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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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*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<sup>+</sup> 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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## TABLE OF CONTENTS

	<u>Page</u>
Front Pages	
Abstract.....	ii
Acknowledgements.....	iii
Table of Contents.....	vi
List of Figures.....	ix
List of Tables.....	x
List of Plates.....	xi
Definition of Terms.....	xii
1.0 Introduction.....	2
1.1 Yeasts used for this investigation.....	2
1.2 Sugar utilisation of <i>Pachysolen tannophilus</i> and <i>Pichia stipitis</i> .....	3
1.3 Organic acid utilisation of <i>Pachysolen tannophilus</i> and <i>Pichia stipitis</i> .....	5
1.4 Carbon catabolite repression and hexokinases.....	7
1.5 2-Deoxyglucose selection of mutants lacking glucose phosphorylating-enzymes.....	12
1.6 Industrial applications.....	13
1.7 Objectives.....	15
2.0 Materials and Methods.....	16
2.1 Media.....	16
2.2 Growth conditions on solid media.....	18
2.3 Growth conditions in liquid media.....	18
2.4 Special conditions for ultraviolet (UV) mutagenesis.....	18
2.5 Special conditions for semi-anaerobic fermentations.....	18
2.6 Yeast strains and maintenance.....	19

TABLE OF CONTENTS CONT'D

	<u>Page</u>
2.7 Anaerobic utilisation of sugars.....	23
2.8 UV mutagenesis and screening by indicator plate.....	23
2.9 UV mutagenesis and 2-deoxyglucose selection.....	23
2.10 Estimation of dry cell mass.....	24
2.11 Performance of semi-anaerobic fermentations.....	25
2.12 HPLC methods.....	26
2.13 Preparation of cell free extracts.....	27
2.14 Hexokinase assays.....	27
2.15 Protein assays.....	28
2.16 Nomarski optics microscopic photography.....	28
3.0 Results.....	29
3.1 Growth of <i>Pachysolen tannophilus</i> and <i>Pichia stipitis</i> wild-type strains on different organic acids.....	29
3.2 Anaerobic utilisation of sugars.....	29
3.3 Mutants isolated on the indicator plate.....	32
3.4 Mutants isolated on the modified indicator plate.....	33
3.5 Mutants isolated by selection on malic acid and 2-deoxyglucose.....	33
3.6 Relationship of dry weight and cell number to optical density.....	34
3.7 Growth of strains on solid media plus sugars.....	35
3.8 Growth of strains on malic acid plus 2-deoxyglucose.....	35
3.9 Growth of strains on xylose plus 2-deoxyglucose.....	37
3.10 Response of strains to indicator plus organic acids and glucose.....	37
3.11 Response of strains to indicator plus organic acids and xylose.....	40

## TABLE OF CONTENTS CONT'D

	<u>Page</u>
3.12 Semi-anaerobic fermentations.....	64
3.13 Semi-anaerobic fermentations in minimal malate glucose.....	64
3.14 Semi-anaerobic fermentations in minimal malate.....	66
3.15 Semi-anaerobic fermentations in minimal succinate glucose.....	66
3.16 Hexokinase assays.....	67
4.0 Discussion.....	68
4.1 Sugar utilisation of <i>Pachysolen tannophilus</i> and <i>Pichia stipitis</i> .....	68
4.2 Organic acid utilisation of <i>Pachysolen tannophilus</i> and <i>Pichia stipitis</i> .....	70
4.3 Hexokinases, 2-deoxyglucose resistance and gluconeogenesis.....	73
4.4 Utility of the indicator plate.....	74
4.5 Summary.....	76
4.6 Further work.....	78
5.0 Appendices.....	79
5.1 Method of regeneration of reverse phase columns.....	79
5.2 Plot of abs <sub>600</sub> vs dry cell mass.....	80
5.3 Plot of malate concentration against HPLC response factors.....	81
5.4 Plot of glucose concentration against HPLC response factors.....	82
5.5 Bovine serum albumin concentration vs absorbance.....	83
6.0 Bibliography.....	84

## LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1 Fermentation of <i>Pachysolen tannophilus</i> 2530 (wild-type) on MM...	42
2 Fermentation of <i>Pachysolen tannophilus</i> P510-5A on MMG.....	43
3 Fermentation of <i>Pachysolen tannophilus</i> P509-3C on MMG.....	44
4 Fermentation of <i>Pachysolen tannophilus</i> 335 on MMG.....	45
5 Fermentation of <i>Pachysolen tannophilus</i> P509-1B on MMG.....	46
6 Fermentation of <i>Pachysolen tannophilus</i> D/X A on MMG.....	47
7 Fermentation of <i>Pichia stipitis</i> Y7124 (wild-type) on MMG.....	48
8 Fermentation of <i>Pichia stipitis</i> 77 on MMG.....	49
9 Fermentation of <i>Pichia stipitis</i> 238 on MMG.....	50
10 Fermentation of <i>Pichia stipitis</i> 287 on MMG.....	51
11 Fermentation of <i>Pichia stipitis</i> 411 on MMG.....	52
12 Fermentation of <i>Pachysolen tannophilus</i> 2530 (wild-type) on MM...	53
13 Fermentation of <i>Pachysolen tannophilus</i> P510-5A on MM.....	54
14 Fermentation of <i>Pichia stipitis</i> Y7124 (wild-type) on MM.....	55
15 Fermentation of <i>Pichia stipitis</i> 77 on MM.....	56
16 Fermentation of <i>Pichia stipitis</i> 238 on MM.....	57
17 Fermentation of <i>Pichia stipitis</i> 287 on MM.....	58
18 Fermentation of <i>Pichia stipitis</i> 411 on MM.....	59
19 Fermentation of <i>Pachysolen tannophilus</i> 2530 (wild-type) on MSG.	60
20 Fermentation of <i>Pachysolen tannophilus</i> P509-1B on MSG.....	61
21 Fermentation of <i>Pichia stipitis</i> Y7124 (wild-type) on MSG.....	62
22 Fermentation of <i>Pichia stipitis</i> 411 on MSG.....	63

## LIST OF TABLES

<u>Table</u>	<u>Page</u>
1 The $pK_a$ of organic acids used in this study.....	7
2 Media used in this study.....	16
3 <i>Pachysolen tannophilus</i> and <i>Pichia stipitis</i> strains used in this study.....	19
4 <i>Pachysolen tannophilus</i> 2530 grown on solid media plus organic acids.....	30
5 <i>Pichia stipitis</i> Y7124 grown on solid media plus organic acids.....	30
6 Ability of strains to utilise sugars anaerobically.....	32
7 <i>Pachysolen tannophilus</i> strains on solid media plus sugars.....	36
8 <i>Pichia stipitis</i> strains on solid media plus sugars.....	36
9 <i>Pachysolen tannophilus</i> strains on solid media plus malate and 2-deoxyglucose.....	38
10 <i>Pachysolen tannophilus</i> strains on solid media plus xylose and 2-deoxyglucose.....	38
11 <i>Pachysolen tannophilus</i> strains on indicator plus organic acids and glucose (plate colour).....	39
12 <i>Pichia stipitis</i> strains on indicator plus organic acids and glucose (plate colour).....	39
13 <i>Pachysolen tannophilus</i> strains on indicator plus xylose and organic acids (plate colour).....	41
14 <i>Pichia stipitis</i> strains on indicator plus xylose and organic acids (plate colour).....	41
15 Hexose-ATP-kinase activities of <i>Pichia stipitis</i> strains.....	67
16 Hexose-ATP-kinase activities of <i>Pachysolen tannophilus</i> strains..	69

