) Cho & Komor, 1983; (9) Kessel & Lubin, 1962; (10) Rowland & Tristram, 1975; (11) Wood & Zadworny, 1979; (12); Tristram & Neale, 1968; (13) Stalmach *et al.*, 1983; (14) Milner *et al.*, 1988; (15) Dabrowa & Howard, 1976; (16) Law & Mukkada, 1979; (17) Ratzkin *et al.*, 1978; (18) Cairney *et al.*, 1984; (19) Anderson *et al.*, 1980; (20) Bae & Miller, 1992; (21) Townsend & Wilkinson, 1992; (22) Winkler & Daugherty, 1984; (23) Kay & Gronlund, 1969a, 1969b, 1969c, 1969d

Species	Transport System	Proline Induced	Ammonium/Amino Acid Sensitive	K_m μM	V_{max}	Energy Source[a]	Reference
Acinetobacter johnsonii	L-proline specific carrier	Yes	No	2.5	0.21b	H^+/Na^+	1
Agrobacterium tumefaciens	Non-specific amino acid carrier	No	Yes	60	15c		2
Aspergillus nidulans	L-proline specific carrier	Yes	Partial				3,4
	General amino acid carrier	No	Yes				3,4
Candida albicans	L-proline specific carrier	Yes	No	1	1.4	H^+/K^+	5,6,7
	Non-specific amino acid carrier	No	Yes				5,7
Chlorella vulgaris	Non-specific amino acid carrier	Yes	Yes	106		H^+	8
Escherichia coli	Proline porter I	Yes	Yes	2	47	H^+/Na^+	9,10,11,12
	Proline porter II	No	Yes	200		H^+	13,14
Histoplasma capsulatum	L-proline specific carrier	Yes	Partial	20			15
Leishmania tropica	Non-specific amino acid carrier	No	Yes	60	5.6	H^+	16
Pseudomonas aeruginosa	L-proline specific carrier	Yes	No	1	18		23
	Low affinity proline carrier	Yes	Partial	20	90		23
Rickettsia prowazekii	L-proline specific carrier	Yes	No	14	64	H^+	22
Salmonella typhimurium	Proline porter I	Yes	Yes	3.6	0.7	Na^+	17,18
	Proline porter II	No	Yes	40	10	H^+	19
Staphylococcus aureus	L-proline specific carrier	Yes	Partial	2	15	H^+	20,21
	Low affinity proline carrier	No	Partial	200	6	H^+	20,21

Neame, 1962; Abadom & Scholefield, 1962; Spencer & Brody, 1964; Begin & Scholefield, 1965; Munck, 1966; Hillman *et al.*, 1968) through a common carrier that also mediates transport of other neutral amino acids.

In higher plants uptake of L-proline, together with other amino acids, is driven by the electrical gradient of protons and is associated with proton co-transport (Etherton, 1980; Robinson & Beevers, 1981; Despeghel & Delrot, 1983; Sauer *et al.*, 1983; Wyse & Komor, 1984). This H^+ potential is maintained by the metabolic energy of respiration. The exact mechanism of fuelling the H^+ gradient and its uniqueness in amino acid transport remains elusive, as experiments showing that transport can be driven by pH and/or electrical gradients in metabolically poisoned cells, proved to be unsuccessful.

The active transport of L-proline and other amino acids by the cells of animal tissues is associated with a co-transport of Na^+ and antiport of K^+ cations (Rosenbusch *et al.*, 1967; Eddy, 1968; Margolis & Lajtha, 1968; Tews & Harper, 1969). Electrochemical gradient of Na^+/K^+ cations is maintained by the energy derived from hydrolysis of oxidative phosphorylated ATP (Eddy, 1968).

2.3 Metabolism of proline

The imino acid proline plays three physiological roles:

- it is a precursor for protein synthesis where the rigid pyrrolidine ring of proline provides structural constrains to the movement of the polypeptide chain (Csonka & Baich, 1983),
- it can be degraded and used as a sole carbon, nitrogen or energy source by a number of organisms (Adams & Frank, 1980),
- proline can serve as an osmoprotectant for some bacteria and higher plants, where it is accumulated under conditions of osmotic stress thanks to its high solubility in

water. In this role, proline protects the cell against osmotic stress by accumulating in the cytosol to restore the optimum osmotic differential between the cell and the environment (Singh *et al.,* 1973; Jager & Meyer, 1977; Csonka, 1981; Stewart, 1981; McCue & Hanson, 1990).

Proline catabolic and anabolic pathways are common to most organisms. However the presence of a secondary amino group in the proline ring excludes this imino acid from transamination and decarboxylation reactions characteristic of the biochemistry of other amino acids (Maloy, 1987).

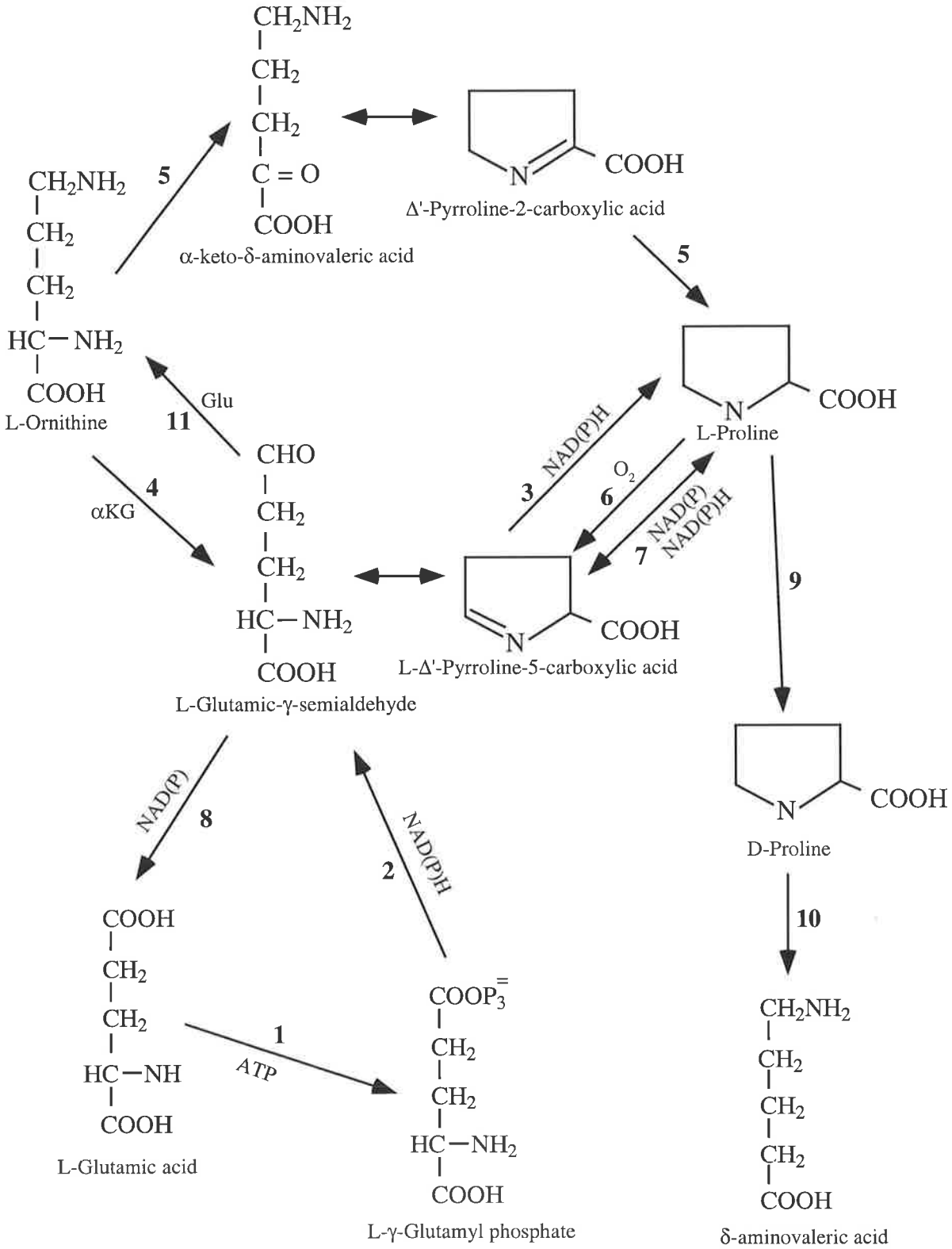
2.3.1 Anabolism of proline

Two pathways lead to the biosynthesis of L-proline, one involving the precursor L-glutamate and the other the precursor L-ornithine. These can interchange through a common intermediate, L-glutamic-γ-semialdehyde.

2.3.1.1 The glutamate pathway

The first reaction in the biosynthesis of proline from glutamate is the phosphorylation of glutamate to L-γ-glutamyl phosphate by the γ-glutamyl kinase (refer to Figure 2.2, Reaction 1). This reaction was established on the basis of the isolation of functional γ-glutamyl kinase (catalysing production of γ-glutamyl hydroxamate, ADP and Pi in a reaction mixture containing glutamate, Mg^{2+}, ATP and hydroxylamine) from *E. coli* (Baich & Pierson, 1965; Hayzer & Moses, 1978), *Brevibacterium flavum* (Yoshinaga *et al.*, 1967) and *Pseudomonas aeruginosa* (Krishna & Leisinger, 1979) and by studies of proline auxotrophic mutants of *E. coli* (Baich, 1969, 1971), *Salmonella typhimurium* (Miyake & Demerec, 1960) and *S. cerevisae* (Brandriss, 1979). It is argued that due to its instability, γ-glutamyl phosphate remains bound to the enzyme, γ-glutamyl kinase (Gamper & Moses, 1974) before it is converted by the second enzyme of proline biosynthesis, γ-glutamyl phosphate reductase, to form L-glutamic-

Figure 2.2. Steps in the pathways of proline metabolism in animals, plants and bacteria. Enzyme **1** is γ-glutamyl kinase; **2**, γ-glutamyl phosphate reductase; **3**, P5C reductase; **4**, ornithine transaminase in the ornithine catabolic direction; **5**, ornithine cyclase (deaminating), Clostridial enzyme; **6**, proline oxidase; **7**, proline oxidoreductase; **8**, P5C dehydrogenase; **9**, proline racemase, Clostridial enzyme; **10**, D-proline reductase, Clostridial enzyme; **11**, ornithine transaminase in the direction of ornithine synthesis from P5C. Redrawn from Adams & Frank (1980), modified.


α-keto-δ-aminovaleric acid
Δ'-Pyrroline-2-carboxylic acid
L-Ornithine
L-Proline
L-Glutamic-γ-semialdehyde
L-Δ'-Pyrroline-5-carboxylic acid
D-Proline
L-Glutamic acid
L-γ-Glutamyl phosphate
δ-aminovaleric acid
Glu
αKG
ATP
NAD(P)H
NAD(P)
O2
1
2
3
4
5
6
7
8
9
10
11

γ-semialdehyde (GSA) (Figure 2.2, Reaction 2). The NAD(P)H-dependent γ-glutamyl phosphate reductase has been purified from *E. coli* (Baich, 1971), *Pseudomonas* (Krishna *et al,* 1979), Chinese hamster lung cells (Wasmuth & Caskey, 1976) and higher plants (Fowden, 1965; Morris *et al.*, 1969). Employing proline auxotrophic mutants of *S. cerevisiae,* Brandriss (1979) demonstrated the conversion of γ-glutamyl phosphate to GSA in yeast.

L-glutamic-γ-semialdehyde spontaneously cyclizes to form Δ'-pyrroline-5-carboxylic acid (P5C) which in turn is reduced to L-proline by the P5C reductase (Figure 2.2, Reaction 3). This reaction has been demonstrated in microorganisms (Sanderson, 1972; Bachmann *et al.*, 1976; Brandriss, 1979; Krishna *et al.,* 1979), animals (Valle *et al.,* 1975; Farmer *et al.,* 1979; Smith & Phang, 1979) and plants (Splittstoesser & Splittstoesser, 1973; Rena & Splittstoesser, 1975). The P5C reductases that have been investigated, utilise either NADPH or NADH as reductant, are soluble and have an apparent molecular weight ranging from 94, 000 to 320, 000 (Mazelis & Creveling, 1974; Rossi *et al.*, 1977; Costilow & Cooper, 1978; Hayzer & Moses, 1978; Farmer *et al.*, 1979; Krishna *et al.*, 1979).

2.3.1.2 The ornithine pathway

It has been reported that the amino acid ornithine may serve as a precursor for the synthesis of L-proline. This reaction was observed in numerous animal tissues (Chinese hamster ovary cells, Vale *et al.*, 1973; Smith & Phang, 1979; larvae of blowfly, Yoshida *et al.*, 1977; rat mammary gland, Yip & Knox, 1972; Mezl & Knox, 1977; rat muscle tissue, Herzfeld *et al.*, 1977; human connective tissue, Smith & Phang, 1978; liver fluke, Ertel & Isseroff, 1974), some plants (peanut cotyledons, Mazelis & Fowden, 1969; pumpkin seeds, Splittstoesser & Fowden, 1973) and some microorganisms (*Clostridium sporogenes*, Muth & Costilow, 1974; *Saccharomyces cerevisiae*, Brandriss & Magasanik, 1980, 1981). In this pathway, ornithine

undergoes transamination by the ornithine δ-transaminase to L-glutamic-γ-semialdehyde/P5C which is reduced to proline (Figure 2.2, Reaction 4). Thus the ornithine pathway of proline synthesis joins the glutamate route at the GSA/P5C step. In animals the ornithine δ-transaminase is located in the mitochondria (Peraino & Pitot, 1963; Swick *et al.,* 1970) whereas a cytosolic location of the enzyme was reported for plants (Mazelis & Fowden, 1969).

In several organisms, including *S. cerevisiae*, the ornithine pathway of proline synthesis plays a role in the arginine catabolic pathway. Arginine is broken down to ornithine and urea by an enzyme arginase. Further utilisation of the carbon and nitrogen originating in arginine occurs through degradation of proline (Mazelis & Fowden, 1969; Splittstoesser & Fowden, 1973; Brandriss & Magasanik, 1980). It is believed however, that in microorganisms, including *S. cerevisiae,* the role of the ornithine pathway in the biosynthesis of proline is of minimum significance (Orser *et al.*, 1988).

2.3.1.3 Regulation of the proline anabolic pathway

Studies of proline biosynthesis in *Neurospora crassa* (Vogel & Bonner, 1954), *E.coli* (Baich & Pierson, 1965; Baich, 1969; Hayzer & Leisinger, 1980; Csonka, 1981; Smith *et al.*, 1984; Smith, 1985), *Pseudomonas aeruginosa* (Krishna & Leisinger, 1979), *S. cerevisiae* (Tomenchok & Brandriss, 1987; Li & Brandriss, 1992) and mothbean (Hu *et al.*, 1992) showed that proline biosynthesis from glutamate is regulated through feedback inhibition by the end-product of the reaction, proline. This regulation occurs at the first step of the pathway, the conversion of glutamic acid to γ-glutamyl phosphate, catalysed by γ-glutamyl kinase, the product of the *proB* gene in *E. coli* and *S. typhimurium.* Gene *proB* forms an operon with *proA*, encoding glutamate-γ-semialdehyde dehydrogenase, the second enzyme of the pathway (Mahan & Csonka, 1983; Csonka & Baich, 1983; Deutch *et al.*, 1984). In *S. cerevisiae,* γ-

glutamyl kinase is coded by the *Pro1* gene (Brandriss, 1979; Tomenchok & Brandriss, 1987; Orser *et al.*, 1988). In addition to the inhibition by proline, the catabolic function of *E. coli* γ-glutamyl kinase is impaired by the high concentration of intracellular ADP (Smith *et al.*,1984). Such two-tiered regulation makes proline synthesis responsive to cellular energy levels thus preventing ATP depletion and unnecessary proline overproduction with its subsequent excretion. In animal tissues (Smith *et al.*, 1980) and a protozoan *Tetrahymena pyriformis* (Hill & Chambers, 1967), where arginine serves as the primary precursor for proline synthesis (rather than glutamate, as it is in plants and the microorganisms), proline anabolism is sensitive to ornithine.

The mechanism of proline synthesis control at the molecular level has not been fully elucidated. Because the synthesis of proline anabolic enzymes is not repressed by exogenous proline, it is believed that the proline biosynthetic genes are expressed constitutively (Berg & Rossi, 1974; Hayzer & Leisinger, 1980; Bloom *et al.*, 1983; Brady & Csonka, 1988; Brandriss & Falvey, 1992) and the allosteric control of the first enzyme of the pathway appears to be the sole mechanism regulating the synthesis of proline.

2.3.2 Catabolism of L-proline

2.3.2.1 The P5C pathway

In the majority of organisms, the pathway of proline degradation is a reversal of its synthesis. Two enzymes are involved in proline degradation. First, proline is oxidised to P5C by either an ETC-linked proline oxidase or NAD(P)-linked proline dehydrogenase (Figure 2.2, Reaction 6 and 7). Second, P5C is oxidised to glutamate by NAD(P)-dependent P5C dehydrogenase (Figure 2.2, Reaction 8). The route involving the proline dehydrogenase enzyme has only been demonstrated *in vitro* with enzyme preparations from plants (Mazelis & Fowden, 1971; McNamer & Stewart, 1974; Rena & Splittstoesser, 1975) and certain species of anaerobic bacteria belonging

to the genus *Clostridium* (Costilow & Laycock, 1969; Costilow & Cooper, 1978). The oxidation of proline by the proline oxidase enzyme is common to all studied aerobic organisms: bacteria, (Dendinger & Brill, 1970; Menzel & Roth, 1981a; Meile *et al.*, 1982; Abrahamson *et al.*, 1983; Wood, 1987), *Aspergillus nidulans* (Arst & MacDonald, 1975), *S. cerevisiae*, (Lundgren & Ogur, 1973; Brandriss & Magasanik, 1979; Brandriss, 1987), plants (Mazelis & Fowden, 1971; Bogges *et al.*, 1978; Stewart & Bogges, 1978; Elthon & Stewart, 1982) and animals (Strecker, 1971; Blake, 1972; Mezl & Knox, 1977).

In studied procaryotic organisms, proline oxidase and P5C dehydrogenase activity are associated with the same membrane bound protein. A flavin group is necessary for the oxidase but not the dehydrogenase reaction. Purified enzyme consists of a 124, 000 dalton polypeptide in *E. coli* (Dendinger & Brill, 1970; Scarpulla & Soffer, 1978; Graham *et al.*, 1984), a 132, 000 dalton in *Salmonella typhimurium* (Menzel & Roth, 1981a; Graham *et al.*, 1984) whereas an enzyme of 120, 000 dalton is seen in *Pseudomonas aeruginosa* (Meile *et al.*, 1982). In all eukaryotes [*S. cerevisiae*, (Brandriss & Magasanik, 1979a, 1979b), higher plants (Stewart & Lai, 1974; Bogges *et al.*, 1978; Huang & Cavalieri, 1979; Elthon & Stewart, 1982) and animals (Strecker, 1960; Strecker, 1971)] the oxidation of proline and P5C are associated with separate proteins. Proline oxidation is carried out by a membrane-bound, mitochondrial enzyme which is associated with a flavoprotein on the electron transport chain (Meyer, 1977). P5C dehydrogenase, catalysing the oxidation of P5C to glutamate, is an NAD-dependent enzyme which has been localised to the mitochondrial matrix in plants (Bogges *et al.*, 1975; Balboni & Hecht, 1977; Elthon & Stewart, 1982), rat liver (Brunner & Neupert, 1969) and yeast (Lundgren & Ogur, 1973; Brandriss & Magasanik, 1981).

Oxidation of proline by the NAD(P)-dependent proline dehydrogenase has been demonstrated with extracts from a number of higher plants (Mazelis & Fowden, 1971; Mazelis & Creveling, 1974; Rena & Splittstoesser, 1975), alga *Chlorella vulgaris*

(McNamer & Stewart, 1974) and anaerobic bacterium *Clostridium* sp. (*C. botulinum*, Costilow & Laycock, 1969; *C. sporogenes*, Costilow & Cooper, 1978). *In vitro,* this reaction proceeds under alkaline pH (above 10) and is reversible (P5C is reduced to proline). It has been shown that the optimal pH for proline oxidation can be lowered significantly, to pH 7.2, by removal of the reaction product, P5C, from the reacting medium by reaction of P5C with *o*-aminobenzaldehyde (Costilow & Cooper, 1978).

The diversity in molecular weights and cofactor specificities displayed by the proline dehydrogenases isolated from different organisms and tissues suggests the existence of several isozymes.

2.3.2.2 Regulation of proline-P5C catabolic pathway

The bi-functional degradative enzyme, proline dehydrogenase and P5C dehydrogenase (Pro/P5C-DH), oxidising proline to glutamate in procaryotes is encoded by a single *PutA* gene located at minute 22 on the genetic maps of *E. coli* (Wood, 1981) and *Salmonella typhimurium* (Sanderson & Hartman, 1978; Menzel & Roth, 1981a). In addition to its catalytic function, Pro/P5C-DH protein plays a regulatory role in proline degradation through repression of the *PutA* and *PutP* genes (*PutP* codes for a proline transporter) (Menzel & Roth, 1981b; Wood & Zadworny, 1979). Since the oxidase reaction of the *PutA* product depends on a functional ETC, physical interaction of Pro/P5C-DH with specific sites on the ETC is necessary. Binding of proline to the Pro/P5C-DH may promote its association with the membrane. The model proposed for proline catabolic pathway regulation in *E. coli* and *S. typhimurium* that once Pro/P5C-DH-specific sites on the respiratory membrane are saturated, excess Pro/P5C-DH (not membrane bound) acts as a autoregulatory repressor of the *Put* genes at the transcriptional level (Menzel & Roth, 1981b; Maloy & Roth, 1983; Wood, 1981; Wood *et al.*, 1987).

Both enzymes in the *S. cerevisiae* proline catabolic pathway [proline oxidase, product of the *Put1* gene (Wang & Brandriss, 1986, 1987) and P5C dehydrogenase, encoded by the *Put2* gene (Brandriss & Krzywicki, 1986)] are induced by the substrate, proline. This induction is mediated through a positive activator protein, the product of the *Put3* gene, whose expression is necessary for proline utilisation (Brandriss & Magasanik, 1979a, 1979b; Brandriss, 1987). It is proposed that Put3 is part of a DNA-binding protein complex containing proline responsive elements which binds to the upstream activation sequences of *Put1* and *Put2* promoters thereby only allowing for transcription in the presence of proline (Siddiqui & Brandriss, 1988, 1989; Marczak & Brandriss, 1991; Des Etages *et al.*, 1996; Regenbeberg *et al.*, 1999; Huang & Brandriss, 2000).

There is no comprehensive account of the regulation of proline catabolism in animals or plants. However, research indicates that proline degradation in animals is regulated by the energetic state of the cell, particularly by factors controlling electron flow through the ETC (Hansford & Sacktor, 1970; Phang *et al.*, 1979). In plants, proline catabolism is sensitive to osmotic stress (Boggess *et al.*, 1976; Schobert & Tschesche, 1978) but the precise mechanism of its control has not been elucidated.

2.3.2.3 The δ-amino-valerate pathway

Certain species of anaerobic bacteria from the genus *Clostridium* can utilise proline as the final electron acceptor in a redox reaction between two amino acids (Stickland reaction). Proline acts here as an oxidant and is reduced to the δ-amino-valeric acid which can then be further metabolised for use as a nitrogen and energy source (Seto & Stadtman, 1976). The proline, acting as a final electron sink, plays a significant role in the anaerobic metabolism of many amino acids (Barker *et al.*, 1987).

The first step in L-proline reduction involves its conversion to the D-isomer by the proline racemase (Figure 2.2, Reaction 9; Stadtman & Elliott, 1957; Keenan &

Alworth, 1974). The D-proline ring is then reductively cleaved to δ-aminovalerate by proline reductase (Figure 2.1, Reaction 10; Arkowitz *et al.*, 1994). Proline reductase is a membrane-bound protein composed of 10 subunits of 30 kD each. The enzyme is a selenoprotein containing covalently bound pyruvyl groups which are necessary for the enzyme's catalytic function. It is postulated that the carbonyl group of pyruvate forms a Schiff base with the nitrogen of proline, thus allowing for a nucleophilic displacement at the α-carbon (Hodgins & Abeles, 1967, 1969; Seto & Stadtman, 1976; Seto, 1980). Under physiological conditions, NADH is the primary electron donor for the proline reductase. However the transfer of reducing equivalents is not direct but occurs sequentially through two membrane associated electron carriers, FAD-containing NADH dehydrogenase and an Fe-protein (Seto, 1978; Schwartz & Muller, 1979; Arkowitz *et al.*, 1994). No studies have been carried out concerning the mechanisms of regulation of the proline reduction pathway.

2.4 Developing wine yeast

Present laboratory and industrial (baking, brewing, distilling and wine) strains of the species *Saccharomyces cerevisiae* where selected from organisms naturally occurring on the grape skins. The ability to ferment sugar was a primary criterion in their selection (Kunkee & Goswell, 1977; Kreger van Rij, 1984). Early studies of the genetics and life-cycle of *S. cerevisiae* (Winge, 1935; Lindegren, 1943; Winge & Roberts, 1958) revealed haploid and diploid stages and two mating types, Mat a and Mat α. The genomes of laboratory strains are heterothallic, haploid possessing either Mat a or Mat α characteristics. Diploids are formed when cells of opposite mating type fuse. Homothallic strains arise when this fusion occurs between cells derived from a single spore. Homothallism is conferred by a dominant allele (*HO*) of a single gene and heterothalism by its recessive counterpart (Harashima *et al.*, 1974; Herskowitz & Oshima, 1981). Haploid state of laboratory yeast simplifies studies of

life processes at the molecular and metabolic level. Industrial yeast however, lack a specific mating type, are diploid or polyploid, rarely reproduce sexually and when they do, their spores are characterised by low viability (Fowell, 1969; Anderson & Martin, 1975). By conferring stability against mutation and recombination (Kielland-Brandt *et al.*, 1983; Panchal *et al.*, 1986) or influencing fermentation through a high dosage of desired genes (Stewart *et al.*, 1981), the polyploid state is of benefit to the industrial application of such strains but it also makes analyses of industrial traits and genetic improvement of strains difficult.

2.4.1 Classical genetic techniques for yeast development

A number of genetic techniques can be employed to confer desirable features in *S. cerevisiae*. Clonal selection is a technique, which involves screening of clones derived from a single cell for the characteristic of interest. Here natural genetic variation, which may arise through mutation or mitotic recombination, is exploited. This variation may be enhanced through the application of mutagenesis. The most commonly used mutagens with wine yeast are UV and X rays, ethyl methane sulfonate, N-Methyl-N-nitro-N-nitrosoguanidine, N-nitrosourea and diethylstilbestrol (Tubb & Hammond, 1987). Several desirable characteristics has been enhanced using clonal selection and selection after mutagenesis. These include wine yeasts that are non-foaming (Ouchi & Akiyama, 1971; Eschenbruch & Rassell, 1975), have improved ethanol tolerance (Brown & Oliver, 1982), reduced H_2S production (Rupela & Tauro, 1984), and the ability to lower the fusel oil content of wine and distilled beverages (Rous & Snow, 1983), or brewing yeast with increased flocculation and diacetyl producing abilities (Molzahn, 1977). Mutagenesis has the potential to disrupt or eliminate undesirable characteristics and to enhance favourable properties in industrial yeasts. However, this process is limited as the mutagen treatment often produces additional mutations to the one of interest and these may be undesirable.

Fusion of the Mat a and Mat α in a conventional crossbreeding produces heterozygous diploid hybrids with possibly new, desirable, genetic characteristic. Recombinant progeny are recovered by sporulating the diploid and recovering the haploid ascospores. Undesirable characteristics are removed by backcrossing. Crossbreeding has been used to introduce the killer characteristics into sake (Ouchi & Akiyama, 1976) and wine (Hara *et al.*, 1980) yeast and to produce a flocculent, non-foaming wine yeast with high ethanol production and a high fermentation rate (Romano *et al.*, 1985). The fact that wine yeasts are generally homothallic however, limits the use of crossbreeding.

A non-mating polyploid strains can be forced to produce hybrids with Mat a or Mat α. In this procedure, known as rare mating, a large number of cells of the parental strains are mixed together and a positive selective pressure is applied to identify the rare hybrids (Gunge & Nakatomi, 1971). Rare mating can be used to construct "true" hybrids in which nuclear and cytoplasmic characters are introduced from the donor strain, or for cytoduction, a rare form of hybridisation whereby cytoplasmic genetic elements, or just one or two nuclear chromosomes are transferred into the industrial strain (Conde & Fink, 1976). The rare-mating technique was used to construct killer yeast for the beer (Hammond & Eckersley, 1984) and wine (van der Westhuizen & Pretorious, 1989) industries as well as flocculent wine yeast strains (Thornton, 1985; Vezinhet *et al.*, 1992).

Another technique that is used in asexual crossbreeding between non-mating commercial yeast strains is spheroplast fusion. The yeast cell wall is removed by a lytic enzyme, spheroplasts from different strains are fused together and allowed to regenerate their cell walls on an osmotically stabilised agar medium (van Solingen & van der Platt, 1977). Brewing yeast able to ferment dextrins (Freeman, 1981; Russell *et al.*, 1983), or with improved tolerance to ethanol (Seki *et al.*, 1983) and baker's

yeast able to utilise starch (Bortol *et al.*, 1988) have been created by the spheroplast fusion technique.

2.4.2 Recombinant DNA technology

The genetic techniques described in the preceeding section have a limited potential to create new strains with a markedly different genetic character. This is because these procedures are concerned with the modification and/or abolition of existing genetic traits or the reassortment of a limited pool of genetic variation. In contrast, recombinant DNA technology, coupled with transformation allows for construction of novel strains by the controlled introduction of a wide variety of new genes and enables the directed modification of existing genetic traits.

2.4.2a Recombinant techniques

i) plasmid basics

The first yeast transformation was conducted by Hinnen *et al.* (1978) who designed a protocol to introduce DNA into cells by enzymatically altering the permeability of the cell wall. Since then, alternative protocols involving chemical treatments with alkali metal salts (Ito *et al.*, 1983, 1984; Brzobohaty & Kovac, 1986), formation of spheroplasts (Burgers & Percival, 1987) and electroporation (Hashimoto *et al.*, 1985; Delorme, 1989; Simon & McEntee, 1989) have been developed.

Once the DNA is introduced, an important aspect of transformation is the ability to stably maintain the foreign DNA within the host. Consequently, DNA to be introduced into host cells, needs to be incorporated into a transport vehicle or vector to ensure stable inheritance. In general, two major types of DNA vectors are employed for the introduction of foreign DNA into yeast cells. These are autonomously replicating and integrating vectors. Autonomously replicating plasmids contain sequences, which allow them to replicate independently of the nuclear genome. They are usually found in high copy number within a host but are generally mitotically unstable. Integrating vectors involve site-specific integration of the vector into

chromosomal DNA mediated by homologous recombination, resulting in high heritable stability at low gene dosage.

These fundamental plasmid frameworks have been the basis for subsequent development and enhancement of vector design. For example, improved stability of autonomously replicating plasmids was gained by the cloning of the yeast centromeric region (Clark & Carbon, 1980). Subsequent addition of telomeric sequences by Murray and Szostak (1983) led to the development of yeast artificial chromosomes. As a consequence, DNA of several hundred kilobases can now be cloned. For comprehensive information of available yeast vectors and the particular sequences they comprise refer to reviews by Parent *et al.* (1985), Ausubel (1987), Bitter *et al.* (1987) and Rodriguez and Denhardt (1988).

ii) selectable markers

Another feature of yeast vectors includes sequences, which allow selection of the few cells that have incorporated the vector. Common selective sequences for laboratory yeast are prototrophic markers such as *LEU2, URA3, TRP1* and *HIS3* which complement recessive auxotrophic mutations in the host strain (Beggs, 1978; Struhl & Davis, 1980; Tschumper & Carbon, 1980; Rose *et al.*, 1984). However, dominant selectable markers are generally used for transforming industrial yeast strains due to their polyploid state. Common dominant selectable markers frequently used in the genetic manipulation of industrial strains are hygromycin B resistance encoded by a bacterial phosphotransferase gene (Gritz & Davies, 1983; Kaster *et al.*, 1984), the G418 antibiotic resistance gene (Webster & Dickson, 1983) and the chloramphenicol resistance gene (Cohen *et al.*, 1980; Hadfield *et al.*, 1986) encoded by *E. coli* transposons (Jimenez & Davies, 1980; Webster & Dickson, 1983), as well as the copper resistance gene (*CUP1*) of *Saccharomyces* (Fogel & Welch, 1982; Butt *et al.*, 1984; Henderson *et al.*, 1985; Enari *et al.*, 1987).

iii) promoters

Early expression work focused on the use of strong constitutive promoters (particularly those derived from the yeast glycolytic genes) to produce a high level of product. Examples are: alcohol dehydrogenase *ADH1* (Hitzeman *et al.*, 1981; Bennetzen & Hall, 1982a), phosphoglycerate kinase *PGK* (Dobson *et al.*, 1982; Tuite *et al.*, 1982) and glyceraldehyde-3-phosphate dehydrogenase *GAPDH* (Holland & Holland, 1980; Bitter & Egan, 1984). For further information, Shuster (1989) has reviewed the use of these glycolytic promoters in the expression of heterologous proteins in yeast.

Although aiming for a strong promoter is a frequent goal, the need for promoter control or adjustment of promoter activity can become quite apparent. High expression levels may be desired. However, the synthesis of a cloned gene can place additional stress on the cells. This can result in lower growth rates, biomass yield and can reduce overall productivity. Often the detrimental effects of high expression outweigh the positive effects of increasing the gene transcripts. To reduce the negative effects of cloned gene expression, plasmids with regulated promoters may be employed.

Amongst the most tightly regulated promoters of *S. cerevisiae* are those of the galactose regulated genes, *GAL1, GAL7* and *GAL10*. These genes are involved in the metabolism of galactose, their promoters being strongly repressed in the presence of glucose (St John & Davis, 1981). Other regulated promoters include that of the acid phosphatase gene (*PHO5*), which is regulated by inorganic phosphate concentration (Bostian *et al.*, 1980b; Meyhack *et al.*, 1982) and the alcohol dehydrogenase 2 (*ADH2*) promoter, which is repressed in the presence of high glucose and switched on in low glucose concentrations (Beier & Young, 1982; Beier *et al.*, 1985).

Although there are significant advantages with the use of regulatable promoters, their use may be constrained as a result of the nature of the genes to be expressed or the expression conditions. More recently, hybrid promoters have been constructed for the expression of heterologous proteins in yeast. These take advantage of the intrinsic strength of constitutive promoters as well as the modulation of gene expression that is conferred by regulated promoters. The validity of this technique was established when Guarente *et al.* (1982) showed that the UAS of the *GAL10* promoter could confer galactose inducibility on the *CYC1* promoter when inserted upstream of the *CYC1* mRNA initiation start site. The hybrid promoter showed inducibility in the presence of galactose but selection studies showed that overall transcription levels from the hybrid were determined by *CYC1* function. That is, hybrid promoters display the desired properties of each of the promoter components used. Other examples of hybrid promoters include: *ADH/GAPDH* (Cousens *et al.*, 1987; DeBaetselier *et al.*, 1991), *PHO5/GAP* (Rink *et al.*, 1984; Hinnen *et al.*, 1989) and a temperature regulated promoter based on the mating type expression system (Sledziewski *et al.*, 1988; Kobayashi *et al.*, 1990).

iv) terminators

Yeast transcription terminators are also usually present in expression vectors for efficient mRNA 3' end formation. Transcription termination signals are necessary for termination of transcription, cleavage of the 3' end and subsequent polyadenylation. Terminators from a number of genes have been used in expression vectors: *TRP1* (Hitzeman, 1983), *ADH1* (Urdea *et al.*, 1983) and *GAPDH* (Rosenberg *et al.*, 1984).

v) targeting sequences

Finally, once a sequence has been expressed it may be desirable for the protein to be secreted. This requires the presence of a signal sequence to direct the protein product through the secretory pathway. In secretion vectors, the secretion signal sequence is found downstream to the promoter and preceeding the foreign gene to be expressed.

Some mammalian proteins have been secreted from yeast using their signal sequences (Kingsman *et al.*, 1985b). However, it is generally observed that the efficiency of secretion is low and the processing at the signal sequence can be variable (Kingsman *et al.*, 1988). Heterologous protein secretion is often more efficient when secretion signals from yeast are used. A number of secretion vectors based on yeast signal sequences have been constructed. The leader peptide sequence of the α mating pheromone (Bitter *et al.*, 1984; Ernst, 1988) and the killer toxin of *S. cerevisiae* (Skipper *et al.*, 1985; Cartwright, 1992) have been extensively used in vectors to secrete homologous and heterologous gene products in yeast.

2.4.2b Industrial applications of recombinant technology

The influence of recombinant DNA technology on the beer and wine industries is rapidly growing. Thanks to these techniques, progress in understanding of the biochemistry and physiology of industrial yeast strains is rapid. Through the disruption and/or introduction of genes of interest the process of fermentation can be optimised with regard to the parameter (cost reduction, flavour, aroma, etc.) of concern. The following section lists selected areas which have been targeted by recombinant DNA technology.

- Some strains of *S. cerevisiae* produce several types (classified into K1, K2 and K3 groups) of protein toxins, which kill sensitive strains of the same genus and, less frequently, strains of different genera (Bevan & Makower, 1963; Young & Yagiu, 1978; Tipper & Bostian, 1984). Killer proteins and the immunity to them are encoded by double stranded RNA plasmids (Bostian *et al.*, 1980a). By cloning and expressing genes for the K1 protein in a K2-producing yeast, a modified K1-K2 strain was developed with a broad competitive advantage over

sensitive and killer strains of *S. cerevisiae* without loss of the strains desired wine making characteristics (Boone *et al.*, 1990).

- Flocculation is an ability of certain *S. cerevisiae* strains to agglomerate to form flocs, which precipitate to the bottom of the fermentation vessel. This feature greatly assists in the removal of the yeast from the ferment reducing and/or eliminating the need for centrifugation, use of fining agents and decreasing settling time after completion of fermentation. The condition is encoded by a number of genes (Stratford, 1992), with the dominant flocculation gene, *FLO1*, necessary for the presence of the trait (Johnson & Reader, 1983; Watari *et al.*, 1994a; Bidard *et al.*, 1995). The mechanism of flocculation is under study. It is understood however, that cell-cell recognition through lectin-like binding of surface proteins to cell wall polysaccharides is involved (Miki *et al.*, 1980, 1982; Stratford & Brundish, 1990). The *FLO1* gene has been introduced by chromosomal integration under the control of the *ADH1* promoter into a non-flocculent brewers' yeast resulting in a flocculant transformant strain (Watari *et al.*, 1989; Watari *et al.*, 1994b).

- In brewers' wort, up to 20 percent of the sugar is present as dextrin, which is non-utilisable by *S. cerevisiae*. To produce a low carbohydrate beer, the enzymic conversion of dextrin to glucose, can be effected by addition of amyloglucosidase to fermenting wort. Several species of fungi (*Aspergillus niger* [Yocum, 1986], *Aspergillus awamori* [Nunberg *et al.*, 1984], *Schwanniomyces occidentalis* [Lanchashire *et al.*, 1989], *Saccharomyces cerevisiae var. diastaticus* [Pretorius & Lambrechts, 1991]) synthesise extracellular glucoamylases capable of dextrin and starch hydrolysis. A number of glucoamylase genes have been cloned and

successfully expressed in *S. cerevisiae* leading to the glucoamylase secreting strains (Innis *et al.*, 1985; Meaden & Tubb, 1985; Yocum, 1986).

