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Sugar and organic acid utilisation in *Pachysolen tannophilus* and *Pichia stipitis*

A thesis presented in partial fulfillment of the requirements

for the degree in Masterate in Genetics

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Christopher John Harrod

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Parturiunt montes, nascetur ridiculus mus.

This thesis is dedicated to my mother and father, without whom none of this would have been possible.

ABSTRACT

Wild-type strains of the K^+ yeasts *Pachysolen tannophilus* and *Pichia stipitis* were assessed for their ability to utilise tricarboxylic acid (TCA) cycle intermediates and to ferment glucose and xylose. These two species of yeast were mutagenised using ultraviolet (UV) radiation. The survivors were screened on an indicator plate for mutants with altered rates of malate utilisation, in the presence of glucose. The *Pichia stipitis* wild-type strain was UV mutagenised, and mutants resistant to the lethal analog of glucose, 2-deoxyglucose, were selected.

These mutants, plus several mutants of *Pachysolen tannophilus* isolated in an earlier study, were partially characterised by growth on a variety of solid media. The mutants were also grown on indicator media containing xylose or glucose plus one TCA cycle intermediate: citrate, succinate, fumarate or L-malate. Experiments with the indicator media served both as a method of characterisation of the mutants, and as a method of testing the utility of the indicator plate. The behavior of these mutants when grown under semi-anaerobic conditions in mixtures of glucose and TCA cycle intermediates was investigated. The wild-type *Pachysolen tannophilus* co-utilised malate and glucose, whereas in the wild-type *Pichia stipitis*, malate utilisation was repressed by glucose. Some mutants of both species showed increased utilisation of malate (and succinate, where tested). In *Pachysolen tannophilus*, this behavior correlated with loss of all three hexose-ATP-kinase enzymes, and in *Pichia stipitis* the behavior correlated with the loss of any hexose-ATP-kinase activity.

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DEFINITION OF TERMS

Carbon Catabolite Repression is the ability of catabolic products of glucose or other rapidly metabolisable carbon sources to inhibit the synthesis of certain enzymes (Magasanik 1961, Gancedo & Gancedo 1986).

Catabolite Inactivation is a term coined by Helmut Holzer to describe the glucose induced inactivation of enzymes. Inactivation of enzymes is believed to be by the proteolysis of these enzymes by a serine proteinase (Holzer 1976).

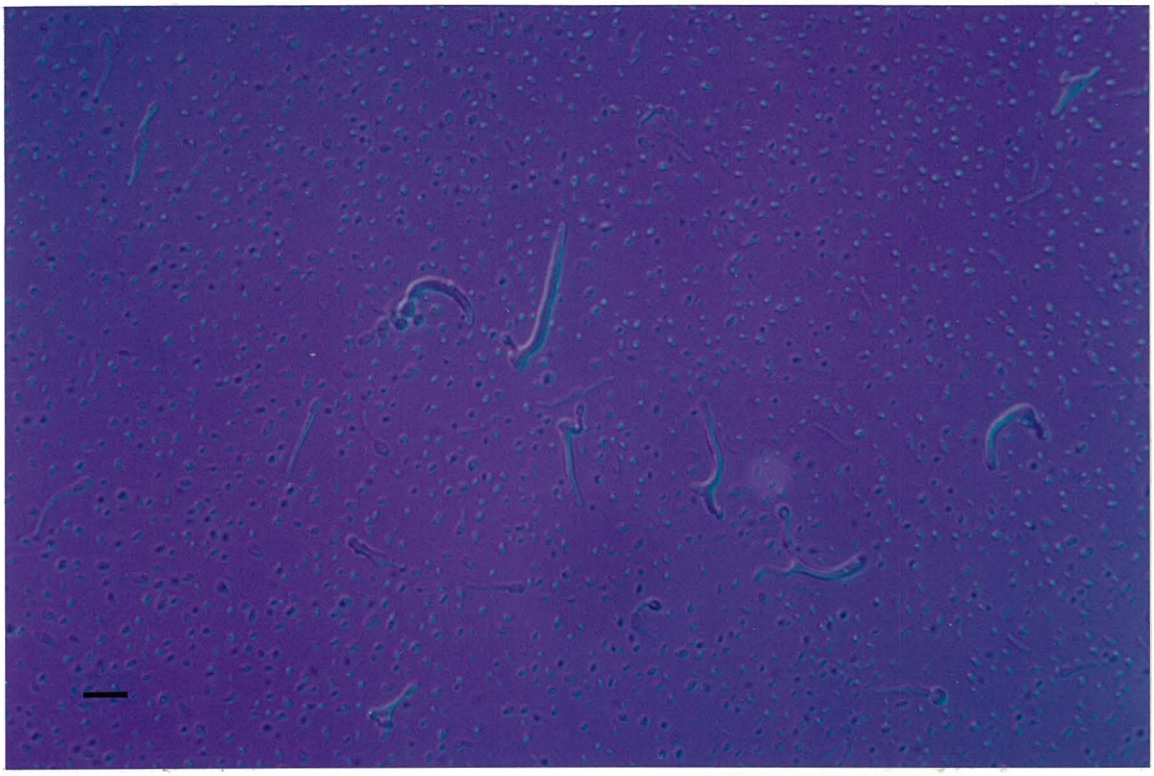
Glucose Control is the phenomena explored in this work and is defined as the mechanism by which glucose present in the media stops the utilisation of organic acids. Glucose control may be an example of catabolite repression, catabolite inactivation or some other hitherto undisclosed mechanism.