## LIST OF PLATES

<u>Plate</u>		<u>Page</u>
1	<i>Pachysolen tannophilus</i> 2530 (wild-type).....	1
2	<i>Pichia stipitis</i> Y7124 (wild-type).....	1
3	Selection of <i>Pichia stipitis</i> mutant 77 on Indicator MG.....	21
4	Selection of <i>Pachysolen tannophilus</i> mutant 335 on Indicator MG (modified).....	21
5	<i>Pichia stipitis</i> mutant 77 grows in clumps in MMG.....	22
6	<i>Pichia stipitis</i> Y7124 (wild-type) showing dancing bodies.....	22
7	Comparison of response of <i>Pichia stipitis</i> strains on Indicator MG.....	31
8	Behavior of <i>Pachysolen tannophilus</i> F/G 2 on different media.....	31

## 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).

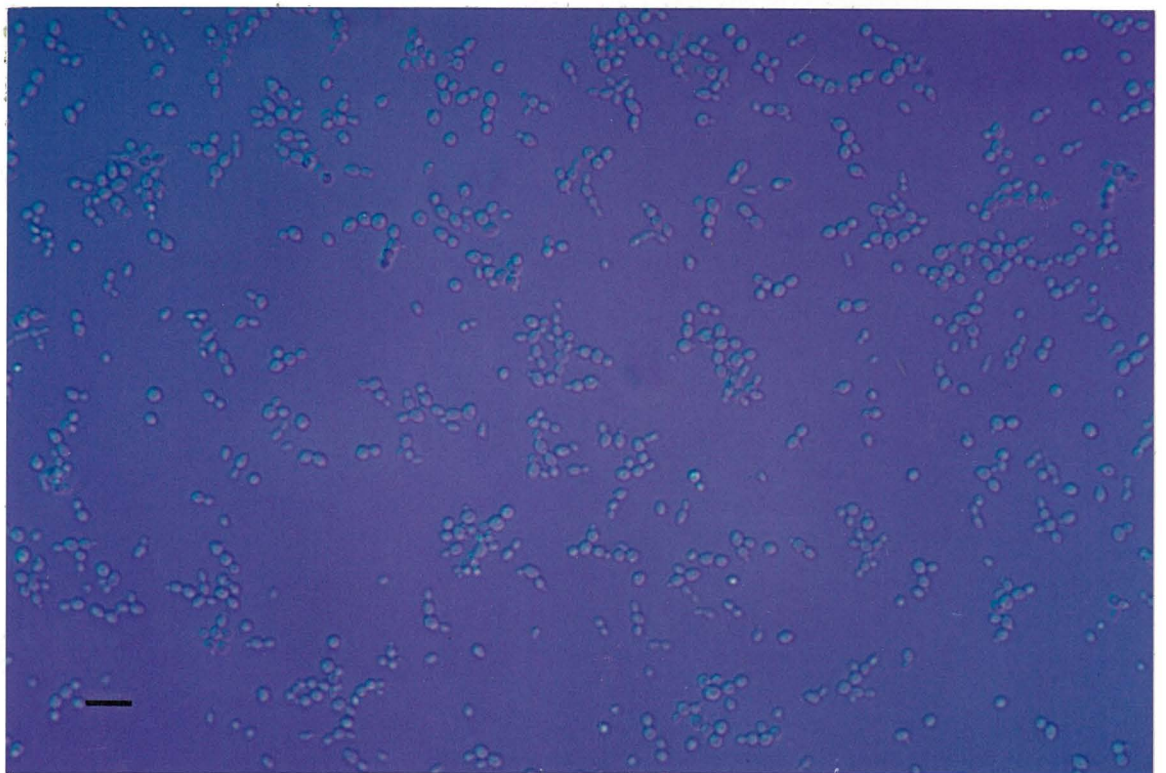
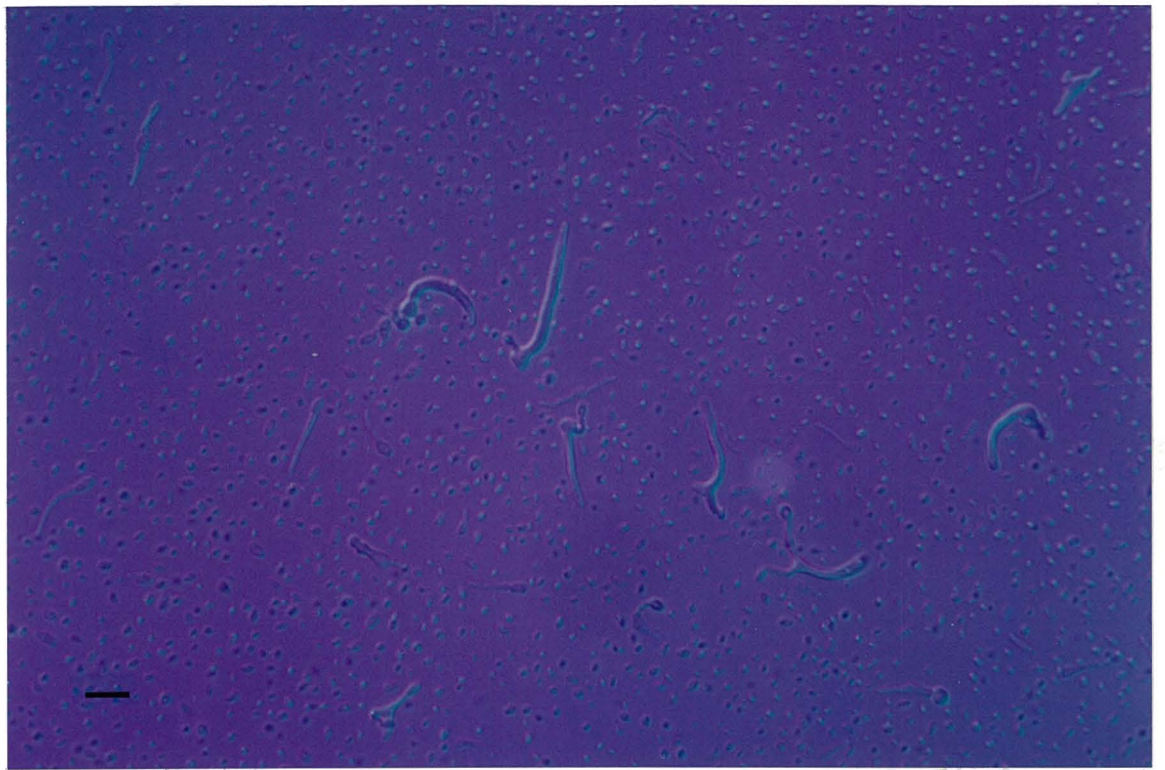
**Glucose Control** is the phenomena explored in this work and is defined as the mechanism by which glucose present in the media stops the utilisation of organic acids. Glucose control may be an example of catabolite repression, catabolite inactivation or some other hitherto undisclosed mechanism.