- The haze forming polysaccharide, β-glucan is often encountered in wort and musts wherein it impairs fermentation and affects the commercial quality of the product. This is often corrected by addition of β-glucanases during fermentation (Aschengreen, 1987). By cloning the β-glucanase structural gene from the fungus *Trichoderma reesei* and introducing it into an *S. cerevisiae* brewing strain, it was possible to produce beer with improved filterability, decreased viscosity and lower β-glucan levels (Arsdell *et al.*, 1987; Enari *et al.*, 1987).

- *Saccharomyces cerevisiae* is also being used for producing non-yeast proteins applicable in the pharmaceutical field. Hinchcliffe *et al.* (1987) genetically modified brewing yeast strain to produce human serum albumin, which is collected from accumulated yeast biomass after completion of fermentation.

- The quality of a finished product of fermentation, beer or wine, is a result of the complex chemistry associated with this process. Many metabolic products of yeast biochemistry and compounds present in wort or must have an influence on the aroma and taste of these beverages. Thanks to recombinant DNA technology, synthesis of these compounds can be controlled with increasing precision.

- The occurrence in beer of diacetyl, an intermediate of valine biosynthesis, is undesirable. Its removal results in increased production costs. A bacterial α-acetolactate decarboxylase gene (encoding an enzyme which converts the diacetyl precursor α-acetolactate to acetoin) has been introduced into brewing yeast to reduce diacetyl levels. Fermentation tests showed that the transformants produced reduced levels of diacetyl without any effect on the other characteristics of the beer (Sone *et al.*, 1987; Suihko *et al.*, 1989; Fujii *et al.*, 1990).

- Flavour of wine is noticeably changed by malolactic fermentation (decarboxylation of L-malate to L-lactate by bacteria), through the reduction in acidity and the products of the bacterial fermentation (Kunkee & Goswell, 1977). However, malolactic fermentation is difficult to conduct due to poor growth of lactic acid bacteria in fermenting wine (Lautensach & Subden, 1984). This challenge was partly overcome by cloning a malolactic gene from *Lactococcus lactis* and its expression from a multicopy plasmid under the control of *ADH1* promoter. The transgenic *S. cerevisiae* strain produced significant amounts of lactate during fermentation. The limiting step however, for *S. cerevisiae* in achieving malolactic fermentation, is malate transport (Ansanay *et al.*, 1993, 1996; Denayrolles *et al.*, 1995).

- The aromatic fragrance of wine is partly caused by monoterpenes present in the must, which exist as free volatile forms or as glycosidically bound non-volatile precursors (Williams *et al.*, 1982). Sugar-bound monoterpenes may present a potential source of new flavours upon hydrolysis. Perez-Gonzalez *et al.*, (1993) introduced and expressed a fungal β-(1,4)-endoglucanase gene in wine yeast.

The transgenic strain produced enzyme which hydrolysed monoterpenyl glycosidic bonds in must producing a wine with increased fruity aroma.

- In beer production, sulfite plays a role in stabilising flavour through the formation of adducts with aldehydes (Dufour, 1991). This compound is also widely used as an antioxidant in food production (Meilgaard, 1975). However, brewers yeast often produce inadequate amounts of sulfite requiring its addition (Brewer & Fenton, 1980). In *S. cerevisiae*, several enzymes are involved in the synthesis of sulfite from its precursor, inorganic sulfate. Sulfite, in turn, serves as a precursor for synthesis of cysteine, methionine and S-adenosylmethionine (Thomas *et al.*, 1990; Mountain *et al.*, 1991). By partial elimination of the *MET10* gene, coding for a subunit of sulfite reductase, Hansen & Kielland-Brandt (1996) developed a *S. cerevisiae* strain with a decreased ability to reduce sulfite to sulfide and therefore increased sulfite accumulation. Beer produced with this strain showed increased flavour stability.

Chapter 3

General studies of L-proline utilisation as a nitrogen source under aerobic and anaerobic conditions by yeasts

3.1 Introduction

Early investigations on the behaviour of amino acids during malt fermentation with *S. cerevisiae* carried out by Jones & Pierce, (1964); Jones *et al.*, (1969); Duteurtre *et al.*, (1971) and later by Horak & Kotyk, (1986), showed that significant utilisation of L-proline from the fermenting medium occurred only after exhaustion of other nitrogen containing compounds and when the medium was well aerated. When anaerobic conditions were applied, the concentration of L-proline remained largely unchanged throughout the fermentation, even after the complete disappearance of the other nitrogen sources. However, when proline was provided as the sole nitrogen source to yeast grown under anaerobic conditions, it's accumulation within the cell was rapid before declining to a plateau phase (Schwencke & Magana-Schwencke, 1969; Duteurtre *et al.*, 1971). These observations led to the postulate that the observed inability to utilise L-proline by yeast under anaerobic conditions is caused by a deficiency in proline catabolism rather than transport. This was confirmed by several studies which demonstrated that L-proline catabolism is governed by the availability of molecular oxygen and the transport of L-proline is subject to regulation controlled by the quality of other nitrogenous compounds present in the fermenting medium (refer to Sections 2.2.2 and 2.3.2).

To date, studies of L-proline transport by *S. cerevisiae* were carried out using bakers' or beer fermenting yeasts under laboratory conditions. The characteristic of wine fermentation with its low pH, high sugar concentration and anaerobiosis, makes it a unique environment for yeast. For this reason, information pertaining to yeast

physiology obtained from other fermenting media or laboratory studies are not necessarily applicable to winemaking.

The following series of experiments were designed to examine several aspects of L-proline biochemistry in a commercial wine making strain of *S. cerevisiae* grown under aerobic and strict anaerobic conditions. These included proline uptake, proline catabolism and the yeasts ability to utilise compounds resulting from L-proline catabolism as nitrogen sources. Experiments were carried out in a fully defined synthetic grape juice medium. A number of *S. cerevisiae* strains and other species of yeast were also tested for their ability to utilise proline as a nitrogen source under anaerobic conditions.

3.2 Materials and methods

3.2.1 Yeast strains and maintenance

The yeast *Saccharomyces cerevisiae* strain AWRI 796, obtained from The Australian Wine Research Institute yeast culture collection, was used in experiments involving L-proline transport, catabolism and for investigations involving compounds of proline biochemistry. It is a widely used strain for the commercial production of wine and is characterised by low H_2S and SO_2 production and the possession of the K2 killer activity. The list of *S. cerevisiae* strains and other yeast species tested for the ability to catabolise L-proline under strict anaerobic conditions is presented in Table 3.1. Cultures were stored at 4^0C on slopes of MYPG medium (malt extract, 3 g/L; yeast extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L; agar, 20 g/L) and were subcultured every three months.

3.2.2 Culture medium

A synthetic grape juice medium (SM) was used in all experiments (Jiranek, 1992). This medium approximates the composition of typical grape juice and contains per liter: glucose, 200 g; L-malic acid, 3 g; potassium hydrogen tartrate, 2.5 g; $MgSO_4$,

1.23 g; K_2HPO_4, 1.14 g; $CaCl_2$, 0.44 g; citric acid, 0.2 g; myo-inositol, 100 mg; pyridoxine HCl, 2 mg; nicotinic acid, 2 mg; calcium pantothenate, 1 mg; thiamine-HCl, 1 mg; *p*-amino-benzoic acid, 0.2 mg; riboflavin, 0.2 mg; folic acid, 0.2 mg; biotin, 0.125 mg; $MnCl_2$, 198.2 μg; $ZnCl_2$, 135.5 μg; $FeCl_2$, 31.96 μg; $Co(NO_3)_2$, 29.1 μg; $NaMoO_4$, 24.2 μg; $CuCl_2$, 13.6 μg; KIO_3, 10.8 μg; H_3BO_3, 5.7 μg; 2 mL of ergosterol/Tween 80 solution prepared by mixing 100 mg of ergosterol with 5 mL of Tween 80 and diluting to 20 mL with hot ethanol. Depending on the experiment, the medium was supplemented with specific nitrogen compounds. The medium was adjusted to pH 3.5 with KOH and filter sterilized through a 0.45 μm membrane.

3.2.3 Fermentation procedure

Starter culture medium, 30 mL, (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L), contained in a 100 mL Erlenmeyer flask loosely plugged with cotton wool and sterilized by autoclaving, was inoculated with a loopful of yeast taken from the MYPG slope. The culture was incubated at 25^{0}C for 16 hours with shaking (150 rpm) and was used to inoculate 50 mL of a pre-experimental culture prepared from SM medium to a density of 2 x 10^6 cells/mL. For aerobic experiments, media were sparged with air using a pump and sparger. For anaerobic experiments, media were placed for a minimum of 15 hours in an anaerobic hood (Coy Laboratory Products Inc., USA) containing 5% hydrogen and 95% nitrogen gas prior to the inoculation. In both instances, cultures were grown with shaking at 25^{0}C until cells reached late log phase (approximately 19 hours). These cultures were used as an inoculum for all experiments at a cell density of 5 x 10^6 cells/mL.

Fermentations were carried out in SM medium contained in 250 mL fermentation flasks fitted with air locks to prevent contamination. Initial volume of medium was 200 mL. For aerobic experiments, media were constantly aerated. Anaerobic experiments were carried out in the anaerobic hood. Anaerobic media were

augmented with 1 mL of 0.1% Resazurin as an indicator of the oxidation state and placed in the hood for at least 15 hours prior to the beginning of the experiment. All experiments were carried out at 25^{0}C.

3.2.4 Cell counts and data analysis

Fermentations were followed by withdrawing medium (0.5 mL) using a medical syringe (volume 1 mL), vortexed and an appropriate dilution made. Cells counts were preformed using a haemocytometer slide (Neubauer, Germany). All data points represent mean values of at least three measurements unless otherwise stated.

3.2.5 Chemical analysis of fermenting media

Ammonium ion concentrations were determined by the enzymatic method obtained from Boehringer-Mannheim and used according to the manufacturers instructions. *Amino acid* concentrations, excluding proline, were determined using the HPLC according to the method of Jiranek, (1992). *L-proline* concentrations were quantified using a modification of the method described by Troll and Lindsley, (1955) preformed as follows: 1.3 mL of fermentation sample was placed in a 1.5 mL Eppendorf tube and the cells pelleted by centrifugation at 12 000 x *g* for 5 minutes. One millilitre of supernatant was then transferred to a boiling tube (20 mm x 140 mm) which contained 3 to 5 glass beads. Fresh ninhydrin solution (5 mL, 2.5 g ninhydrin dissolved in 60 mL of acetic acid and 40 mL of 6M orthophosphoric acid by heating at 70^{0}C) and 5 mL glacial acetic acid were added to the boiling tube which was then thoroughly mixed and covered with a glass marble to prevent evaporation. The boiling tube was heated in a water bath for 60 minutes at 95^{0}C, cooled and shaken with 5 mL of toluene to extract the red colour. Thirty minutes was allowed for the toluene and acid phases to separate clearly and the optical density of the sample was read using a spectrophotometermeter (Beckman, model DU-64) at 520 nm. Standard solutions containing 0.0, 1, 2.5, 5, 10, 20, 40 and 60 µg L-proline were reacted at the same

time as the experimental samples. Proline contents in the samples were calculated from the standard curve.

3.2.6 Transport assay for [^{3}H]-L-proline

Cells were grown aerobically and anaerobically until late log phase in SM medium supplemented with ammonium ions [$(NH_4)_2SO_4$], arginine, glutamate, leucine, threonine, alanine, tyrosine to a total concentration of 500 mg N/L. One hundred millilitres of the prepared cultures were centrifuged at 10 000 x *g* and the cells washed three times in SM medium free of the nitrogen source and starved for 15 minutes at 25^{0}C with shaking. The cells were then transferred to 150 mL of SM medium supplemented with L-proline (500 mg N/L) as the sole source of nitrogen to which [^{3}H]-L-proline (Amersham) was added to give a final concentration of 0.5 μCi/mL. Samples (0.1 mL) were withdrawn at intervals, filtered through a 0.45μm filter (Millipore) and washed with 10 mL of ice-cold, fresh SM medium free of radioactive proline. The filter with cells was transferred to a scintillation vial containing 7 mL of scintillation cocktail (ASC II, Amersham). Radioactivity was counted in a liquid scintillation spectrometer (Packard, Tri-Carb 460 CD), programmed for quench correction.

3.2.7 Protocol for screening yeast species capable of growth on L-proline under anaerobic conditions

Several yeast species and strains of *S. cerevisiae* were tested for the ability to utilise L-proline as the sole source of nitrogen under strict anaerobic conditions. Starter culture medium (refer to Section 3.2.3) was inoculated with a loopful of a tested yeast propagated on a slant. This was then used to inoculate SM medium prepared for microbial growth under anaerobic conditions (refer to Section 3.2.3). Samples were collected at intervals and the culture growth was monitored microscopically.

3.3 Results and Discussion

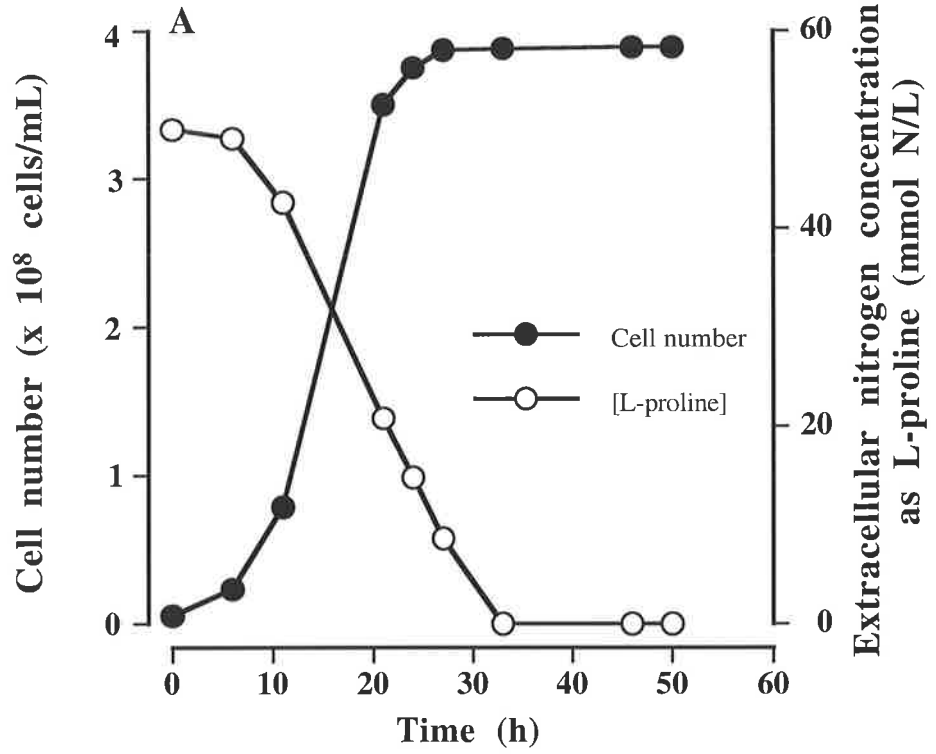
3.3.1 Growth of *S. cerevisiae* AWRI 796 on L-proline as the sole nitrogen source under aerobic and anaerobic conditions

Saccharomyces cerevisiae AWRI 796 was able to utilise L-proline as a sole nitrogen source when grown in well aerated culture medium. The external L-proline concentration gradually decreased as the number of yeast cells increased (Figure 3.1A). When compared to the three control nitrogen sources, ammonia, arginine and tyrosine (Figure 3.1B), growth on proline was slowest but resulted in the highest cell number (3.9×10^8 cells/mL) with growth on ammonia producing the lowest cell number and the highest growth rate (3.3×10^8 cells/mL). When cultured under strict anaerobic conditions the yeasts were not able to grow when L-proline was provided as the sole source of nitrogen. No increase in cell number was observed and the L-proline concentration of the medium remained unchanged throughout the experiment (Figure 3.2A). Three other nitrogen sources supported growth of yeast under anaerobic conditions with the final cell number reaching approximately 42% of that obtained by cultures grown in well aerated medium. Similarly to the aerobic conditions, yeast growth on ammonia was the fastest, however, final cell number was less than those grown on the amino acids tested (Figure 3.2B).

The data compare well with previous studies on L-proline utilisation by *S. cerevisiae* in which all investigated *S. cerevisiae* strains of bakers' (Horak & Rihova, 1982), brewing (Jones, *et al.*, 1969; Duteurtre, *et al.*, 1971) and wine (Bisson, 1991) yeasts were unable to utilise L-proline as a carbon or nitrogen source when grown under anaerobic conditions. Further studies elucidated that the necessity for molecular oxygen is due to the requirement of this element by the first enzyme involved in proline catabolism, proline oxidase. For its function, this enzyme requires the oxidative respiratory transport chain (ETC), where it interacts with cytochrome *c* (refer to Section 2.3.2). When grown aerobically, L-proline can be utilised only after exhaustion of other nitrogen sources present in the medium (Schwencke & Magana-Schwencke, 1969; Duteurtre *et al.*, 1971). This is the result of strong nitrogen

Figure 3.1. Growth of *S. cerevisiae* AWRI 796 on (A) L-proline and (B) selected sources of nitrogen under aerobic conditions.

Cells were grown in SM medium at 25^{0}C in well aerated and shaken 250 mL fermentation flasks. Initial concentration of nitrogen in all experiments was 700 mg N/L.


A
Cell number (x 10^8 cells/mL)
Extracellular nitrogen concentration as L-proline (mmol N/L)
Cell number
[L-proline]
Time (h)
0
1
2
3
4
0
20
40
60
0
10
20
30
40
50
60

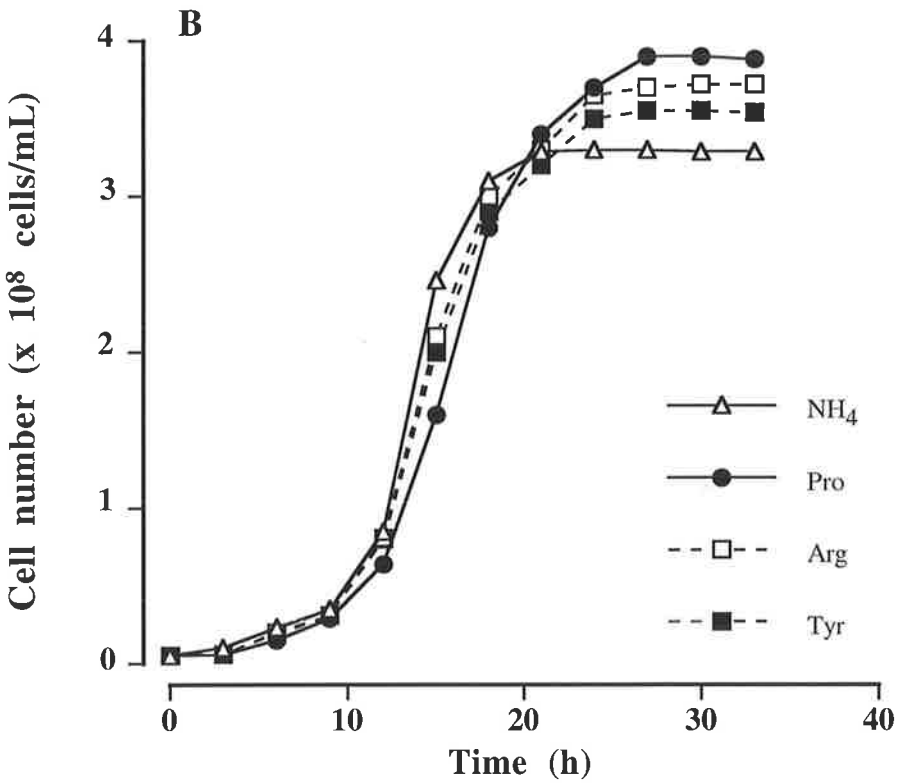
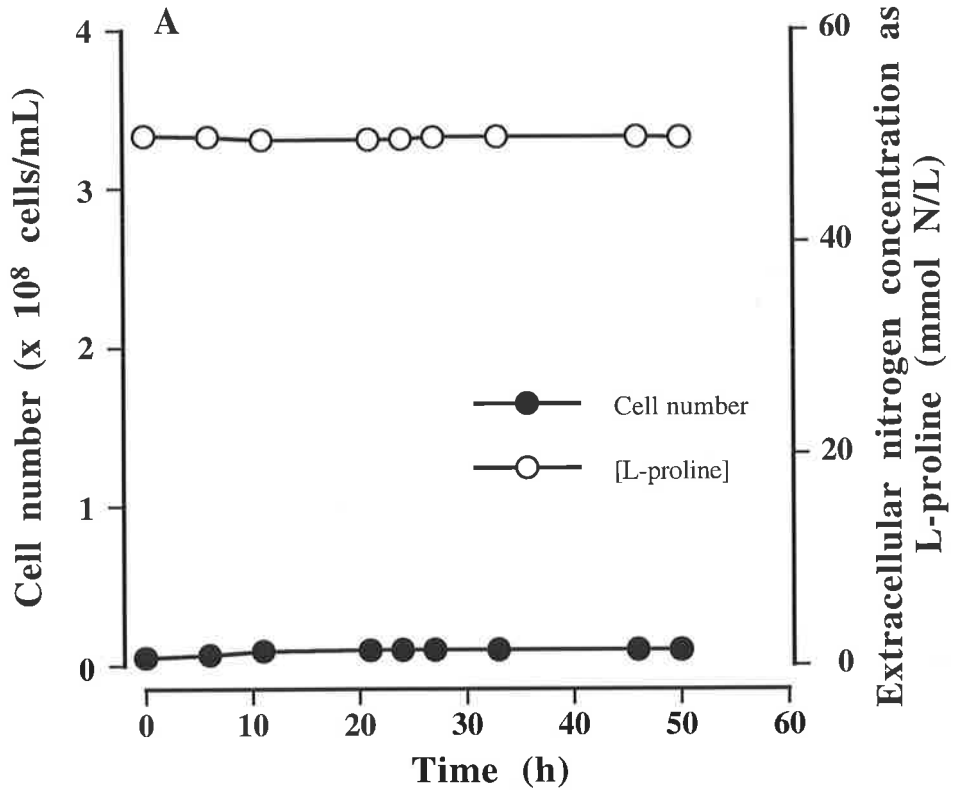
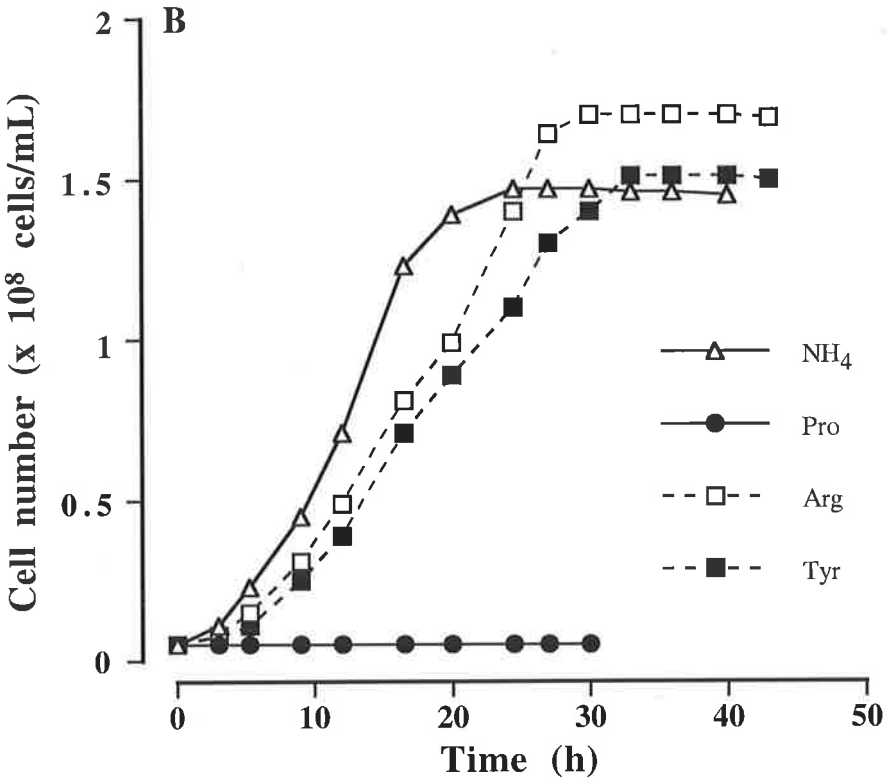

B
Cell number (x 10^8 cells/mL)
NH_4
Pro
Arg
Tyr
Time (h)
0
1
2
3
4
0
10
20
30
40

Figure 3.2. Growth of *S. cerevisiae* AWRI 796 (A) on L-proline and (B) selected sources of nitrogen under strict anaerobic conditions.

Cells were grown with shaking at 25°C in a 250 mL fermentation flasks containing SM medium equilibrated with the atmosphere of 5% hydrogen and 95% of nitrogen gas in an anaerobic hood. Initial concentration of nitrogen was 700 mg N/L.


A
Cell number (x 10^8 cells/mL)
Extracellular nitrogen concentration as L-proline (mmol N/L)
Cell number
[L-proline]
Time (h)
0
1
2
3
4
0
20
40
60
0
10
20
30
40
50
60


B
Cell number (x 10^8 cells/mL)
NH4
Pro
Arg
Tyr
Time (h)
0
0.5
1
1.5
2
0
10
20
30
40
50

catabolite repression of both specific and general L-proline transporters in *S. cerevisiae* by amino acids and ammonium ions (refer to Section 2.2.2).

The strict requirement for molecular oxygen associated with L-proline catabolism has been described for all animal tissues and most microorganisms studied. Interaction of proline oxidase with the ETC occurs at different levels depending on the organism in question. It is understood that in plants L-proline can be degraded via two pathways. One involving oxygen-requiring mitochondrial proline oxidase and the other through NAD(P)-linked cytosolic proline dehydrogenase (Adams & Frank, 1980).

Proline is the only amino acid requiring oxygen for its catabolism by *S. cerevisiae.* These microorganisms can degrade other amino acids and nitrogen-containing compounds under anaerobic conditions.

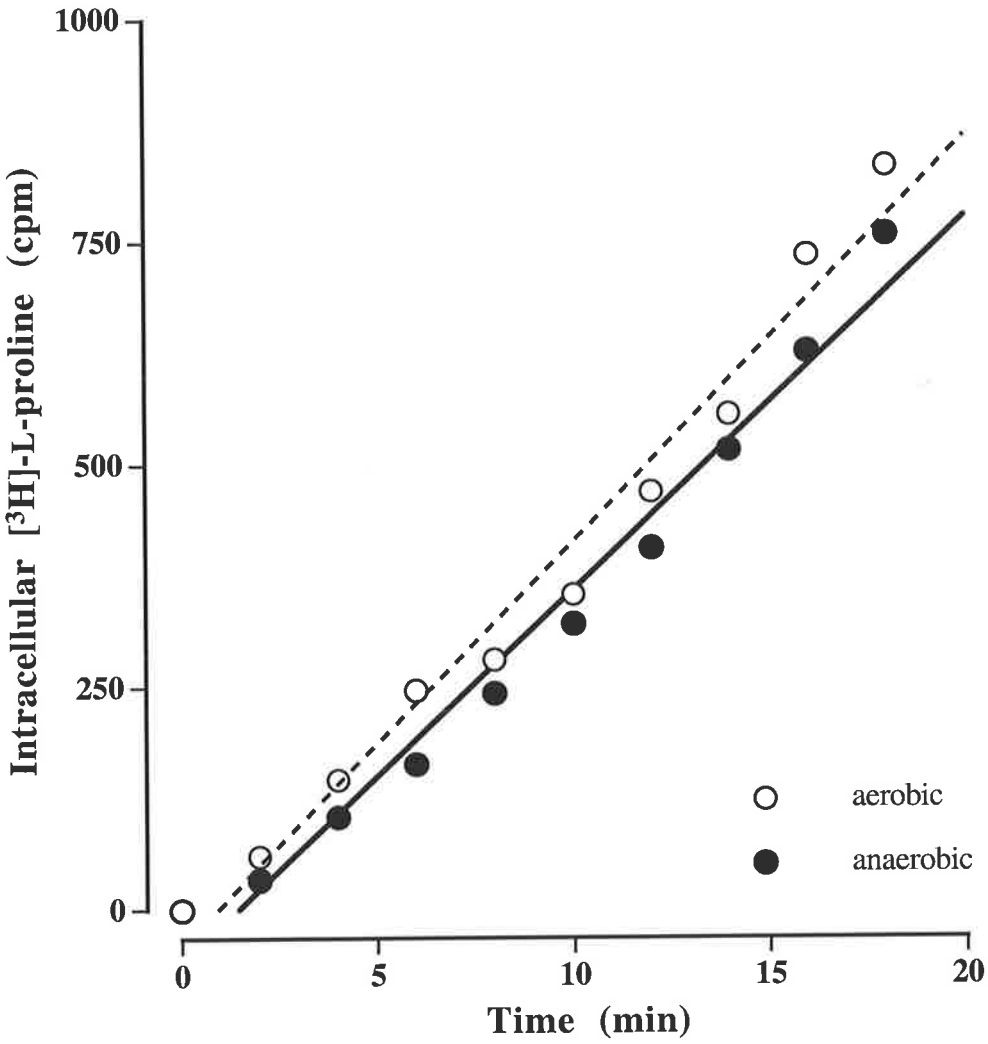
3.3.2 Uptake of L-proline by cells of *S. cerevisiae* AWRI 796 under anaerobic and aerobic conditions

In light of the above findings the transport of L-proline by yeast under well aerated and strict anaerobic conditions was examined. L-proline was provided as the sole nitrogen source. It was found that the rate of [^{3}H]-L-proline accumulation by the *S. cerevisiae* AWRI 796 was the same under both, aerobic and anaerobic conditions (Figure 3.3.). Transport of proline, as measured by the rate of its intracellular accumulation, was linear and proportional to the duration of the experiment.

The ability of *S. cerevisiae* to accumulate proline at similar rates under anaerobic and aerobic conditions when proline was provided as a sole source of nitrogen was reported earlier (Schwencke & Magana-Schwencke, 1969; Duteurtre *et al.*, 1971). Similarly, other proteogenic amino acids and a range of substrates serving as energy, carbon and/or nitrogen sources are transported by yeast to the same extent under these two growth conditions. These compounds are symported into the cell with H^+ ions by secondary active carriers driven by the electrochemical potential gradient of protons

Figure 3.3. Intracellular [^{3}H]-L-proline concentration (counts per minute) in *S. cerevisiae* AWRI 796 grown under aerobic and anaerobic conditions.

Cells were grown aerobically or anaerobically, as described in Materials and Methods, at 25^{0}C in 150 mL of SM medium with 500 mg N/L as L-proline and 0.5 μCi/mL of [^{3}H]-L-proline. 0.1 mL of cell suspension was withdrawn every two minutes, filtered through a 0.45 μm filter and washed with 10.0 mL of ice-cold SM medium free of radioactive proline. Each point represents the mean of five estimates. Statistical analysis of data was preformed using Test for Equality of Slopes of Several Regression Lines (Sokal & Rohlf, 1981).


1000
750
500
250
0
Intracellular [3H]-L-proline (cpm)
0
5
10
15
20
Time (min)
aerobic
anaerobic

($\Delta\mu_{H^+}$). This gradient is generated through active pumping of H^+ ions by the plasma membrane H^+-ATPase (Grenson & Hennaut, 1971; Cooper, 1982b). Experiments with respiratory-deficient mutants of *S. cerevisiae* (Sigler *et al.*, 1980, 1981a, 1981b) showed that the energy derived from glycolysis alone can generate sufficient $\Delta\mu_{H^+}$ and its value is not significantly influenced by the anaerobic conditions. That yeast grown under anaerobic conditions transport this imino acid by the glycolytically derived $\Delta\mu_{H^+}$ is supported by the following observations: alkalification of a yeast suspension upon the addition of proline (Seaston *et. al.*, 1973), abolition of transport by compounds interfering with ATP synthesis, such as sodium azide (Magana-Schwencke & Schwencke, 1969) and the correlation between intracellular ATP levels and proline accumulation (Horak & Kotyk, 1986).

Similarly in *E. coli* (Klein & Boyer, 1972; Kobayashi *et al.*, 1974; Kayama-Gonda & Kawasaki, 1979; Mogi & Anraku, 1984a, 1984b, 1984c; Chen *et al.*, 1985), *Streptococcus faecalis* (Asghar *et al.*, 1973) and *Chlorella vulgaris* (Cho & Komor, 1983) where the energetics of proline transport has been investigated in some detail, the rate of uptake of this imino acid under aerobic and anaerobic conditions was similar. In these microorganisms, as is apparently the case with yeast, proline transport is associated with the depolarisation of the membrane potential, is sensitive to inhibitors of membrane-bound H^+-ATPase and can be driven by the metabolic energy derived from glycolysis.

3.3.3 Uptake of L-proline by *S. cerevisiae* AWRI 796 from a medium containing other nitrogen bearing compounds

The following experiments were designed to investigate the behaviour of *S. cerevisiae* AWRI 796 towards L-proline in the presence of other sources of nitrogen in a synthetic grape juice medium.

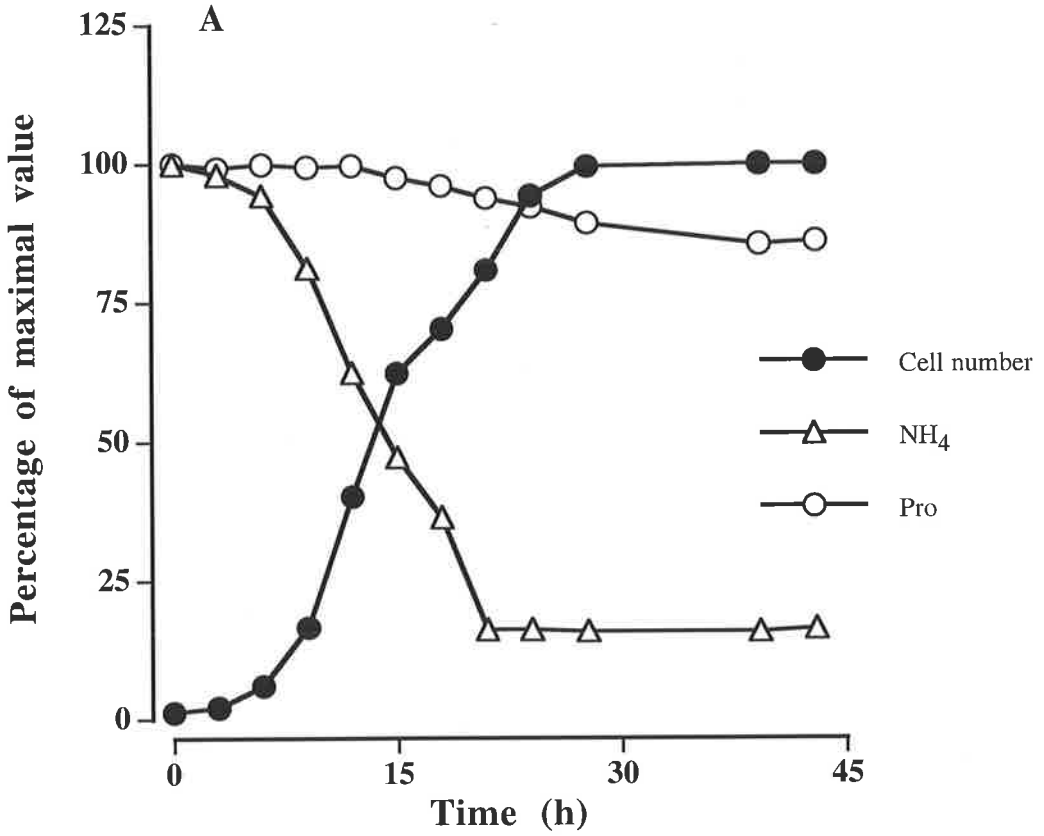
Firstly, it was established that the minimum nitrogen demand for this strain to completely ferment 200 g/L of glucose under aerobic conditions at 25^{0}C was 165 mg N/L, whereas 700 mg N/L provided optimal growth resulting in the highest cell number and rate of sugar utilisation. The previously reported values for nitrogen requirement of different strains of *S. cerevisiae* vary greatly depending on the strain used and the conditions under which fermentation was carried out. Nevertheless 140 to 175 mg N/L has been reported to be the minimal value and from 420 to 878 mg N/L is required for maximal fermentation rates (Agenbach, 1977; Bely *et al.*, 1990; Jiranek, 1992). Thus the demand for nitrogen of *S. cerevisiae* AWRI 796 lies at the upper end of the amount of nitrogen required for fermentation.

When a limited amount of nitrogen was provided in the form of ammonium salt (50 mg/L) or selected amino acids, such as arginine, glutamate, alanine and tyrosine, which constitute the bulk of nitrogen contained in a typical grape juice, it was found that the uptake of proline occurred only after significant depletion of these nitrogen sources (Figure 3.4B, 3.5 and 3.6). This suggests that, under the experimental conditions, transport of proline by the yeast strain AWRI 796 was repressed by the ammonium and amino acids tested. Derepression of proline uptake was associated with a marked decrease in the external concentration of assimilable nitrogen other than proline. Some uptake of proline was observed at the beginning of the stationary phase when an excess of nitrogen was supplied in the form of ammonium ions (Figure 3.4A). In contrast to proline, several other amino acids analysed were co-transported by yeast and their uptake was insensitive to the presence of ammonium ions (Figure 3.5 and 3.7).

Yeast transport L-proline with the low and high-affinity transport systems (refer to Section 2.1.2). The unique feature of these transporters is that both are trans-inhibited by a number of amino acids and are sensitive to ammonium ions (Magana-Schwencke & Schwencke, 1969; Brandriss & Magasanik, 1979a, 1979b; Lasko & Brandris, 1981). Consequently, proline is absorbed last from the fermenting medium. This has

Figure 3.4. Concentration of L-proline in relation to the concentration of ammonium ions in a medium supporting growth of *S. cerevisiae* AWRI 796.

Cells were grown aerobically and with shaking at 25°C in SM media supplemented with (A) 700 mg N/L as NH_4 plus 500 mg N/L as L-proline and (B) 50 mg N/L as NH_4 plus 500 mg N/L as L-proline.


A
125
100
75
50
25
0
Percentage of maximal value
0
15
30
45
Time (h)
Cell number
NH4
Pro

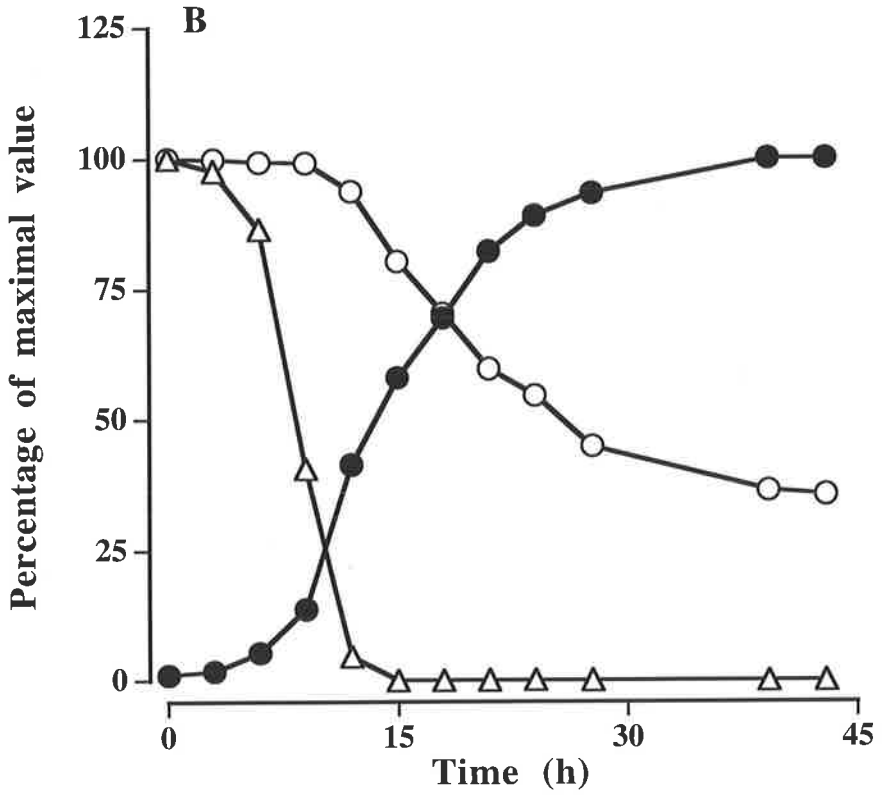

B
125
100
75
50
25
0
Percentage of maximal value
0
15
30
45
Time (h)

Figure 3.5. Course of changes in L-proline concentration during growth of *S. cerevisiae* AWRI 796 in the medium containing other amino acid nitrogen sources.