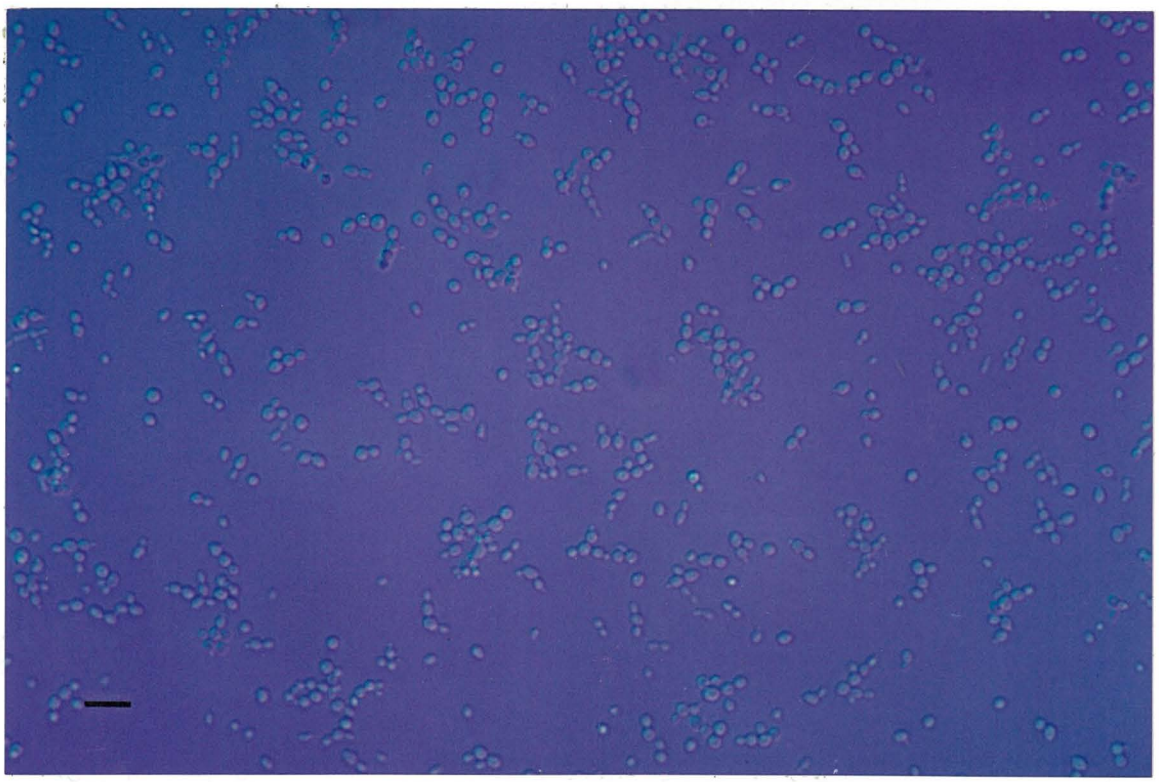
Glucose, Fructose, Xylose, Malic Acid, Malate: Unless stated specifically otherwise these refer to D-glucose, D-fructose, D-xylose, L-malic acid and L-malate, respectively.