**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 parallelipedus* 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 Pignat (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 adaptation 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<sup>+</sup> yeasts. The K<sup>+</sup> yeasts are characterised by their ability to grow on a number of TCA cycle acids as sole carbon sources; K<sup>-</sup> yeasts cannot grow under the same conditions (Barnett & Kornberg 1960). Cell free extracts of K<sup>+</sup> and K<sup>-</sup> 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<sup>-</sup> 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 bailii*, 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 undissociated 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<sup>+</sup> 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<sub>a</sub> of organic acids used in this study.

Acid	Temperature	pK <sub>a1</sub>	pK <sub>a2</sub>	pK <sub>a3</sub>
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<sub>a3</sub> 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.

## 2.0 MATERIALS AND METHODS

### 2.1 Media

Table 2. Media used in this study

	YM+M Agar	YEP (xylose) Agar	Sugar Anaerobiosis Medium	Indicator Medium	Indicator Medium (modified)	Indicator M Medium	YNB-Sugar Agar	YNB-Acid Agar	MMG	MM	MSG
Yeast extract (g/l)	3	10	5	-	-	-	-	-	-	-	-
Malt extract (g/l)	3	-	-	-	-	-	-	-	-	-	-
Bacto peptone (g/l)	5	20	-	-	-	-	-	-	-	-	-
Yeast nitrogen base <sup>1</sup> (g/l)	-	-	-	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Bromocresol green <sup>2</sup> (ml/l)	-	-	-	55	55	55	-	-	-	-	-
Glucose (g/l)	10	-	-	-	-	-	-	-	100	-	100
Xylose (g/l)	-	20	-	-	-	-	-	-	-	-	-
Malic acid (g/l)	10	-	-	-	-	10	-	-	3	3	-
Succinic acid (g/l)	-	-	-	-	-	-	-	-	-	-	3
Appropriate sugar <sup>3</sup> (g/l)	-	-	*5	80	5	-	20	-	-	-	-
Appropriate acid <sup>4</sup> (g/l)	-	-	-	10	20	-	-	20	-	-	-
Agar (g/l)	20	20	-	20	20	20	20	20	-	-	-

Yeast Nitrogen Base<sup>1</sup>: without amino acids.

Bromocresol Green<sup>2</sup>: 0.1 g of BCG dissolved in 10 ml of 0.1 M KOH and made up to 250 ml with deionised distilled water.

Appropriate Sugar<sup>3</sup>: Depending on the type of YNB-Sugar plate or the required constituents of an indicator plate, any of the following sugars could be added at the listed amount: D-glucose, D-xylose, D-mannose, D-fructose, maltose.

Appropriate Acid<sup>4</sup>: Depending on the type of YNB-Acid plate or the required constituents of an indicator plate, any of the following organic acids could be added at the listed amount: citric acid, succinic acid, fumaric acid, L-malic acid.

\*<sup>5</sup>: Sufficient sugar was added to make a 50 mM solution: 9.0 g/l of glucose or 7.5 g of xylose.

Unless stated otherwise all media was made up using AnalAr grade reagents. Constituents were dissolved in deionised distilled water. The pH of all media was brought to 3.8 with KOH and HCl.

Where a medium contained agar it was made in two parts, all soluble components at pH 3.8 in one part, agar in the other, each in a half volume of water. These were autoclaved separately, mixed and then poured.

Where a medium contained both xylose and agar it was made in three parts, xylose in one, agar in the second and all other components at pH 3.8 in the third. These were each in a third volume of water, autoclaved separately, mixed and poured.

All media was autoclaved at 110°C and at 10 lbs pressure. Heat labile compounds were sterilised by being passed through a sterile 0.2 µm filter.

Constituents of all media used in this study are given in Table 2.

## 2.2 Growth conditions on solid media

Yeasts were applied to agar plates by spreading, or by patching or streaking using spatulate toothpicks. These plates were incubated for between two and six days at 30°C. Cells spread on indicator plates immediately following UV mutagenesis were incubated in the dark to counter the yeasts light mediated DNA repair mechanisms.

## 2.3 Growth conditions in liquid media

For any given experiment a 10 ml aliquot of the media to be used was inoculated from a stock culture and shaken for two days at 30°C.

## 2.4 Special conditions for ultraviolet (UV) mutagenesis

A two day culture was counted using an Improved Neubauer Haemocytometer and, diluted between 100-fold and 1000-fold in sterile distilled water to give cell numbers of approximately  $10^4$  to  $10^5$  cells per ml. Cells were exposed to 40 seconds of UV light in a UV light box 32 cm distant from a Philips Fluorescent UV tube (574 P/40 OA, TUV, 15W).

## 2.5 Special conditions for semi-anaerobic fermentations

A two day culture was diluted 10-fold and the  $OD_{600}$  measured on a Bausch and Lomb Spectronic 20 Spectrophotometer. The concentration of dry cell mass equivalent was calculated (Section 2.10) and sufficient of the two day culture was added to the flask of medium to give a final yeast concentration of  $4.5 \times 10^{-4}$  mg / ml dry cell mass (approximately  $1.45 \times 10^5$  cells per ml of *Pachysolen tannophilus* and  $4.38 \times 10^4$  cells per ml of *Pichia stipitis*). Flasks were of 1 litre capacity and

contained 400 ml of the appropriate medium. The flasks were then sealed with a fermentation trap and incubated at 25°C while shaking at 100 r.p.m. These cultures were grown for five to seven days and were sampled once or twice daily by removal of fermentation trap and pipetting out a 10 ml aliquot.