The initial total nitrogen concentration excluding L-proline was 60 mg N/L. Concentration of nitrogen as L-proline was 700 mg N/L. Growth conditions were as described in Figure 3.4.

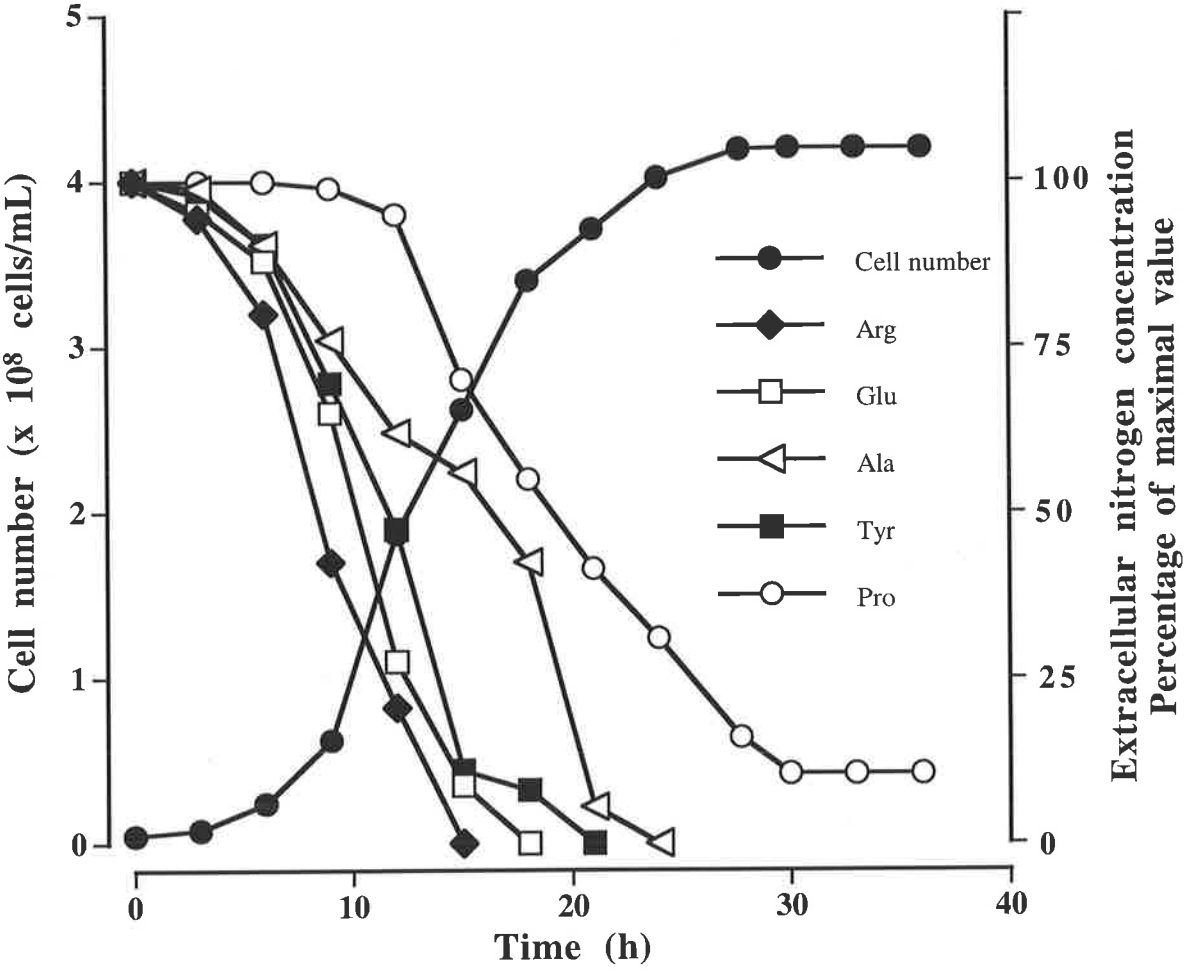

Cell number (x 10⁸ cells/mL)
Extracellular nitrogen concentration
Percentage of maximal value
Time (h)
Cell number
Arg
Glu
Ala
Tyr
Pro
0
1
2
3
4
5
0
25
50
75
100
0
10
20
30
40

Figure 3.6. Rate of [^{3}H]-L-proline accumulation in relation to the extracellular concentration of assimilable nitrogen by *S. cerevisiae* AWRI 796.

Yeast were grown aerobically at 25^{0}C in SM medium. Nitrogen source and concentrations were as shown in Figure 3.5. Radioactive proline was estimated following the procedure described in materials and methods and Figure 3.3.

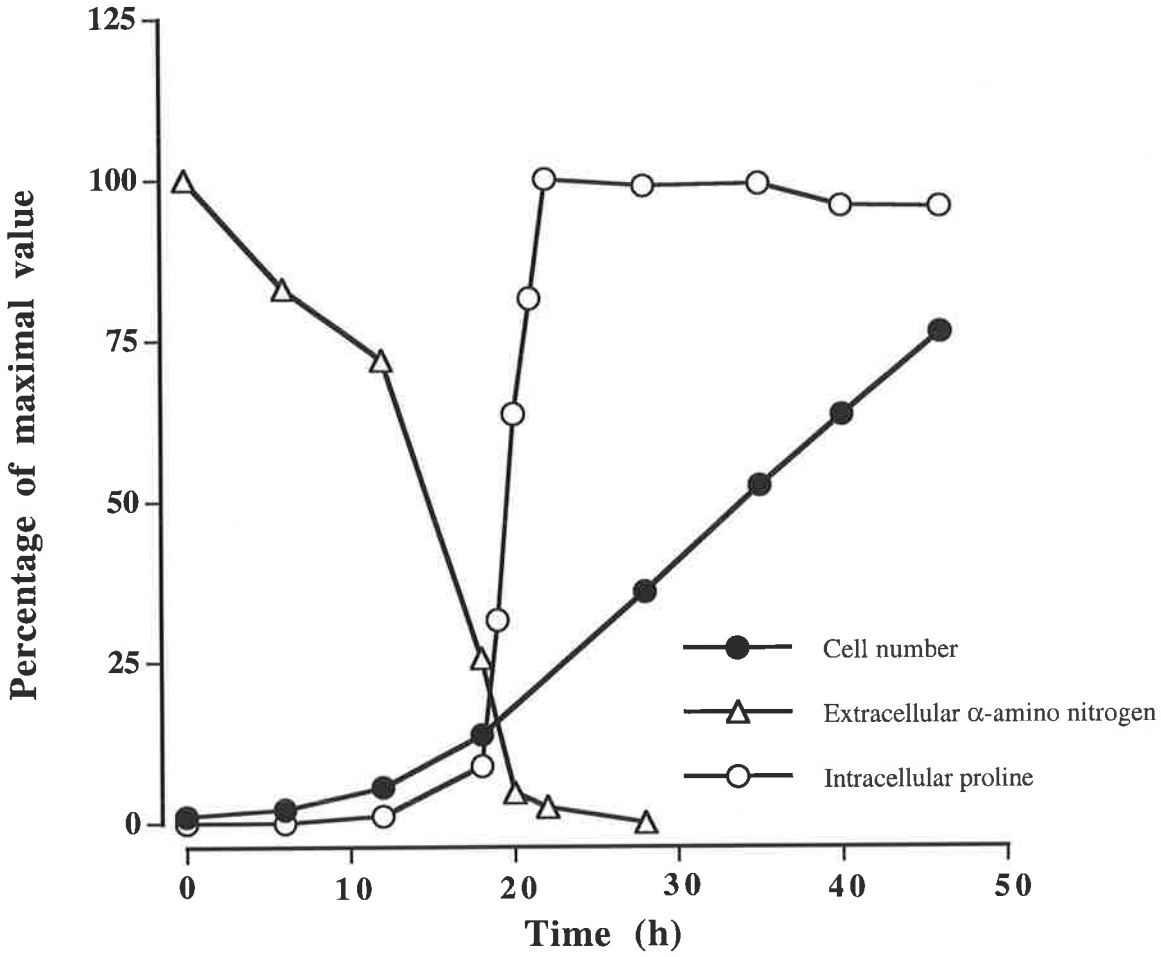
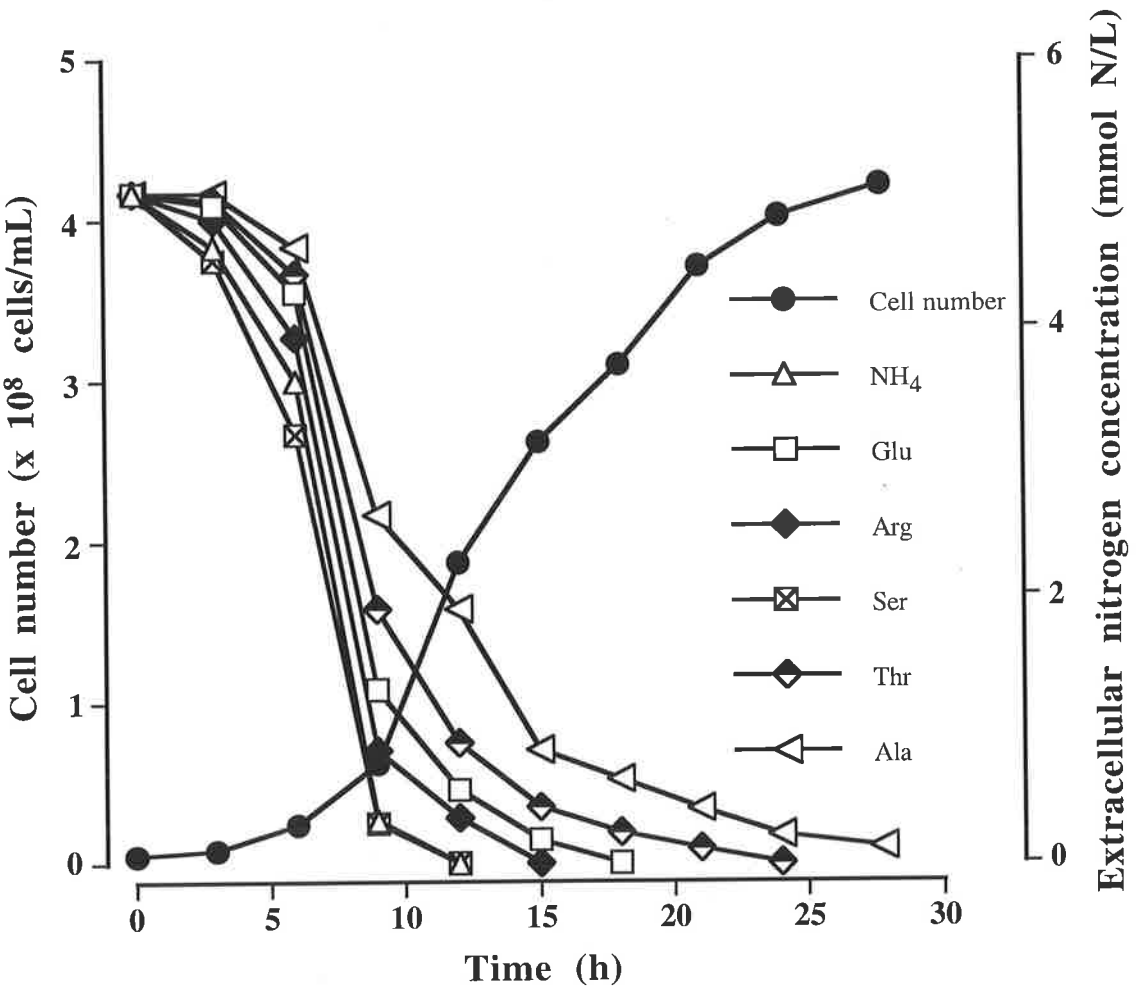

Percentage of maximal value
125
100
75
50
25
0
0
10
20
30
40
50
Time (h)
Cell number
Extracellular α-amino nitrogen
Intracellular proline

Figure 3.7. Utilisation of ammonium ions and selected amino acids supplied as a nitrogen source during fermentation of SM medium by *S. cerevisiae* AWRI 796. Experimental conditions were as described for Figure 3.4.


Cell number (x 10^8 cells/mL)
Extracellular nitrogen concentration (mmol N/L)
Time (h)
Cell number
NH_4
Glu
Arg
Ser
Thr
Ala
0
1
2
3
4
5
6
0
5
10
15
20
25
30

been observed for both, brewing (Harris & Merritt, 1961; Jones & Pierce, 1964; Rose & Keenan, 1981) and wine yeasts (Bisson, 1991; Jiranek, 1992).

It is interesting to note that some transport of proline occurred at the end of log phase and at the beginning of the stationary phase of growth even when excess nitrogen was supplied in the form of NH_4. A similar observation was made by Horak & Rihova (1982) with a strain of bakers' yeast. This late derepression of proline uptake is difficult to explain on the basis of the data obtained. It is possible that with time, inhibition of proline transport is relaxed, especially when the external proline concentration is high. Such derepression would enable proline to be transported and perhaps used directly for protein synthesis or storage. Uptake of exogenous proline would present an energetically more favourable alternative to *de novo* synthesis of proline.

It has been observed that the order of absorption of nitrogen containing compounds, largely amino acids, either from fermenting wort (Jones & Pierce, 1964) or must (Jiranek, 1992), bears close similarity for a number of *S. cerevisiae* strains. Based on the time of their uptake from fermenting medium, these compounds were assembled into four distinctive groups (Table 2.2). When yeast utilise a compound as a nitrogen source, the energetics associated with transport and liberation of the nitrogen moiety from that compound plays a critical role in the preference for its utilisation. For this reason compounds such as ammonium ions, glutamate, glutamine or arginine (Group A) are preferred over phenylalanine (Group B) or glycine (Group C). Ammonia, glutamate and glutamine lie at the core of nitrogen metabolism in yeast (refer to Figure 2.1) whereas arginine provides four nitrogen atoms per co-transported proton. In contrast, phenylalanine and glycine require two protons for their transport. This represents an energetically less favourable nitrogen source as the yeast cell must extrude excess protons in order to avoid acidification. Extrusion of a proton demands utilisation of one molecule of ATP.

In this light, proline presents a rather poor nitrogen source for yeast. Recovering the nitrogen atom from proline involves an enzyme which requires a functional electron transport chain, presence of molecular oxygen and possible energetic output associated with the transport of proline into the mitochondria. The proton gradient driven proline transport into the mitochondria has been reported for rat liver (Cybulski & Fisher, 1977; Meyer, 1977) and mung bean (Cavalieri & Huang, 1980). Thus multi-level regulation of proline transport and degradation by yeast ensures that this imino acid is utilised as a last resort in its quest for nitrogen.

3.3.4 Growth of *S. cerevisiae* AWRI 796 on L-proline metabolites, Δ'-pyrroline-5-carboxylate (P5C), δ-aminovalerate (AVA), D-proline and L-glutamate

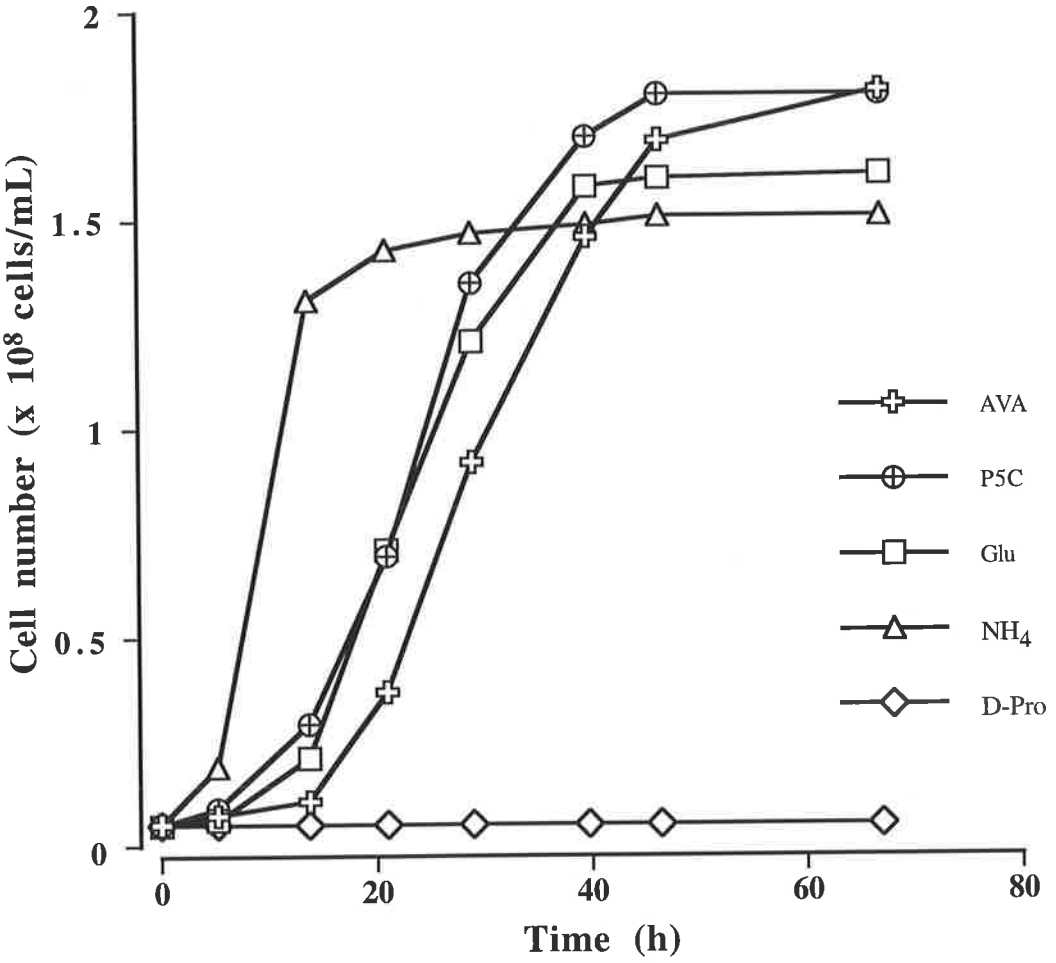
In yeast, as in all studied eukaryotes and most prokaryotes, catabolism of L-proline is oxidative and proceeds to glutamate via P5C. In some anaerobic bacteria, however, the D-proline ring is reductively cleaved to AVA. D-proline is formed from L-proline by a racemase. In eukaryotes proline conversion occurs in mitochondria, whereas in bacteria it is cytoplasmic (refer to Section 2.3.2).

Results showed that both, P5C and AVA supported yeast growth under anaerobic conditions (Figure 3.8). The cultures grown on P5C and AVA reached the same cell number at stationary phase, although the lag phase of cells grown on AVA was extended compared to growth on P5C. For comparison, growth on glutamate closely resembled that on P5C except for the lower final cell number for cells grown on glutamate. The lowest cell number was observed with cells grown on NH_4, these however, exhibited the fastest growth. No growth on D-proline was observed.

This set of experiments was designed to investigate the ability of wine yeasts to utilise two primary compounds resulting from proline catabolism, namely P5C and AVA, as a sole nitrogen source under anaerobic conditions. Although the enzyme oxidising

Figure 3.8. Growth of *S. cerevisiae* AWRI 796 on L-proline catabolites provided as the sole source of nitrogen. Growth on ammonium ions was monitored for comparison.

Yeast were grown at 25^{0}C in SM medium under strict anaerobic conditions as described in materials and methods. AVA, δ amino-valeric acid; P5C, Δ-pyrroline-5-carboxylic acid; Glu, glutamic acid; D-Pro, D-proline.


Cell number (x 10^8 cells/mL)
2
1.5
1
0.5
0
0
20
40
60
80
Time (h)
AVA
P5C
Glu
NH_4
D-Pro

P5C to glutamate, P5C dehydrogenase (P5C-DH), functions without involvement of the electron transport chain in yeast, it is known to be located in the mitochondria (Cooper, 1982a). Reports show that yeast can utilise this compound as a sole nitrogen source in the presence of oxygen (Lundgren & Ogur, 1973), therefore the question was asked if P5C supplied as a sole nitrogen source would support yeast growth under anaerobiosis. No reported data exists to indicate that *S. cerevisiae* can catabolise AVA.

It is known that the mitochondrial morphology of yeast cells grown under anaerobic conditions differ considerably from those grown in the presence of oxygen (Plattner & Schatz, 1969; Plattner *et al.*, 1971; Visser *et al.*, 1995). Despite these differences however, mitochondria of anaerobically grown cells contain most of the anabolic and catabolic enzymes which are present in respiring yeasts. These include the enzymes of the Krebs cycle, sterol metabolism and amino acid metabolism. These enzymes are synthesized as a response to the available substrate and/or biochemical processes required (Damsky *et al.*, 1969; Shimizu *et al.*, 1973; Jenkins *et al.*, 1984; Visser *et al.*, 1994). It is only the enzymes associated with the respiratory transport chain which are not found in anaerobically grown yeast (De Winde & Grivell, 1993, 1995). As P5C oxidation is catalysed by the NAD-linked P5C-DH located in the mitochondrial matrix, anaerobic conditions did not interfere with this reaction, resulting in full utilisation of nitrogen contained in the P5C molecules. It is expected that reoxidation of NAD occurs through the malate-asparate shuttle - a process commonly employed by eukaryotic cells for transfer of oxidising/reducing power, as NAD and NADH can not penetrate the inner mitochondrial membrane. Alternatively, P5C-DH might be present in the yeast cell cytosol. Functional cytosolic P5C-DH was reported to be present in locust flight muscle (Brosemer & Veerabhadrappa, 1965) and in rat liver cells (Brunner & Neupert, 1969). The reaction of P5C oxidation by these cytosolic enzymes occurred twice as fast with NADP as a cofactor compared with NAD, indicating the possible existence of two enzymes: a mitochondrial P5C dehydrogenase with higher affinity for NAD and a cytosolic P5C

dehydrogenase with the higher affinity for NADP. Existence of a yeast cytosolic P5C-DH requires confirmation.

There are no reports of studies of δ-aminovalerate metabolism in *S. cerevisiae*. This non-proteogenic amino acid is formed in large amounts during the anaerobic degradation of proteins by many proteolytic gastrointestinal, ruminal and oral cavity *Clostridium* species. These bacteria use D-proline as an oxidant and reduce it to AVA (Stadtman, 1956; Dreyfus *et al.*, 1968; Mead, 1971; Albone *et al.*, 1976; Barker, 1981; Kasai & Kiriyama, 1988). AVA is also formed as an intermediate product of lysine, ornithine and arginine catabolism by certain procaryotes (*Pseudomonas* sp., Ichihara *et al.*, 1960; Miller & Rodwell, 1971 and *E. coli*, Roberts, 1954) and eukaryotes (mouse liver cells, Roberts, 1954; *Aspergillus*, Roberts, 1954; *Hansenula saturnus* Rothstein, 1965 and *Candida guilliermondii* var. *membranaefaciens*, Der Garabedian, 1986). Several organisms are reported to be capable of further catabolism of AVA. In *Pseudomonas* AVA is transaminated to form glutamic acid via α-ketoglutarate (Ichihara *et al.*, 1960). A similar pathway is suspected to occur in the yeasts *Candida guilliermondii* (Der Garabedian, 1986) and *Hansenula saturnus* (Rothstein, 1965). The first step of AVA degradation by strictly anaerobic *Clostridium aminovalericum*, a bacterium which uses this amino acid as a sole source of carbon and nitrogen, also involves transamination to form glutaric semialdehyde and glutamate ultimately leading to the formation of ammonia, acetate, propionate and valerate (Hardman & Stadtman, 1960; Barker *et al.*, 1987).

Thorough investigation of AVA catabolism by *S. cerevisiae* was outside the scope of this work. It can be hypothesised however, that since *S. cerevisiae* can utilise AVA as the sole nitrogen source, its catabolism involves transamination via α-ketoglutarate and formation of glutamate.

3.3.5 Screening various yeast species and strains for the ability to grow on L-proline under anaerobic conditions

The results of screening tests for the ability of a number of yeast species to grow anaerobically on L-proline when provided as the only nitrogen source proved to be unsuccessful (Table 3.1). None of the species or strains tested were able to utilise this imino-acid in the absence of oxygen. This finding confirms previous reports (Cooper, 1982a) indicating that proline catabolism in yeasts is associated with the availability of molecular oxygen

3.3.6 Conclusions

The transport of proline by the *S. cerevisiae* strain AWRI 796 is inhibited by ammonia and other nitrogen containing compounds present in the medium and its catabolism is governed by the availability of molecular oxygen. It is of significance to this project that anaerobic conditions do not interfere with either the rate of proline transport by yeast cells or the utilisation of proline catabolites.

None of the strains of *S. cerevisiae* or other species of yeast investigated could utilise L-proline under anaerobic conditions.

Genus	Species
Dekkera	*anomala* *bruxellensis*
Hansenispora	*valbyensis*
Hansenula	*anomala* *saturnus var. saturnus*
Kloeckera	*apiculata*
Kluyveromyces	*marxianus*
Pichia	*membranaefaciens*
Saccharomyces	*cerevisiae* *bayanus*

Table 3.1. Species of yeasts tested for their ability to utilise L-proline as the sole nitrogen source under anaerobic conditions.

Chapter 4

Purification and partial characterisation of L-proline:NAD(P)$^+$-oxidoreductase (EC 1.5.1.2) from soybean leaves

4.1 Introduction

Experiments described in the previous chapter demonstrated that anaerobic growth conditions do not interfere with proline transport by *S. cerevisiae*. However, catabolism of this imino acid is prevented by the absence of oxygen. A pathway for proline degradation, which eliminates the need for molecular oxygen, was therefore introduced into yeast cells as a possible alternative mechanism for proline catabolism.

As discussed in Section 2.3.2, there are two established pathways for L-proline degradation. One pathway occurs in some species of clostridia and involves the conversion of L-proline to D-proline with subsequent reduction to δ-aminovalerate (AVA). The second pathway involves the oxidation of proline to Δ'-pyrroline-5-carboxylate (P5C) with further oxidation to glutamate. Although usually involving the enzyme proline oxidase, which is linked to the electron transport chain, the reaction also occurs in the cytoplasm of higher plants, some unicellular algae and bacteria. The cytosolic reaction is carried out by the NAD(P) linked bifunctional L-proline:NAD(P)$^+$-oxidoreductase (PDH/P5CR), the enzyme which also catalyses the reduction of P5C to proline.

Thus two possible routes exist when considering anaerobic proline catabolism: 1) reduction to AVA and 2) oxidation to P5C by a cytosolic dehydrogenase. Introduction of one of these pathways into yeast could enable the anaerobic degradation of proline. The pathway leading to the formation of AVA involves

several enzymes for which catabolic function depends on their precise spatial arrangement on cytoplasmic membranes (refer to Section 2.3.2.3). Introduction of such a complex pathway into the yeast cell could prove difficult. Conversely, introduction of a single cytoplasmic enzyme, namely NAD(P) linked PDH/P5CR, represents more attainable solution.

Recently, a soybean gene encoding PDH/P5CR was cloned and sequenced by functional complementation in *E. coli* (Delauney & Verma, 1990). The purpose of this series of experiments was to isolate soybean PDH/P5CR and to determine the ability and conditions of the enzyme to catalyse the oxidation of L-proline with NAD(P) as an electron acceptor *in vitro*. If successful, expressing the gene in *S. cerevisiae* may provide a pathway for the oxidation of proline under anaerobic growth conditions.

4.2 Materials and methods

4.2.1 Plant material

Soybean (*Glycine max*) seeds were sown in moist vermiculite contained in plastic trays (60 x 80 x 15 cm). Trays were placed in a glasshouse (18 h photoperiod, 25^{0}C) and watered daily. Plants were grown for 15 days.

4.2.2 Enzyme purification

All operations were carried out at 4^{0}C unless otherwise stated. Soybean leaves (500 g) were frozen in liquid nitrogen and ground. The resulting powder was homogenised in 2 L of Buffer 1 (0.2 M potassium phosphate, pH 7.2, 1 mM Na_2EDTA and 10 mM β-mercaptoethanol) in a Waring homogeniser (Model 37 B L18, USA) and filtered through several layers of cheesecloth. The extract was heated to 60^{0}C for 1 minute, immediately cooled and filtered through Whatmann filter No. 1. Filtrate was centrifuged for 30 minutes at 25 000 x *g*. The supernatant solution was

decanted and 15 mL of a 1% protamine sulphate solution added, with stirring, for each 100 mL of extract. The suspension was stirred gently for one hour and then centrifuged for 30 minutes at 25 000 x *g*. The resulting supernatant was fractionated with solid $(NH_4)_2SO_4$. The fractions precipitating between 0-25% and 25- 50% saturation were collected by centrifugation (30 minutes at 25 000 x *g*) and dissolved in 200 mL of Buffer 2 (50 mM phosphate, pH 7.2 and 10 mM β-mercaptoethanol). The 25-50% fraction (20 mL) was concentrated by passing it through a YM 10 ultrafiltration membrane mounted in the Stirred Cell Ultrafiltration Apparatus (Amicon Inc., U.S.A.). Pressure was applied by introducing nitrogen gas into the headspace at 300 kPa. The resulting 3 mL of concentrated suspension was passed through a high resolution gel filtration column (Sephacryl S-300 HR, bed volume 140 mL, void volume 58 mL, column size 21 x 400 mL; Pharmacia, Sweden) and eluded with Buffer 2. Fractions of 1 mL volume were collected. The fraction with the highest activity of proline dehydrogenase was poured onto an affinity chromatography column (2'5' ADP-Sepharose 4B column, bed volume 3.4 mL, void volume 1.1 mL, column size 10 x 43 mm, Pharmacia, Sweden), which was rinsed with 10 mL of Buffer 3 (0.1 M Tris; 1 mM EDTA, pH 8.3). The elution of enzyme was achieved with a 0 to 1 M NaCl concentration gradient in Buffer 3.

4.2.3 Assay procedures

4.2.3.1 P5C reductase assay

The activity of P5C reductase was measured by monitoring the loss of absorbancy at 340 nm. The standard reaction mixture contained: 0.2 mM Tris-HCl buffer (pH 7.5), 0.1 mM NADH, 3.0 mM L-P5C and enzyme to a final volume of 1 mL. The reaction was initiated by the addition of enzyme fraction and carried out for 30 minutes.

4.2.3.2 Proline dehydrogenase assay

Proline dehydrogenase activity was assayed by measuring NADH formation at 340 nm or by formation of a colored complex between P5C and *o*-aminobenzaldehyde (*o*-

AB). When the formation of NADH was measured, reaction mixtures of 1 mL contained: 20 mM L-proline, 10 mM NAD and enzyme in 67 mM CAPS buffer (pH 10.1) unless otherwise stated. The reaction was initiated by addition of the enzyme fraction and carried out for a specified interval of time.

When the P5C-*o*-AB complex was measured, reaction mixtures of 1 mL contained: 0.25 M potassium phosphate buffer of desired pH, which had been saturated with *o*-aminobenzaldehyde, 20 mM L-proline, 10 mM NAD and enzyme fraction. These were incubated for 20 minutes and the reaction was stopped by addition of 1 mL of 10% trichloroacetic acid in ethanol. After centrifugation, the absorbance at 444 nm was determined using a double-beam spectrophotometer (Varian, model DMS-200). All enzymatic reactions were carried out at 25^{0}C. An enzyme unit is defined as the activity which produces a change in optical density of 0.05 per minute at 340 nm, 1 cm light path (Peisach & Strecker, 1962). Protein concentration was determined by the standard method of Lowry *et al.,* (1951), with bovine serum albumin as the standard.

4.2.4 Identification of the product of the L-proline oxidation

Two methods were used to identify the product of proline oxidation: Fast Atom Bombardment Mass Spectrometry (FAB MS) and Thin Layer Chromatography (TLC). The complete reaction mixture was made up as described in Section 4.2.3.2. A control mixture consisted of the same constituents except that the enzyme was inactivated by boiling for ten minutes before its addition to the reaction. A second control was also used in which NAD was omitted from the mixture. All three mixtures were incubated for 40 minutes after which the reaction was halted by the addition of 0.5 mL of 95% ethanol. The mixtures were kept on ice for 10 minutes, centrifuged for 15 minutes at 12 000 x *g* and the precipitate discarded.

When employing the TLC technique, the supernatant from each mixture, together with standard solutions of L-proline, glutamic acid and L-P5C, were spotted using a

microcapillary onto a TLC plastic silica gel sheet (Merck, 60 F_{254}). Descending chromatography was performed in a pre-equilibrated tank with the solvent system 1-butanol : acetic acid : water (12/3/5, v/v/v). After migration, the TLC sheet was allowed to dry, sprayed with ninhydrin solution and developed in an 80^{0}C oven.

FAB MS analysis was undertaken with a Finnigan TSQ 70 triple stage quadruple mass spectrometer. Xenon was used as the bombardment gas. The ionisation voltage was 8 KeV, the ion current < 0.5 mA and the collision cell pressure 1.8 millitorr. Glycerol was used as the matrix.

4.3 Results

4.3.1 Copurification of P5C reductase (P5CR) and proline dehydrogenase (PDH) activities

A partially purified preparation of the bi-functional enzyme catalysing reduction of Δ'-pyrroline-5-carboxylic acid and oxidation of proline was obtained from soybean leaves. A summary of the purification steps is presented in Table 4.1. The relative activities of the proline dehydrogenase and P5C reductase remained reasonably constant through the first three purification steps. The final two steps of purification, the Sephacryl S-300 column and the affinity chromatography column, resulted in the sharp increase in the relative P5C reductase activity producing a high P5CR/PDH ratio. Enzyme eluted in a single peak by NaCl gradient from the affinity chromatography column (Figure 4.1), resulting in 295.4 and 62.9-fold purification for P5CR and PDH, respectively. The specific activities of the proline dehydrogenase in the final purification (2.2 units/mg) was significantly lower than that for the P5C reductase (180.2 units/mg). Enzyme activity of the final purification step was stable for 2 months when stored at -80^{0}C in 5% glycerol and 2.0 mM dithiothreitol. Freezing and thawing, resulted in considerable losses in activity. Enzyme prepared by Step 5 of purification was used for further experiments.

Table 4.1. Partial purification of soybean L-proline:NAD(P)$^+$-oxidoreductase. The purification steps and enzyme assays are explained in Materials and Methods, Sections 4.2.2 and 4.2.3.

			Proline dehydrogenase		P5C reductase		Relative activities	
Purification step	Total volume (mL)	Total protein (mg)	Specific activity units/mg protein	Purification	Specific activity units/mg protein	Purification	PDH	P5CR
1. Crude homogenate	600.0	2850.0	0.035	1.0	0.61	1.0	1.0	17.4
2. Heat precipitation at 60^0C	520.0	1530.0	0.103	2.9	1.74	2.9	1.0	16.9
3. $(NH_4)_2SO_4$ 25-50% precipitate	60.0	506.0	0.19	5.4	2.96	4.9	1.0	15.6
4. Sephacryl S-300 column	16.0	28.0	1.7	48.6	55.6	91.1	1.0	32.7
5. 2'5'-ADP Sepharose 4B	3.00	8.0	2.2	62.9	180.2	295.4	1.0	82.0

Figure 4.1. Elution profile of soybean L-proline:$NAD(P)^+$-oxidoreductase from affinity chromatography column.

The column was eluted with a gradient concentration of NaCl (0.0-1.0 M) in Tris-EDTA buffer (pH 8.3) and effluent collected as 1.0 mL fractions. Reaction mixture for determination of PDH activity is described in section 4.2.3.2.