Hexokinase(s) Most yeasts appear to have a system of three enzymes for adding phosphate to hexose sugars - the first step in glycolysis - two hexose-ATP-kinases that phosphorylate both glucose and fructose, usually termed hexokinases; and one hexose-ATP-kinase that phosphorylates glucose specifically, termed a glucokinase. In this study all three are collectively referred to as hexokinases.

Plate 1. *Pachysolen tannophilus* 2530 (wild-type). Grown in MEA (as per Difco bacto peptone plus 20 g agar per litre) for 3 days at 25°C. Large structures are ascophores. Photographed with Nomarski Optics plus blue colour and polarising filters. Bar is twenty microns long.

Plate 2. *Pichia stipitis* Y7124 (wild-type). Grown for three days in MMG at 25°C. The cells grow in chains. Photographed with Nomarski Optics plus blue colour and polarising filters. Bar is twenty microns long.





1.0 INTRODUCTION

1.1 Yeasts used for this investigation

Pachysolen tannophilus

Isolated from tanning liquors of vegetable origin and first described by Boiden and Adzet (1957). This is a haploid homothallic yeast. After three days growth at 25°C the vegetative cells are spheroidal to ellipsoidal (1.5 - 5.0) x (2.0 - 7.0) µm and usually have one or two buds. Asexual reproduction is by the production of buds. Ascospores form when a vegetative cell produces a stout tube (the ascophore), generally from one end (Plate 1). The tube may vary in length from quite short to 60 µm and may be straight or curved. The tube enlarges at one end to form the ascus which may contain up to four ascospores which are hat shaped. When the ascus disintegrates the spores are released and a V-shaped notch is visible in the end of the ascophore where the ascus formed. Asci may be conjugated or unconjugated depending on the strain (after Kurtzman 1984a). A well developed system of genetic manipulation has been documented (James & Zahab 1982).

Pichia stipitis

This organism was isolated originally from the larvae of *Cetonia* sp., *Dorcus parallelipipedus* and *Laphria* (the larvae of two types of scarab beetles and the robber fly, respectively); all of which were found in an old fruit-tree stump near Lyon, in France. First described by Pignal (1967). This is a haploid homothallic yeast. After three days at 25°C the cells are spheroidal to ovoidal (2.2 - 4.5) x (2.5 - 6.0) µm and occur singly or in pairs (Plate 2). Asexual reproduction is by budding. Ascospores are formed by conjugation between a parent cell and a bud or between independent cells or the ascus may be unconjugated. Asci produce one

to four hat shaped ascospores which are released soon after formation (after Barnett *et al.* 1983, Kurtzman 1984b).

1.2 Sugar utilisation of *Pachysolen tannophilus* and *Pichia stipitis*

Pachysolen tannophilus (Slininger *et al.* 1987) and *Pichia stipitis* (Prior *et al.* 1989) are both able to ferment xylose. This pentose sugar is the major constituent of enzyme or mild acid hydrolysates of plant tissue. The ability to ferment xylose is an unusual property for yeasts (Slininger *et al.* 1987), and may represent an adaption to the environment from which they were first isolated. As noted elsewhere (Section 1.1), these two yeasts were found associated with plant material which had been treated with acid (*Pachysolen tannophilus* in tanning liquors), or with enzymes (*Pichia stipitis* from insect larvae).