## 2.6 Yeast strains and maintenance

Table 3. The *Pachysolen tannophilus* and *Pichia stipitis* strains used in this study.

Strain	Genotype	Source
<i>Pachysolen tannophilus</i>		
2530	wild-type	NRCC (Al James)
P510-5A	<i>glu 1</i>	Neil Wedlock
P509-3C	<i>hvk 2</i>	Neil Wedlock
P509-1B	<i>hvk 2 glu 1</i>	Neil Wedlock
D/X A	<i>hvk 1 hvk 2 glu 1</i>	Neil Wedlock
F/G	<i>hvk 1 hvk 2 glu 1 y</i>	Neil Wedlock
335	ND	this study
<i>Pichia stipitis</i>		
Y7124	wild-type	Susan Rodriguez
77	ND	this study
238	ND	this study
287	ND	this study
411	ND	this study

ND: Not determined.

The yeast strains used in this study are listed in Table 3. For long term storage of strains, the yeasts were streaked on 2.5 ml slopes of the appropriate agar incubated for two days at 30°C with loosened lids. The lids were tightened and the slopes stored at 5°C. The *hxx*<sup>-</sup> strains supplied by Neil Wedlock were stored on YEP(xylose) agar and all other strains were stored on YM+M agar. For short term storage, yeasts were incubated on agar plates, sealed with parafilm and stored at 5°C.

*Pachysolen tannophilus* mutants P510-5A, P509-1B, P509-3C and D/X A were isolated for their ability to grow on xylose in the presence of the lethal glucose analog 2-deoxyglucose. *Pachysolen tannophilus* F/G 2 was derived from D/X A by ultraviolet (UV) mutagenesis and selection on YNB glucose xylose plates (Wedlock *et al.* 1989).

During this work *Pichia stipitis* Y7124 was never seen to produce spores. This strain was pre-incubated on plates of rich medium, streaked on eight different types of sporulation medium and incubated both aerobically and anaerobically for extended periods. Despite these efforts the strain could not be induced to sporulate. Consequently, genetic analysis of the mutants generated during this study was not possible. It has been noted that this strain is not very amenable to genetic analysis (Does & Bisson 1989a) presumably because for this reason. Strain Y7124 has been shown to be haploid during this study, as it was easy to isolate auxotrophs: after UV irradiation six auxotrophs were generated out of approximately eight thousand survivors.

Plate 3. Screening for mutants on the original indicator plate: Indicator MG. *Pichia stipitis* Y7124 (wild type) and mutant 77 were mixed and spread on this plate to demonstrate the ease with which mutants can be detected. The small turquoise colonies are mutant 77. The plate was incubated at 30°C for six days.

Plate 4. Screening for mutants on the new indicator plate: Indicator MG (modified). *Pachysolen tannophilus* strains 2530 (wild type) and mutant 335 were mixed and spread on this plate to demonstrate how mutants appeared on this plate. The white colonies are mutant 335. The plate had a four day incubation at 30°C.