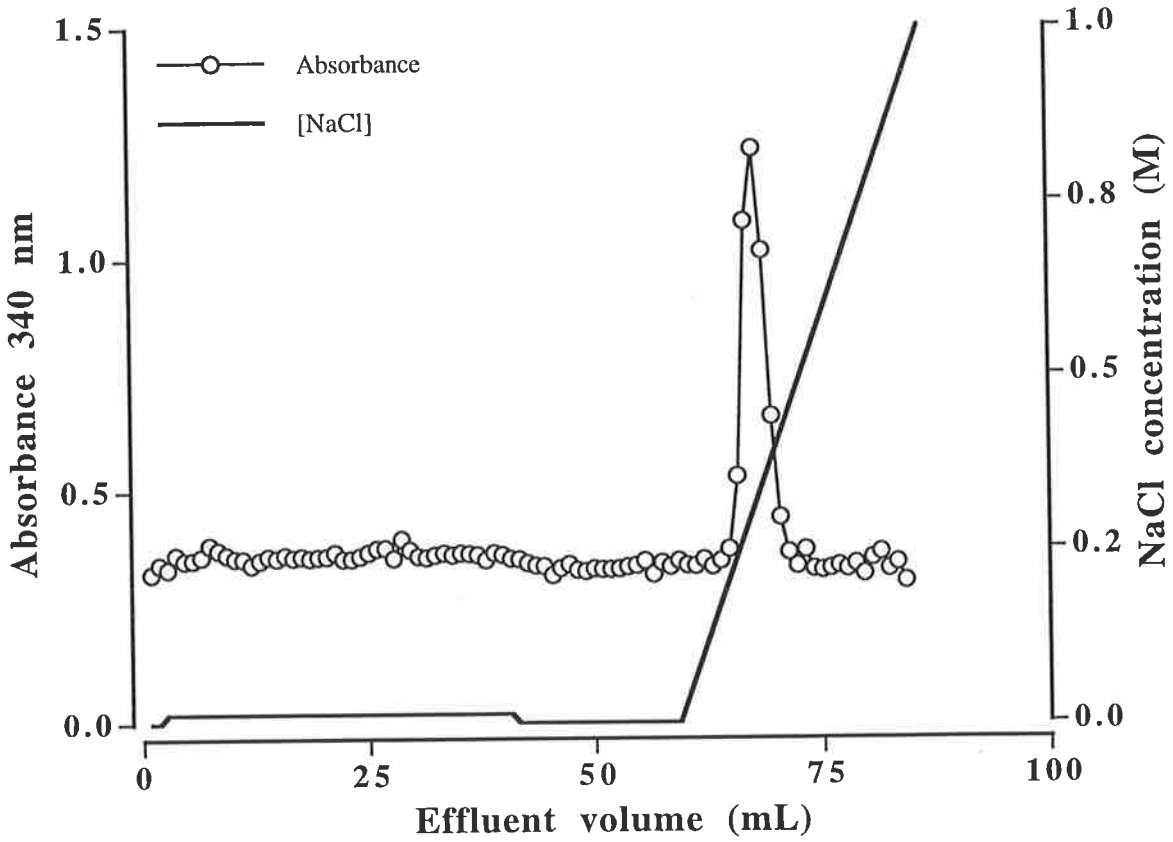
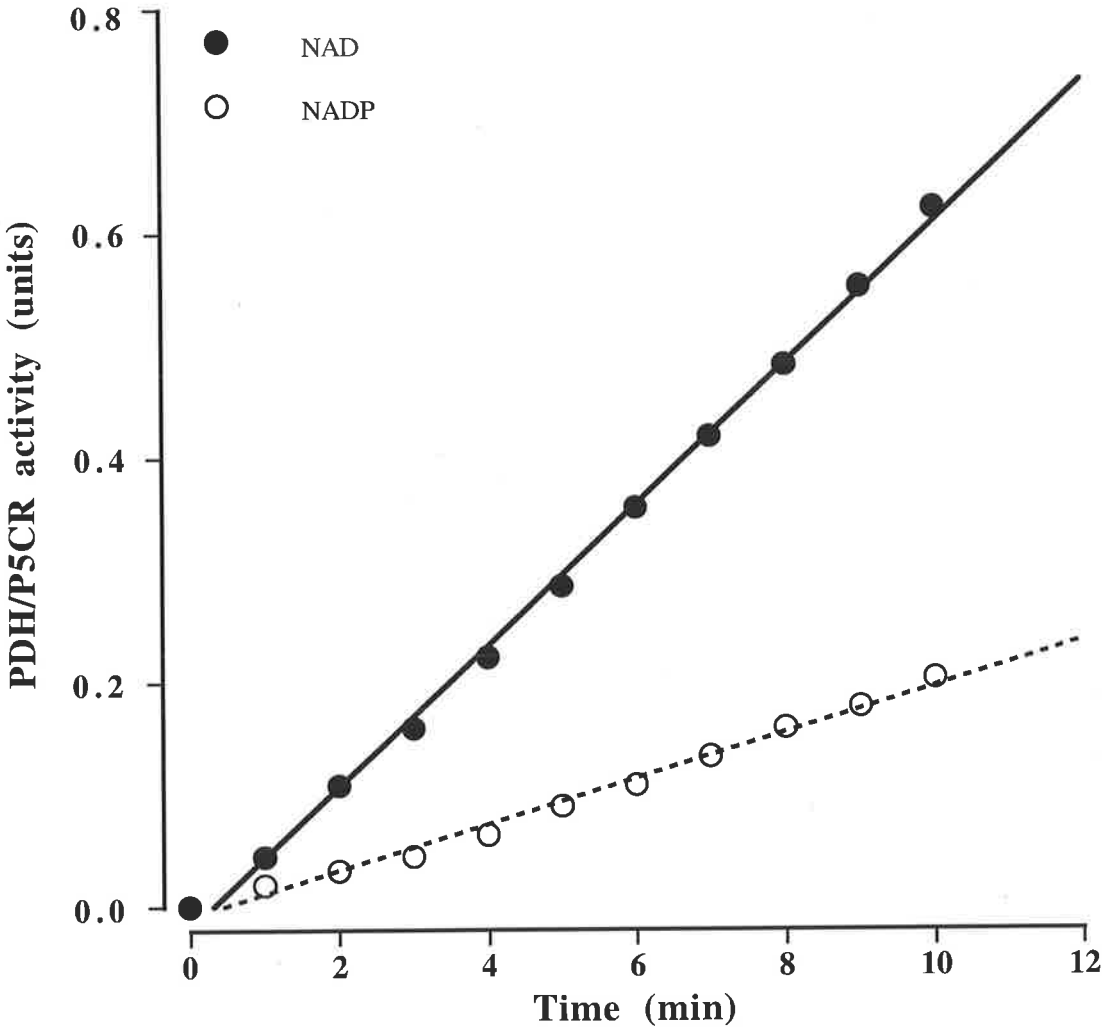

Absorbance
[NaCl]
Absorbance 340 nm
NaCl concentration (M)
Effluent volume (mL)
1.5
1.0
0.5
0.0
1.0
0.8
0.5
0.2
0.0
0
25
50
75
100

Figure 4.2. L-proline dependent reduction of oxidised pyridine nucleotides by the soybean L-proline:NAD(P)$^+$-oxidoreductase.

The reaction was carried out in quartz cuvettes. The reaction mixture was as described in section 4.2.3.2. NAD(P) concentration was 10.0 mM. Change in the absorbancy was recorded every minute.


0.8
0.6
0.4
0.2
0.0
NAD
NADP
PDH/P5CR activity (units)
0
2
4
6
8
10
12
Time (min)

4.3.2 Activity of proline dehydrogenase and cofactor specificity

The oxidation of proline was a linear function of both the concentration of enzyme and time for the first 10 minutes of the reaction. NAD was the preferred cofactor. When NADP was used the reaction rate was at most 30% of that with NAD (Figure 4.2). The P5C reductase used NADH and NADPH as electron donors, but showed a 4.5-fold greater activity with NADH (not shown).

4.3.3 pH optima

The optimal pH for proline dehydrogenase activity was dependent on the assay method (Figure 4.3). When activity was measured by monitoring NADH formation, the optimal pH occurred between 9.9 and 10.2. Activity was first detected at pH 8.1, showing a sharp increase at pH 8.4. After reaching its pH optimum, the enzyme activity dropped off sharply with no activity detectable above pH 11.1. When the concentration of L-proline was doubled some PDH activity was detected as low as pH 7.6. Further increase in proline concentration did not allow the reaction to occur at pH lower than 7.6. In contrast, when the activity was measured by monitoring the formation of P5C-*o*-aminobenzaldehyde complex, the optimal pH was 7.5. No activity was detected at pH lower than 6.2 or higher than 9.0.

The P5C reductase exhibited a broad pH optimum from 6.9 to 7.6. Activity declined more rapidly at the alkaline side. No reductase activity was detected at pH lower than 5.1 or higher than 8.7. (Results not shown).

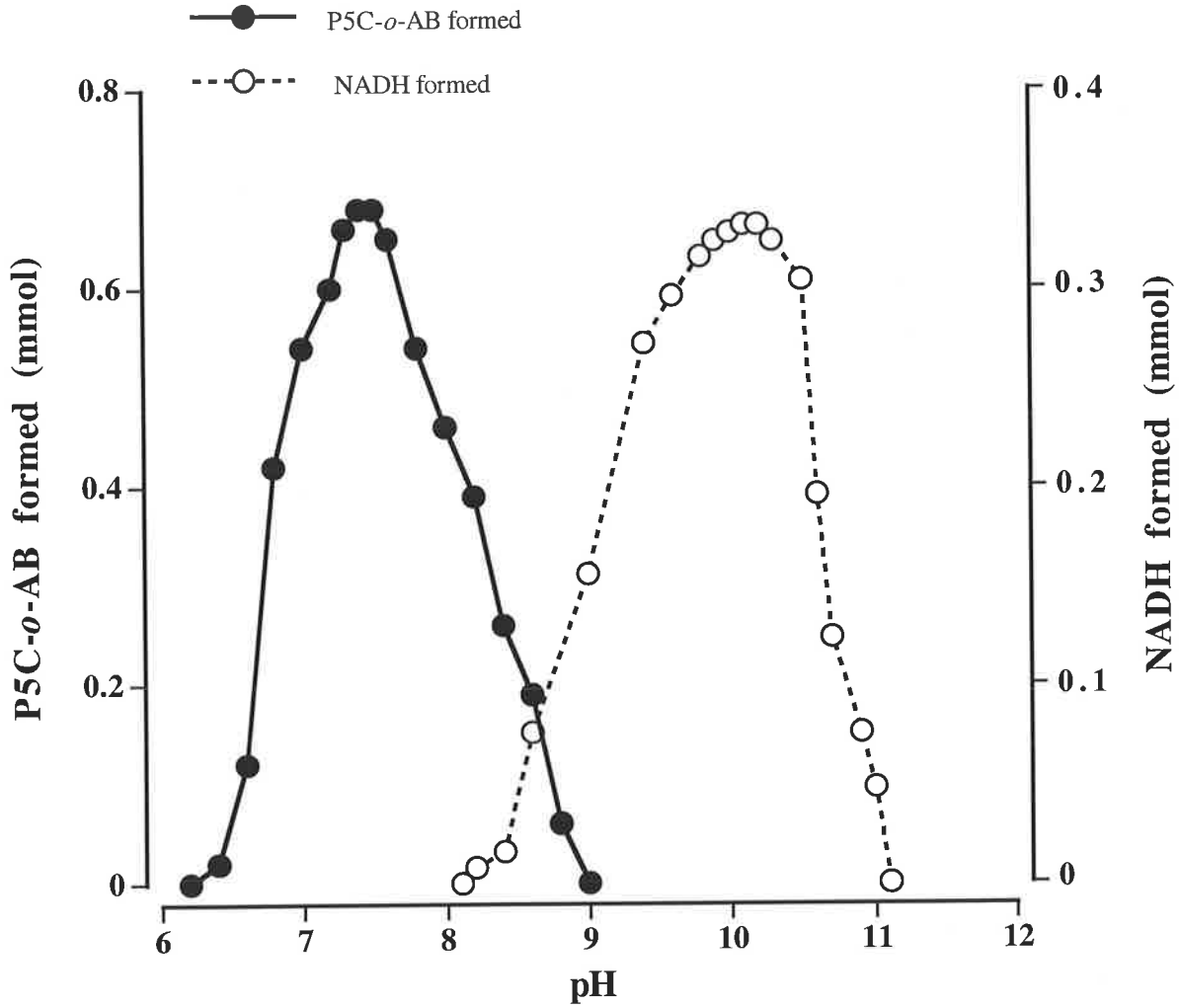
4.3.4 Identification of the product of proline oxidation

4.3.4.1 TLC analysis

The chromatographic migration of compounds present in the test reaction mixture (proline, NAD, enzyme), and two controls (containing boiled enzyme or no NAD) revealed that only in the presence of the active enzyme and NAD was a product

Figure 4.3. Effect of pH on the activity of soybean proline dehydrogenase.

The conditions employed were as described in Materials and Methods, except that for estimation of pH optima in the reactions of P5C-*o*-AB formation, pH was varied by the use of suitable mixtures of KH_2PO_4 and K_2HPO_4 at constant total phosphate concentration of 0.1 M. In the estimation of NADH formation, reactions were carried out for 20 minutes and the pH was varied by the use of a suitable sodium carbonate buffer to a final concentration of 0.25 M. The pH was determined with a pH meter.


P5C-o-AB formed
NADH formed
P5C-o-AB formed (mmol)
NADH formed (mmol)
0.8
0.6
0.4
0.2
0
0.4
0.3
0.2
0.1
0
6
7
8
9
10
11
12
pH

formed which upon reaction with ninhydrin gave a pink colour characteristic of P5C and migrated to the same distance as a control containing synthetic P5C.

4.3.4.2 FAB MS analysis

Comparison of mass spectra between test sample, controls and reference sample (synthetic P5C), revealed a spectrum in the test sample identical to that produced by the reference sample. This spectrum was absent from two control samples.

4.4 Discussion

4.4.1 Enzyme purification

A partially purified preparation from soybean leaves showed P5C reductase and proline dehydrogenase activities. Both activities co-purified with a constant ratio (P5CR/PDH 17.2/1) through the first four steps of purification and eluted in a single peak from the affinity chromatography column. The last step resulted in a sharp increase in the reductase to dehydrogenase ratio (82/1) producing a 295.4 and 62.9-fold purification, respectively. The change in the ratio of the two activities caused by the affinity chromatography probably resulted from the exposure of the enzyme to the low ionic strength buffer on the column. Similar changes in P5C reductase/proline dehydrogenase ratio associated with exposure of the enzyme to low ionic strength buffer were observed in an enzyme isolated from *Clostridium* sp. (Costilow & Cooper, 1978; Monticello & Costilow, 1981). Rena & Splittstoesser (1975) also observed rapid changes in the reductase/dehydrogenase ratio during storage of pumpkin extract at low ionic strength buffer.

The ratios of the two activities in extracts from *Clostridium*, pumpkin and soybean were large. The reductase/dehydrogenase ratio was about 1.5 in crude and purified preparations from pumpkin (Rena & Splittstoesser, 1975). The ratio in the crude clostridial extract was about 30 and increased to 96 during purification, (Monticello & Costilow, 1981), whereas the average ratio in the crude soybean extract was 17.4 and

increased to 82 during purification, indicating that the enzymes from these three different sources are quite different with respect to relative activities.

The co-purification of P5C reductase and proline dehydrogenase activities has been reported for several higher plants and some green algae (Dashek & Erickson, 1981). Detailed analysis of pumpkin and clostridial enzymes carried out by Rena & Splittstoester (1975) and Costilow & Cooper (1978) respectively, revealed that both activities are catalysed by the same protein or protein complex. The fact that both P5C reductase and proline dehydrogenase of soybean co-purified (216.4 and 120 times), eluted in single peaks from Sephacryl S-300 and Sepharose 4B columns, prefered reduced or oxidised forms of NAD over NADP suggest that, as with pumpkin and clostridial enzymes, both these activities are catalysed by a single protein or protein complex. This has also been observed for most studied dehydrogenases, including the amino acid dehydrogenases which catalyse reversible product/substrate-substrate/product redox reactions (Tsukada, 1966; Boyer, 1975).

4.4.2 Cofactor specificity

The co-purified soybean proline dehydrogenase and P5C reductase showed preference for NAD and NADH respectively as co-factors. When phosphorylated pyridine nucleotide was used, the significant decrease in proline oxidation and P5C reduction rates was noted. Observations regarding the reductase activity of the PDH/P5CR protein agrees with those of Miller & Stewart (1976). These workers found significantly higher activity (about 4 times) of soybean P5C reductase when NADH was used instead of NADPH. Similarly, pumpkin (Rena & Splittstoesser, 1975) and wheat (Mazelis & Creveling, 1974) P5C reductases had an approximately 2.5-fold greater activity with NADH than NADPH. P5C reductase of reversible PDH/P5CR of *Chlorella autotrophica, Chlorella saccharophila* (Laliberte & Hellebust, 1989) and *Clostridium sporogenes* (Costilow & Cooper, 1978), were also highly specific for NADH, whereas P5C reductase of PDH/P5CR of peanut (Mazelis & Fowden, 1969, 1971), barley (Krueger *et al.*, 1986), tobacco (Noguchi *et al.*, 1966),

halophyte, *Mesembryanthemum nodiflorum* (Treichel, 1986), and non reversible P5C reductase of *Escherichia coli* (Rossi *et al.*, 1977) and *Drosophila melanogaster* (Farmer *et al.* 1979), used NADPH more effectively. P5C reductase of *Saccharomyces cerevisiae*, which does not display proline oxidation activity, showed equal affinity for NADH and NADPH, (Matsuzawa & Ishiguro, 1980). In contrast to preference differences for reduced pyridine nucleotides by P5C reductases, all studied proline dehydrogenases of PDH/P5CR protein, displayed specific affinity for NAD, a property that is characteristic of other amino acid dehydrogenases. In general, amino acid dehydrogenases are specific for NAD whereas reduction reactions require NADH or NADPH as the co-factor (Smith *et al.*, 1975; Ohshima & Soda, 1990) agreeing with the notion that most catabolic reactions in the cell require NAD as an electron acceptor.

4.4.3 Product of L-proline oxidation

The TLC and FAB MS analysis revealed that the product of L-proline oxidation by the soybean proline dehydrogenase is Δ'-pyrroline-5-carboxylic acid. This result confirms earlier studies showing that in higher plants (Dashek & Erickson, 1981), animal tissues and microorganisms (Adams & Frank, 1980), Δ'-pyrroline-5-carboxylic acid is the first product of L-proline degradation.

4.4.4 L-proline dehydrogenase activity

In the presence of isolated soybean PDH/P5CR, L-proline was converted to pyrroline-5-carboxylate, indicating that the isolated protein possesses proline dehydrogenase activity. This activity is reported to be associated with proteins isolated only from plants, algae (Dashek & Erickson, 1981) and the anaerobic bacterium, *Clostridium sporogenes* (Costilow & Cooper, 1978). Enzymes catalysing reduction of P5C to proline isolated from *E. coli* (Meister *et al.*, 1957), calf liver (Peisach & Strecker, 1962), rat tissues (Herzfeld *et.al.*, 1977), *Pseudomonas aeruginosa* (Krishna *et al.*, 1979), *Drosophila melanogaster* (Farmer *et al.*, 1979) and

S. cerevisiae (Matsuzawa & Ishiguro, 1980), do not display proline dehydrogenase activity. Isolated P5C reductases differ markedly in molecular weights, ranging from 95 to 320 kdalton. It is of interest to note that the lower molecular weight enzymes do not display proline dehydrogenase activity (Rossi *et al.*, 1977; Matsuzawa & Ishiguro, 1980; Monticello & Costilow, 1981; Deutch *et al.*, 1982). No in-depth studies have been carried out to determine the reason for this distinction. As these reversible and non-reversible P5C reductases are composed of a variable number of subunits, the number and size of which are species specific, it is likely that the proline dehydrogenase activity is rendered by specific, spatial arrangement of the enzyme molecule which is preserved only in some organisms.

4.4.5 pH optima

L-proline dehydrogenase of soybean showed maximal activity at a high, alkaline pH (10.1). This pH optimum of *in vitro* PDH activity is similar to the L-amino acid dehydrogenases isolated from other organisms (Table 4.2). Although the reported pH optima for these enzymes were observed with *in vitro* reactions, no information exists to indicate that these enzymes require alkaline pH for their function *in vivo*, nor has a satisfactory explanation been proposed for the necessity of high pH for the *in vitro* reaction, despite the fact that several genes encoding these enzymes were cloned and expressed in *E. coli* for commercial production of desirable compounds (Hummel & Kula, 1989).

Intracellular pH is of vital importance to all organisms, not only for maximising enzyme activities but also for control of many cellular processes such as solute transport across membranes, DNA transcription and protein synthesis. Therefore, a variety of mechanisms have evolved by which cytoplasmic pH can be maintained within a narrow physiological range. This pH value in most microorganisms, including *E. coli, Bacillus* spp., *Pseudomonas* spp. and *S. cerevisiae* oscillates between 6.4 and 7.6, (Acidophils can exhibit intracellular pH values of 5.9, whereas alkalophils can tolerate pH 9; Booth, 1985; Bakker, 1990; Bruno *et al.*, 1992;

Table 4.2. pH optima for *in vitro* oxidation reactions of reversible amino acid dehydrogenases.

Enzyme	Optimal pH for *in vitro* reaction	Organism	Reference
Alanine dehydrogenase	10.5	*Bacillus subtilis*	Yoshida & Freese, 1965
	10.6	*Bacillus spaericus*	Ohshima & Soda, 1979
	10.1	*Desulfovibrio desulfuricans*	Germano & Anderson, 1968
Leucine dehydrogenase	11.0	*Bacillus stearothermophilus*	Ohshima *et al.*, 1985
	10.8	*Bacillus sphaericus*	Ohshima *et al.*, 1978
Phenylalanine dehydrogenase	10.5	*Brevibacterium* sp.	Hummel *et al.*, 1984
Proline dehydrogenase	10.3	*Arachis hypogaea*	Mazelis & Fowden, 1971
	10.2	*Clostridium sporogenes*	Costilow & Cooper, 1978
	10.3	*Cucurbita moschata*	Rena & Splittstoesser, 1975
	10.1	*Glycine max*	This studies
	10.2	*Triticum vulgare*	Mazelis & Creveling, 1974
Threonine dehydrogenase	10.3	*Escherichia coli* K-12	Boyan & Dekker, 1981
	10.5	*Pseudomonas cruciviae*	Misono *et al.*, 1993

Breeuwer *et al.*, 1996). Therefore it is highly unlikely, that the observed high pH for the catalytic functions of amino acid dehydrogenases *in vitro*, is necessary for their function *in vivo*.

The significant drop in the optimal pH for proline oxidation (from 10.3 to 7.5), which was observed following the removal of the reaction product, P5C when measuring P5C-*o*-AB complex, indicates that the reaction is strongly inhibited by the accumulation of its product. This agrees with the observation of Costilow & Laycock (1971), who found that during the oxidation of proline catalysed by the clostridial proline dehydrogenase, the presence of pyruvate, lactic acid dehydrogenase and *o*-aminobenzaldehyde dramatically increased the amount of P5C-*o*-AB in the reaction mixture. These reagents would be expected to pull the reaction toward P5C by its removal and the regeneration of NAD.

In most redox reactions catalysed by dehydrogenases, the equilibrium in the oxidation direction is highly unfavourable. The reaction rate is further hampered by severe product inhibition. This is particularly true for the oxidation of amino acids to corresponding oxo-acids and alcohols to ketones (Germano & Anderson, 1968; Smith *et al.*, 1975; Lee & Whitesides, 1986; Hummel & Kula, 1989; Ohshima & Soda, 1979, 1990). In the majority of alcohol oxidation reactions *in vitro*, the shift in the equilibrium towards the product is achieved by continuous extraction of the product (Liese *et al.*, 1996). The oxidation of malate to oxaloacetate by the malate dehydrogenase shows a similar shift (Whiskitch, 1977; Elthon & Stewart, 1982). In the NAD-linked oxidation of ethanol to acetaldehyde, the equilibrium of the reaction lies heavily on the side of ethanol and NAD. The reaction can, however, be completely displaced in the direction of acetaldehyde by alkaline conditions and by trapping the formed acetaldehyde (Beutler, 1984).

It can be hypothesised that with the *in vitro* oxidation of proline, as with other amino acids, the alkaline pH generates a shift in the equilibrium of the reaction in the

direction of oxidation. This may be explained by the effect of high pH on the formation of the ionized carboxyl group and the un-ionized amino group of proline and/or a change in the enzyme configuration which increases affinity of the enzyme for the substrate which results in the shift of the equilibrium towards product. It has been shown by Peisach & Strecker (1962) that ionisation of the components of the P5C reduction reaction is relevant to the rate of the reaction. Alkaline pH also increases the affinity of the plasma membrane H^+-ATPase for its substrate (Serrano, 1984). The alkalisation of the reaction mixture of glycerol oxidation leads to a 4-fold increase in the rate of the reaction (McGregor *et al.*, 1974). It is also probable that the high pH optima displayed by the amino acids dehydrogenases reflect a common reaction mechanism or a similar role of these enzymes in cellular metabolism.

4.4.6 Conclusions

Partially purified soybean L-proline:$NAD(P)^+$-oxidoreductase showed both P5C reductase and L-proline dehydrogenase activity. The requirement for high alkaline conditions for proline oxidation *in vitro* by the enzyme was alleviated by the removal of the product of the reaction, P5C. This, together with reports in the literature that show that microbial amino acid dehydrogenases are reversible *in vivo*, while *in vitro* the dehydrogenases behave in a manner similar to L-proline dehydrogenase of soybean, suggests a possible role for NAD dependent oxidation of proline by this enzyme *in vivo*.

Chapter 5

Cloning and expression of soybean L-proline: $NAD(P)^+$-oxidoreductase in *Saccharomyces cerevisiae* DT1100

5.1 Introduction

It has been shown that partially purified proline oxidoreductase (PDH/P5CR) of soybean catalyses oxidation of L-proline in an NAD-linked reaction *in vitro*. With the removal of the reaction product, P5C, the pH optimum for this reaction was 7.5 (refer to Sections 4.3.3 and 4.4.5). It is also known that *S. cerevisiae* is able to transport proline under anaerobic conditions (Duteurtre *et al.*, 1971; this work, Section 3.3.2). Based on these findings an attempt was made to clone and express the soybean gene encoding proline oxidoreductase with the ultimate goal of inducing anaerobic proline catabolism in yeast.

The interconversion of proline and P5C is common to all studied prokaryotes and eukaryotes (Adams & Frank, 1980; Phang, 1985). It was reasoned that P5C reductase would be functionally conserved in *S. cerevisiae* and soybean. Complementation of a P5C reductase deficient yeast strain would provide the best strategy for identifying the gene of interest. An *S. cerevisiae* strain, DT1100, deficient in P5C reductase activity, was described by Tomenchok & Brandris (1987). Recently, using DT1100, the human P5C reductase was isolated by complementation studies (Dougherty *et al.*, 1992). A similar method was also used to isolate the soybean PDH/P5CR, but in this case an *E.coli* proline auxotroph mutant was utilised (Delauney & Verma, 1990).

Since the sequence of the soybean PDH/P5CR gene is already known (Delauney & Verma, 1990), the polymerase chain reaction (PCR) was chosen as the most appropriate method for isolating the PDH/P5CR gene.

5.2 Materials and Methods

5.2.1 Cloning soybean proline oxidoreductase

5.2.1.1 Plant material

Soybean plants were cultivated as described in section 4.2.1.

5.2.1.2 Isolation of total RNA from plant material

All glassware used for RNA isolation was baked at 160^{0}C for 10 hours. Solutions were made using di-ethyl-pyro-carbonate (DEPC) treated water to remove RNase activity. Treated water was prepared by the addition of DEPC to a concentration of 0.1%, incubating at 37^{0}C for 12 hours and then autoclaving twice to remove the DEPC. Plasticware was soaked in 0.4N KOH for 20 minutes and then rinsed with DEPC treated water.

Approximately 3 g of young leaves were cut from soybean plants using a surgical scalpel and immediately placed in a pre-cooled mortar containing liquid nitrogen. Leaves were ground into a fine powder. Three millilitres of RNA extraction buffer (REB; 100 mM Tris-HCl, pH 8.4; 4% Sarkosyl; 10 mM EDTA) was added and plant tissue was homogenised, transferred to a 15 mL Corex^R centrifuge tube, allowed to thaw and centrifuged at 14 000 x *g* at 4^{0}C for 5 minutes. The supernatant was transferred to a 10 mL ultracentrifuge tube. For each millilitre of supernatant, 1 g of CsCl was added and mixed thoroughly. Three millilitres of CsCl cushion solution (0.965 g/mL CsCl in TE buffer: 10 mM Tris-HCl, pH 7.5; 1 mM EDTA) was carefully added into the bottom of the tube with a Pasteur pipette. Contents of the tube were centrifuged for 16 hours at 92 000 x *g* at 4^{0}C. After centrifugation, the surface layer was removed with a sterilised cotton bud and the supernatant poured out. The pellet was resuspended in ice-cold 0.4 mL REB buffer and 0.4 mL of phenol/chloroform/iso-amyl alcohol solution (25:24:1, equilibrated with Tris-HCl buffer, pH 8.0) was added. The mixture was transferred to a 1.5 mL Eppendorf tube, vortexed for 30 seconds and centrifuged for 5 minutes at 12 000 rpm. The upper layer was transferred to a fresh Eppendorf tube and the RNA was precipitated with

ethanol. After precipitation, RNA was dried under vacuum and resuspend in 20 µL of TE buffer. Quality of the obtained RNA was analysed by electrophoresis on a 1.5% agarose gel at a constant current of 80 mA. The gel was stained with ethidium bromide and photographed on a short wave UV light box. RNA concentration was determined by spectrophotometry at 260 nm, using the relationship of one A_{260} unit to 40.0 µg/µL single stranded RNA (Sambrook *et al.*, 1989).

5.2.1.3 Isolation of polyA$^+$RNA from total RNA samples

PolyA$^+$RNA was purified from total RNA using the Promega PolyA Tract mRNA Isolation System according to the manufacturers recommendations.

5.2.1.4 cDNA synthesis

Fifteen micrograms of RNA was heated at 70^{0}C for 10 minutes with 2 µg of dT Primer in an Eppendorf tube then chilled on ice. Four microlitres of 5X buffer (250 M Tris-HCl, pH 8.3; 0.375 M KCl; 15 mM $MgCl_2$), 3 µL of 0.1 M DTT and 8 µL of 1.25 mM dNTP's were added to the tube and the mixture was gently mixed. After incubation at 45^{0}C for 2 minutes, 2 µL of reverse transcriptase enzyme with RNase H$^-$ (Superscript II, GibcoBRL, USA) was added and gently mixed. The mixture was incubated at 45^{0}C for 1 hour and 30 µL of H_2O was added. Synthesized DNA was precipitated with 5 µL of 3 M Na-Acetate, pH 4.8, 120.0 µL of cold, 99% ethanol. The tube was placed at -80^{0}C overnight. Contents were warmed on ice and centrifuged at 12 000 rpm for 15 minutes at 4$^-$C. The supernatant was discarded and the pellet washed with 1 mL of 70% ethanol. The pellet was resuspended in 5 µL of H_2O and stored at -80^{0}C.

5.2.1.5 Polymerase chain reaction

Primers for PCR amplification were designed using the soybean proline oxidoreductase sequence published by Delauney & Verma (1990). The primers were of the following sequence:

Upper: CCG GAT CCC TTA ACC TAA AAT GGA AAT C

Lower: GAA GAT CTC TCA AAG AAG AGC CCC AAG T

Reactions were carried out in 0.5 mL Eppendorf tubes. The reaction mixture contained: 5 µL of 10 x reaction buffer (Promega), 3 µL of $MgCl_2$, 8 µL of dNTP's each of 1.25 mM, 0.3 µg of each primer, 2 units of *Taq* polymerase (Promega), 5 µg of cDNA and nanopure H_2O to the total volume of 50 µL. Reaction mixtures were topped up with a drop of parafin oil and placed in the programmable Thermal Cycler (MJ Research Inc. Watertown, Mass.). DNA was denatured initially at 94^{0}C for 2 minutes, followed by 35 cycles consisting of 1 minute denaturation at 94^{0}C, 2 minutes annealing at 50^{0}C and 2 minutes extension at 72^{0}C. The PCR was completed with a final extension step of 5 minutes at 72^{0}C and cooling to 25^{0}C.

5.2.1.6 Gel electrophoresis

To visualise the quality of DNA obtained by the PCR, gel electrophoresis was carried out through 1 % (w/v) agarose horizontal slab gels. Samples were mixed with 1/5 volume of 6 x Ficoll loading buffer (100 mM Tris-HCl; 200 mM EDTA; 15% Ficoll type 400; 25% bromophenol blue; 0.25% xylene cyanol FF, pH 8.0) prior to loading. The running buffer was Tris-borate (TBE: 90 mM Tris-borate; 1 mM Na_2EDTA, pH 8.0). DNA was visualised by staining the gels subsequent to electrophoresis in a solution of ethidium bromide (0.5 µg/mL). Gels were photographed on Polaroid 665 positive film using a Polaroid Landpak camera.

5.2.1.7 Isolation of DNA from agarose gels

The 'Geneclean' protocol (BIO 101) was used for the recovery of DNA from the gel. Gel slices were excised under longwave UV light and dissolved at 60^{0}C in 6 M NaI (700 µL). Glass milk (5 µL) was added and the solution incubated on ice for 1 hour. The glass milk was pelleted by centrifugation at 12 000 rpm, the supernatant was removed by aspiration and the pellet washed thoroughly three times with 1 mL of wash buffer (50 mM NaCl; 10 mM Tris-HCl, pH 7.5; 2.5 mM EDTA; 50% v/v ethanol). Following the final wash, the pellet was dried briefly under vacuum and the DNA eluted by resuspension in TE buffer (20 µL) and incubation at 55^{0}C for 3 minutes. DNA was stored at -20^{0}C.

5.2.1.8 Ligation of PCR product into vector

The PCR amplified fragment was ligated into the pGEM^R-T vector (Promega). All ligation steps were performed according to the manufacturer instructions.

5.2.1.9 Transformation of *E. coli*

Transformation of the *E. coli* strain DH5α was performed according to the method of Hanahan (1983).

5.2.1.9a Preparation of competent cells for transformation

A single colony of *E. coli* DH5α was inoculated into 5 mL of SOB medium (20 g/L Tryptone; 5 g/L Yeast extract; 0.5 g/L NaCl [pH 7.0]) and grown overnight at 37^{0}C with rapid shaking. The starter culture (500 µL) was used to inoculate 25 mL SOB supplemented with 20 mM $MgSO_4$ and the cells grown to OD_{600} 0.6 at 37^{0}C with shaking. The culture was incubated on ice for 10 minutes and the cells pelleted by centrifugation at 2 500 rpm for 12 minutes at 4^{0}C (JA 20 rotor in Beckman 121 centrifuge). The supernatant was removed and the cells resuspended in 8.5 mL of TFB buffer (10 mM MES, pH 6.3; 45 mM $MnCl_2$; 100 mM $RbCl_2$; 10 mM $CaCl_2$; 3

mM Hexylamine-$CoCl_2$) and incubated on ice for 10 minutes. Cells were pelleted as above and resuspended in 2 mL of TFB. Seventy microlitres of di-methyl sulfoxide (DMSO) was added and the cells incubated on ice. After 5 minutes, 157 µL of 1 M dithiothreitol was added and mixed. After a further 10 minutes, 75 µL of DMSO was added and mixed.

5.2.1.9b Transformation procedure

Two hundred microlitres of competent cells were gently dispensed into a 50 mL glass test tube and kept on ice. Five microlitres of ligation mix was added and gently mixed. The cells were incubated on ice for 30 minutes, heat shocked in a 42^{0}C water bath for 2 minutes then returned to ice for 5 minutes. Eight hundred microlitres of SOC medium (1 mL SOB, 10 µL 2 M $MgSO_4$; 7 µL 50% glucose) was added and incubated at 37^{0}C for 45 minutes. 200 µL of transformed cells were spread onto Petri plates containing LB 1.5% agar medium (1% Bacto-tryptone; 0.5% Bacto-yeast extract; 1% NaCl; pH 7.2) supplemented with 50 µg/mL ampicillin; 10 mM isopropyl beta-thiogalactopyranoside (IPTG) and 0.4% bromo-(5)-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal). Plates were incubated overnight at 37^{0}C and putative recombinant colonies identified by their white colour.

5.2.1.10 Small scale isolation of plasmids

A single colony of *E. coli* DH5α containing the plasmid of interest was inoculated into 4.0 mL of Terrific Broth (0.5% Bacto-yeast extract; 1% Bacto-tryptone; 0.5% NaCl) containing 50.0 µg/mL of ampicillin. The culture was grown overnight at 37^{0}C with rapid shaking. After centrifugation, cells were resuspended in 200 µL of Plasmid 1 solution (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA) and incubated on ice for 10 minutes. Three hundred microlitres of Plasmid 2 solution (0.2 N NaOH;

1% SDS) was added, mixed gently by inversion then incubated on ice for 5 minutes. Two hundred microlitres of potassium acetate (3 M, pH 4.8) was added, mixed and the solution incubated on ice for 15 minutes. The tubes were centrifuged at 12 000 rpm for 10 minutes and the supernatant transferred to a clean tube. DNA was precipitated by the addition of 2.5 volumes of ice cold ethanol followed by incubation at -20^{0}C overnight. The DNA was pelleted by centrifugation at 12 000 rpm for 15 minutes, dried briefly under vacuum then resuspended in 500 µL of nanopure water. RNase A was added to a final concentration of 100 µg/mL and the solution incubated for 2 hours at 37^{0}C, extracted with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) and then with an equal volume of chloroform. The aqueous upper layer was transferred to a fresh tube and the DNA precipitated with 7.5 M ammonium acetate (pH 4.8) and ethanol. The DNA was dissolved in 40 µL of nanopure water and was of sufficient purity for all molecular techniques including sequencing.

5.2.1.11 Sequencing reactions

Recombinant pGEM^R-T plasmids were isolated as described above and the concentration of the DNA adjusted to approximately 0.25 ηg/µL with nanopure water. The subsequent sequencing reactions were set up using the 'Primer Ready Dye-Deoxy Terminator Cycle Sequencing Kit' according to the manufacturer (Applied Biosystems). Separation of the sequencing reaction products was carried out on an ABI automated DNA sequencer (Applied Biosystems). The sequence data was analysed using Sequence Editor software (SEQ-ED; Applied Biosystems) designed for Macintosh computers.

5.2.2 Yeast transformation and fermentation trials

5.2.2.1 Yeast strain

Saccharomyces cerevisiae strain DT1100: *MATα ura3-52 pro3,* (Tomenchok & Brandriss, 1987) was used for all experimental work.

5.2.2.2 Media and growth conditions

All conditions and techniques for aerobic and anaerobic yeast growth were as described in Section 3.2.3. In all experiments *S. cerevisiae* DT1100 and transformed strains were grown in minimal medium (MM; 1.5 g/L yeast nitrogen base without amino acids and $(NH_4)_2SO_4$, Difco; 20 g/L glucose; 0.023 g/L uracil; supplemented with either 2 g/L of $(NH_4)_2SO_4$ or 1 g/L of L-proline as a nitrogen source. When suppression of the expression of the introduced soybean PDH/P5CR was required, the medium was supplemented with 50 µg/mL of methionine (Cherest *et al.*, 1985).

5.2.2.3 Cloning vector

Plasmid pDS848 (YCplac33-MET3) was used to transform *S. cerevisiae* DT1100. This *URA3* marked centromeric plasmid is characterised by a 596bp fragment of the *S. cerevisiae MET3* promoter. It also contains the Amp^R bacterial marker (Gietz & Sugino, 1988).

5.2.2.4 Transformation protocol

The soybean PDH/P5CR sequence cloned into the $pGEM^R$-T was digested with *Bam*HI and *Sac*I restriction endonucleases and subcloned into the pDS848. All cloning steps were performed according to Sambrook *et al.* (1989).

Alkali cation transformation of yeast was performed according to the method of Ito *et al.* (1983), with slight modification. Yeast cells were grown to late exponential phase at 28°C in liquid MM medium supplemented with di-ammonium sulphate, uracil and L-proline. Cells were harvested by centrifugation at 2 500 rpm for 5 minutes, washed once in 10 mL TE buffer, reharvested and suspended in 20 mL LiOAc/TE buffer (10 mM Tris-HCl, pH 7.5; 0.1 M lithium acetate; 1 mM EDTA). Cells were gently shaken at 30°C for 1 hour. Approximately 8 x 10^7 cells were harvested and suspended in 100 µL of LiOAc/TE buffer. Plasmid DNA (10 µg) with 5 µg salmon

sperm carrier DNA was added in a total of 10 µL to the cells and the suspension was incubated at 28°C for 30 minutes. Seven hundred microlitres of PEG reagent (40% polyethylene glycol 4000; 0.1 M lithium acetate; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA) was added and the mixture was vortexed before incubation at 28°C for 1 hour. Cells were heat shocked by incubation at 42°C for 5 minutes, harvested and resuspended in 1 mL sterile water.

For the selection of transformants, the cell suspension was spread on MM medium supplemented with di-ammonium sulphate lacking uracil, proline and methionine.

5.2.2.5 Isolation of plasmid DNA from yeast

Plasmid DNA was isolated from yeast according to the method described in Current Protocols in Molecular Biology, Chapter 13, Unit 13.11 (Ausubel *et al.,* 1994).

5.2.2.6 Yeast DNA isolation

Untransformed *S. cerevisiae* DT1100 strain and DT1100 transformed with YCplac33 without PDH/P5CR insert (DT1100-YC) were grown in MM medium supplemented with di-ammonium sulphate, proline and uracil. DT1100 transformed with YCplac33-PDH/P5CR was grown in MM medium supplemented with di-ammonium phosphate. Yeasts were grown for 24 hours at 25°C. Cells were pelleted by centrifugation at 3 000 rpm for 5 minutes, washed once in zymolyase buffer (1.2 M sorbitol; 0.1 M sodium citrate; 0.06 M EDTA; pH 7.0) and resuspended in 500 µL zymolyase buffer containing zymolyase to a total concentration of 1 mg/mL. The suspension was then incubated with shaking at 30°C for 30 minutes. Spheroplasts were pelleted at 3 000 rpm for 5 minutes, then resuspended in 100 µL TE buffer. To this suspension, 300 µL of lysis buffer (0.5 M Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 2% SDS) was added and the mixture incubated at 60°C for 15 minutes. Following this incubation, 200 µL of 5 M potassium acetate was added and the suspension incubated on ice for

30 minutes. After centrifugation at 12 000 rpm for 20 minutes, the supernatant was carefully decanted into a fresh tube. To this supernatant, an equal volume of absolute ethanol was added and the mixture was allowed to sit for 1 hour at room temperature.