Pichia stipitis can transport glucose into the cell using by a constitutive low affinity proton symport (Kilian & van Uden 1988). By analogy with other fermentative yeasts studied, it is believed that *Pichia stipitis* has a high affinity glucose transport system which is glucose repressible. This seems a reasonable assumption because a) all fermentative yeasts studied conform to this pattern and b) amongst these yeasts two were the *Pichia* species *Pichia guillermondi* and *Pichia strasburgensis* (Does & Bisson 1989b). *Pichia stipitis* can transport xylose into the cell either by a high affinity proton symport or by a low affinity proton symport, both of which are constitutive. The low affinity proton symport for glucose is the same as that for xylose (Kilian & van Uden 1988).

In *Pachysolen tannophilus* little is known about the mode of transport of xylose and glucose into the cell.

In the yeast *Saccharomyces cerevisiae*, it has been demonstrated that the high affinity transport of glucose requires the activity of at least one hexokinase (Bisson & Fraenkel 1983). It is probable that the same system operates in *Pachysolen tannophilus* and *Pichia stipitis* given the highly conserved nature of glucose transporters (Gould & Bell 1990).

In both yeasts catabolism of glucose is by the Emden-Meyerhof-Parnas (EMP) pathway. Metabolites passing through the EMP pathway are converted to pyruvate. In yeasts growing at high glucose concentrations, or under semi-anaerobic conditions, pyruvate is converted to ethanol and liberated into the medium (Lagunas 1986). Because few organisms, other than yeasts, can metabolise ethanol, this ability confers an ecological advantage over other microorganisms in the competition for available substrates (Lagunas 1981). Secretion of ethanol may confer additional advantages - at high concentrations, ethanol inhibits the growth of most other microorganisms. In this way yeasts with high ethanol tolerance can gain a growth advantage over their competitors. Yeasts growing in low concentrations of glucose, under aerobic conditions, metabolise pyruvate via the tricarboxylic acid (TCA) cycle into carbon dioxide and water (Lagunas 1986).

In yeasts capable of metabolising xylose, this compound is converted to xylulose-5-phosphate which then enters the Pentose Phosphate (PP) pathway. Xylulose-5-phosphate is converted to fructose-6-phosphate and glyceraldehyde-3-phosphate (with some fructose-6-phosphate cycling through the oxidation part of the PP pathway to regenerate NADPH). These two metabolites then enter the EMP pathway, both above and below the step catalysed by phosphofructokinase (PFK), where they are catabolised (Prior *et al.* 1989). An alternative pathway may exist in some yeasts - the Phosphoketolase (PPK) pathway. In those yeasts which do have the PPK pathway, it is probably the major route of xylose catabolism (Evans

& Ratledge 1984). *Pachysolen tannophilus* contains the key enzyme in this pathway, xylulose-5-phosphate phosphoketolase, though there is some uncertainty about the levels of activity present (Evans & Ratledge 1984). It is unknown if this second pathway operates in *Pichia stipitis* (Prior *et al.* 1989).

1.3 Organic acid utilisation of *Pachysolen tannophilus* and *Pichia stipitis*

Pachysolen tannophilus and *Pichia stipitis* are both K^+ yeasts. The K^+ yeasts are characterised by their ability to grow on a number of TCA cycle acids as sole carbon sources; K^- yeasts cannot grow under the same conditions (Barnett & Kornberg 1960). Cell free extracts of K^+ and K^- yeasts had similar activities of the TCA cycle enzymes and it was proposed that the observed difference was due to permeability barriers - that TCA cycle intermediates could not penetrate the cell in significant amounts (Barnett & Kornberg 1960).

The yeasts *Zygosaccharomyces bailii* and *Schizosaccharomyces pombe* provide evidence in support of the permeability barrier theory. Both of these K^- yeasts are exceptional in that they can co-utilise malic acid in the presence of glucose. This property has been shown to be facilitated by an active transport system for malate across the membrane (Baranowski & Radler 1984, Osothsilp & Subden 1986). In both cases transport was shown to be pH and temperature sensitive as well as protein synthesis dependent. Furthermore, in the case of *Zygosaccharomyces bailli*, the transport mechanism was shown to be stereospecific. In both species, growth on mixtures of TCA cycle intermediates indicated that these compounds inhibited malate uptake by competing for access to the transport system. By contrast, growth in mixtures of organic acids and sugars showed that the malic acid transport system of *Schizosaccharomyces pombe* was constitutive, but that of *Zygosaccharomyces bailii* was catabolite repressible, particularly by fructose.