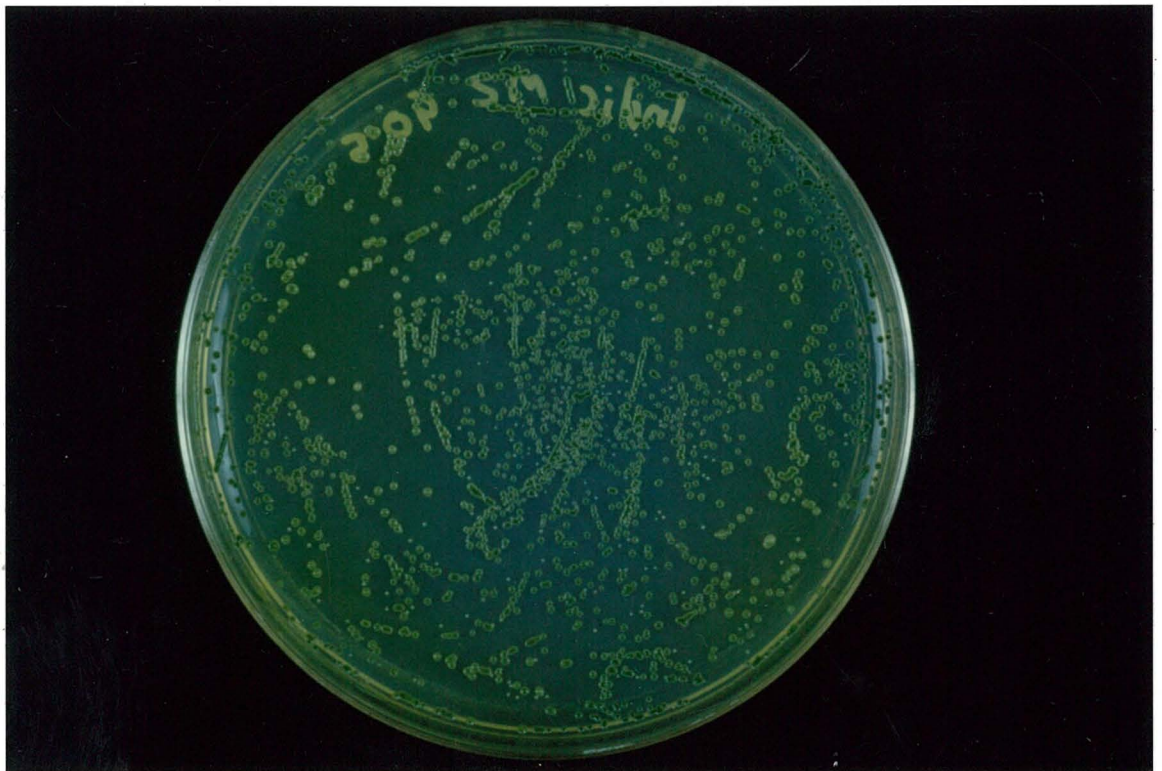
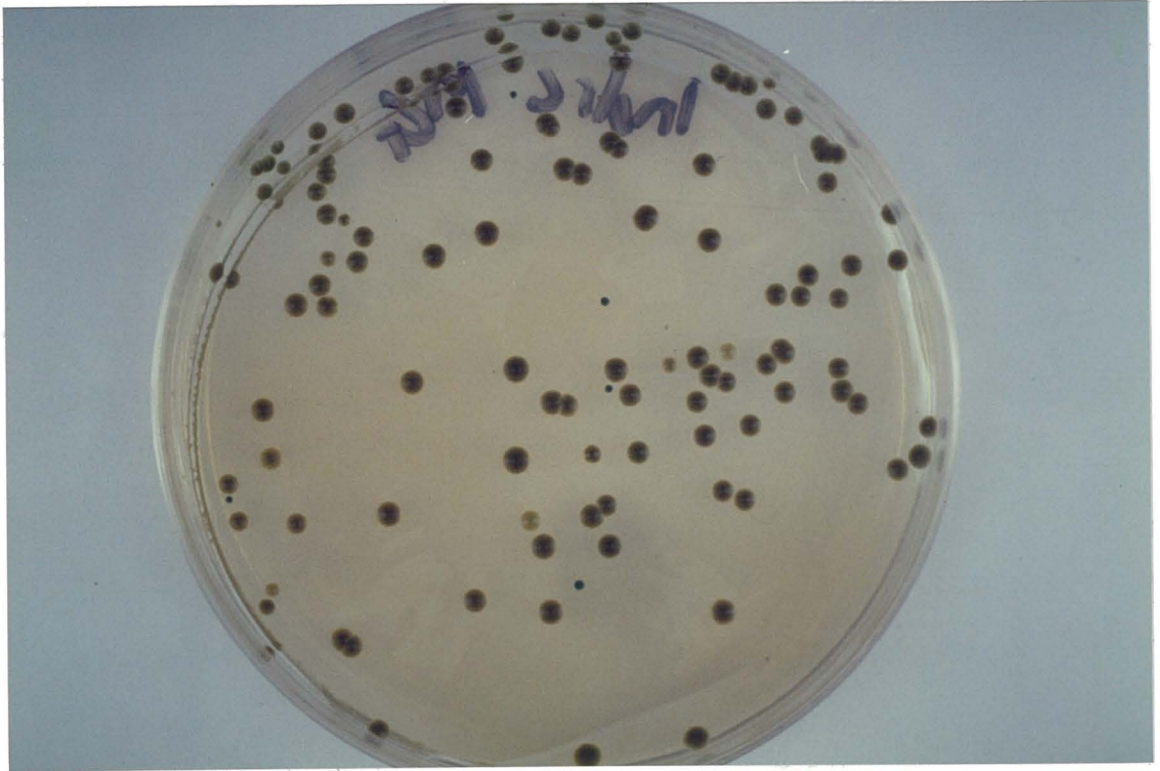