After centrifugation at 12 000 rpm for 10 minutes, the resultant DNA pellet was vacuum dried and resuspended in 200 µL TE buffer. The salt concentration of the aqueous phase was adjusted by the addition of 50 µL of 5 M potassium acetate followed by incubation on ice for 30 minutes. After centrifugation at 12 000 rpm for 20 minutes, the supernatant was carefully decanted again into a fresh tube. To this supernatant, an equal volume of absolute ethanol was added and the mixture was left for 20 minutes at -80^{0}C. Centrifugation at 12 000 rpm resulted in the pelleting of the DNA which was resuspended in a fixed volume of R40 (40 µg/mL RNase in TE buffer). The concentration of the DNA was determined by spectrophotometry.

5.2.2.7 Yeast RNA isolation

Total RNA was isolated by a procedure based on that by Schmitt *et al.* (1990). The yeast culture was grown in the MM medium, supplemented with the desired nitrogen source and methionine when required, for 24 hours at 24^{0}C. 10 mL samples were taken and the cells harvested by centrifugation at 3 000 rpm for 5 minutes and resuspended in 400 µL AE buffer (50 mM sodium acetate, pH 5.3; 10 mM EDTA). After transfer to an Eppendorf tube, 40 µL of 10% SDS was added and the tube vortexed. An equal volume of phenol (equilibrated with AE buffer) was added, the tube vortexed again and then incubated at 65^{0}C for 4 minutes. The tube was then rapidly chilled in liquid nitrogen until phenol crystals appeared and centrifuged for 2 minutes at maximum speed in an Eppendorf centrifuge. The upper aqueous layer was transferred to a fresh tube to which an equal volume of phenol/chloroform was added and left at room temperature for 5 minutes. To the extracted upper aqueous phase, 40

μL of 3 M sodium acetate, pH 5.3 and 2.5 volumes of ethanol were added. Tubes were left overnight at -20^{0}C before the RNA was pelleted by centrifugation. The RNA pellet was washed once with 70% ethanol, dried and resuspended in a fixed volume of RNase free nanopure water.

Following RNA extractions, the absorbency values at 260 nm and 280 nm of each RNA sample was determined on a Shimadazu UV-Vis spectrophotometer UV-160A. The A_{260}/A_{280} ratios of the RNA samples were consistently in the range of 1.8 to 2.2. The relationship of one A_{260} unit to 40 μg/μL RNA was used in the calculation of RNA concentrations (Sambrook *et al.*, 1989).

5.2.2.8 Electrophoresis of RNA

Equipment was cleaned with 0.25 N NaOH to remove RNases before use. Total RNA was run on a 1.5% agarose denaturing RNA gel [0.7 g agarose; 36 mL nanopure H_2O; 5 mL 1 x MOPS/EDTA buffer (0.5 M MOPS, 0.01 M EDTA); 9 mL 37% formaldehyde]. Gels were poured and pre-run in 1 x MOPS/EDTA buffer at 60 volts for 30 minutes.

Each RNA sample (5 μg) was dried under vacuum and resuspended in 4.5 μL RNA buffer A (294 mL 10 x MOPS/EDTA; 706 mL nanopure H_2O) and 9.5 μL formamide/ formaldehyde. The sample was incubated at 70^{0}C for 10 minutes and then chilled on ice for 2 minutes. RNA loading buffer (322 mL RNA buffer A; 178 mL 37% formaldehyde; 500 mL deionised formamide; 3 mg xylene cyanol; 3 mg bromocresol green; 250 mg sucrose), 3 μL, was then added before loading into the gel. Samples were run at 60 volts for 30 minutes and then 100 volts for a further 1-2 hours. Gels were stained with ethidium bromide and photographed under UV light.

5.2.2.9 Transfer of DNA to nylon membranes

DNA fragments separated by agarose gel electrophoresis were transferred to Hybond-N nylon membrane (Amersham) by capillary blotting (Southern, 1975). Gels were made ready for transfer by soaking in denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 20 minutes. Upon completion of the transfer, membranes were briefly rinsed in 2 x SSC (0.15 M NaCl; 0.015 M sodium citrate) and the DNA fixed to the membrane by placing it in 0.4 M NaOH for 20 minutes. Finally, the filter was soaked in neutralising solution (0.5 M Tris-HCl, pH 7.0; 1.5 M NaCl; 0.001 M EDTA, pH 7.2) for 5 minutes prior to hybridisation.

5.2.2.10 Transfer of RNA to nylon membranes

RNA separated by denaturing electrophoresis was transferred to membranes by capillary blotting (Southern, 1975). Gels were prepared for transfer by soaking in 20 x SSC for 30 minutes. RNA was transferred from the gel to a Hybond-N nylon membrane overnight by capillary blotting in 20 x SSC. After transfer, membranes were rinsed in 2 x SSC and UV cross-linked for 7 minutes before being baked at 40^0C for 1 hour.

5.2.2.11 Radio-labelling of samples

A probe for hybridisation studies was prepared by random primer radio-labelling of double stranded DNA with (α^{32}P) dCTP. The DNA template (1 µg) plus 9-mer random primer (0.1 µg/mL) in a total of 8 µL, were boiled for 5 minutes then chilled on ice for a further 5 minutes. This was then added to 12.5 µL of 2 x oligolabelling mix (dATP, dTTP, dGTP, 60 µM each; 150 mM Tris-HCl, pH 7.6; 150 mM NaCl; 30 mM $MgCl_2$; 300 µg/mL BSA), 3 µL of (α^{32}P) dCTP and 1.5 µL of Klenow enzyme (1 unit/µL). The sample was then incubated at 37^0C for 60 minutes. The

radio-labelled DNA fragments were purified using a Sephadex G-100 column and denatured by boiling for 5 minutes before adding to the hybridisation solution.

5.2.2.12 DNA hybridisation

Membranes were briefly rinsed in 2 x SSC before being placed in a "Hybaid" hybridisation bottle and prehybridised in 10 mL of prehybridisation solution (3 mL 5 x HSB[3M NaCl, 100 mM PIPES, 25 mM EDTA, pH 6.8]; 3 mLDenhardts III [2% polyvinyl pyrolidone 360, 2% Ficoll 400, 2% bovine serum albumin] 2 mL nanopure H_2O; 1 mL 10% SDS; 1 mL carrier DNA [5 g salmon sperm in 100 mL H_2O]) at 65^0C for 2 hours. The prehybridisation solution was replaced with freshly prepared hybridisation solution (3 mL 5 x HSB; 2 mL Denhardts III; 3 mL 25% dextran sulphate; 1 mL nanopure H_2O; 0.5 mL carrier DNA) plus denatured radioactive probe. The membrane was left to hybridise with the probe for 20 hours at 65^0C with constant rotation. The membrane was removed from the bottle and washed with agitation for 20 minutes at 65^0C in a series of four washing steps using washing solutions, 1-4, (wash solution 1: 2 x SSC, 0.1% SDS [1 g sodium dodecyl sulphate; 9 mL nanopure H_2O]; wash solution 2: 1 x SSC, 0.1% SDS; wash solution 3: 0.5 x SSC, 0.1% SDS; wash solution 4: 0.2 x SSC, 0.1% SDS) to remove background radioactivity. The membrane was then exposed to X-ray film between intensifying screens at -80^0C. The exposure time was determined by the amount of detectable radioactivity on the film.

5.2.2.13 RNA hybridisation

The hybridisation membrane was rinsed in 2 x SSC before being placed in a "Hybaid" hybridisation bottle and prehybridised in 10 mL of RNA hybridisation solution (2 mL Denhardts III; 1 mL SDS; 1 mL carrier DNA; 5 mL 20 x SSPE [3.6 M NaCl; 0.2 M NaH_2PO_4; 0.02 M EDTA]; 10 mL formamide) at 42^0C for 24 hours. After this time, the solution was replaced with fresh hybridisation solution (10 mL) plus denatured radioactive probe. The membrane was left to hybridise with the probe for 24 hours at 42^0C with constant rotation in a "Hybaid" bottle. The membrane was removed from

the bottle and washed with agitation for 20 minutes at 65^{0}C with each of the wash solutions (1 - 4) to remove background radioactivity. The membrane was then exposed to X-ray film at -80^{0}C for 48 hours.

5.2.2.14 Preparation of yeast cell free extracts for enzyme assays

Yeast cell free extracts were prepared as described by Jiranek (1992). All steps were carried out at 4^{0}C. Cells from 100 mL of late log phase culture were collected by centrifugation, washed twice with an equal volume of 0.25 M phosphate buffer (pH 7.3) containing 0.1 mM EGTA and resuspended in 5 mL of the same clarified (0.45 μm membrane) buffer containing glycerol at 20% (w/w). After the addition of 10 g of glass beads (Braun, 0.45-0.5 mm), cells were shaken in a cell homogeniser (Braun, model MSK) with CO_2 cooling at 2000 rpm until *ca.* 99% disruption was achieved. Glass beads were removed by filtration through a glass sinter and cellular debris was removed from the filtrate by centrifugation at 40 000 x *g* for 30 minutes. Dialysis was performed for two periods of 75 minutes, each against 1000 volumes of fresh resuspension buffer. The supernatant was stored on ice before being assayed. Proline dehydrogenase and P5C reductase assays were carried out as described in section 4.2.3.

5.3 Results

5.3.1 Cloning the soybean proline oxidoreductase (PDH/P5CR) gene

Total RNA isolated from leaves of two week old soybean seedlings was used as a template for preparing complementary DNA. The cDNA served as a template in PCR using specific primers designed from the sequence of the soybean PDH/P5CR gene (Delauney & Verma, 1990). The PCR reaction produced a band of the expected size of approximately 980 bp (Figure 5.1).

The PCR fragment was ligated into pGEM^R-T vector and transformed into *E. coli* DH5α. Twenty one white colonies were obtained and two carried an insert of the expected length for the soybean PDH/P5CR gene. These plasmids were named pSM-PDH/P5CR-1 and pSM-PDH/P5CR-2. The insert from pSM-PDH/P5CR-1 was partially sequenced from both ends. Sequence revealed 100% homology at the amino acid level with the published sequence of soybean PDH/P5CR gene (Delauney & Verma, 1990; Figure 5.2).

5.3.2 Expressing cloned soybean P5CR gene in *S. cerevisiae*

5.3.2.1 Construction of the yeast expression vector

A *Sac* I, *Bam* HI fragment of pSM-PDH/P5CR-1 carrying the coding region of the soybean PDH/P5CR gene was fused to the *MET3* promoter of the YCplac33 yeast expression vector. The resultant plasmid was named YCplac33-PDH/P5CR. The YCplac33-PDH/P5CR plasmid was transformed into *S. cerevisiae* DT1100 mutant (Dougherty *et al.*, 1992).

5.3.2.2 Analysis of transformants

Approximately 10^7 competent cells were spread onto plates containing minimal medium lacking uracil supplemented with L-proline and methionine (non-inducible medium). Seventy nine colonies capable of growth on this medium were identified. These colonies were named *S. cerevisiae* DT1100-A. All DT1100-A colonies were replica plated onto uracil, proline and methionine-free minimal medium (inducible

Figure 5.1. RT-PCR products of soybean total cDNA.

Total cDNA was electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualised on a UV light box.

Lanes:

1: PCR with cDNA templete and primers flanking the soybean PDH/P5CR gene.

2: PCR in the absence of cDNA.

3: molecular weight marker (pTZ 18U cut with *Hind* III).

4: molecular weight marker (pTZ 18U cut with *Dra* I and *Rsa* I).

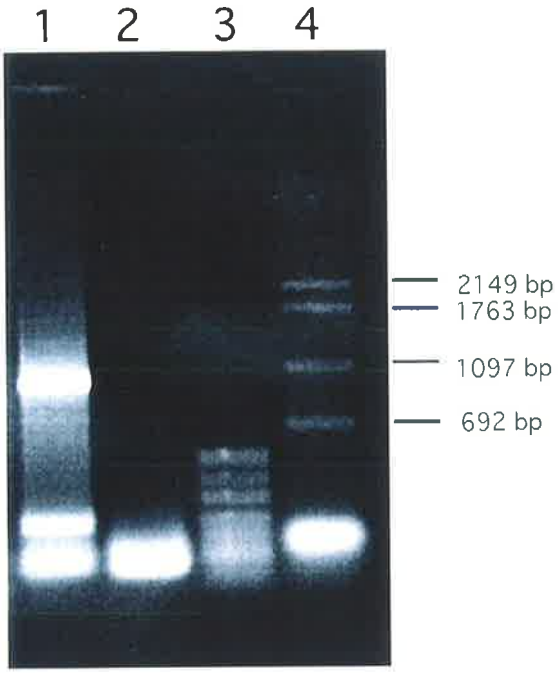

1
2
3
4
2149 bp
1763 bp
1097 bp
692 bp

Figure 5.2. Nucleotide sequence of the soybean PDH/P5CR gene and the deduced amino acid sequence of the putative PDH/P5C reductase protein.

Sequence typed in black is that published by Delauney & Verma (1990). Red indicates partially sequenced PDH/P5CR gene described here. Primers are boxed in.

```
AGCAGCAACATTTGCGCAAATCGAAATTGATTCTCTGCCACTTAACCTAAA                51
                                                CCGGATCCCTTAACCTAAA
CGCATGCTCCCGGCCGCCATGGCCACGGGATTCCGGATCCCTTAACCTAAA

ATG GAA ATC TTT CCG ATT CCC GCC GAA TCC TAC ACT CTC                 90
ATG GAA ATC
ATG GAA ATC TTT CCG ATT CCC GCC GAA TCC TAC ACT CTC
M   E   I   F   P   I   P   A   E   S   Y   T   L

GGC TTC ATC GGC GCC GGA AAA ATG GCC GAG TCC ATT GCC                 129
GGC TTC ATC GGC GCC GGA AAA ATG GCC GAG TCC ATT GCC
G   F   I   G   A   G   K   M   A   E   S   I   A

CGA GGC GCC GTC CGC TCC GGC GTC CTG CCG CCT TCT CGC                 168
CGA GGC GCC GTC CGC TCC GGC GTC CTG CCG CCT TCT CGC
R   G   A   V   R   S   G   V   L   P   P   S   R

ATT CGG ACC GCC GTC CAC TTC AAC CTC GCC CGC CGT GGC                 207
ATT CGG ACC GCC GCT CAC TTC AAC CTC GCC CGC CGT GGC
I   R   T   A   V   H   F   N   L   A   R   R   G

GCC TTC GAG TCC TTC GGC GTC ACC GTT CTC CCT TCA AAC                 246
GCC TTC GAG TCC TTC GGC GTC ACC GTT CTC CCT TCA AAC
A   F   E   S   F   G   V   T   V   L   P   S   N

GAC GAC GTC GTT CGC GAA AGC GAC GTC GTC GTT TTG TCG                 285
GAC GAC GTG GTT CGC GAA AGC GAC GCT GTG GTT TTG TCG
D   D   V   V   R   E   S   D   V   V   V   L   S

GTC AAA CCT CAA TTA GTG AAA GAC GTG GTG TCG AAA TTG                 324
GTC AAG CCT CAA TTA GTG AAA GAC GTG GTG TCG AAA TTG
V   K   P   Q   L   V   K   D   V   V   S   K   L

ACG CCG CTT TTG ACG AAG CAC AAG CTT TTG GTT TCG GTC                 363
ACG CCG CTT TTG ACG AAG CAC AAG CTT TTG GTT TCG GTC
T   P   L   L   T   K   H   K   L   L   V   S   V

GCT GCT GGT ACC AAA TTG AAA GAT CTT CAG GAA TGG GCT                 402
GCT GCT GGT ACC AAA TTG AAA GAT CTT CAG GGA TGG GCT
A   A   G   T   K   L   K   D   L   Q   P   W   A

GGG AAC GAC AGA TTT ATA AGA GTG ATG CCT AAT ACC CCA                 441
GGG AAC GAC AGA TTT ATA AGA GTG ATG CCT AAT ACC CCA
G   N   D   R   F   I   R   P   M   P   N   T   P

GCA GCT GTT GGC CAG GCA GCA TCT GTA ATG AGC TTG GGG                 480
GCA GCT GTT GGC CAG GCA GCA
A   A   V   G   Q   A   A   S   V   M   S   L   G

GGA TCT GCA ACG GAA GAA GAT GGA AAT ATT ATA GCC CAA                 519
G   S   A   T   E   E   D   G   N   I   I   A   Q

TTA TTT GGG TCA ATT GGC AAA ATA TGG AAA GCT GAG GAA                 558
L   F   G   S   I   G   K   I   W   K   A   E   E
```

```
AAG TAT TTT GAT GCA ATA ACT GGC CTG AGT GGC AGT GGT      597
                                  AGC GGC AGT GGT
K   Y   F   D   A   I   T   G   L   S   G   S   G

CCT GCT TAT GTT TAT TTA GCA ATA GAG GCT TTG GCT GAT      636
CCT GCC TAT GTT TAT TTA GCA ATA GAG GCT TTG GCT GAT
P   A   Y   V   Y   L   A   I   E   A   L   A   D

GGA GGA GTA GCA GCT GGT TTA CCA CGT GAT CTT TCA TTA      675
GGA GGA GTA GCA GCT GGT TTA CCA CGT GAT CTT TCA TTA
G   G   V   A   A   G   L   P   R   D   L   S   L

AGT CTA GCT TCT CAA ACT GTA TTA GGG GCA GCA TCA ATG      714
AGT CTA GCT TCT CAA ACT GTA TTA GGG GCA GCC TCA ATG
S   L   A   S   Q   T   V   L   G   A   A   S   M

GTC TCC CAG ACT GGG AAG CAC CCG GGA CAG CTC AAA GAT      753
GTC TCC CAG ACT GGG AAG CAC CCG GGA CAG CTC AAA GAT
V   S   Q   T   G   K   H   P   G   Q   L   K   D

GAC GTT ACT TCT CCA GGT GGG ACA ACA ATT ACG GGC ATT      792
GAC GTT ACT TCT CCA GGT GGG ACA ACA ATT ACG GGC ATT
D   V   T   S   P   G   G   T   T   I   T   G   I

CAT GAG TTA GAA AAT GGT GGG TTC CGT GGA ACA CTG ATG      831
CAT GAG TTA GAA AAT GGT GGG TTC CGT GGA ACA CTG ATG
H   E   L   E   N   G   G   F   R   G   T   L   M

AAT GCT GTT GTT GCT GCT GCT AAG CGC AGC CGA GAG CTT      870
AAT GCT GTT GTT GCT GCT GCT AAG CGC AGC CGA GAG CTT
N   A   V   V   A   A   A   K   R   S   R   E   L

TCC TGA  ATCAAGAGATTAGGATTCTACTTTTTTTTTAGACGAAAATCTG    919
TCC TGA  ATCAAGAGATTAGGATTCTACTTTTTTTTTAGACCAAAATCTG
S   *

ATTTCGGTCGAATTTTCAGTCACTATTATGAGAACTTGGGGCTCTTCTTTG     970
                                  GGGGCTCTTCTTTG
ATTTCGGTCGAATTTTCAGTCACTATTATGAGAACTTGGGGCTCTTCTTTG

AGAGGAGTAAACAAGAATGTTTGGCCATAACAGTCTTTTCGAGCTAGCATC    1021
AGAGATCTTC
AGAGATCTTC

ATGAATGCATCCGAGTCTATATGAGAACTTGAAGTTTCCTTGCAAAAGAAG    1072
AGTACTAGCAGTAAAAATAAAATAAAACCACTTGGCC (A) 42            1151
```

Figure 5.3. Transformation of *S. cerevisiae* DT1100 with YCplac33-PDH/P5CR.

Approximately 10^7 cells were plated onto minimal medium plates supplemented with :

Plate A: di-ammonium sulphate, L-proline and methionine.

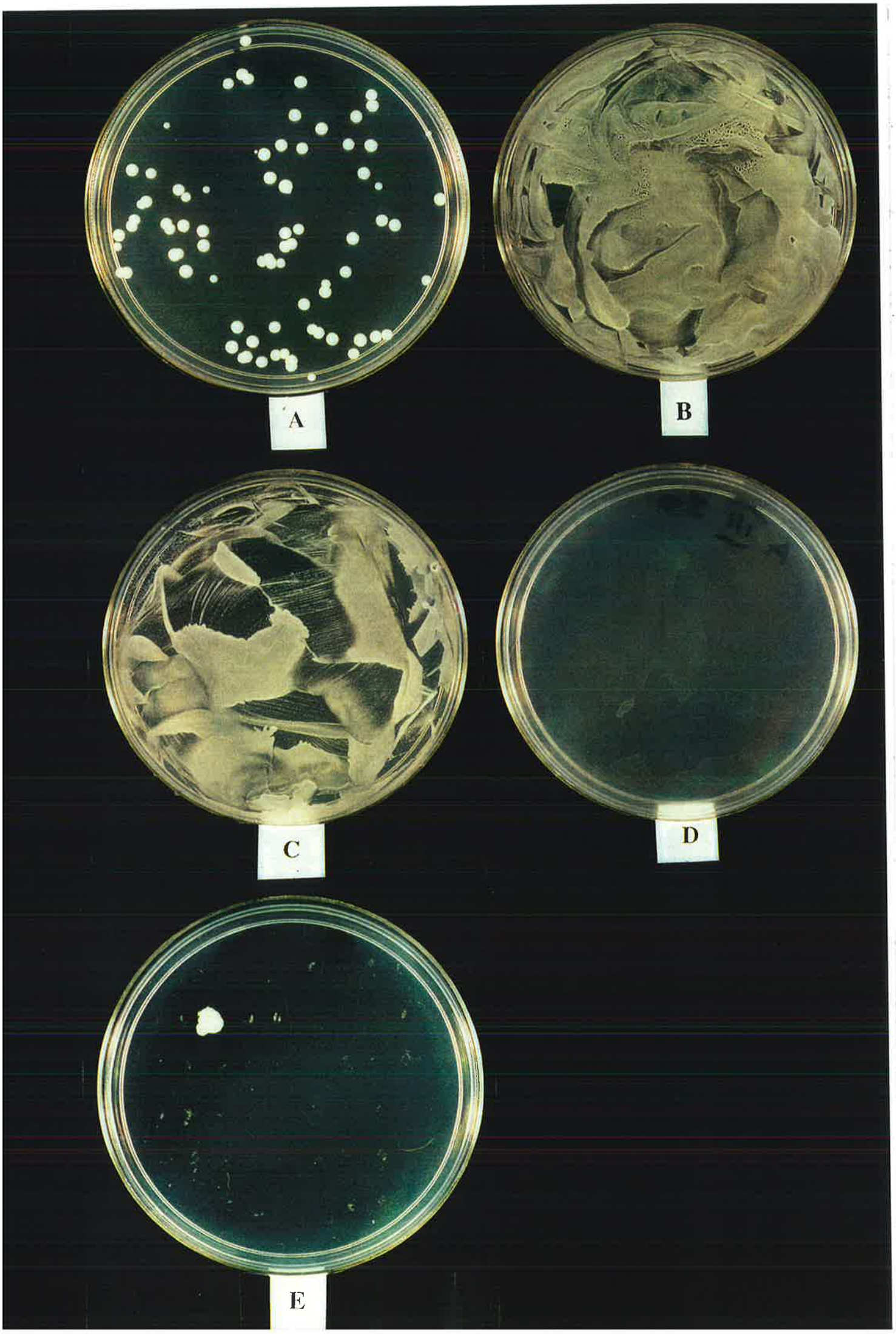
Plate B (control): di-ammonium sulphate, uracil, L-proline and methionine.

Plates C and D (controls) were spread with untransformed *S. cerevisiae* DT1100. Plates contained minimal media supplemented with:

Plate C: di-ammonium sulphate,uracil and L-proline.

Plate D: L-proline.

Plate E: replica-plate of Plate A onto minimal medium without L-proline, uracil and methionine, supplemented with di-ammonium sulphate.


A
B
C
D
E

medium). One of the 79 colonies were able to grow on this medium (Figure 5.3). Transformation was repeated several times. In total 522 Ura^+ transformants were obtained. Of these, five colonies were identified which complemented the *pro*$^-$ mutation. These Pro^+ colonies, were named *S. cerevisiae* PU1. As all 522 Ura^+ transformants were also expected to be Pro^+, the nature of the Pro^+ phenotype that arose in only five colonies was further investigated. The Pro^+ phenotype could result from several factors. These are: changes within the yeast genome, changes associated with the vector sequence or changes to the introduced PDH/P5CR gene. To differentiate between these, the following experiments were performed:

1) Growth of S. cerevisiae PU1 strain on a permissive and inducible medium

PU1 cells were grown in a nonselective medium (minimal medium supplemented with uracil, proline and di-ammonium sulphate) to isolate cells that had lost plasmid. Such Ura^- colonies were also Pro^-, indicating that the gene complementing proline auxotrophy was located on the plasmid. This was further confirmed by the growth of PU1 only on the inducible, minimal medium (without uracil, proline and methionine supplement). PU1 did not grow when expression of introduced PDH/P5CR was suppressed by the addition of methionine.

2) Recovery and reintroduction of the YCplac33-PDH/P5CR plasmid

Recovered plasmids from PU1 strain were used to transform DT1100. One hundred and thirty Ura^+ colonies were identified. All Ura^+ colonies were also Pro^+ (Figure 5.4) demonstrating that the Pro^+ phenotype arose as a result of a mutation within the introduced plasmid.

3) Southern blot analysis of the total DNA of S. cerevisiae DT1100-A and PU1 strains

DT1100-A and PU1 strains were grown in 200 mL of inducible minimal medium supplemented with proline. Total DNA was extracted from both strains and analysed

Figure 5.4. Transformation of *S. cerevisiae* DT1100 with YCplac33-PDH/P5CR plasmid recovered from PU1 cells.

S. cerevisiae DT1100 was transformed with YCplac33-PDH/P5CR plasmid recovered from PU1 strain. Approximately 10^7 cells were plated onto minimal medium supplemented with di-ammonium sulphate, L-proline and methionine (Plate A). Transformants were replica plated onto minimal medium without methionine supplemented with di-ammonium sulphate (Plate B).


A
B

Figure 5.5. Southern hybridisation detecting soybean PDH/P5CR sequence in YCplac33-PDH/P5CR plasmid in transformants of DT1100-A and PU1 strains.

Panel A: total yeast DNA samples electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualised on a UV light box.

Panel B: autoradiogram of gel depicted in Panel A after hybridisation with a radio-labelled PDH/P5CR sequence recovered from pSM-PDH/P5CR-1.

Lanes: 1: λ *Hind* III marker

2: pTZ 18U marker cut with *Dra*I and *Rsa*I

3: untransformed DT1100

4: transformed DT1100 with YCplac33 without PDH/P5CR insert

5 - 9: PU1

10 - 14: DT1100-A

15: 10-fold dilution of DNA from lane 5

16: 10-fold dilution of DNA from lane 10

17: 100-fold dilution of DNA from lane 5

18: 100-fold dilution of DNA from lane 10


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A
6742 bp
B
6742 bp

by Southern hybridisation. Results are shown in Figure 5.5. Panel A shows gel electrophoresis of isolated DNA digested with *Bam*H I restriction endonuclease. Panel B shows Southern transfer of DNA probed with radio-labelled PDH/P5CR gene recovered from pSM-PDH/P5CR-1 plasmid.

The lanes containing PU1 DNA show a band of approximately 6740 bp, close to the expected size of pYCplac33-PDH/P5CR-1 plasmid whereas in DT1100-A lanes, containing the same plasmid, this band is not visible (Panel A). PU1 and DT1100-A DNA probed with radio-labelled PDH/P5CR sequence show marked difference in hybridisation intensity (Panel B). PU1 DNA gave an intense band at approximately 6740 bp when hybridised with P^{32}-PDH/P5CR probe. DT1100-A DNA also shows a band but is characterised by a higher molecular size and a significantly reduced intensity, indicating the difference in the copy number and size between PU1 and DT1100-A plasmids. A 10-fold dilution of DT1100-A DNA did not give a hybridisation signal whereas 10-fold diluted PU1 DNA produces a clearly visible band.

4) Hybridisation analysis of total RNA extracted from PU1 and DT1100 strains

Total RNA extracted from DT1100 and PU1strains, grown in various liquid media, was electrophoresed, transferred to Hybond-N^+ membrane and probed with radio-labelled PDH/P5CR sequence excised from pSM-PDH/P5CR-1 plasmid. The membrane was exposed to autoradiographic film. Results are shown in Figure 5.6. Lanes 1 to 3 contain RNA extracted from PU1 strain grown in inducible, minimal medium (supplemented with di-ammonium phosphate and lacking methionine). To see if the expression of introduced PDH/P5CR gene is influenced by proline and/or uracil, the minimal medium was supplemented with proline, lane 2 and with proline and uracil, lane 3. Lane 4 contains RNA extracted from PU1 strain grown in non-inducible minimal medium, containing methionine, supplemented with proline and lane 5 non-inducible minimal medium supplemented with proline and uracil. Lane 6

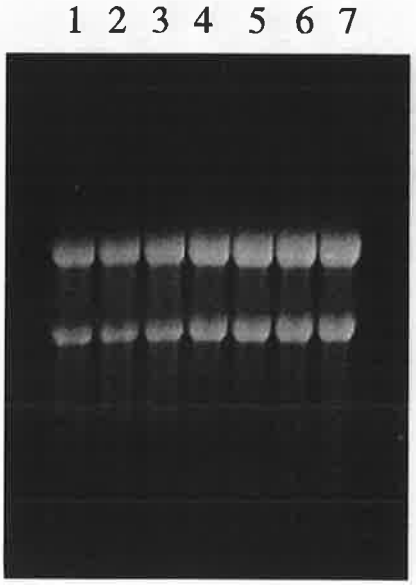
Figure 5.6. RNA hybridisation analysis of soybean PDH/P5CR gene expression in PU1 grown aerobically in the minimal medium with varying supplement. DT1100 strain was used as a control.

Panel A: total yeast RNA electrophoresed on 1.5% agarose gel. The gel was stained with ethidium bromide.

Panel B: Northern hybridisation performed on samples depicted in Panel A, probed with radio-labelled PDH/P5CR sequence.

	Strain:	Medium supplement:
Lanes: 1:	PU1	di-ammonium sulphate
2:	PU1	L-proline
3:	PU1	L-proline, uracil
4:	PU1	methionine, di-ammonium sulphate, L-proline
5:	PU1	methionine, di-ammonium sulphate, L-proline, uracil
6:	DT1100	di-ammoinum sulphate, L-proline, uracil
7:	DT1100 transformed with YCplac33;	di-ammonium sulphate, L-proline, uracil

A



B

2.9 kbp ——

1.8 kbp ——

contains RNA isolated from untransformed DT1100 strain and lane 7 that of DT1100 strain transformed with Ycplac33 plasmid without soybean PDH/P5CR insert.

Northern hybridisation revealed a single band in lanes 1, 2 and 3 corresponding to the expected size of the PDH/P5CR mRNA. There was no detectable hybridising transcript in Lanes 4, 5, 6 and 7 despite the presence of approximately similar amounts of RNA in each lane. The presence of proline in the medium did not influence the level of transcription of the PDH/P5CR gene (Lanes 2 and 3).

5.3.3 Assay for proline dehydrogenase activity in crude extracts from *S. cerevisiae* PU1 and DT1100 strains

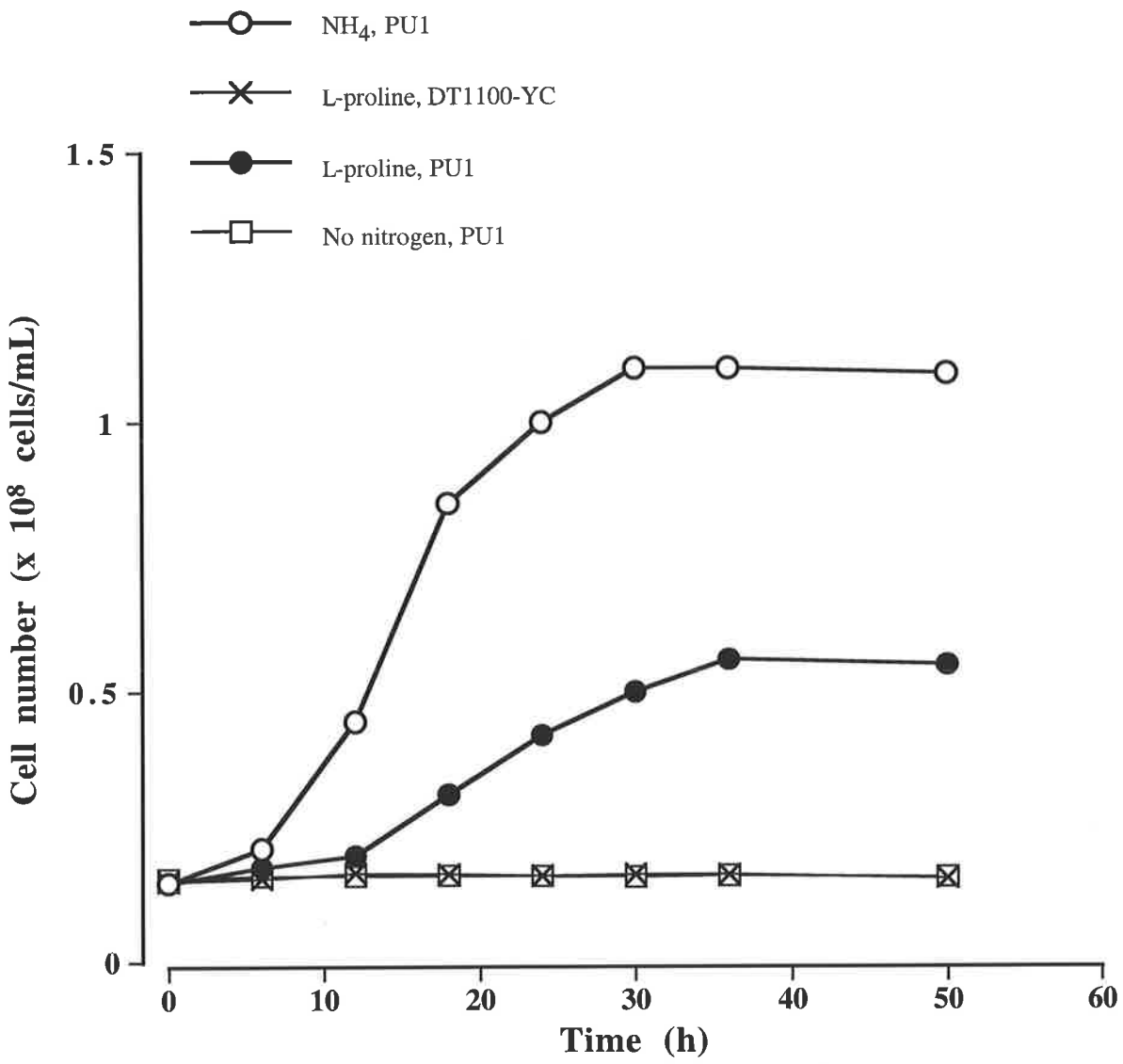
Partially purified cell extract of *S. cerevisiae* PU1 was assayed for proline dehydrogenase activity. Cell free extracts exhibited reduction of NAD in the presence of L-proline. This indicates that the translated product of the cloned soybean PDH/P5CR gene was a functional protein displaying proline dehydrogenase activity. Crude extracts of DT1100 strain showed no detectable proline dehydrogenase activity.

5.3.4 Growth characteristics of *S. cerevisiae* PU1 and DT1100 strains under strict anaerobic conditions

The ability of PU1 and DT1100 strains to utilise L-proline as the sole nitrogen source under anaerobic conditions was investigated. It was found that the inducible, minimal medium supplemented with L-proline supported growth of the PU1 strain. The anaerobic growth of PU1 on proline was characterised by a prolonged lag phase, lasting 37 hours, and a significantly smaller final culture cell number, reaching 46% of PU1 cells growth on ammonium ions (Figure 5.7). L-proline did not support growth of DT1100 under similar conditions.

Figure 5.7. Anaerobic growth of *S. cerevisiae* PU1 and DT1100-YC on minimal medium supplemented with Tween 80 and nitrogen source as indicated in the legend.

Media were inoculated with 5 x 10^6 starter culture cells prepared anaerobically. Growth was conducted at 25^0C in 100 mL conical flasks placed in an anaerobic hood containing the atmosphere of 5% hydrogen and 95% nitrogen gas.


NH4, PU1
L-proline, DT1100-YC
L-proline, PU1
No nitrogen, PU1
Cell number (x 10^8 cells/mL)
1.5
1
0.5
0
0
10
20
30
40
50
60
Time (h)

5.4 Discussion

5.4.1 Complementation of a *Saccharomyces cerevisiae* P5C reductase mutant by the soybean proline oxidoreductase

The proline oxidoreductase gene of soybean was cloned by the polymerase chain reaction technique. This enzyme catalyses the final step in the proline synthetic pathway, being the reduction of P5C to proline and, *in vitro,* it also catalyses the reverse reaction, oxidation of proline to P5C. Partial sequence of the cloned PDH/P5CR gene showed 100% homology at the amino acid level with the soybean PDH/P5CR gene isolated by Delauney & Verma (1990). The gene was cloned into a yeast expression vector (YCplac33) and expressed in a proline auxotrophic *S. cerevisiae* strain (DT1100) lacking P5C reductase activity. Only 0.96% of transformants, characterised by the Ura^+ phenotype, exhibited proline prototrophy (PU1 strains). This was an unexpected result since all transformants harbouring the YCplac33-PDH/P5CR plasmid were expected to be prototrophic for proline. A number of factors could lead to the Pro^+ phenotype. These include changes within the yeast chromosome, mutation of the introduced soybean gene encoding proline reductase or changes associated with the vector carrying the cloned gene. Results showing simultaneous loss of the Ura^+ and Pro^+ phenotypes from PU1 colonies grown in a medium permissive for plasmid loss (minimal plus di-ammonium phosphate, uracil and proline), indicates the necessity of the YCplac33-PDH/P5CR plasmid for manifestation of the Pro^+ phenotype. Complementation of proline auxotrophy in all DT1100 colonies transformed with plasmid cured from PU1 and growth of PU1 only in medium that activate expression of the plasmid borne soybean PDH/P5CR, indicate that the Pro^+ phenotype in PU1 strains was conferred by the enzyme encoded by the soybean PDH/P5CR gene.

Further evidence for soybean PDH/P5CR complementing proline auxotrophy in DT1100 was provided by DNA and RNA hybridisation analysis. When total RNA extracted from DT1100, DT1100-YC and PU1, was probed with the labelled PDH/P5CR sequence, only RNA extracted from the PU1 strain grown in the medium

allowing transcription of the introduced soybean PDH/P5CR gene was detected. RNA of DT1100, DT1100-YC and PU1 grown in noninducible medium, did not produce a hybridisation band.

Probing a Southern blot of a gel containing DNA extracted from DT100-YC, DT1100-A, PU1 and the untransformed DT1100 strain, with labelled PDH/P5CR sequence revealed bands present only in lanes containing PU1 and DT1100-A DNA. The DT1100-A DNA showed a band of the expected size for the Ycplac33-PDH/P5CR plasmid, whereas a band produced by the PU1 DNA was of a higher molecular weight. Interestingly, hybridisation to PU1 DNA produced a significantly stronger signal than that of DT1100-A DNA, an approximately 10-fold increase in PDH/P5CR DNA present in the PU1 cells. Such an increase likely results from changes to the number of copies of Ycplac33 plasmid carrying PDH/P5CR gene.