This last observation is consistent with the fact that *Zygosaccharomyces bailii* is fructophilic and would therefore catabolise fructose in preference to organic acids.

The yeast *Hansenula anomala* has been shown to have a transport system specific for dicarboxylic acids (Côrte-Real & Leão 1990). It did not transport citric acid (a tricarboxylic acid). The transport system was found to be inducible and catabolite repressible. There was evidence that the transport was a proton symport and that the acids were transported in anionic form. *Hansenula anomala* transported citric acid into the cell via a proton symport distinct from the dicarboxylic acid transporter.

In solution, acids exist as an equilibria between the dissociated and undissocciated form. The degree of dissociation of an acid is dependant on the pH (the log of the H^+ concentration) of the solution. At low (acid) pH, most of the acid is in the undissociated uncharged form. At high (alkali) pH, most of the acid is in the dissociated ionic form. For a given temperature, the pH at which half of the acid molecules have lost a proton is termed the pk_a. Acids capable of losing several protons have a pk_a for each i.e. pk_{a1}, pk_{a2}, pk_{a3} ... etc. The available protons are listed in order of increasing resistance to dissociation. The pk_a values for the organic acids used in this study are given in Table 1, (Source: The Handbook of Biochemistry: Selected data for molecular biology, 1968).

In K^+ yeasts it is probable that the main level of regulation of organic acid catabolism is by carbon catabolite repression of the enzymes of the TCA cycle. Recent work with *Pichia stipitis* (Rodriguez & Thornton 1990) indicates it is very strongly catabolite repressed by glucose, even at low levels (5 g/l or 28 mM).

If K^+ yeasts are to have an application in deacidification of foods they are likely to be used on substrates rich in sugars. If this is the case then catabolite repression of the TCA cycle becomes of extreme concern.

Table 1. The pk_a of organic acids used in this study.

Acid	Temperature	pk_{a1}	pk_{a2}	pk_{a3}
Citric acid	25°C	3.128	4.761	6.396
	30°C	3.116	4.755	6.406
Succinic acid	25°C	4.2066	5.635	-
	30°C	4.1980	5.641	-
Fumaric acid	25°C	3.095	4.602	-
	30°C	NA	NA	NA
Malic acid	25°C	3.458	5.097	-
	30°C	3.452	5.099	-

-: There is no pk_{a3} for this acid. NA: Data is not available.

1.4 Carbon catabolite repression and hexokinases

Definition

Carbon catabolite repression is the mechanism by which an organism adapts its cellular metabolism to utilise the most appropriate carbon source. This phenomena is defined as the inhibition of synthesis of certain enzymes by glucose or other rapidly metabolisable carbon sources (Gancedo & Gancedo 1986). The term carbon catabolite repression was first used by Magasanik (1961) to describe the behaviour of bacteria. The phenomena occurs in many other living organisms including yeast (Gancedo & Gancedo 1986). The most important and frequently

studied of the rapidly metabolisable carbon sources is glucose, and for this reason the phenomena is often called glucose repression.

Carbon catabolite repression is controlled by a large number of genes (Zimmermann & Scheel 1977, Ciriacy 1977, Entian & Zimmermann 1982), many of which are epistatic to one another (Gancedo & Gancedo 1986). Mutants may be isolated which are derepressed for some, but not all, pathways which are under carbon catabolite repression (Entian & Frölich 1984). These two facts indicate that the control of carbon catabolite repression is a complex system, not easily dissected or understood. Carbon catabolite repression is believed to be a regulatory cascade which ultimately leads to protein interactions with DNA, thereby affecting gene transcription (Gancedo & Gancedo 1986). However, the mechanism by which this occurs is not yet fully known (Gancedo & Gancedo 1986).

Enzymes under carbon catabolite control

Enzymes in a number of pathways involving energy metabolism, and glucose utilisation or synthesis, are affected by carbon catabolite repression. These include the tricarboxylic acid cycle (Polakis and Bartley 1965), the glyoxylate cycle (Barnett & Kornberg 1960), the high affinity transport system for glucose (Bisson 1988), hexokinase P II synthesis (Entian 1981), amylase production (van Uden *et al.* 1980, McCann & Barnett 1984) and the utilisation of methanol (Sibirny *et al.* 1988).

Hexokinases

Hexokinases are responsible for the hexose-ATP-kinase activity in yeast cells (Lobo & Maitra 1977a). These enzymes catalyse the first step in glycolysis: the addition of a phosphate group to the sixth carbon of a hexose sugar, the phosphate being donated by ATP. It has been observed by a large number of workers that

yeasts lacking hexose-ATP-kinase activity are freed from the effects of carbon catabolite repression (Entian & Mecke 1982).

In *Saccharomyces cerevisiae,* there are three of these enzymes: hexokinase P I, hexokinase P II (Lobo & Maitra 1977a) and glucokinase (Maitra & Lobo 1983). Hexokinase P I is constitutive (Fernández *et al.* 1985) and catalyses the phosphorylation of glucose, fructose and mannose (Entian & Mecke 1982). For this enzyme the ratio of fructose to glucose phosphorylation activity (F/G) is approximately 3.0 (Entian & Mecke 1982). Hexokinase P I is coded for by the HXK 1 gene (Lobo & Maitra 1977a).

Hexokinase P II is an isoenzyme of P I and phosphorylates the same substrates. Hexokinase P II is inducible by glucose (Fernández *et al.* 1985). The ratio of fructose to glucose phosphorylation (F/G) is approximately 1.2 (Fernández *et al.* 1985). In *Saccharomyces cerevisiae,* hexokinase P II constitutes the major hexose-ATP-kinase activity within the cells during the exponential growth on glucose (Fernández *et al.* 1985). The synthesis of hexokinase P II is under the control of carbon catabolite repression (Entian 1981). This enzyme is coded for by the gene HXK 2 (Lobo & Maitra 1977a), and is believed to mediate carbon catabolite repression (Entian & Mecke 1982, Ma & Botstein 1986). Hexokinase P I, unlike hexokinase P II, has no role in carbon catabolite repression (Entian *et al.* 1984).

The third hexose-ATP-kinase is termed a glucokinase in the literature, although it phosphorylates both glucose and mannose (Fernández *et al.* 1985). This enzyme is inducible, and provides the major hexose-ATP-kinase activity within cells grown on ethanol or other gluconeogenic substrates (Fernández *et al.* 1985). This enzyme is coded for by the gene GLK 1 (Maitra & Lobo 1983).

In the yeast *Pachysolen tannophilus* there are three hexose-ATP-kinase enzymes: hexokinase A, hexokinase B and glucokinase (Wedlock *et al.* 1989). Hexokinase A is inducible by glucose, phosphorylates both glucose and fructose, and has a F/G ratio of between 1.3 and 1.5 (Wedlock and Thornton 1989, Wedlock *et al.* 1989). This enzyme, along with glucokinase, provides the major hexose-ATP-kinase activity within cells during exponential growth on glucose (Wedlock *et al.* 1989). Hexokinase A mediates the carbon catabolite repression of xylose utilisation (Wedlock & Thornton 1989). Hexokinase A of *Pachysolen tannophilus* appears to be closely analogous to hexokinase P II of *Saccharomyces cerevisiae*. Hexokinase B of *Pachysolen tannophilus* is constitutive and phosphorylates fructose and glucose with a F/G ratio of 3.0 (Wedlock *et al.* 1989). In this, hexokinase B of *Pachysolen tannophilus* resembles hexokinase P I of *Saccharomyces cerevisiae*. The glucokinase of *Pachysolen tannophilus* is constitutive and phosphorylates glucose.

In the yeast *Schwaniomyces occidentalis* there is only one enzyme with hexose-ATP-kinase activity. This enzyme phosphorylates glucose and fructose, and mediates carbon catabolite repression (McCann *et al.* 1987).