The YCplac33 vector used to transform *S. cerevisiae* DT1100 contained a DNA segment with a functional yeast centromere sequence (CEN) and relied on a yeast autonomously replicating sequence (ARS) as a DNA replication origin (Gietz & Sugino, 1988). Plasmids containing these two elements possess many properties of chromosomes. They are relatively stably maintained through mitotic and meiotic divisions and segregate through meiosis (Stinchcomb *et al.*, 1982). A third important property of the CEN sequences is copy number control. Plasmids containing a functional centromere are kept to an average of one to two copies per cell (Clarke & Carbon, 1980; Bloom *et al.*, 1983). It is known however, that plasmid DNA rearrangements do occur. Deletions in the functional CEN region are particularly common and appear to be triggered by the process of transformation itself. These deletions vary over a size range of 20 to 2000 bp and cause plasmid reversion to high copy number. For instance, the approximate number of ARS plasmids is 20 copies per cell (Tschumper & Carbon, 1983; Clancy *et al.*, 1984; Kingsman *et al.*, 1985a).

Indeed, the close examination of the hybridised fragments of PU1 and DT1100-A DNA with labelled PDH/P5CR sequence, revealed a difference in the length of the plasmid, with the PU1 hybridisation band characterised by a smaller molecular weight. This result indicates the occurrence of a deletion in the YCplac33-PDH/P5CR vector, most likely in the CEN region, converting it into a autonomously replicating sequence (ARS) plasmid and resulting in increased copies of the cloned soybean PDH/P5CR gene in *S. cerevisiae* PU1 cells. It is proper to speculate therefore that the Pro^+ phenotype in the PU1 strain was brought about by the increased number of PDH/P5CR gene product. High level of expression was also necessary to complement proline auxotrophy in *S. cerevisiae* DT1100 by the human P5C reductase (Dougherty *et al.,* 1992) and *E. coli* by the soybean PDH/P5CR (Delauney & Verma, 1990).

The expression of foreign genes in yeast and the intracellular assembly of functional protein is affected by a range of factors operating at the level of transcription, translation and post-translational processing. As the final yield of a protein is dependent on the level of mRNA, which in turn is determined by the rate of initiation and turnover of transcription, disturbances to those processes may lead to low protein production. The length of a transcript may also be affected by the presence of additional sequences in foreign genes which may cause delay or premature termination of transcription. The efficiency of translation can also be compromised by hindered initiation affected by the different structure of the mRNA leader sequence and the AUG context of the foreign transcript (Kozak, 1989). The bias commonly observed between organisms for a preferred subset of codons, especially for highly expressed genes, may also contribute to the final low protein yield and quality (Bennetzen & Hall, 1982b; Pedersen, 1984; Hoekema *et al.*, 1987; Kurland, 1987). The low yield of foreign protein influenced by the codon content was observed to occur more often when yeast cells were grown in minimal medium (Sharp & Cowe, 1991). Finally, the activity of the introduced soybean protein could also be affected by faulty folding, incorrect post-translational processing, its instability in yeast cells or negative

interaction between native, truncated yeast P5C reductase subunits with soybean subunits.

5.4.2 Growth of *S. cerevisiae* PU1 on L-proline as the sole source of nitrogen under strict anaerobic conditions

Growth under anoxic conditions of transgenic *S. cerevisiae* strain PU1, harbouring the proline oxidoreductase from soybean, was supported with L-proline provided as the sole source of nitrogen. The rate of growth and the final biomass of PU1 grown on L-proline however, was significantly reduced when compared to that when nitrogen was supplied in the form of ammonium ions. This reduced growth may reflect inefficiency of the overall metabolism of PU1 cells brought about by the introduced soybean protein. This could be associated with the expression of the soybean gene and the assembly of PDH/P5CR protein as well as the energetics of the proline oxidation reaction itself when cells are grown on proline as the sole source of nitrogen.

It is well documented that the elevated expression of a foreign gene can place a significant metabolic strain on the transgenic cell through a number of factors associated with transcription and translation (Hinnen *et al.*, 1989). The maintenance of a high number of plasmid copies is in itself sufficient to reduce the growth rate of the yeast host cell (Walmsley *et al.*, 1983). As a result of the intense expression of a foreign gene, the high level of foreign mRNA, especially when containing rare codons, could cause growth inhibition by depleting tRNA and inhibiting the translation of host mRNA containing rare codons. This effect was particularly noticed when yeast were grown on minimal medium (Purvis *et al.*, 1987). However, significantly higher growth of PU1 on minimal medium supplemented with di-ammonium sulphate, when compared to growth on proline (Figure 5.7), indicates that the reduced growth on proline was caused by the metabolism of this nitrogen source rather than the biochemistry associated with the expression of the introduced soybean gene.

In addition to the energy expenditure associated with the synthesis of introduced soybean PDH/P5CR, the reaction catalysed by this enzyme, which utilises NAD as a cofactor, may influence the overall redox balance of the cell, especially when proline is used as the sole nitrogen source. For metabolic reactions, cells utilise energy stored in the form of ATP and reducing power in the form of nucleotide phosphates, mostly NADPH. Under the strict anaerobic conditions applied during this study, the only source for cellular synthesis of ATP and NADPH, through the pentose phosphate pathway, was the fermentative oxidation of glucose. The initial oxidation of glucose requires NAD as an electron acceptor. The resulting NADH is re-oxidised in the reaction leading to the formation of ethanol, thus oxidation of glucose to pyruvate is redox-balanced by the synthesis of ethanol. Further synthesis of most biomolecules is a highly oxidative process with NAD acting as an oxidant (Nordstrom, 1966; Lagunas & Gancedo, 1973). For these anabolic reactions to proceed, redox balance must be maintained. In yeast, this is accomplished by the oxidation of NADH in the reaction of glycerol formation. Glycerol is produced, in an energetically wasteful step, through the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate via NAD-dependent glycerol-3-phosphate dehydrogenase followed by dephosphorylation of glycerol-3-phosphate by a glycerol-3-phosphatase (Norbeck *et al.*, 1996). Glycerol, which serves as an electron sink, is excreted from the cell. It has been shown that in *S. cerevisiae*, glycerol synthesis is the key reaction in the reoxidation of NADH under anaerobic conditions thus maintaining intracellular redox balance (Nordstrom, 1966; Oura, 1977). Mutants lacking functional glycerol-3-phosphate dehydrogenase are unable to grow fermentatively on glucose. Their growth can be restored by the addition of exogenous NADH oxidant (Ansell *et al.*, 1997).

NAD-dependent oxidation of proline by the introduced soybean PDH/P5CR would present PU1 cells with an additional demand for NAD, as all the nitrogen for biosynthesis had to be derived from proline. It is known that the need for oxidative power, in the form of NAD, is much higher in yeast grown on minimal media, as

compared to rich medium, even when nitrogen is supplied as readily assimilable ammonium salts. This is reflected by the elevated production of glycerol (Lagunas & Gancedo, 1973; Van Dijken & Scheffers, 1986). In order to balance the NAD/NADH ratio, PU1 cells would have to increase synthesis of glycerol which would lead to the decrease in available energy - one mole of ATP is used for synthesis of one mole of glycerol. This would manifest itself in the reduced growth rate and reduced final biomass of the culture (Fig. 5.7).

5.4.3 Conclusions

Soybean proline oxidoreductase complemented a proline reductase deficient mutant of *S. cerevisiae* DT1100. The introduced enzyme enabled yeast to utilise L-proline as the sole source of nitrogen under strict anaerobic conditions. The growth rate and the final cell count of the transgenic strain (*S. cerevisiae* PU1), when grown anaerobically on L-proline, was markedly reduced. This could reflect biochemical constraints associated with the expression and synthesis of the foreign protein and/or the inefficiency of the pathway of anaerobic proline degradation.

Chapter 6

General conclusions and further prospects

Saccharomyces cerevisiae strain PU1 was genetically modified to utilize L-proline, the dominant nitrogen source in most grape musts (Chapters 1 and 2.1), as a sole source of nitrogen under strict anaerobic conditions. This was achieved by introducing the soybean gene encoding proline oxidoreductase (PDH/P5CR) into yeast cells. By a NAD-linked reaction, the proline oxidoreductase oxidizes L-proline to Δ'-pyrroline-5-carboxylate, which in turn, is oxidized to glutamate - a key compound in the nitrogen metabolism of yeasts. Wild type *S. cerevisiae* can not catabolise proline anaerobically due to the functional link of proline oxidase to the oxygen-dependent electron transport chain (ETC), and therefore the need for molecular oxygen.

It has been shown by *in vitro* studies that NAD-linked proline PDH/P5CR is present only in certain anaerobic bacteria, some algae and higher plants. It is also argued that in plants, as in animals, proline degradation is catabolised by the ETC-linked proline oxidase (Chapter 2, Section 2.3.2). Before this work was undertaken, *in vivo* catabolism of proline involving PDH/P5CR had been documented only for the anaerobic bacterium *Clostridium sporogenes* (Chapter 2, Section 2.3.2.1). Growth of PU1 on L-proline as the sole nitrogen source under strict anaerobic conditions indicates that PDH/P5CR of plants can function in a proline catabolic role *in vivo* in yeast. Anaerobiosis or low oxygen concentration may be encountered by the aquatic algae during thermal stratification of water (Harris, 1986) or in root systems of plants growing in swampy soils. In such conditions the only route for proline catabolism would proceed through NAD-linked PDH/P5CR providing these organisms with proline nitrogen. Strictly O_2-dependent metabolism of animals may explain the absence of functional PDH/P5CR in these organisms, whereas the exclusive O_2-dependent catabolism of proline by yeast may reflect the evolutionary history of this group of organisms. It is likely that, in the case of *S. cerevisiae,* before it was

employed in winemaking by mankind, its niche included the surfaces of sugar-rich fruits which are likely to be well exposed to air, where evolution of the alcoholic fermentation pathway would present *S cerevisiae* with the advantage over less alcohol-tolerant species in competition for nutrients.

When the PU1 strain was provided with L-proline as the sole nitrogen source, it showed a reduced rate of growth and reduced final cell number when compared to growth on ammonium ions (Chapter 5). The impaired growth of PU1 on proline could result from energetic demands associated with deriving all required nitrogen from proline and, at the same time, synthesis of proline dehydrogenase encoded by the introduced soybean gene. It is possible that providing the PDH/P5CR carrying strain with a medium containing a range of assimilable nitrogen compounds together with proline as a supplement rather than as a sole nitrogen source, could result in efficient growth.

While proline uptake in yeast is redox insensitive, it is under the control of nitrogen catabolite repression (NCR, Chapter 3). Strains insensitive to NCR could be obtained by altering the proline transporter of *S. cerevisiae* or by introducing a foreign transporter insensitive to the quality of nitrogen contained in the growth medium, such as those present in *Candida albicans* or *Pseudomonas aeruginosa,* (see Chapter 2 Section 2.2.2c). Such a modification would allow the concurrent uptake of proline with other nitrogen containing compounds.

It has been shown that addition of assimilable nitrogen to nitrogen-deficient musts effectively reduces production of hydrogen sulfide and prevents stuck fermentation (Agenbach, 1977; Vos & Gray, 1979; Jiranek, 1992). To allow the assimilation of L-proline by yeast growth under oenological conditions, further work should deal with combining a nitrogen-insensitive proline transporter and a NAD-linked PDH/P5CR into a wine yeast. Utilising a low nitrogen-demanding *S. cerevisiae* strain could further optimize fermentation, in relation to the nitrogen content of the must. Such

modified yeasts would eliminate the need for nitrogen supplementation of the majority of musts. It would also enable the supplementation of nitrogen deficient musts with proline. Currently, nitrogen deficient musts are supplemented with ammonium salt, a practice which may lead to production of ethyl carbamate (a suspected carcinogen) and/or growth of undesirable microbial flora (Bisson, 1991). By using proline as a nitrogen supplement, these detrimental factors would be eliminated as microbe communities of fermenting grape juice can not utilise proline and its catabolism does not produce undesirable compounds which may have health implications.

An anaerobic proline degrading pathway may also be introduced into a wine yeast strain possessing particularly desirable winemaking characteristics. Dominance of such a strain during the fermentation process could be controlled by the amount of proline present in the must. Furthermore, musts with a high proline to total nitrogen content would give a selective advantage to such yeast. This could reduce the need for SO_2 addition, which is normally added to grape musts as an antiseptic substance to inhibit growth of indigenous yeast.

Proline is accumulated as a compatible solute by a number of organisms, including grape vine (*Vitis vinifera*), in response to the high ionic and/or water deficit stress (Schobert, 1977; Stewart & Hanson, 1980; Itai & Paleg, 1982; LaRosa *et al.,* 1991; Saradhi & Saradhi, 1991; Delauney & Verma, 1993; Kelly & Register, 1996). The stress resistance of plants can be markedly increased by the genetic modifications of proline synthetic pathway leading to the higher proline accumulation (Hanson *et al.,* 1994; Kishor *et al.,* 1995; Nolte *et al.,* 1997; Peng *et al.*, 1996). Increased proline accumulation in vegetative tissues leads to its increase concentration in fruits (Stewart & Hanson, 1980; Flasinski & Rogozinska, 1985). Using molecular genetics techniques, proline metabolism of grape vine can be modified resulting in drought and salinity (conditions often encountered in the southern and western part of Australia) resistant varieties. Increased proline nitrogen in the berries of such modified vines, till now unutilized during wine fermentation, may serve as a nitrogen source in

fermentation employing a proline-utilizing strain of *S. cerevisiae*. Such integration of viticulture and wine-making may lead to a reduction or elimination of nitrogen supplementation of fermenting musts, a practice which is under growing scrutiny due to pressure for reduced use of additives and chemicals in food and beverage manufacture.

References

Abadom P.N., P.G. Scholefield (1962). Aminon acid transport in brain cortex slices. II. Competition between amino acids. Can. J. Biochem. Physiol. 40: 1591-1602.

Abrahamson J.L.A., L.G. Baker, J.T. Stephenson, J.M. Wood (1983). Proline dehydrogenase from *Escherichia coli* K12. Properties of the mambrane-associated enzyme. Eur. J. Biochem. 134: 77-82.

Adams E., L. Frank (1980). Metabolism of proline and hydroxyprolines. Ann. Rev. Biochem. 49: 1005-1061.

Agenbach W.A. (1977). A study of must nitrogen content in relation to incomplete fermentations, yeast production and fermentation activity. In: Proceedings of the South African Society for Enology and Viticulture; 21-22 November 1977; Cape Town, S. Afr. pp 66-87. South African Society for Enology and Viticulture, Stellenbosch, S. Afr.

Albone E.S., S.P. Robins, D. Patel (1976). 5-aminovaleric acid, a major free amino acid component of the anal sac secretion of the red fox, *Vulpes vulpes*. Comp. Biochem. Physiol. 55B: 483-486.

Amerine M.A., H.W. Berg, R.E. Kunkee, C.S. Ough, V.L. Singleton, A.D. Webb (1980). The composition of grapes. In: The Technology of Wine Making. 4th ed. pp 77-139. AVI Publishing Company, Westport, Connecticut.

Ames G.S., C.S. Mimura, S.R. Holbrook, V. Shyamala (1992). Traffic ATPases: a superfamily of transport proteins operating from *Escherichia coli* to humans. Adv. Enzymol. 65: 1-47.

Anderson R.G., B.H. Kirsop (1974). The control of volatile ester synthesis during the fermentation of wort of high specific gravity. J. Inst. Brew. 80: 48-55.

Anderson E., P.A. Martin (1975). The sporulation and mating of brewing yeast. J. Inst. Brew. 81: 242-247.

Anderson R.R., R. Menzel, J.M. Wood (1980). Biochemistry and regulation of a second L-proline transport system in *Salmonella typhimurium.* J. Bacteriol. 141: 1071-1076.

Andreasen A.A., T.J.B. Stier (1954). Anaerobic nutrition of *Saccharomyces cerevisiae.* II. Unsaturated fatty acids requirement of growth in a defined medium. J. Cell. Comp. Physiol. 43: 271-281.

Ansanay V., S. Dequin, B. Blondin, P. Barre (1993). Cloning sequence and expression of the gene encoding the malolactic enzyme from *Lactococcus lactis.* FEBS Lett. 332: 74-80.

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

Ansell R., K. Granath, S. Hohmann, J.M. Thevelein, L. Adler (1997). The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J. 16: 2179-2187.

Arkowitz R.A., S. Dhe-Paganon, R.H. Abeles (1994). The fate of the carboxyl oxygens during D-proline reduction by Clostridial proline reductase. Arch. Biochem. Biophis. 311: 457-459.

Arsdell J.N., S. Kwok, V.L. Schweickart, M.B. Lander, D.H. Gelfand, M.A. Innis (1987). Cloning, characterisation and expression in *Saccharomyces cerevisiae* of endoglucanase I from *Trichoderma reesei.* Bio/Technology 5: 60-64.

Arst H.N. Jr., D.J. Cove (1973). Nitrogen metabolite repression in *Aspergillus nidulans.* Mol. Gen. Genet. 126: 111-141.

Arst H.N. Jr., D.W. MacDonald (1975). A gene cluster in *Aspergillus nidulans* with an internally located *cis*-acting regulatory region. Nature 254: 26-31.

Arst H.N. Jr., D.W. MacDonald, S.A. Jones (1980). Regulation of Proline Transport in *Aspergillus nidulans.* J. Gen. Microbiol. 116: 285-294.

Asghar S.S., E. Levin, F.M. Harold (1973). Accumulation of neutral amino acids by *Streptococcus faecalis.* J. Biol. Chem. 248: 5225-5233.

Aschengreen N.H. (1987). Enzyme technology. Proc. Eur. Brew. Conv. 21st Cong. Madrid, pp 221-231.

Ausubel F.M. (1987). Yeast Cloning Vectors and Genes: Current Protocols in Molecular Biology. Wiley and Sons, Inc., New York.

Ausubel F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (1994). Current Protocols in Molecular Biology. New York, Wiley.

Bachmann B.J., K.B. Low, A.L. Taylor (1976). Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. 40: 116-167.

Bae J.H., K.J. Miller (1992). Identification of two proline transport systems in *Staphylococcus aureus* and their possible roles in osmoregulation. Appl. Environ. Microbiol. 58: 471-475.

Baich A. (1969). Proline synthesis in *Escherichia coli.* A proline inhibitable glutamic acid kinase. Biochim. Biophys. Acta 192: 462-467.

Baich A. (1971). The biosynthesis of proline in *Escherichia coli.* Phosphate dependent glutamic g-semialdehyde dehydrogenase (NADP), the second enzyme in the pathway. Biochim. Biophys. Acta 244: 129-134.

Baich A., D.J. Pierson (1965). Control of proline synthesis in *Escherichia coli.* Biochim. Biophys. Acta 104: 397-404.

Bakker E.P. (1990). The role of alkali-cation transport in energy coupling of neutrophilic and acidophilic bacteria: an assessment of methods and concepts. FEMS Microbiol. Rev. 75: 319-334.

Balboni E., R.I. Hecht (1977). Studies on the inner mitochondrial membrane localization of proline dehydrogenase. Biochim. Biophys. Acta 462: 171-176.

Ballarin-Denti A., J.A. den Hollander, D. Sanders, C.W. Slayman, C.L. Slayman (1984). Kinetics and pH-dependence of glycine-proton symport in *Saccharomyces cerevisiae.* Biochim. Biophys. Acta 778: 1-16.

Barker H.A. (1981). Amino acid degradation by anaerobic bacteria. Ann. Rev. Biochem. 50: 23-40.

Barker H.A., L. D'Ari, J. Kahn (1987). Enzymatic reactions in the degradation of 5-aminovalerate by *Clostridium aminovalericum.* J. Biol. Chem. 262: 8994-9003.

Beggs J.D. (1978). Transformation of yeast by a replicating hybrid plasmid. Nature 275: 104-109.

Begin N., P.G. Scholefield (1965). The uptake of amino acids by mouse pancreas *in vitro.* III. The kinetic characteristics of the transport of L-proline. Biochim. Biophys. Acta 104: 566-573.

Behki R.M. (1967). Metabolism of amino acids in *Agrobacterium tumefaciens.* III. Uptake of L-proline. Can. J. Biochem. 45: 1819-1830.

Beier D.R., E.T. Young (1982). Characterisation of a regulatory region upstream of the *ARD2* locus of *Saccharomyces cerevisiae.* Nature 300: 724-728.

Beier D.R., A. Sledziewski, E.T. Young (1985). Deletion analysis indentifies a region upstream of *ADH2* gene of *Saccharomyces cerevisiae* which is required for *ADR1* derepression. Mol. Cell. Biol. 5: 1743-1749.

Bely M., J.M. Sablayrolles, P. Barre (1990). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in enological conditions. J. Ferment. Bioeng. 70: 246-252.

Bennetzen J.L., B.D. Hall (1982a). The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase I. J. Biol. Chem. 257: 3018-3025.

Bennetzen J.L., B.D. Hall (1982b). Codon selection in yeast. J. Biol. Chem. 257: 3026-3031.

Berg C.M., J.J. Rossi (1974). Proline excretion and indirect suppression in *Escherichia coli* and *Salmonella typhimurium.* J. Bacteriol. 118: 928-939.

Beutler H.O. (1984). Two-and one-carbon compounds, 4.1: Ethanol. In: Methods of Enzymatic Analysis, Vol. VI, Section 4: Metabolites 1: Carbohydrates. 3rd ed. H.U. Bergmeyer (ed.). Verlag Chemie, pp 598-606.

Bevan E.A., M. Makower (1963). The physiological basis of the killer character in yeast. Proc. 11th Int. Cong. Genetics, Hague, pp 202-205.

Bidard F., M. Bony, B. Blondin, S. Dequin, P. Barre (1995). The *Saccharomyces cerevisiae FLO1* flocculation gene encodes for a cell surface protein. Yeast 11: 809-822.

Bisson L.F. (1991). Influence of nitrogen on yeast and fermentation of grapes. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wines (Seattle, WA, 1991), J.M. Rantz (ed.), pp 172-184. Dvies, CA: American Society for Enology and Viticulture.

Bitter G.A., K.K. Chen, A.R. Banks, P.H. Lai (1984). Secretion of foreign proteins from *Saccharomyces cerevisiae* directed by α-factor gene fusion. Proc. Natl Acad. Sci. USA 81: 5330-5334.

Bitter G.A., K.M. Egan (1984). Expression of heterologous genes in *Saccharomyces cerevisiae* from vectors utilising the glyceraldehyde-3-phosphate dehydrogenase gene promoter. Gene 32: 263-274.

Bitter G.A., K.M. Egan, R.A. Koski, M.O. Jones, S.G. Elliot, J. Giffin (1987). Expression and secretion vectors for yeast. In: Methods in Enzymology. R. Wu, L. Grossman, K. Moldove (eds). Academic Press, New York, pp 516-544.

Bizeau C. (1963). Etude des facteurs limitant la croissance des levures dans les mouts de 'Clairette' et de 'Muscat Blanc a Petits Grains'. Ann. Technol. Agric. 12: 247-276.

Blake R.L. (1972). Animal model for hyperprolinaemia: deficiency of mouse proline oxidase activity. Biochem. J. 129: 987-989.

Bloom K.S., M. Fitzgerald-Hayes, J. Carbon (1983). Structural analysis and sequence organization of yeast centromeres. Cold Spring Harbor Symp. Quant. Biol. 47.

Boggess S.F., D.E. Koeppe, C.R. Stewart (1978). Oxidation of proline by plant mitochondria. Plant Physiol. 62: 22-25.

Bogges S.F., L.G. Paleg, L.G. Aspinall (1975). Pyrroline-5-carboxylic acid dehydrogenase in barley, a proline accumulating species. Plant Physiol. 56: 259-262.

Boggess S.F., C.R. Stewart, D. Aspinall, L.G. Paleg (1976). Effect of water stress on proline synthesis from radioactive precursors. Plant Physiol. 58: 398-401.

Bogonez E., J. Satrustegui, A. Machado (1985). Regulation by ammonium of glutamate dehydrogenase ($NADP^+$) from *Saccharomyces cerevisiae*. J. Gen. Microbiol. 131: 1425-1432.

Boone C., A.M. Sdicu, J. Wagner, R. Degre, C. Sanchez, H. Bussey (1990). Integration of the yeast K1 killer toxin gene into the genome of marked wine yeast and its effect on vinification. Am. J. Enol. Vitic. 41: 37-42.

Booth I.R. (1985). Regulation of Cytoplasmic pH in Bacteria. Microbiol. Rev. 49: 359-378.

Bortol A., C. Nudel, A.M. Giulietti, J.F.T. Spencer, D.M. Spencer (1988). Industrial yeast strain improvement. Construction of strains having the killer character and capable of using starch. Appl. Microbiol. Biotech. 28: 577-579.

Bostian K.A., J.E. Hopper, D.J. Rogers, D.J. Tipper (1980a). Translational analysis of killer associated virus like particle dsRNA genome of *Saccharomyces cerevisiae*; M-dsRNA encodes toxin. Cell 19: 403-414.

Bostian K.A., J.M. Lemire, L.E. Cannon, H.O. Halvorson (1980b). *In vitro* synthesis of repressible yeast acid phosphatase; identification of multiple mRNA and products. Proc. Natl Acad. Sci. USA 77: 4504-4508.

Boyer P.D. (ed.) (1975). The enzymes. 3rd (ed.), vol. XI. Academic Press, New York.

Boylan S.A., E.E. Dekker (1981). L-Threonine dehydrogenase. Purification and properties of the homogeneous enzyme from *Escherichia coli* K-12. J. Biol. Chem. 256: 1809-1815.

Brady R.A., L.N. Csonka (1988). Transcriptional regulation of the *proC* gene of *Salmonella typhimurium*. J. Bacteriol. 170: 2379-2382.

Brandriss M.C. (1979). Isolation and Preliminary Characterisation of *Saccharomyces cerevisiae* Proline Auxotrophs. J. Bacteriol. 138: 816-822.

Brandriss M.C. (1987). Evidence for positive regulation of the proline utilization pathway in *Saccharomyces cerevisiae*. Genetics 117: 429-435.

Brandriss M.C., D.A. Falvey (1992). Proline biosynthesis in *Saccharomyces cerevisiae*: analysis of the *PRO3* gene, which encodes Δ'-pyrroline-5-carboxylate reductase. J. Bacteriol. 174: 3782-3788.

Brandriss M.C., K.A. Krzywicki (1986). Amino-terminal fragments of Δ'-pyrroline-5-carboxylate dehydrogenase direct β-galactosidase to the mitochondrial matrix in *Saccharomyces cerevisiae*. Mol. Cel. Biol. 6: 3502-3512.

Brandriss M.C., B. Magasanik (1979a). Genetics and physiology of proline utilisation in *Saccharomyces cerevisiae*: enzyme induction by proline. J. Bacteriol. 140: 498-503.

Brandriss M.C., B. Magasanik (1979b). Genetics and physiology of proline utilisation in *Saccharomyces cerevisiae*: mutation causing constitutive enzyme expression. J. Bacteriol. 140: 504-507.

Brandriss M.C., B. Magasanik (1980). Proline: an essential intermediate in the degradation of arginine in *Saccharomyces cerevisiae*. J. Bacteriol. 143: 1403-1410.

Brandriss M.C., B. Magasanik (1981). Subcellular compartmentation in control of converging pathways for proline and arginine metabolism in *Saccharomyces cerevisiae*. J. Bacteriol. 145: 1359-1364.

Breeuwer P., J.L.Drocourt, F.M. Rombouts, T. Abee (1996). A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. Appl. Environ. Microbiol. 62: 178-183.

Brewer J.D., M.S. Fenton (1980). The formation of sulphur dioxide during fermentation. Proc. Conv. Inst. Brew., Australia and New Zealand section 16: 155-164.

Brosemer R.W., P.S. Veerabhadrappa (1965). Pathway of proline oxidation in insect flight muscle. Biochim. Biophys. Acta 110: 102-112.

Brown S.W., S.G. Oliver (1982). Isolation of ethanol tolerant mutants of yeast by continuous selection. Eur. J. Appl. Microbiol. Biotechnol. 16: 119-122.

Brunner G., W. Neupert (1969). Localisation of proline oxidase and Δ'-pyrroline-5-carboxylic acid dehydrogenase in rat liver. FEBS Lett. 3: 283-286.

Bruno M.E.C., A. Kaiser, T.J. Montville (1992). Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. Appl. Environ. Microbiol. 58: 2255-2259.

Brzobohaty B., L. Kovac (1986). Factors enhancing genetic transformation of intact yeast cells modify cell wall porosity. J. Gen. Microbiol. 132: 3089-3093.

Burgers P.M.J., K.J. Percival (1987). Transformation of yeast spheroplasts without cell fusion. Anal. Biochem. 163: 391-397.

Bussey H., H.E. Umbarger (1970). Biosynthesis of the branched-chain amino acids in yeast: A trifluoroleucine-resistant mutant with altered regulation of leucine uptake. J. Bacteriol. 103: 286-294.

Butt T.R., E. Sternberg, J. Herd, S.T. Crooke (1984). Cloning and expression of a copper metallothionein gene. Gene 27: 23-33.

Cairney J., C.F. Higgins, I.R. Booth (1984). Proline uptake through the major transport system of *Salmonella typhimurium* is coupled to sodium ions. J. Bacteriol. 160: 22-27.

Calahorra M., J. Ramirez, M. Opekarova, A. Pena (1989). Leucine transport in plasma membrane vesicles of *Saccharomyces cerevisiae*. FEBS Lett. 247: 235-238.

Calderbank J., M.H.J. Keenan, A.H. Rose, G.D. Holman (1984). Accumulation of amino acids by *Saccharomyces cerevisiae* Y185 with phospholipids enriched in different fatty-acyl residued: A statistical analysis of data. J. Gen. Microbiol. 130: 2817-2824.

Cartwright C.P., Y.S. Zhu, D.J. Tipper (1992). Efficient secretion in yeast based on fragments from K1 killer pretoxin. Yeast 8: 261-272.

Castino M., A. Piracci, G. Spera (1981). Proline in the D.O.C. wines: Colli Albini, Frascati, Marino, Velletri, Trebbiano e Merlot d'Aprilia, Cesanese del Piglio, Montepulciano d' Abruzzo, Verdicchio di Jesi. Vini D'Italia pp 345-353.

Castor J.G.B. (1953). The free amino acids of musts and wines. II. The fate of amino acids of must during alcoholic fermentation. J. Food Res. 18: 146-151.

Castor J.G.B., T.E. Archer (1959). The free amino acids of musts and wines. III. Effect of added ammonia and of fermentation temperature on the fate of amino acids during fermentation. J. Food Res. 24: 167-175.

Cavalieri A.J., A.H.C. Huang (1980). Carrier protein-mediated transport of neutral amino acids into mung bean mitochondria. Plant Physiol. 66: 588-591.

Chan P.Y., E.A. Cossins (1976). General properties and regulation of arginine transporting systems in *Saccharomyces cerevisiae*. Plant Cell Physiol. 17: 341-353.

Chen C.-C., T. Tsuchiya, Y. Yamane, J.M. Wood, T.H. Wilson (1985). Na^+ (Li^+)-proline cotransport in *Escherichia coli*. J. Membr. Biol. 84: 157-164.

Cherest H., N.N. Thao, Y. Surdin-Kerjan (1985). Transcriptional regulation of the *MET3* gene of *Saccharomyces cerevisiae*. Gene 34: 269-281.

Cho B.-H., E. Komor (1983). Mechanism of proline uptake by *Chlorella vulgaris*. Biochim. Biophys. Acta 735: 361-366.

Christensen H.N., M. Liang (1966). Transport of diamino acids into the Ehrlich cell. J. Biol. Chem. 241: 5542-5551.

Clancy S., C. Mann, R.W. Davis, M.P. Calos (1984). Deletion of plasmid sequences during *Saccharomyces cerevisiae* transformation. J. Bacteriol. 159: 1065-1067.

Clarke L., J. Carbon (1980). Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature 257: 504-509.

Cohen J.D., T.R. Eccleshall, R.B. Needleman, H. Federoff, B.A. Buchferer, J. Marmur (1980). Functional expression in yeast of the *E. coli* plasmid gene encoding chloramphenicol acetyltransferase. Proc. Natl Acad. Sci. USA 77: 1078-1082.

Conde J., G.R. Fink (1976). A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc. Natl Acad. Sci. USA 73: 3651-3655.

Cooper T.G. (1982a). Nitrogen metabolism in *Saccharomyces cerevisiae* . In: The Molecular Biology of the Yeast *Saccharomyces*. Metabolism and Gene Expression. J.N. Strathern, E.W. Jones, J.B. Broach (eds). pp 39-99. Cold Spring Harbor Laboratory, New York.

Cooper T.G. (1982b). Transport in *Saccharomyces cerevisiae*. In: The Molecular Biology of the Yeast *Saccharomyces*. Metabolism and Gene Expression. J.N. Strathern, E.W. Jones, J.B. Broach (eds.). pp 399-461. Cold Spring Harbor Laboratory, New York.

Cordonnier R. (1966). Etude des proteins et des substances azotees. Leur evolution au cours des traitements oenologique. Conditions de le stabilite proteique des vius. Bull. O. I. V. 39: 1475-1489.

Correa I., M.C. Polo, L. Amigo, M. Ramos (1988). Separation des proteines des mouts de raison au moyen de techniques electrophoretiques. Connaiss. Vigne Vin 22: 1-9.

Costilow R.N., L. Laycock (1969). Reactions involving the conversion of ornithine to proline in clostridia. J. Bacteriol. 100: 662-667.

Costilow R.N., L. Laycock (1971). Ornithine cyclase (deaminating). Purification of a protein that converts ornithine to proline and definition of the optimal assay conditions. J. Biol. Chem. 246: 6655-6660.

Costilow R.N., D. Cooper (1978). Identity of proline dehydrogenase and Δ'-pyrroline-5-carboxylic acid reductase in *Clostridium sporogenes*. J. Bacteriol. 134: 139-146.

Courchesne W.E., B. Magasanik (1983). Ammonia regulation of amino acid permeases in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 3: 627-683.

Cousens L.S., J.R. Shuster, C. Gallegos, L. Ku, M.M. Stempien, M.S. Urdea, R. Sanches-Pescador, A. Taylor, P. Tekamp-Olson (1987). High expression of pro-insulin in yeast *Saccharomyces cerevisiae*. Gene 61: 265-275.

Crabeel M., M. Grenson (1970). Regulation of histidine uptake by specific feedback inhibition of two histidine permeases in *Saccharomyces cerevisiae*. Eur. J. Biochem. 14: 197-204.

Csonka L.N. (1981). Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium*. Mol. Gen. Genet. 182: 82-86.

Csonka L.N., A. Baich (1983). Proline biosynthesis. In. Amino acids: Biosynthesis and Genetic Regulation. K.M. Herrmann, R.L.Sommerville (eds). Addison-Wesley, Reading, Mass. USA, pp 35-51.

Cybulski R.L., R.R. Fisher (1977). Mitochondrial neutral amino acid transport: evidence for a carrier mediated mechanism. Biochemistry 16: 5116-5120.

Dabrowa N., D.H. Howard (1976). Uptake of L-proline by *Histoplasma capsulatum*. Can. J. Microbiol. 22: 1188-1190.

Dabrowa N., D.H. Howard (1981). Proline uptake in *Candida albicans*. J. Gen. Microbiol. 127: 391-397.

Damsky C.H., W.M. Nelson, A. Claude (1969). Mitochondria in anaerobically-grown, lipid-limited brewer's yeast. J. Cell. Biol. 43: 174-179.

Darte C., M. Grenson (1975). Evidence for three glutamic acid transporting systems with specialized physiological functions in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Comm. 67: 1028-1033.

Dashek W.V., S.S. Erickson (1981). Isolation, assay, biosynthesis, metabolism, uptake and translocation, and function of proline in plant cells and tissues. Bot. Rev. 47: 349-385.

Daudt C.E., C.S. Ough (1980). A method for detecting volatile amines in grapes and wines. Am. J. Enol. Vitic. 31: 356-359.

David M.H., B.H. Kirsop (1973). Yeast growth in relation to the dissolved oxygen and sterol content of wort. J. Inst. Brew. 79: 20-25.

DeBaetselier A., A. Vasavada, P. Dohet, V. Ha-Thi, M. De Beukelaer, T. Erpicum, L. De Clerk, J. Hanotier, S. Rosberg (1991). Fermentation of a yeast producing *A. niger* glucose oxidase: scale up, purification and characterisation of the recombinant enzyme. Biotech. 9: 559-561.

Delauney A.J., D.P.S. Verma (1990). A soybean gene encoding Δ^{1}-pyrroline-5-carboxylate reductase was isolated by functional complementation in *Escherichia coli* and is found to be osmoregulated. Mol. Gen. Genet. 221: 299-305.

Delauney A.J., D.P.S. Verma (1993). Proline biosynthesis and osmoregulation in plants. Plant J. 4: 215-223.

Delorme E. (1989). Transformation of *Saccharomyces cerevisiae* by electroporation. Appl. Env. Microbiol. 55: 2242-2246.

Denayrolles M., M. Aigle, A. Lonvaud-Funel (1995). Functional expression in *Saccharomyces cerevisiae* of the *Lactococcus lactis mleS* gene encoding malolactic enzyme. FEMS Microbiol. Lett. 125: 37-44.

Dendinger S., W. J. Brill (1970). Regulation of proline degradation in *Salmonella typhimurium*. J. Bacteriol. 103: 144-152.

Der Garabedian P.A., (1986). *Candida* δ-aminovalerate:α-ketoglutarate aminotransferase: Purification and enzymologic properties. Biochemistry 25: 5507-5512.

Des Etages S.A., D.A. Falvey, R.J. Reece, M.C. Brandriss (1996). Functional analysis of the PUT3 transcriptional activator of the proline utilization pathway in *Saccharomyces cerevisiae*. Genetics 142: 1069-1082.

Despeghel J.P., S. Delrot (1983). Energetics of amino acid uptake by *Vicia faba* leaf tissue. Plant Physiol. 71: 1-6.

Deutch A.H., K.E. Rushlow, C.J. Smith (1984). Analysis of the *Escherichia coli proBA* locus by DNA and protein sequencing. Nucleic Acids Res. 12: 6337-6355.

Deutch A.H., C.J. Smith, K.E. Rushlow, P.J. Kretschmer (1982). *Escherichia coli* Δ'-pyrroline-5-carboxylate reductase: gene sequence, protein overproduction and purification. Nucleic Acids Res. 10: 7701-7714.

De Winde J.H., L.A. Grivell (1993). Global regulation of mitochondrial biosynthesis in *Saccharomyves cerevisiae*. Progr. Nucleic Acids Res. Mol. Biol. 46: 51-91.

De Winde J.H., L.A. Grivell (1995). Regulation of mitochondrial biogenesis in *Saccharomyces cerevisiae* – intricate interplay between general and specific transcription factors in the promoter of the QCR8 gene. Eur. J. Biochem. 233: 200-208.

Dobson M.J., N.A. Tuite, N.A. Roberts, A.J. Kingsman, S.M. Kingsman, R.E. Perkins, S.C. Conroy, B. Dunbar, L.A. Fothergill (1982). Conservation of high efficiency promoter sequences in *Saccharomyces cerevisiae*. Nucl. Acids Res. 10: 2625-2637.

Dougherty K.M., M.C. Brandriss, D. Valle (1992). Cloning human pyrroline-5-carboxylate reductase cDNA by complementation in *Saccharomyces cerevisiae*. J. Biol. Chem. 267: 871-875.