In *Saccharomyces cerevisiae* there is *in vitro* evidence that hexokinase P II acts as a protein kinase (Herrero *et al.* 1989). If this occurs *in vivo* then hexokinase P II may mediate carbon catabolite repression by phosphorylating a messenger protein. In eukaryotes, such events commonly initiate regulatory cascades, which in turn control gene expression. For example such an event is triggered by protein kinase C (Kikkawa & Nishizuka 1986). Recently published work suggests the ability of hexokinase P II to mediate carbon catabolite repression is dependant on its hexose phosphorylation activity. Mutant alleles of the HXK 2 gene have been isolated which produce normal-size, stable proteins which lack hexose phosphorylation activity (Ma *et al.* 1989b). Detailed analysis of these mutants

revealed all lesions were within or close to the active site of the enzyme, or at the hinge region (which allows induced fit of the enzyme to hexoses) (Ma *et al.* 1989a). These two observations imply that carbon catabolite repression is dependant on the integrity of the hexose phosphorylating active site of the enzyme. This work is in complete contrast to the findings of Entian and Frölich (1984), who believe the regulatory and hexose phosphorylating sites of hexokinase P II are separate and distinct.

Catabolite inactivation and glucose control

Many of the enzymes which are regulated by carbon catabolite repression are also rapidly inactivated upon the addition of glucose (Gancedo & Schwerzmann 1976, Busturia & Lagunas 1986). The phenomena by which this occurs is termed catabolite inactivation (Holzer 1976). The mechanism appears to involve proteolysis of the enzymes by a proteinase. Carbon catabolite repression and catabolite inactivation are closely allied, complimentary phenomena. Upon addition of glucose to yeast cells, the former switches off the production of certain enzymes, while the latter inactivates many of the same enzymes present within the cell (Holzer 1976). Coordination of these two phenomena allows the cell to adapt quickly, and completely to the new environment. Carbon catabolite repression and catabolite inactivation are controlled by a number of the same genes (Entian 1977).

These two phenomena, similar but distinct, are frequently confused in the literature. For the purposes of this work, the term 'glucose control' has been used to denote the regulation of cellular metabolism by carbon catabolite repression, catabolite inactivation or some other hitherto undisclosed mechanism.

1.5 2-Deoxyglucose selection of mutants lacking glucose-phosphorylating enzymes

To isolate mutants with increased organic acid utilisation it is desirable to eliminate carbon catabolite repression. In *Saccharomyces cerevisiae* a central control element of carbon catabolite repression is hexokinase P II (Entian & Mecke 1982, see also Section 1.4). In *Pachysolen tannophilus* this element is termed hexokinase A (Wedlock & Thornton 1989). A direct selection for yeast mutants lacking glucose-phosphorylating enzymes has been reported (Lobo & Maitra 1977b). This strategy involves incubating cells in the presence of 2-deoxyglucose, a lethal analog of glucose, and a carbon source (required for cell growth). Such a strategy also leads to mutants with decreased ability to utilise glucose. These mutants have potential application in the wine and food industries (Section 1.6).

The analog of glucose, 2-deoxyglucose, once transported into the cell and phosphorylated to 2-deoxyglucose-6-phosphate is lethal. The analog interferes with the production of cell walls by being incorporated into cell-wall mannan, and possibly by blocking synthesis of cell-wall polysaccharides and glycoproteins. This causes the cells to lyse during growth.

During stationary phase fermentation and respiration are inhibited by 2-deoxyglucose. The analog also lowers the production of energy by the cells, interferes with enzyme synthesis, and is incorporated into uridine and guanosine nucleotides (Barnett 1976). This lethal analog of glucose has been credited with the triggering of carbon catabolite repression (Witt *et al.* 1966). However, such a conclusion fails to take into account the pleiotropic effects of 2-deoxyglucose (or its phosphorylated form) on yeast cells.

1.6 Industrial applications

With the dwindling of oil reserves, greater attention is being paid to the search for alternative fuel sources. One of the most promising areas of research is into the production of ethanol (Titchener 1982). One possible source of ethanol is by fermentation of xylose, the major constituent of plant hydrolysates (Slininger *et al.* 1987). Consequently, considerable research has been done on yeasts capable of fermenting this pentose sugar. As has been noted (Section 1.2), both species of yeast used in this study are capable of xylose fermentation.

The results of such research with *Pachysolen tannophilus* have been reviewed by Slininger *et al.* (1987). The ability of *Pichia stipitis* to produce ethanol by fermentation of xylose has also been studied. In this role it is superior to *Pachysolen tannophilus* for at least three reasons. Firstly it does not require the addition of expensive vitamins for growth. *Pachysolen tannophilus* requires both biotin and thiamin for growth (Dellweg *et al.* 1984). Secondly *Pichia stipitis* can produce ethanol under strictly anaerobic conditions, so no ethanol is lost by respiration of the yeast. *Pachysolen tannophilus* cannot grow under strictly anaerobic conditions because its xylose reductase enzyme uses NADPH as a coenzyme almost exclusively, and action of the xylose pathway produces an excess of NADH which blocks other metabolic pathways. The xylose reductase of *Pichia stipitis* also uses NADH as a co-enzyme allowing better fermentation of xylose (Dellweg *et al.* 1984). Thirdly *Pichia stipitis* produces less xylitol than *Pachysolen tannophilus* (Dellweg *et al.* 1984).

In the areas of industry and food manufacture, common use is made of living organisms to produce commercially useful products. Of particular interest here are organisms that utilise or produce large quantities of carboxylic (organic) acids. For example, in the production of yoghurt and cheese, lactic acid bacteria convert

the sugar lactose to lactic acid, thereby lowering the pH and causing the casein in the milk to precipitate out (curdle). Acetic acid bacteria aerobically convert ethanol (produced by anaerobic fermentation of various substrates by brewer's yeast) into acetic acid (vinegar), which is used in foods and in industry. Citric acid is produced commercially using the fungi *Aspergillus niger*. The lactic acid produced by bacterial fermentation is used as a preservative in some foodstuffs - for example in pepperoni sausage and in silage (for cattle).

In wines the degree of 'tartness' is closely related to the concentration of malic and tartaric acids. Overly tart wines contain excessive amounts of these acids and are termed 'sharp', 'green', 'acidulus' or 'unripe'. Tartaric acid may be removed, in the form of potassium bitartrate, by precipitation at low temperatures (Beelman & Gallander 1979). Malic acid, however, remains highly soluble. It may be removed instead, by the action of bacteria capable of malolactic fermentation, or by its decomposition by yeasts (Snow & Gallander 1979, Gallander 1977, Rankine 1966).

There are further potential uses for organisms that utilise organic acids. At the present time fruit juice manufacturers have no use for pomace - a dry paste, rich in cellulose, sugar and organic acids - which is the main byproduct of fruit juice extraction. This material is unacceptable as cattle fodder because of the high acid content. The activity of acid utilising organisms could make such waste products available as food. Such organisms can be grown on waste organic acids from any source and then rendered down as Microbial Biomass Protein (MBP) - a source of food for humans (Moo-Young & Gregory 1986).

In fruit juices there are often high levels of malate, succinate and citrate which make juice unpalatable. Commercially this is masked by using cane sugar. If organisms can be used to reduce the level of acid in fruit juices, adding sucrose

will become unnecessary, thereby decreasing production costs and satisfying the growing consumer preference for 'no added sugar' products.

1.7 Objectives

An understanding of the sugar and organic acid metabolism of K^+ yeasts is necessary before they can be used commercially. Consequently, the following objectives were set for this study:

1) Determine which of the TCA cycle intermediates will support growth of the K^+ yeasts *Pachysolen tannophilus* and *Pichia stipitis* .

2) Using the original indicator medium, isolate mutants capable of altered rates of malate utilisation in the presence of glucose.

3) Determine whether increased malate utilisation is accompanied by increased utilisation of the other TCA cycle intermediates which support growth.

4) Determine the pattern of utilisation of malate and glucose for the wild-type and mutant strains of both species.