Dreyfus P.M., H.L. Levy, M.L. Efron (1968). Concerning amino acids in human saliva. Experientia 24: 447-448.

Dubois E., M. Grenson, J.M. Wiame (1974). The participation of the anabolic glutamate dehydrogenase in the nitrogen catabolite repression of arginase in Saccharomyces cerevisiae. Eur. J. Biochem. 48: 603-616.

Dubois E., S. Vissers, M. Grenson, J-M. Wiame (1977). Glutamine and ammonia in nitrogen catabolite repression of Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 75: 233-239.

Dufour J.P., (1991). Influence of industrial brewing and fermentation working conditions on beer SO_2 level and flavour stability. Proc. Eur. Brew. Conv. Cong., Lisbon, pp 209-216.

Duteurtre B., C. Bourgeois, B. Chollot (1971). Study of the assimilation of proline by brewing yeast. J. Inst. Brew. 77: 28-35.

Eddy A.A. (1968). A net gain of sodium ions and a net loss of potassium ions accompanying the uptake of glycine by mouse ascites-tumour cells in the presence of sodium cyanide. Biochem. J. 108: 195-206.

Eddy A.A. (1982a). Mechanism of solute transport in selected eukaryotic micro-organisms. Adv. Microbiol. Physiol. 23: 1-78.

Eddy A.A. (1982b). Symport mechanisms as the basis of solute transport in yeast and mouse ascites-tumor cells. In: Membranes and Transport. A.N. Martonosi (ed.), Vol. 2. Plenum Press, New York, p. 47-53.

Elthon T.E., C.R. Stewart (1982). Proline oxidation in corn mitochondria. Plant. Physiol. 70: 567-572.

Enari T.M., J. Knowles, U. Lehtinen, M. Nikkola, M. Penttila, M.L. Suihko, S. Home, A. Vilpola (1987). Glucanolytic brewers yeast. Proc. Eur. Brew. Conv. 21st Cong., Madrid, pp 529-536.

Ernst J.F. (1988). Efficient secretion and processing of heterologous proteins in Saccharomyces cerevisiae is mediated solely by the presegment of α-factor precursor. DNA 5: 355-360.

Ertel J., H. Isseroff (1974). Proline in fascioliasis: I. Comparative activities of ornithine-δ-transaminase and proline oxidase in Fasciola and mammalian livers. J. Parasitol. 60: 574-577.

Eschenbruch R., J.M. Rassell (1975). The development of non-foaming yeast strains for wine making. Vitis 14: 43-47.

Etherton B. (1980). Amino acid transport in higher plants. In Plant Membrane Transport: Current Conceptual Issues. R.M. Spanswick, W.J. Lucas, J. Dainty (eds). Proceedings of the Internatioonal Workshop. Toronto, Canada, July 22-27, pp 261-269.

Etievant P., P. Schlich, J.C. Bouvier, P. Symonds, A. Bertrand (1988). Varietal and geographic classification of french red wines in terms of elements, amino acids and aromatic alcohols. J. Sci. Food Agric. 45: 25-41.

Farmer J.L., W.S. Bradshaw, C.S. Smith III (1979). Characteristics of Δ'-pyrroline-5-carboxylate reductase from Drosophila melanogaster. Comp. Biochem. Physiol. 62B: 143-146.

Finch L.R., F.J.R. Hird (1960a). Uptake of amino acids by isolated segmemnts of rat intestine. I. Survey of factors affecting measurement of uptake. Biochim. Biophys. Acta 43: 268-277.

Finch L.R., F.J.R. Hird (1960b). Uptake of amino acids by isolated segments of rat intestine. II. Survey of affinity for uptake from rates of uptake and competition for uptake. Biochim. Biophys. Acta 43: 278-289.

Flasinski S., J. Rogozinska (1985). Effect of water deficit on proline accumulation, protein and chlorophyll content during flowering and seed formation in winter rape. Acta Agrobot. 38: 11-21.

Fogel S., J. Welch (1982). Tandem gene amplification mediates copper resistance in yeast. Proc. Natl Acad. Sci. USA 79: 5342-5346.

Fowden L. (1965). Amino acid biosynthesis. In: biosynthetic pathways in higher plants. J.B. Pridham, T. Swain (Eds). Academic Press, N.Y. pp 87-99.

Fowell R.R. (1969). Life cycle in yeast. In: The Yeast, Volume 1. A.H. Rose, J.S. Harrison (eds). Academic Press, London, pp 303-383.

Fox M., S. Their, L. Rosenberg, S. Segal (1964). Ionic requirements for amino acid transport in the rat kidney cortex slice. I. Influence of extracellular ions. Biochim. Biophys. Acta 79: 167-176.

Freeman R.F. (1981). Construction of brewing yeasts for the production of low carbohydrate beers. Proc. Eur. Brew. Conv. Cong. 18th, Copenhagen, 497-504.

Fujii T., K. Kondo, F. Shimizu, H. Sone, J. Tanaka, T. Inoue (1990). Application of a ribosomal integration vector in the construction of a brewer's yeast having α-acetolactate decarboxylase activity. Appl. Env. Micro. 56: 997-1003.

Gamper H., V. Moses (1974). Enzyme organization in the proline biosynthetic pathway of *Escherichia coli*. Biochim. Biophys. Acta 354: 75-87.

Germano G.J., K.E. Anderson (1968). Purification and properties of L-alanine dehydrogenase from *Desulfovibrio desulfuricans*. J. Bacteriol. 96: 55-60.

Gietz R.D., A. Sugino (1988). New yeast-*Escherichia coli* shuttle vector constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74: 527-534.

Gits J.J., M. Grenson (1967). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. III. Evidence for a specific methionine-transporting system. Biochim. Biophys. Acta 135: 507-516.

Gits J.J., M. Grenson (1969). Regulation of methyl amino acids in *Saccharomyces cerevisiae*. Arch. Internat. Physiol. Biochim. 77: 153-154.

Graham S.B., J.T. Stephenson, J.M. Wood (1984). Proline dehydrogenase from *Escherichia coli* K12. Reconstitution of a functional membrane association. J. Biol. Chem. 259: 2656-2661.

Greasham R.L., A.G. Moat (1973). Amino acid transport in a polyaromatic amino acid auxotroph of *Saccharomyces cerevisiae*. J. Bacteriol. 115: 975-981.

Gregory M.E., M.H.J. Keenan, A.H. Rose (1982). Accumulation of L-asparagine by *Saccharomyces cerevisiae*. J. Gen. Microbiol. 128: 2557-2562.

Grenson M. (1966). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. II. Evidence for a specific lysine-transporting system. Biochim. Biophys. Acta 127: 339-346.

Grenson M. (1983a). Inactivation-reaction process and repression of permease formation regulate several ammonia-sensitive permeases in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 133: 135-139.

Grenson M. (1983b). Study of the positive control of the general amino-acid permease and other ammonia-sensitive uptake systems by the product of the *NPR1* gene in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 133: 141-144.

Grenson M. (1992). Amino acid transporters in yeast: structure, function and regulation. In De Pont (Ed.), Molecular Aspects of Transport Proteins. Elsevier Science Publishers B. V., pp 219-245.

Grenson M., B. Acheroy (1982). Mutations affecting the activity and the regulation of the general amino-acid permease of *Saccharomyces cerevisiae*. Localisation of the cis acting dominant PGR regulatory mutation in the structural gene of this permease. Mol. Gen. Genet. 188: 261-265.

Grenson M., E. Dubois (1982). Pleiotropic deficiency in nitrogen-uptake systems and derepression of nitrogen-catabolic enzymes in *npr-1* mutants of *Saccharomyces cerevisiae*. Eur. J. Biochem. 121: 643-647.

Grenson M., C. Hou (1972). Ammonia inhibition of the general amino acid permease and its suppresion in NADPH-specific glutamate dehydrogenaseless mutants of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Comm. 48: 749-756.

Grenson M., C. Hennaut (1971). Mutation affecting activity of several distinct amino acid transport systems in *Saccharomyces cerevisiae*. J. Bacteriol. 105: 477-482.

Grenson M., C. Hou, M. Crabeel (1970). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. IV. Evidence for a general amino acid permease. J. Bacteriol. 103: 770-777.

Grenson M., F. Muyldermans, K. Broman, S. Vissers (1987). 4-Amino butyric acid (GABA) uptake in baker's yeast *Saccharomyces cerevisiae* is mediated by

the general amino acid permease, the proline permease and GABA-specific permease integrated into the GABA-catabolic pathway. Biochem. (Life Sci. adv.) 6: 35-39.

Gritz L., J. Davies (1983). Plasmid encoded hygromycin B resistance: The sequence of hygromycin B phosphotransferase gene and its expression in *E. coli* and *Saccharomyces cerevisiae*. Gene 25: 179-188.

Guarente L., R.R. Yoccum, P. Gifford (1982). A *GAL10/CYC1* hybrid yeast promoter identifies the *GAL4* site. Proc. Natl Acad. Sci., USA 79: 1279-1286.

Gunge N., Y. Nakatomi (1971). Genetic mechanisms of rare matings of the yeast *Saccharomyces cerevisiae* heterozygous for mating type. Genetics 70: 41-58.

Hadfield C., A.M. Cashmore, P.A. Mecock (1986). An efficient chloramphenicol resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli.* Gene 45: 149-158.

Hama H., T. Shimamoto, M. Tsuda, T. Tsuchiya (1987). Properties of Na^+-coupled serine-threonine transport system in *Escherichia coli.* Biochim. Biophys. Acta 905: 231-239.

Hammond J.R.M., K.W. Eckersley (1984). Fermentation properties of brewing yeast with killer character. J. Inst. Brew. 90: 167-177.

Hanahan D. (1983). Studies on transformants of *Escherichia coli* with plasmids. J. Mol. Biol. 166: 557-580.

Hansen J., M.C. Kielland-Brandt (1996). Inactivation of *MET10* in brewer's yeast specifically increases SO_2 formation during beer production. Nature Biotech. 14: 1587-1591.

Hansford R.G., B. Sacktor (1970). The control of the oxidation of proline by isolated flight muscle mitochondria. 245: 991-994.

Hanson A.D., B. Rathinasabapathi, J. Rivoal, M. Burnet, M.O. Dillon, D.A. Gage (1994). Osmoprotective compounds in the Plumbaginaceae - a natural experiment in metabolic engineering of stress tolerance. Proc. Natl Acad. Sci. USA 91: 306-310.

Hara S., Y. Iimura, K. Otsuka (1980). Breeding of useful killer wine yeast. Am. J. Enol. Vitic. 32: 28-33.

Harashima S., Y. Nogi, Y. Oshima (1974). The genetic system controlling homothallism in *Saccharomyces* yeasts. Genetics 77: 639-650.

Hardman J.K., T.C. Stadtman (1960). Metabolism of ω-amino acids. II. Fermentation of Δ-aminovaleric acid by *Clostridium aminovalericum*. J. Bacteriol. 79: 549-552.

Harris G.P. (1986). Phytoplankton ecology: structure, function and fluctuation. Chapman and Hall, London, New York, pp384.

Harris G., N.R. Merritt (1961). Comparison of the course of fermentation in a continuous and a batch brewing process. J. Inst. Brew. 67: 482-487.

Hashimoto H., H. Morikawa, Y. Yamada, A. Kimura (1985). A novel method for transformation of intact cells by electroinjection of plasmid DNA. Appl. Microbiol. Biotechnol. 21: 336-339.

Hayzer D.J., T. Leisinger (1980). The gene-enzyme relationships of proline biosynthesis in *Escherichia coli*. J. Gen. Microbiol. 118: 287-293.

Hayzer D.J., V. Moses (1978). The enzymes of proline biosynthesis in *Escherichia coli*: their molecular weights and the problem of enzyme aggregation. Biochem. J. 173: 219- 228.

Heirholzer G., H. Holzer (1963). Repression der synthese von DPN-abhangiger glutaminsauredehydrogenase in *Saccharomyces cerevisiae* durch ammoniumionen. Biochem. Z. 339: 175-185.

Henderson R.C.A., B.S. Cos, R. Tubb (1985). The transformation of brewing yeast with a plasmid containing the gene for copper resistance. Curr. Genet. 9: 135-138.

Henschke P.A., V. Jiranek (1991). Hydrogen sulfide formation during fermentation: Effect of nitrogen composition in model grape must. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wines (Seattle, WA, 1991),

edited by J.M. Rantz, pp 255-258. Dvies, CA: American Society for Enology and Viticulture.

Henschke P.A., V. Jiranek (1993). Metabolism of nitrogen compounds. In: Wine Microbiology and Biotechnology, Chapter 4. G.H. Fleet (ed.), Harwood Academic publishers, Chur, Switzerland, pp 77-164

Herskowitz I., Y. Oshima (1981). Control of cell type in *Saccharomyces cerevisiae*: mating-type interconversion. In: The Molecular Biology of the Yeast *Saccharomyces cerevisiae*: Life Cycle and Inheritance. J.N. Strathen, E.W. Jones, J.R. Broach (eds). Cold Spring Harbor Laboratory Press, New York, pp 181-209.

Herzfeld A., V.A. Mezl, W.E. Knox (1977). Enzymes metabolizing Δ'-pyrroline-5-carboxylate in rat tissues. Biochem. J. 166: 95-103.

Higgins C.F., M.P. Gallagher, S.C. Hyde, M.L. Mimmack, S.R. Pearce (1990). Periplasmic binding protein-dependent transport systems: the membrane-associated components. Philos. Trans. R. Soc. London Ser. B 326: 353-365.

Hill D.L., P. Chambers (1967). The biosynthesis of proline by *Tetrahymena pyriformis.* Biochim. Biophys. Acta 148: 435-447.

Hillman R.E., I. Albrecht, L.E. Rosenberg (1968). Identification and analysis of multiple glycine transport systems in isolated mammalian renal tubules. J. Biol. Chem. 243: 5566-5571.

Hinchcliffe E., E. Kenny, A. Leaker (1987). Novel products from surplus yeast via recombinant DNA technology. Eur. Brew. Con. Brew. Yeast Symp., Vuocranta (Helsinki), Monograph XII, pp 139-151.

Hinnen A., J.B. Hicks, G.R. Fink (1978). Transformation of yeast. Proc. Natl. Acad. Sci., USA. 75: 1929-1933.

Hinnen A., B. Meyhack, J. Heim (1989). Heterologous gene expression in yeast. In P.J. Barr, A.J. Brake, P. Valenzuela (Eds.), *Yeast genetic engineering*, Butterworths, pp 193-213.

Hitzeman R.A., F.A. Hagie, H.L. Levine, G.V. Goeddel, G. Ammerer, B.D. Hall (1981). Expression of human gene for interferon in yeast. Nature 293: 717-727.

Hitzeman R.A. (1983). Secretion of a human gene for interferon in yeast. Nature 293: 717-722.

Hodgins D., R.H. Abeles (1969). Studies of the mechanism of action of D-proline reductase: the presence on covalently bound pyruvate and its role in the catalytic process. Arch. Biochem. Biophys. 130: 274-285.

Hoekema A., R.A. Kastelstein, M. Vasser, H.A. de Boer (1987). Codon replacement in the *PGK1* gene of *Saccharomyces cerevisiae*: experimental approach to study the role of biased codon usage in gene expression. Mol. Cell. Biol. 7: 2914-2924.

Hoffmann, W. (1985). Molecular characterisation of the *CAN1* locus in *Saccharomyces cerevisiae*. J. Biol. Chem. 260: 11831-11837.

Holland J.P., M.J. Holland (1980). Structural comparison of 2 non-tandemly repeated yeast glyceraldehyde-3-phosphate dehydrogenase genes. J. Biol. Chem. 255: 2596-2605.

Horak J. (1986). Amino acid transport in eucaryotic microorganism. Biochim. Biophys. Acta 864: 223-256.

Horak J., A. Kotyk (1986). Energetics of L-proline uptake by *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 857: 173-179.

Horak J., L. Rihova (1982). L-proline transport in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 691: 144-150.

Houtman A.C., C.S. du Plessis (1981). The effects of juice clarity and several conditions promoting yeast growth on fermentation rate, the production of aroma components and wine quality. S. Afr. J. Enol. Vitic. 2: 71-81.

Houtman A.C., C.S. du Plessis (1986). Nutritional deficiencies of clarified white grape juices and their correction in relation to fermentation. S. Afr. J. Enol. Vitic. 7: 39-46.

Hu C.A., A.J. Delauney, D.P.S. Verma (1992). A bifunctional enzyme (Δ'-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc. Natl Acad. Sci. USA 89: 9354-9358.

Huang H.L., M.C. Brandriss (2000). The regulator of the yeast proline utilization pathway is differentially phosphorylated in response to the quality of the nitrogen source. Mol. Cel. Biol. 20: 892-899.

Huang A.H.C., A.J. Cavalieri (1979). Proline oxidase and water stress-induced proline accumulation in spinach leaves. Plant Physiol. 63: 531-535.

Huang Z., C.S. Ough (1989). Effect of vineyard location, varieties and rootstock on the juice amino acid copmosition of several cultivars. Am. J. Enol. Vitic. 40: 135-139.

Hummel W., M.R. Kula (1989). Dehydrogenases for the synthesis of chiral compounds. Eur. J. Biochem. 184: 1-13.

Hummel W., N. Weiss, M.R. Kula (1984). Isolation and characterization of a bacterium possessing L-phenylalanine dehydrogenase activity. Arch. Microbiol. 137: 47-52.

Ichihara A., E.A. Ichihara, M. Suda (1960). Metabolism of L-lysine by bacterial enzymes. IV. δ-aminovaleric acid-glutamic acid transaminase. J. Biochem. 48: 412-420.

Ingledew W.M., R. Kunkee (1985). Factors influencing sluggish fermentations of grape juice. Am. J. Enol. Vitic. 36: 65-76.

Innis M., M.J. Holland, P.C. McCabe, G.E. Cole, V.P. Wittman, K.W.K. Gelfand, J.P. Holland, J.H. Meade (1985). Expression, glycosylation and secretion of an *Aspergillus* glucoamylase by *S. cerevisiae*. Science 228: 2-9.

Itai C., L.G. Paleg (1982). Responses of the water stressed *Hordeum distichum* L. and *Cucumis sativus* to proline betaine. Plant Sci. Lett. 25: 329-335.

Ito H., Y. Fukuda, K. Murata, A. Kimura (1983). Transformation of intact cells treated with alkali cations. J. Bacteriol. 153: 163-168.

Ito H., Y. Fukuda, K. Murata, A. Kimura (1984). Transformation of intact cells treated with alkali cations or thiol compounds. Agri. Biol. Chem. 48: 341-347.

Jager H.J., H.R. Meyer (1977). Effect of Water Stress on Growth and Proline Metabolism of *Phaseolus vulgaris.* Oecologia (Berl.) 30: 83-96.

Jauniaux J.-C., M. De Baerdemaeker, M. Grenson (1989). Primary structure of the *Saccharomyces cerevisiae* general amino acid permease (GAP) deduced from nucleotide sequence of its cloned gene. Arch. Int. Physiol. Biochim. 97: B37

Jauniaux J.-C., M. Grenson (1990). *GAP1,* the general amino acid permease gene of *Saccharomyces cerevisiae.* Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases and nitrogen catabolite repression. Eur. J. Biochem. 190: 39-44.

Jauniaux J.-C., M. Vandenbol, S. Vissers, K. Broman, M. Grenson (1987). Nitrogen catabolite regulation of proline permease in *Saccharomyces cerevisiae.* Eur. J. Biochem. 164: 601-607.

Jayakumar A., R. Prasad (1978). Mechanism of active transport of proline in *Candida albicans* cells - a pathogenic strain of yeast. In: Biomembranes - Proceedings of the National Symposium on Biological Membranes and Model Systems. S.V. Talekar, P. Balaram, S.K. Podder and C.L. Khetrapal (eds). pp 140-146. Phonexis Press, India.

Jayakumar A., M. Singh, R. Prasad (1979). An inducible proline transport system in *Candida albicans.* Biochim. Biophys. Acta 556: 144-150.

Jenkins R.O., T.G. Cartledge, D. Lloyd (1984). Respiratory adaptation of anaerobically grown *Saccharomyces uvarum*: changes in distribution of enzymes. J. Gen. Microbiol. 130: 2809-2816.

Jimenez A., J. Davies (1980). Expression of a transposable antibiotic resistance element in *Saccharomyces cerevisiae.* Nature 287: 869-871.

Jiranek V. (1992). Hydrogen sulfide formation in *Saccharomyces cerevisiae* and its regulation by assimilable nitrogen. PhD Thesis, University of Adelaide, Australia.

Jiranek V., P. Langridge, P.A. Henschke (1991). Yeast nitrogen demand: selection criterion for wine yeast for fermenting low nitrogen musts. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wines, 18-19 June; Seattle, WA, J.M. Rantz (ed.). pp 266-269. American Society for Enology and Viticulture; Davies, CA, USA.

Johnston J.R., H.P. Reader (1983). Genetic control of flocculation. In: Yeast Genetics: Fundamental and Applied Aspects. J.F.T. Spencer, D.M. Spencer, A.R.W. Smith (eds). Springer-Verlang, New York, pp 205-224.

Joiris C.R., M. Grenson (1969). Specificity and regulation of a permease of dicarboxylic amino-acids in *Saccharomyces cerevisiae*. Arch. Internat. Physiol. Biochim. 77: 154-156.

Jones M., J.S. Pierce (1964). Absorption of amino acids from wort by yeast. J. Inst. Brew. 70: 307-315.

Jones M., M.J. Pragnell, J.S. Pierce (1969). Absorption of amino acids by yeast from a semi-defined medium simulating wort. J. Ins. Brew. 75: 520-536.

Jung K.D., U. Luttge (1980). Amino acid uptake by *Lemna gibba* by a mechanism with affinity to neutral L- and D-amino acids. Planta 150: 230-235.

Kalman M., D.R. Gentry, M. Cashel (1991). Characterization of the *Escherichia coli* K12 *gIts* glutamate permease gene. Mol. Gen. Genet. 225: 379-386.

Kasai T., S. Kiriyama (1988). Identification of δ-aminovaleric acid in feces of SPF-rats and its absence in those of germ-free rats. J. Nutr. Sci. Vitaminol. 34: 261-264.

Kaster K.R., S.G. Burgett, T.D. Ingolia (1984). Hygromycin B resistance and dominant selectable marker in yeast. Curr. Genet. 8: 353-358.

Kay W.W., A.F. Gronlund (1969a). Proline transport by *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 193: 444-455.

Kay W.W., A.F. Gronlund (1969b). Isolation of amino acid transport-negative mutants of *Pseudomonas aeruginosa* and cells with reprssed transport activity. J. Bacteriol. 98: 116-123.

Kay W.W., A.F. Gronlund (1969c). Amino Acid pool formation in *Pseudomonas aeruginosa.* J. Bacteriol. 97: 282-291.

Kay W.W., A.F. Gronlund (1969d). Amino Acid Transport in *Pseudomonas aeruginosa.* J. Bacteriol. 97: 273-281.

Kayama-Gonda Y., T. Kawasaki (1979). Role of lithium ions in proline transport in *Escherichia coli.* J. Bacteriol. 139: 560-564.

Keenan M.V., W.L. Alworth (1974). The inhibition of proline racemase by a transition state analogue: Δ-1-pyrroline-2-carboxylate. Biochem. Biophys. Res. Commun. 57: 500-504.

Kelly R, E. Register (1996). Isolation and sequence analysis of the cDNA encoding delta 1-pyrroline-5-carboxylate reductase from *Zalerion arboricola.* Gene 172: 149-153.

Kessel D., M. Lubin (1962). Transport of proline in *Escherichia coli.* Biochim. Biophys. Acta 57: 32-43.

Kielland-Brandt M.C., T. Nilsson-Tillgren, J.G. Litske-Peterson, S. Holmberg, C. Gjermansen (1983). Approaches to the genetic analysis and breeding of brewers yeast. In: Yeast Genetics, Fundamental and Applied Aspects. J.F.T. Spencer, D.M. Spencer (eds). Springer-Verlang, New York, pp 421-437.

King J., R. Hirji (1975). Amino acid transport systems of cultured soybean root cells. Can. J. Bot. 53: 2088-2091.

Kingsman S.M., A.J. Kingsman, M.J. Dobson, J. Mellor, N.A. Roberts (1985a). Heterologous gene expression in *Saccharomuces cerevisiae.* In: Biotechnol. Genet. Eng. Revs. G.E. Russell (ed.), Vol. 3, pp 377-416.

Kingsman S.M., A.J. Kingsman, J. Mellor (1985b). The production of mammalian proteins in *Saccharomyces cerevisiae.* TIBTECH. 5: 53-62.

Kingsman A.J., C. Stanway, S.M. Kingsman (1988). The expression of homologous and heterologous genes in yeast. Antonie van Leeuwenhoek J. Microbiol. 53: 325-333.

Kinraide T.B., B. Etherton (1980). Electrical evidence for different mechanisms of uptake for basic, neutral and acidic amino acids in oat coleoptiles. Plant Physiol. 65: 1085-1089.

Kishor P.B.K., Z. Hong, G.H. Miao, C.A.A. Hu, D.P.S. Verma (1995). Overexpression of D'-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol. 108: 1387-1394.

Kitamoto K., K. Yoshizawa, Y. Ohsumi, Y. Anraku (1988). Dynamic aspects of vacuolar and cytosolic amino acids pools of *Saccharomyces cerevisiae*. J. Bacteriol. 170: 2683-2686.

Klein L., P.D. Boyer (1972). Energization of active transport by *Escherichia coli*. J. Biol. Chem. 247: 7257-7265.

Kliewer W.M. (1970). Free amino acids and other nitrogenous substances of table grape varieties. J. Food Sci. 34: 274-278.

Kluba R.M., L.R. Mattick, L.R. Hackler (1978). Changes in the free and total amino acid composition of several *Vitis labruscana* varieties during maturation. Am. J. Enol. Vitic. 29: 102-111.

Knatchbull F.B., J.C. Slaughter (1987). The effect of low CO_2 pressure on the absorption of amino acids and production of flavour-active volatiles by yeast. J. Inst. Brew. 93: 420-424.

Kobayashi H., E. Kin, Y. Anraku (1974). Transport of sugars and amino acids in Bacteria. Source of energy and energy coupling reactions of the active transport systems for isoleucine and proline in *E. coli*. J. Biochem. 76: 251-261.

Kobayashi H., N. Nakazawa, S. Harashima, Y. Oshima (1990). A system for temperature controlled expression of a foreign gene with dual mode in *Saccharomyces cerevisiae*. J. Ferm. Bioeng. 69: 322-327.

Koch J., E. Sajak (1959). A review and some studies on grape protein. Am. J. Enol. Vitic. 10: 114-123.

Konings W.N., B. Pollman, A.J.M. Driessen (1992). Can the excretion of metabolite by bacteria be manipulated? FEMS Microbiol. Revs 88: 93-108.

Kotyk A., M. Dvorakova (1990). Transport of L-Tryptophan in *Saccharomyces cerevisiae*. Folia Microbiology 35: 209-217.

Kotyk A., M. Dvorakova, J. Koryta (1990). Deuterons cannot replace protons in active transport processes in yeast. FEBS Letters 264: 203-205.

Kozak M. (1989). The scanning model for translation: an update. J. Cell. Biol. 108: 229-241.

Kreger van Rij N.J.W. (1984). The Yeast: A Taxonomic Study, 3rd edition. Elsevier, Amsterdam.

Krishna R.V., P. Beilstein, T. Leisinger (1979). Biosynthesis of proline in *Pseudomonas aeruginosa*. Properties of γ-glutamyl phosphate reductase and Δ'-pyrroline-5-carboxylate reductase. Biochem. J. 181: 223-230.

Krishna R.V., T. Leisinger (1979). Biosynthesis of proline in *Pseudomonas aeruginosa*. Partial purification and characterization of γ-glutamyl kinase. Biochem. J. 181: 215-222.

Krueger R., H.J. Jager, M. Hintz, E. Pahlich (1986). Purification to homogeneity of pyrroline-5-carboxylate reductase of barley. Plant Physiol. 80: 142-144.

Kunkee R.E. (1991). Relationship between nitrogen content of must and sluggish fermentation. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wines (Seattle, WA, 1991). J.M. Rantz (ed), pp 148-154. Dvies, CA: American Society for Enology and Viticulture.

Kunkee R.E., R.W. Goswell (1977). Table wines. In: The Yeast, Volume 3. A.H. Rose & J.S. Harrison (eds). Academic Press, London, pp 315-385.

Kurland C.G. (1987). Strategies for efficiency and accuracy in gene expression 1. The major codon preference: a growth optimization strategy. Trends Biochem. Sci. 12: 126-128.

Lafon-Lafourcade S. (1986). Applied microbiology. Experientia 42: 904-914.

Lafon-Lafourcade S., E. Peynaud (1959). Dosage microbiologique des acides amines des mouts de raisin et des vins. Vitis 2: 45-56.

Lafon-Lafourcade S., G. Guimberteau (1962). Evolution des aminoacides au cours de la maturation des raisins. Vitis 3: 130-135.

Lagunas R., J.M. Gancedo (1973). Reduced pyridine-nucleotides balance in glucose-growing *Saccharomyces cerevisiae*. Eur. J. Biochem. 37: 90-94.

Laliberte G., J.A. Hellebust (1989). Pyrroline-5-carboxylate reductase in *Chlorella autotrophica* and *Chlorella saccarophila* in relation to osmoregulation. Plant Physiol. 91: 917-923.

Lanchashire W.E., A.T. Carter, J.J. Howard, R.J. Wilde (1989). Superattenuating brewing yeast. Proc. Eur. Brew. Conv. 22nd Cong., Zurich, pp 491-498.

Large P.J. (1986). Degradation of organic nitrogen compounds by yeasts. Yeast 2: 1-34.

LaRosa P.C., D. Rhodes, J.C. Rhodes, R.A. Bressan, L.N. Csonka (1991). Elevated accumulation of proline in NaCl adapted tobacco cells is not due to altered Δ'-pyrroline-5-carboxylate reductase. Plant Physiol. 96: 245-250.

Larue F., S. Lafon-Lafourcade (1989). Survival factors in wine fermentation. In: Alcohol tolerance in yeasts and bacteria. N.J. van Uden (Ed.), pp 193-215. CRC Press, Inc.: Boca Raton, Fla.

Lasko P.F., M.C. Brandriss (1981). Proline transport in *Saccharomyces cerevisiae*. J. Bacteriol. 148: 241-247.

Lautensach A., R.E. Subden (1984). Cloning of malic acid assimilating activity from *Leuconostoc oenos* in *E. coli*. Microbios 39: 29-39.

Law S.S., A.J. Mukkada (1979). Transport of L-proline and its regulation in *Leishmania tropica* Promastigotes. J. Protozool. 26: 295-301.

Lee W.J. (1987). Utilisation of nucleosides and nucleobases by the larger yeast, *Saccharomtces carlsbergensis*. Am. Soc. Brew. Chem. 45: 128-131.

Lee L.G., G.M. Whitesides (1986). Preparation of optically active 1,2-diols and α-hydroxy ketones using glycerol dehydrogenase as catalyst: limits to enzyme-catalyzed synthesis due to noncompetitive and mixed inhibition by product. J. Org. Chem. 51: 25-37.

Li W., M.C. Brandriss (1992). Proline biosynthesis in *Sachcaromyces cerevisiae*: molecular analysis of the PRO1 gene, which encodes gamma-glutamyl kinase. J. Bacteriol. 174: 4148-4156.

Liese A., M. Karutz, J. Kamphuis, C. Wandrey, U. Kragl (1996). Enzymatic resolution of 1-phenyl-1,2-ethanediol by enantioselective oxidation - overcoming product inhibition by continuous extraction. Biotech. Bioeng. 51: 544-550.

Lindegren C.C. (1943). A new method for hybridising yeast. Proc. Natl. Acad. Sci. USA 29: 306-308.

Lowry O.H., N.J. Rosebrough, A.L. Farr, R.J. Randall (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lundgren D.W., M. Ogur (1973). Inhibition of yeast Δ'-pyrroline-5-carboxylate dehydrogenase by common amino acids and the regulation of proline catabolism. Biochim. Biophys. Acta 297: 246-257.

Magana-Schwencke N., J. Schwencke (1969). A proline transport system in *Saccharomyces chevalieri.* Biochim. Biophys. Acta 173: 313-323.

Magana-Schwencke N., J. Kuznar, J. Schwencke (1973). Imino acid transport in yeast: The uptake of sarcosine. Biochim. Biophys. Acta 318: 281-288.

Mahan M.J., L.N. Csonka (1983). Genetic analysis of the *proBA* genes of *Salmonella typhimurium*: physical and genetic analyses of the cloned $proB^+A^+$ genes of *Escherichia coli* and of a mutant allele that confers proline overproduction and enhanced osmotolerance. J. Bacteriol. 156: 1249-1262.

Maloy S.R. (1987). The proline utilization operon. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, H.E. Umbarger (eds). American Society for Microbiology, Washington, DC, pp 1513-1519.

Maloy S.R., J.R. Roth (1983). Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put*::Mu *d*(Ap, *lac*) operon fusions. J. Bacteriol. 154: 561-568.

Marczak J.E., M.C. Brandriss (1991). Analysis of constitutive and noninducible mutations of the PUT3 transcriptional activator. Mol. Cell. Biol. 11: 2609-2619.

Margolis R.K., A. Lajtha (1968). Ion dependence of amino acid uptake in brain slices. Biochim. Biophys. Acta 163: 374-385.

Matsuzawa T., I. Ishiguro (1980). Δ'-pyrroline-5-carboxylate reductase from baker's yeast. Purification, properties and its application in the assays of L-Δ'-pyrroline-5-carboxylate and L-ornithine in tissue. Biochim. Biophys. Acta 613: 318-323.

Maw G.A. (1965). The role of sulfur in yeast and in brewing. Wallerstein. Lab. Comm. 28: 49-70.

Mazelis M., L. Fowden (1969). Conversion of ornithine into proline by enzymes from germinating peanut cotyledons. Phytochemistry 8: 801-809.

Mazelis M., L. Fowden (1971). The metabolism of proline in higher plants. II. L-proline dehydrogenase from cotyledons of germinating peanut (*Arachis hypogea* L.) seedlings. J. Exp. Bot. 22: 137-148.

Mazelis M., R.K. Creveling (1974). L-proline dehydrogenase of *Triticum vulgare* germ: purification, properties and cofactor interactions. Phytochemistry 13: 559-565.

McCue K.F., A.D. Hanson (1990). Drought and salt tolerance: towards understanding and application. Trends Biotechnol. 8: 358-362.

McGregor W.G., J. Phillips, C.H. Suelter (1974). Purification and Kinetic Characterization of a Monovalent Cation-activated Glycerol Dehydrogenase from *Aerobacter aerogenesI.* J. Biol. Chem. 249: 3132-3139.

McKelvey J., R. Rai, T.G. Cooper (1990). GABA transport in *Saccharomyces cerevisiae.* Yeast 6: 263-270.

McNamer A.D., C.R. Stewart (1974). Nicotinamide adenine dinucleotide-dependent proline dehydrogenase in *Chlorella.* Plant Physiol. 53: 440-444.

Mead G.C. (1971). The amino acid-fermenting Clostridia. J. Gen. Microbiol. 67: 47-56.

Meaden P.G., R.S. Tubb (1985). A plasmid vector system for the genetic manipulation of brewing strains. Proc. Eur. Brew. Con. 20th Cong., Helsinki, pp 219-226.

Meile L., L. Soldati, T. Leisinger (1982). Regulation of proline catabolism in *Pseudomonas aeruginosa.* Arch. Microbiol. 132: 189-193.

Meilgaard M.C. (1975). Flavor chemistry of beer: part II: flavor and threshold of 239 aroma volatiles. MBAA Tech. Quart. 12: 151-168.

Menzel R., J. Roth (1981a). Purification of the putA gene product. A bifunctional membrane-bound protein from *Salmonella typhimurium* responsible for the two-step oxidation of proline to glutamate. J. Biol. Chem. 256: 9755-9761.

Menzel R., J. Roth (1981b). Regulation of the genes for proline utilization in *Salmonella typhimurium*: autogenous repression by the putA gene product. J. Mol. Biol. 148: 21-44.

Messenguy F., D. Colin, J.P. Ten Have (1980). Regulation of compartmentation of amino acid pools in *Saccharomyces cerevisiae* and its effects on metabolic control. Eur. J. Biochem. 108: 439-447.

Meyer J. (1977). Proline transport in rat liver mitochondria. Arch. Biochem. Biophys. 178: 387-395.

Meyhack B., W. Bjawa, H. Rudolph, A. Hinnen (1982). Two yeast acid phosphatase structural genes are the results of tandem duplication and show different degrees of homology in their promoter and coding sequence. EMBO J. 1: 675- 680.

Mezl V.A., W.E. Knox (1977). Metabolism of arginine in lactating rat mammary gland. Biochem. J. 164: 105-113.

Miki B.L.A., N.H. Poon, A.P. James, V.L. Seligy (1980). Flocculation in *Saccharomyces cerevisiae*: mechanism of cell-cell interactions. In: Current Developments in Yeast Research. G.G. Stewart, I. Russell (eds). Pergamon Press, Canada, pp 165-170.

Miki B.L.A., N.H. Poon, V.L. Seligy (1982). Repression and induction of flocculation interactions in *Saccharomyces cerevisiae*. J. Bacteriol. 150: 890-899.

Miller D.L., V.W. Rodwell (1971). Metabolism of basic amino acids in *Pseudomonas putida*. Intermediates in L-arginine catabolism. 246: 5053-5058.

Miller P.M., C.R. Stewart (1976). Pyrroline-5-carboxylic acid reductase from soybean leaves. Phytochemistry 15: 1855-1857.

Milner J.L., S. Grothe, J.M. Wood (1988). Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. J. Biol. Chem. 263: 14900-14905.

Misono H., I. Kato, K. Packdibamrung, S. Nagata (1993). $NADP^+$-dependent D-threonine dehydrogenase from *Pseudomonas cruciviae* IFO 12047. Appl. Environ. Microbiol. 59: 2963-2968.

Miyake T., M. Demerec (1960). Proline mutants of *Salmonella typhimurium.* Genetics 45: 755-762.

Mogi T., Y. Anraku (1984a). Mechanism of proline transport in *Escherichia coli* K-12. 1. Effect of a membrane potential on the kinetics of 2H+/proline symport in cytoplasmic membrane vesicles. J.Biol. Chem. 259:7791-7796.

Mogi T., Y. Anraku (1984b). Mechanism of proline transport in *Escherichia coli* K-12. 2. Effect of alkaline cations on binding of proline to a H+/proline symport carrier in cytoplasmic membrane vesicles. J. Biol. Chem. 259:7797-7801.

Mogi T., Y. Anraku (1984c). Mechanism of proline transport in *Escherichia coli* K-12. 3. Inhibition of membrane potential-driven proline transport by syn-coupled ions and evidence for symmetrical transition states of the $2H^+$/proline symport carrier. J. Biol. Chem. 259: 7802-7806.

Molzahn S.W. (1977). A new approach to the application of genetics to brewing yeast. J. Am. Soc. Brew. Chem. 34: 54-59.

Moneton P., P. Sarthou, F. Le Goffie (1986). Role of the nitrogen source in peptide transport in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 36: 95-98.

Monk P.R. (1982). Effect of nitrogen and vitamin supplements on yeast growth and rate of fermentation of Rhine Riesling grape juice. Food Tech. Aust. 34: 328-332.

Monk P.R., D. Hook, B.M. Freeman (1987). Amino acid metabolism by yeast. In: Proceedings of the Sixth Australian Wine Industry Technical Conference; 14-17 July 1986; Adelaide, S.A. T.H. Lee (Ed.). pp 129-133. Australian Industrial Publishers, Adelaide, S.A.

Monteiro F.F., L.F. Bisson (1991). Biological assay of nitrogen content of grape juice and prediction of sluggish fermentations. Am. J. Enol. Vitic. 42: 47-57.

Monteiro F.F., L.F. Bisson (1992). Nitrogen supplementation of grape juice. I. Effect on amino acid utilization during fermentation. Am. J. Enol. Vitic. 43: 1-10.

Monticello D.J., R.N. Costilow (1981). Purification and partial characterization of proline dehydrogenase from *Clostridium sporogenes*. Can. J. Microbiol. 27: 942-948.

Morris C.J., J.F. Thompson, C.M. Johnson (1969). Metabolism of glutamic acid and N-acetylglutamic acid in leaf discs and cell-free extracts of higher plants. Plant Physiol. 44: 1023-1026.

Mountain H.A., A.S. Bystrom, J.T. Larsen, C. Korch (1991). Four major transcriptional responses in the methionine/threonine biosynthetic pathway of *Saccharomyces cerevisiae*. Yeast 7: 781-803.

Munck B.G. (1966). Amino acid transport by the small intestine of the rat. The existence and specificity of the transport mechanism of imino acids and its relation to the transport of glycine. Biochim. Biophys. Acta 120: 97-103.

Murray A.W., J.W. Szostak (1983). Construction of artificial chromosomes in yeast. Nature, 305: 189-193.

Muth W.L., R.N. Costilow (1974). Ornithine cyclase (deaminating). II. Properties of the homogeneous enzyme. J. Biol. Chem. 249: 7457-7462.

Naider F., J.M. Becker, E. Katzir-Katchalski (1974). Utilization of methionine-containing peptides and their derivatives by a methionin-requiring auxotroph of *Saccharomyces cerevisiae*. J. Biol. Chem. 249: 9-20.

Neame K.D. (1962). Uptake of L-histidine, L-proline, L-tyrosine and L-ornithine by brain, intestinal mucosa, testis, kidney, spleen, liver, heart muscle, skeletal muscle and erythrocytes of the rat *in vitro*. J. Physiol. 162: 1-12.

Noguchi M., A. Kowai, E. Tamaki (1966). Studies on nitrogen metabolism in tobacco plants. VII. Δ'-pyrroline-5-carboxylate reductase from tobacco leaves. Agr. Biol. Chem. 30: 452-456.

Nolte K.D., A.D. Hanson, D.A. Gage (1997). Proline accumulation and methylation to proline betaine in citrus - implications for genetic engineering of stress resistance. J. Am. Soc. Hortic. Sci. 122: 8-13.

Norbeck J., A.K. Pahlman, N. Akhtar, A. Blomberg, L. Adler (1996). Purification and characterisation of two isoenzymes of DL-glycerol 3-phosphatase from *Saccharomyces cerevisiae*. Identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 271: 13875-13881.

Nordstrom K. (1966). Yeast growth and glycerol formation. Acta Chem. Scand. 20: 1016-1025.

Nunberg J.H., J.H. Meade, G. Cole, F.C. Lawyer, P. McCabe, V. Schweickart, R. Tal, V.P. Wittman, J.E. Flatgaard, M.A. Innis

(1984). Molecular cloning and characterisation of the glucoamylase gene of *Aspergillus awamori*. Mol.Cell. Biol. 4: 2306-2311.

Ohshima T., H. Misono, K. Soda (1978). Properties of crystalline leucine dehydrogenase from *Bacillus sphaericus*. J. Biol. Chem. 253: 5719-5725.

Ohshima T., S. Nagata, K. Soda (1985). Purification and characterization of thermostable leucine dehydrogenase from *Bacillus stearothermophilus*. Arch. Microbiol. 141: 407-411.

Ohshima T., K. Soda (1979). Purification and properties of alanine dehydrogenase from *Bacillus sphaericus*. Eur. J. Biochem. 100: 29-39.

Ohshima T., K. Soda (1990). Biochemistry and biotechnology of amino acids dehydrogenases. Adv. Biochem. Eng/Biotechnol. 42: 187-208.

Olivera H., A. Gonzalez, A. Pena (1993). Regulation of the amino acid permeases in nitrogen-limited continuous cultures of the yeast *Saccharomyces cerevisiae*. Yeast 9: 1065-1078.

Orser C.S., B.W. Goodner, M. Johnston, S.B. Gelvin, L.N. Csonka (1988). The *Escherichia coli proB* gene corrects the proline auxotrophy of *Saccharomyces cerevisiae pro1* mutants. Mol. Gen. Genet. 212: 124-128.

Ouchi K., H. Akiyama (1971). Non-foaming mutants of sake yeasts. Selection by cell agglutination method and by froth flotation method. Agric. Biol. Chem. 35: 1024-1032.

Ouchi K., H. Akiyama (1976). Breeding of useful killer sake yeast by repeated back-crossing. J. Ferment. Technol. 54: 615-623.

Ough C.S. (1968). Proline content of California grapes and wines. Vitis 7: 321-331.

Ough C.S. (1969). Ammonia content of California grapes. Am. J. Enol. Vitic. 20: 213-220.

Ough C.S., M.A. Amerine (1988). Nitrogen compounds. In: Methods for Analysis of Musts and Wines, 2nd ed, pp 172-195. Wiley-Interscience, New York.

Ough C.S., E.A. Crowell (1980). Nitrate determination in California USA musts and wines. Am. J. Enol. Vitic. 31: 344-346.

Ough C.S., E.A. Crowell, L.A. Mooney (1988). Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acid, urea and ammonia effects of fortification on nitrocellular and extracellular precursors. Am. J. Enol. Vitic. 39: 243-249.

Ough C.S., A. Kriel (1985). Ammonia concentrations of musts from different grape cultivars and vineyards in the Stellenbosch area. S. Afr. J. Enol. Vitic. 6: 7-11.

Ough C.S., T.H. Lee (1981). Effect of vineyard nitrogen fertilization level on the formation of some fermentation esters. Am. J. Enol. Vitic. 32: 125-127.

Ough C.S., R.M. Stashak (1974). Further studies on proline concentration in grapes and wines. Am. J. Eno. Vitic. 25: 7-12.

Oura E. (1977). Reaction products of yeast fermentations. Proc. Biochem. 12: 19-21.

Oxender D.L. (1972). Membrane transport. Annu. Rev. Biochem. 41: 777-841.

Panchal C.J., C.A. Bilinski, I. Russell, G.G. Stewart (1986). Yeast stability in the brewing and industrial fermentation industries. Crit. Rev. Biotech. 4: 253- 262.

Parent S.A., C.M. Fenimore, K.A. Bostian (1985). Vector systems for the expression analysis and cloning of DNA sequences in *Saccharomyces cerevisiae.* Yeast 3: 175-185.

Pedersen S. (1984). *Escherichia coli* ribosomes translate *in vivo* with variable rate. EMBO J. 3: 2895-2898.

Peisach J., H.J. Strecker (1962). The interconversion of glutamic acid and proline. V. The reduction of Δ'-pyrroline-5-carboxylic acid to proline. J. Biol. Chem. 237: 2255-2260.

Pekur G.N., N.I. Bur'yan, N.M. Pavlenko (1981). Characteristics of nitrogen metabolism in wine yeast under different fermentation conditions. Appl. Biochem. Microbiol. 17: 248-252.

Peng Z., Q. Lu, D.P. Verma (1996). Reciprocal regulation of delta 1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. Mol. Gen. Genet. 253: 334-341.

Peraino C., H.J. Pitot (1963). Ornithine-δ-transaminase in the rat. I. Assay and some general properties. Biochem. Biophys. Acta 73: 222-231.

Perez-Gonzalez J.A., R. Gonzalez, A. Querol, J. Sendra, D. Ramon (1993). Construction of a recombinant wine yeast strain expressing β-(1,4)-Endoglucanase and its use in microvinification processes. Appl. Env. Microbiol. 59: 2801-2806.

Perl M., E.B. Kearney, T.P. Singer (1976). Transport of riboflavin into yeast cells. J. Biol. Chem. 251: 3221-3228.

Phang J.M. (1985). The Regulatory Functions of Proline and Pyrroline-5-carboxylic Acid. Curr. Top. Cell. Regul. 25: 91-132.

Phang J.M., J.S. Downing, G.C. Yeh, R.J. Smith, J.A. Williams (1979). Stimulation of the hexose-monophosphate pentose pathway by D'-pyrroline-5-carboxylic acid in human fibroblasts. Biochem. Biophys. Res. Commun. 87: 363-370.

Plattner H., G. Schatz (1969). Promitochondria of anaerobically grown yeast. III. Morphology. Biochemistry 8: 339-343.

Plattner H., M. Salpeter, J. Saltzgaber, W. Rouslin, G. Schatz (1971). Pro-mitochondria of anaerobically-grown yeast: evidence for their conversion into functional mitochondria during respiratory adaptation. In: Boardman N.K., Linnane A.W. and Smillie R.M. (Eds). Autonomy and biogenesis of mitochondria and chloroplasts, pp 175-184. North-Holland Publishing Company, Amsterdam.

Pretorius I.S., M.G. Lambrechts (1991). The glucoamylase multigene family in *Saccharomyces cerevisiae* var. *diastaticus*: an overview. Critic. Rev. Biochem. Mol. Biol. 26: 53-76.

Purvis I.J., A.J.E. Bettany, L. Loughlin, A.J.P. Brown (1987). Translation and stability of an *Escherichia coli* β-galactosidase mRNA expressed under the control of pyruvate kinase sequences in *Sacchsromyces cerevisiae*. Nucleic Acid Res. 15: 7963-7974.

Ramos E.H., L.C. de Bongioanni, A.O.M. Stoppani (1980). Kinetics of L-[^{14}C] Leucine transport in *Saccharomyces cerevisiae*. Biochim. Biophus. Acta 599: 214-231.

Rapp A., H. Franck (1971). Uber die Bildung von Athanol und einigen Aromastoffen bei Modellgarversuchen in Abhangigkeit von der Aminosaurekonzentration. Vitis 9: 299-311.

Rapp A., G. Versini (1991). Influence of nitrogen compounds in grapes on aroma compounds of wine. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wine (Seattle, WA, 1991), edited by J. Rantz, pp 156-164. Davis, CA: American Society for Enology and Viticulture.

Ratzkin B., M. Grabnar, J. Roth (1978). Regulation of the major proline permease gene of *Salmonella typhimurium*. J. Bacteriol. 133: 737-743.

Reed G., H.J. Peppler (1973). Wine yeast. In: Yeast Technology. 206 pp. AVI Publishing Co., Westport, CT.

Regenbeberg B., L. During-Olsen, M.C. Kielland-Brandt, S. Holmberg (1999). Substrate specificity and gene expression of amino-acid permeases in *Saccharomyces cerevisiae*. Curr. Genet. 36: 317-328.

Reizer J., A. Reizer, M.H. Saier (1990). The Na^+/pantothenate symporter (PanF) of *Escherichia coli* is homologous to the Na^+/proline symporter (PutP) of *E. coli* and the Na^+/glucose symporter of mammals. Res. Microbiol. 141: 1069-1072.

Rena A.B., W.E. Splittstoesser (1975). Proline dehydrogenase and Δ'-pyrroline-5-carboxylic acid reductase from pumpkin cotyledons. Phytochemistry 14: 657-662.

Rink H., M. Liersch, P. Sieber, F. Meyer (1984). A large fragment approach to DNA synthesis: total synthesis of a gene for the protease inhibitor eglin c from the leech *Hirud medicinalis* and its expression in *E. coli.* Nucl. Acids Res. 12: 6369-6387.

Roberts E., (1954). Studies of transamination. Arch. Biochem. Biophys. 48: 395-401.

Robinson S.P., H. Beevers (1981). Amino acid transport in germinating castor bean seedlings. Plant Physiol. 68: 560-566.

Rodopulo A.K., I.A. Egorov, A.F. Pisarnitski, A.A. Martakov, T.N. Levchenko (1969). Amino acid composition of sherries. Prikl. Biobkim. Mikrobiol. 5: 186-189.

Rodriguez R.L., D.T. Denhardt (1988). Vectors: A Survey of Molecular Cloning Vectors and their Uses. Butterworth Publishers, USA.

Rogers T.O., H.C. Lichstein (1969). Characterisation of the biotin transport system in *Saccharomyces cerevisiae.* J. Bacteriol. 100: 557-564.

Romano P., M.G. Soli, G. Suzzi, L. Garzia, C. Zambonelli (1985). Improvement of a wine *Saccharomyces cerevisiae* strain by a breeding program. Appl. Environ. Microbiol. 50: 1064-1067.

Roon R.J., H.L. Even (1973). Regulation of the nicotinamide adenine dinucleotide- and nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenases of *Saccharomyces cerevisiae.* J. Bacteriol. 116: 367-372.

Roon R.J., H. Even, F. Larimore (1974). Glutamate synthase: Properties of the reduced nicotinamide adenine dinucleotide-dependent enzyme from *Saccharomyces cerevisiae.* J. Bacteriol. 118: 89-95.

Rose A.H., M.H.J. Keenan (1981). Amino acid uptake by *Saccharomyces cerevisiae.* In: Proceedings of the European Brewing Convention Congress, Copenhagen, 1981, Denmark, pp. 207-216. IRL Press. London, U.K.

Rose M., P. Grisafi, D. Botstein (1984). Structure and function of the yeast *URA3* gene: expression in *Escherichia coli.* Gene 29: 113-124.

Rosenberg S., P.J. Barr, R.C. Najarian, R.A. Hallewell (1984). Synthesis in yeast of a functional oxidation resistant mutant of human α-antitrypsin. Nature, 312: 77-80.

Rosenbusch J.P., B. Flanagan, G. Nichols (1967). Active transport of amino acids into bone cells. Biochim. Biophys. Acta 135: 732-740.

Rossi J.J., J. Vender, C.M. Berg, W.H. Coleman (1977). Partial purification and some properties of Δ'-pyrroline-5-carboxylate reductase from *Escherichia coli*. J. Bacteriol. 129: 108-114.

Rothstein M. (1965). Intermediates of lysine dissimilation in the yeast, *Hansenula saturnus*. Arch. Biochem. Biophys. 111: 467-476.

Rous C.V., R. Snow (1983). Reduction of higher alcohols by fermentation with a leucine auxotrophic mutant of wine yeast. J. Inst. Brew. 89: 274-278.

Rowland I., H. Tristram (1975). Specificity of the *Escherichia coli* Proline Transport System. J. Bacteriol. 123: 871-877.

Rupela O.P., P. Tauro (1984). Isolation and characterisation of low hydrogen sulphide producing wine yeasts. Enz. Microb. Technol. 6: 419-421.

Russell I., I.F. Hancock, G.G. Stewart (1983). Construction of dextrin fermentative yeast strains that do not produce phenolic off flavours in beer. J. Am. Soc. Brew. Chem. 41: 45-51.

Rytka J. (1975). Positive selection of general amino acid permease mutants in *Saccharomyces cerevisiae*. J. Bacteriol. 121: 562-570.

Sambrook J., E.F. Fritsch, T. Maniatis (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sanderson K.E. (1972). Linkage map of *Salmonella typhimurium,* edition IV. Bacteriol. Rev. 36: 558-586.

Sanderson K.E., P.E. Hartman (1978). Linkage map of *Salmonella typhimurium*, 5th ed. Microbiol. Rev. 42: 471-519.

Saradi A., P.P. Saradhi (1991). Proline accumulation under heavy metal stress. J. Plant Physiol. 138: 554-558.

Sato T., Y. Ohsumi, Y. Anraku (1984). Substrate specificities of active transport systems for amino acids in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. J. Biol. Chem. 259: 11505-11508.

Sauer N., E. Komor, W. Tanner (1983). Regulation and characterization of two inducible amino-acid transport systems in *Chlorella vulgaris*. Planta 159: 404-410.

Scarpulla R.C., R.L. Soffer (1978). Membrane-bound proline dehydrogenase from *Escherichia coli*. Solubilization, purification and characyerisation. J. Biol. Chem. 253: 5997-6001.

Schmitt M.E., T.A. Brown, L. Trumpower (1990). A rapis and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucl. Acids Res. 18: 3091.

Schobert B. (1977). Is there an osmotic regulatory mechanism in algae and higher plants?. J. Theor. Biol. 68: 17-26.

Schobert B., H. Tschesche (1978). Unusual solution properties of proline and its interaction with proteins. Biochim. Biophys. Acta 541: 270-277.

Schultz S.G., R. Zalusky (1965). Interactions between active sodium transport and active amino-acid transport in isolated rabbit ileum. Nature 205: 292-294.

Schwartz A.C., W. Muller (1979). NADH-dependent reduction of D-proline in *Clostridium sticklandii*. Reconstruction from three fractions containing NADH dehydrogenase, D-proline reductase and a third protein factor. Arch. Microbiol. 123: 203-208.

Schwencke J., N. Magana-Schwencke (1969). Derepression of a proline transport system in *Saccharomyces cerevisiae* by nitrogen starvation. Biochim. Biophys. Acta 173: 302-312.

Seaston A., C. Inkson, A.A. Eddy (1973). The absorption of protons with specific amino acids and carbohydrates by teast. Biochem. J. 134: 1031-1043.

Seki T., S. Myoga, S. Limtong, S. Uedono, J. Kumnuanta, H. Taguchi (1983). Genetic construction of yeast strains for high ethanol production. Biotechnol. Lett. 5: 351-356.

Serrano R. (1984). Plasma membrane ATPase of fungi and plants as a novel type of proton pump. Curr. Top. Cell. Regul. 23: 87-126.

Seto B. (1978). A pyruvate-containing peptide of proline reductase in *Clostridium sticklandii*. J. Biol. Chem. 253: 4525-4529.

Seto B. (1980). Chemical characterization of an alkali-labile bond in the polypeptide of proline reductase from *Clostridium sticklandii*. J. Biol. Chem. 255: 5004-5006.

Seto B., T.C. Stadtman (1976). Purification and properties of proline reductase from *Clostridium sticklandii*. J. Biol. Chem. 251: 2435-2439.

Sharp P.M., E. Cowe (1991). Synonymous codon usage in *Saccharomyces cerevisiae*. Yeast 7: 657-678.

Shimizu I., J. Nagai, H. Hatanaka, H. Katsuki (1973). Mevalonate synthesis in the mitochondria of yeast. Biochim. Biophys. Acta 296: 310-320.

Shtarkshall R.A., L. Reinhold, H. Harel (1970). Transport of amino acids in barley leaf tissues. Evidence for a specific uptake mechanism and the influence of "aging" on accumulation capacity. J. Exp. Bot. 21: 915-925.

Shukla H., L. Viswanathan, M. Singh (1982). A specific inducible serine transport system in distiller's yeast *Saccharomyces cerevisiae*. Biochem. Internat. 5: 253- 262.

Shuster J.R. (1989). Regulated transcriptional systems for the production of proteins in yeast: regulation by carbon source. In: Yeast Genetic Engineering. P.J. Barr, A.J. Brake, P. Valenzuela (eds.). Butterworths, Boston, pp 83-108.

Siddiqui A.H., M.C. Brandriss (1988). A regulatory region responsible for proline-specific induction of the yeast *PUT2* gene is adjacent to its TATA box. Mol. Cell. Biol. 8: 4634-4641.

Siddiqui A.H., M.C. Brandriss (1989). The *Saccharomyces cerevisiae PUT3* activator protein associated with proline-specific upstream activation sequences. Mol. Cell. Biol. 9: 4706-4712.

Sigler K., A. Knotkova, J. Paca, M. Wurst (1980). Extrusion of metabolites from baker's yeast during glucose-induced acidification. Folia Microbiol. 25: 311-317.

Sigler K., A. Knotkova, A. Kotyk (1981a). Factors governing substrate-induced generation and extrusion of protons in the yeast *Saccharomyces cerevisiae.* Biochim. Biophys. Acta 643: 572-582.

Sigler K., A. Kotyk, A. Knotkova, M. Opekarova (1981b). Processes involved in the creation of buffering capacity and in substrate-induced proton extrusion in the yeast *Saccharomyces cerevisiae.* Biochim. Biophys. Acta 643: 583-592.

Simon J.R., K. McEntee (1989). A rapid and efficient procedure for the transformation of intact *Saccharomyces cerevisiae* by electroporation. Biochem. Biophys. Res. Commun. 164: 1157-1164.

Singh T.N., D. Aspinall, L.G. Paleg, S.F. Boggess (1973). Stress metabolism. II. Changes in proline concentration in excised plant tissues. Aus. J. Biol. Sci. 26: 57-63.

Skipper N., M. Sutherland, R.W. Davies, D. Kilburn, R.C. Miller, A. Warren, R. Wong (1985). Secretion of bacterial cellulase by yeast. Science 230: 958-961.

Sledziewski A., A. Bell, K. Kelsay, V.L. Mackay (1988). Construction of temperature regulated yeast promoters using the MATα2 repression system. Biot/Technology 6: 411-416.

Smith L.T. (1985). Characterization of a g-glutamyl kinase from *Escherichia coli* that confers proline overproduction and osmotic tolerance. J. Bacteriol. 164: 1088-1093.

Smith E.L., B.M. Austen, K.M. Blumenthal, J.F. Nyc (1975). Glutamate Dehydrogenases. In: The Enzymes. P.D. Boyer (ed.), 3rd Ed. Vol. 11, pp 293-367, Academic Press, New York.

Smith C.J., A.H. Deutch, K.E. Rushlow (1984). Purification and characteristics of a γ-glutamyl kinase involved in *Escherichia coli* proline biosynthesis. J. Bacteriol. 157: 545-551.

Smith R.J., S.J. Downing, J.M. Phang, R.F. Lodato, T.T. Aoki (1980). Pyrroline-5-carboxylate synthase activity in mammalian cells. Proc. Natl Acad. Sci. USA 77: 5221-5225.

Smith R.J., J.M. Phang (1978). Proline metabolism in cartilage: the importance of proline biosynthesis. Metabolism 27: 685-694.

Smith R.J., J.M. Phang (1979). The importance of ornithine as a precursor for proline in mammalian cells. J. Cell. Physiol. 98: 475-482.

Sokal R.R., F.J. Rohlf (1981). Biometry. The Principles and Practice of Statistics in Biological Research. 2nd edition. W.H. Freeman and Company, New York, pp 859.

Sone H., K. Kondo, T. Fujui, F. Shimuzu, J. Tanaka, T. Inoue (1987). Fermentation properties of brewers yeast having α-acetolactate decarboxylase gene. Proc. Eur. Brew. Conv. 21st Cong., Madrid, pp 545-552.

Soussi-Boudekou S., B. Andre (1999). A co-activator of nitrogen-regulated transcription in *Saccharomyces cerevisiae*. Mol. Microbiol. 31: 753-762.

Southern E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Spencer R.P., K.R. Brody (1964). Intestinal transport of cyclic and noncyclic imino acids. Biochim. Biophys. Acta 88: 400-406.

Splittstoesses W.E., L. Fowden (1973). Ornithine transaminase from *Cucurbita maxima* cotyledons. Phytochem. 12: 785-790.

Splittstoesser S.A., W.E. Splittstoesser (1973). Pyrroline-5-carboxylate reductase from *Cucurbita* cotyledons. Phytochemistry 12: 1565-1568.

Stadtman T.C. (1956). Studies on the enzymic reduction of amino acids: A proline reductase of an amino acid-fermenting *Clostridium* strain HF. Biochem. J. 62: 614-621.

Stadtman T.C., P. Elliot (1957). Studies on the enzymic reduction of amino acids. II. Purification and properties of a D-proline reductase and a proline racemase from *Clostridium sticklandii*. J. Biol. Chem. 228: 983-997.

Stalmach M.E., S. Grothe, J.M. Wood (1983). Two proline porters in *Escherichia coli* K-12. J. Bacteriol. 156: 481-486.

Stein W.D. (1986). Transport and Diffusion Across Cell Membranes. Academic Press, New York, NY.

Stein W.D. (1990). Channels, Carriers and Pumps. An Introduction to Membrane Transport. Academic Press, New York.

Stewart C.R. (1981). Proline accumulation: Biochemical aspects. In: The Physiology and Biochemistry of Drought Resistance in Plants. L.G. Paleg, D. Aspinall (eds), pp 243-260. Academic Press, Sydney.

Stewart C.R., S.F. Boggess (1978). Metabolism of 5-3H proline by barley leaves and its use in measuring the effect of water stress on proline oxidation. Pl. Physiol. 61: 654-657.

Stewart C.R., A.D. Hanson (1980). Proline accumulation as a metabolic response to water stress. In: Adaptation of Plants to Water and high Temperature Stress. N.C. Turmer, P.J. Kramer (eds). John Wiley & Sons, New York, pp 173-189.

Stewart C.R., E.Y. Lai (1974). Δ'-Pyrroline-5-carboxylic acid dehydrogenase in mitochondrial preparations from plant seedlings. Plant Sci. Lett. 3: 173-181.

Stewart G.G., I. Russell, C. Panchal (1981). The genetic manipulation of industrial yeast strains. In: Current developments in yeast research. G.G. Stewart, I. Russell (eds.). Pergamon Press, Toronto. Pp 14-24.

Stinchcomb D.T., C. Mann, R.W. Davis (1982). Centromeric DNA from *Saccharomyces cerevisiae*. J. Mol. Biol. 158: 157-179.

St John T.P., R.W. Davis (1981). The organisation and transcription of the galactose gene cluster of *Saccharomyces cerevisiae*. J. Mol. Biol. 152: 285-315.

Stratford M. (1992). Yeast flocculation: Reconciliation of physiological and genetic view points. Yeast 8: 25-38.

Stratford M., H.M. Brundish (1990). Yeast flocculation: cationic inhibition. Yeast 6: 77-86.

Stratford M., A.H. Rose (1985). Hydrogen sulphide production from sulphite by Saccharomyces cerevisiae. J. Gen. Microbiol. 131: 1417-1424.

Strecker H.J. (1960). The interconversion of glutamic acid and proline. II. The preparation and properties of Δ'-pyrroline-5-carboxy*lic acid. J. Biol. Chem. 235: 2045-2050.*

Strecker H.J. (1971). The preparation of animal proline oxidase (rat liver) and its use for the preparation of Δ'-pyrroline-5-carboxylate. In: Methods in Enzymology, Vol. 17B: Metabolism of Amino Acids and Amines, Section IV: Proline and Hydroxyproline. H. Tabor, C. White-Tabor (eds). Academic Press, pp 251-261

Struhl K., R.W. Davis (1980). A physical, genetic and transcriptional map of yeast *HIS3* gene of *Saccharomyces cerevisiae*. J. Mol. Biolo. 136: 309-332.

Suihko M.L., M. Penttila, H. Sone, S. Home, K. Blomqvist, J. Tanaka, T. Inoue, J. Knowles (1989). Pilot brewing with α-acetolactate decarboxylase active yeast. Proc. Eur. Brew. Conv. 22nd Cong., Zurich, pp 483-490.

Swick R.W., S.L. Tollaksen, S.L. Nance, J.F. Thomson (1970). The unique distribution of ornithine aminotransferase in rat liver mitochondria. Arch. Biochem. Biophys. 136: 212-218.

Tanaka J., G.R. Fink (1985). The histidine permease gene *HIP1* of *Sacharomyces cerevisiae*. Gene 38: 205-241.

Tews J.K., A.E. Harper (1969). Transport of nonmetabolizable amino acids in rat liver slices. Biochim. Biophys. Acta 183: 601-610.

Ter Schure E.G., H.H. Sillje, E.E. Vermeulen, J.W. Kalhorn, A.J. Verkleij, J. Boonstra, C.T. Verrips (1998). Repression of nitrogen catabolic genes by ammonia and glutamine in nitrogen-limited continuous cultures of *Saccharomyces cerevisiae*. Microbiology 144: 1451-1462.

Thomas D., R. Barbey, Y. Surdin-Kerjan (1990). Gene-enzyme relationship in the sulfate assimilation pathway of *Saccharomyces cerevisiae*. Study of the 3'-phosphoadenylysulfate reductase structural gene. J. Biol. Chem. 265: 15518-15524.

Thornton R.J. (1985). The introduction of flocculence into a homothallic wine yeast. Am. J. Enol. Vitic. 36: 47-49.

Tipper D.J., K.A. Bostian (1984). Double stranded ribonucleic acid and killer systems in yeast. Microbiol. Rev. 48: 125-156.

Tomenchok D.M., M.C. Brandriss (1987). Gene-enzyme relationships of the proline biosynthetic pathway in *Saccharomyces cerevisiae*. J. Bacteriol. 169: 5364-5372.

Townsend D.E., B.J. Wilkinson (1992). Proline transport in *Staphylococcus aureus*: a high-affinity system and a low-affinity system involved in osmoregulation. J. Bacteriol. 174: 2702-2710.

Treichel, S. (1986). The influence of NaCl on Δ'-pyrroline-5-carboxylate reductase in proline-accumulating cell suspension cultures of *Mesembryanthemum nodiflorum* and other halophytes. Physiol. Plant. 67: 173-181.

Tristram H., S. Neale (1968). The activity and specificity of the proline permease in wild-type and analogue-resistant strains of *Escherichia coli*. J. Gen. Microbiol. 50: 121-137.

Troll W., J. Lindsley (1955). A photometric method for the determination of proline. J. Biol. Chem. 215: 655-660.

Tromp A. (1984). The effect of yeast strain, grape solids, nitrogen and temperature on fermentation rate and wine quality. S. Afr. J. Enol. Vitic. 5: 1-6.

Tschumper G., J. Carbon (1980). Sequence of a yeast DNA fragment containing a chromosomal replicator on the *TRP* gene. Gene 10: 157-166.

Tschumper G., J. Carbon (1983). Copy number control by a yeast centromere. Gene 23: 221-232.

Tsukada K. (1966). Δ-amino acid dehydrogenases of *Pseudomonas fluorescens*. J. Biol. Chem. 241: 4522-4528.

Tubb R.S., J.R.M. Hammond (1987). Yeast genetics. In: Brewing microbiology. F.G. Priest, I. Campbell (eds.). Elsevier Applied Science Publishers, Essex, England, pp 47-82.

Tuite M.R., M.J. Dobson, N.A. Roberts, R.M. King, D.C. Burke, S.M. Kingsman, A.J. Kingsman (1982). Regulated high efficiency expression of human interferon-alpha in *Saccharomyces cerevisiae*. EMBO J. 1: 603-608.

Urdea M.S., J.P. Merryweather, G.T. Mullenbach, D. Coit, U. Heberlein, P. Valenzuela, P.J. Barr (1983). Chemical synthesis of a gene for human epidermal growth factor uragastrone and its expression in yeast. Proc. Natl Acad. Sci. USA 80: 7461-7465.

Valle D., R.M. Blaese, J.M. Phang (1975). Increased sensitivity of lynphocete D'-pyrroline-5-carboxylate reductase to inhibition by proline with transformation. Nature 253: 214-216.

Valle D., S.J. Downing, S.C. Harris, J.M. Phang (1973). Proline biosynthesis: multiple defects in chinese hamster ovary cells. Biochem. Biophys. Res. Com. 53: 1130-1136.

Vandenbol M., J-C. Jauniaux, S. Vissers, M. Grenson (1987). Isolation of the *NPR1* gene responsible for the reactivation of ammonia-sensitive amino-acid permeases in *Saccharomyces cerevisiae*. Eur. J. Biochem. 164: 607-612.

Vandenbol M., J-C. Jauniaux, M. Grenson (1989). Nucleotide sequence of the *Saccharomyces cerevisiae PUT4* proline-permease-encoding gene: similarities between CAN1, HIP1 and PUT4 permeases. Gene 83: 153-159.

Van der Westhuizen T.J., I.S. Pretorius (1989). Genetic characterisation and breeding of wine yeast. S. Afr. Soc. Enol. Vitic. Cong. 13th, Cape town.

Van Dijken J.P., W.A. Scheffers (1986). Redox balances in the metabolism of sugars by yeast. FEMS Microbiol. Revs. 32: 199-224.

Van Solingen P., J.B. van der Platt (1977). Fusion of yeast spheroplasts. J. Bacteriol. 130: 946-947.

Van Uden N.J., (1989). Effects of alcohols on membrane transport in yeast. In: Alcohol tolerance in yeasts and bacteria. N.J. van Uden (ed.), pp 135-146. CRC Press, Inc.: Boca Raton, Fla.

Van Veen H.W., T. Abee, A.W.F. Kleefsman, B. Melgers, G.J.J. Kortstee, W.N. Konings, A.J.B. Zehnder (1994). Energetics of Alanine, Lysine, and Proline Transport in Cytoplasmic Membranes of the Polyphosphate-Accumulating *Acinetobacter johnsonii* Strain 210A. J. Bacteriol. 176: 2670-2676.

Verma R.S., T.V.G. Rao, R. Prasad (1984). An inducible, specific and derepressible transport of L-serine in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 778: 289-297.

Vezinhet F., P. Barre, M. Laurent, M. Valade (1992). Introduction of flocculation into an industrial yeast strain by transfer of a single chromosome. J. Inst. Brew. 98: 315-319.

Visser W., A.A. van der Bann, W. Batenburg-van der Vegte, W.A. Scheffers, R. Kramer, J.P. van Dijken (1994). Involvement of

mitochondria in the assimilatory metabolism of anaerobic *Saccharomyces cerevisiae* cultures. Microbiology 140: 3039-3046.

Visser W., E.A. van Spronsen, N. Nanninga, J.T. Pronk, J.G. Kuenen, J.P. van Dijken (1995). Effects of growth conditions on mitochondrial morphology in *Saccharomyces cerevisiae*. Antonie van Leeuwenhoek 67: 243-253.

Vogel H.J., D.M. Bonner (1954). On the glutamate-proline-ornithine interrelation in *Neurospora crassa*. Proc. Natl Acad. Sci. USA 40: 688-694.

Vos P.J.A., E. Crous, L. Swart (1980). Fermentation and the optimal nitrogen balance of musts. Die Wynboer 582: 58-60.

Vos P.J.A., R.S. Gray (1979). The origin and control of hydrogen sulfide during fermentation of grape must. Am. J. Enol. Vitic. 30: 187-197.

Vos P.J.A., W. Zeeman, H. Heymann (1979). The effect on wine quality of di-ammonium phosphate addition to musts. In: Proceedings of the South African Society for Enology and Viticulture; 3-4 October 1978; Cape Town, South Africa. Stellenbosh: SASEV; pp 87-104.

Walmsley R.M., D.C. Gardner, S.G. Oliver (1983). Stability of a cloned gene in yeast grown in chemostat culture. Mol. Gen. Genet. 194: 361-365.

Wang S.-S., M.C. Brandriss (1986). Proline utilization in *Saccharomyces cerevisiae*: analusis of the cloned *PUT1* gene. Mol. Cell. Biol. 6: 2638-2645.

Wang S.-S., M.C. Brandriss (1987). Proline utilization in *Saccharomyces cerevisiae*: sequence, regulation and mitochondrial localization of the *PUT1* gene product. Mol. Cell. Biol. 7: 4431-4440.

Wasmuth J.J., C.T. Caskey (1976). Biochemical characterization of azedine carboxylic acid-resistant chinese hamster cells. Cell 8: 71-77.

Watari J., Y. Takata, M. Ogawa, N. Nishikawa, M. Kamimura (1989). Molecular cloning of a flocculation gene in *S. cerevisiae*. Agri. Biol. Chem. 53: 901-903.

Watari J., Y. Takata, M. Ogawa, H. Sahara, S. Koshino, M.L. Onnela, U. Airaksinen, R. Jaatinen, M. Penttilla, S. Keranen (1994a). Molecular cloning and analysis of the yeast flocculation gene *FLO1*. Yeast 10: 211-225.

Watari J., M. Nomura, H. Sahara, S. Koshino, S. Keranen (1994b). Construction of flocculent brewers yeast by chromosomal integration of the yeast flocculation gene *FLO1*. J. Inst. Brew. 100: 73-77.

Watson T.G. (1976). Amino-acid pool composition of *Saccharomyces cerevisiae* as a function of growth rate and amino acid nitrogen source. J. Gen. Microbiol. 96: 263-268.

Webster T.D., R.C. Dickson (1983). Direct selection of *Saccharomyces cerevisiae* resistant to the antibiotic G418 following transformation with a DNA vector carrying the kanamycin resistance gene of Tn903. Gene 26: 243-252.

Weimken A., M. Durr (1974). Characterisation of the amino acid pools in the vacuolar compartment of *Saccharomyces cerevisiae*. Arch. Microbiol. 101: 45-57.

Wiame J.M., M. Grenson, H.N. Arst Jr. (1985). Nitrogen catabolite repression in yeast and filamentous fungi. Advan. Micro. Phys. 26: 1-88.

Williams P.J., C.R. Strauss, B. Wilson, R.A. Massy-Westropp (1982). Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wine. Phytochemistry 21: 2013-2020.

Wilson D.B. (1978). Cellular transport mechanisms. Annu. Rev. Biochem. 47: 933-965.

Winge O. (1935). On haplophase and diplophase in some Saccharomycetes. C. R. Lab. Carlsberg Ser. Physiol. 22: 77-112.

Winge O. & C. Roberts (1958). In: The Chemistry and Biology of Yeast. H. Cook (ed.). Academic Press, New York, pp 123-156.

Winkler H.H., R.M. Daugherty (1984). Proline transport and metabolism in *Rickettsia prowazekii*. J. Bacteriol. 158: 460-463.

Wiskich J.T. (1977). Mitochondrial metabolite transport. Annu. Rev. Plant. Physiol. 28: 45-69.

Wood J.M. (1981). Genetics of L-proline utilization in *Escherichia coli*. J. Bacteriol. 146: 895-901.

Wood J.M. (1987). Membrane association of proline dehydrogenase in *Escherichia coli* is redox dependent. Proc. Natl Acad. Sci. USA 84: 373-377.

Wood J.M., K.A.C.C. Taylor, D.J. McClellan, G.G. Lawrie, R.L. Krogsrud (1987). Isolation and characterization of monoclonal antibodies to proline dehydrogenase from *Escherichia coli* K-12. Biochem. Cell Biol. 65: 507-513.

Wood J.M., D. Zadworny (1979). Characterization of an inducible porter required for L-proline catabolism by *Escherichia coli* K12. Can. J. Biochem. 57: 1191-1199.

Woodward J.R., H.L. Kornberg (1980). Membrane proteins associated with amino acid transport by yeast *Saccharomyces cerevisiae*. Biochem. J. 192: 659-664.

Wyse R.E., E. Komor (1984). Mechanism of amino acid uptake by sugarcane suspension cells. Plant Physiol. 76: 865-870.

Yip M.C.M., W.E. Knox (1972). Function of arginase in lactating mammary gland. Biochem. J. 127: 893-899.

Yocum R.R. (1986). Genetic engineering of industrial yeast. Proc. Bio. Expo. 86, Butterworths, Stoneham, pp 171-180.

Yoshida A., E. Freese (1965). Enzymic properties of alanine dehydrogenase of *Bacillus subtilis*. Biochim. Biophys. Acta 96: 248-262.

Yoshida K., A. Wadano, K. Miura (1977). Metabolism of proline and its physiological significance in a blowfly *Aldrichina grahami*. Insect Biochem. 7: 51-56.

Yoshinaga F., Y. Takeda, S. Okumura (1967). Glutamate kinase activity in *Brevibacterium flavum*: relationship between L-proline and L-glutamine biosynthesis.

Young T.W., M. Yagiu (1978). A comparison of the killer character in different yeast and its classification. Antonie van Leewenhoek J. Microbiol. 44: 59-77.