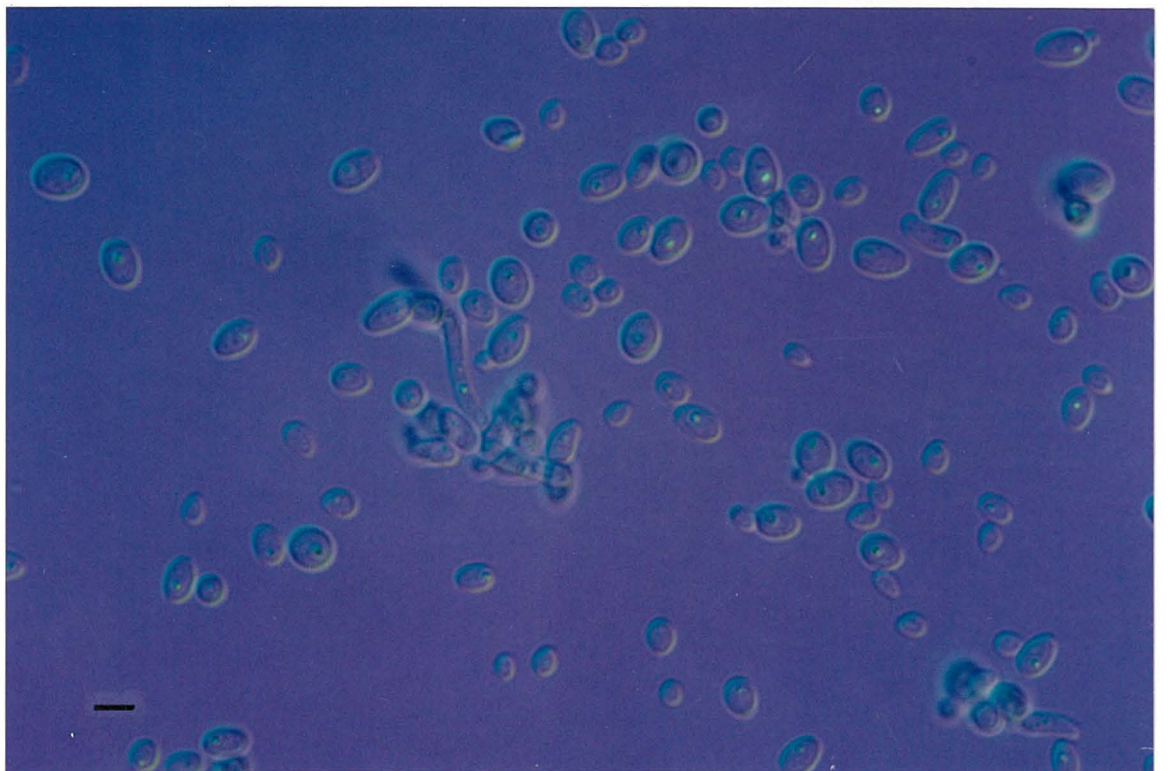
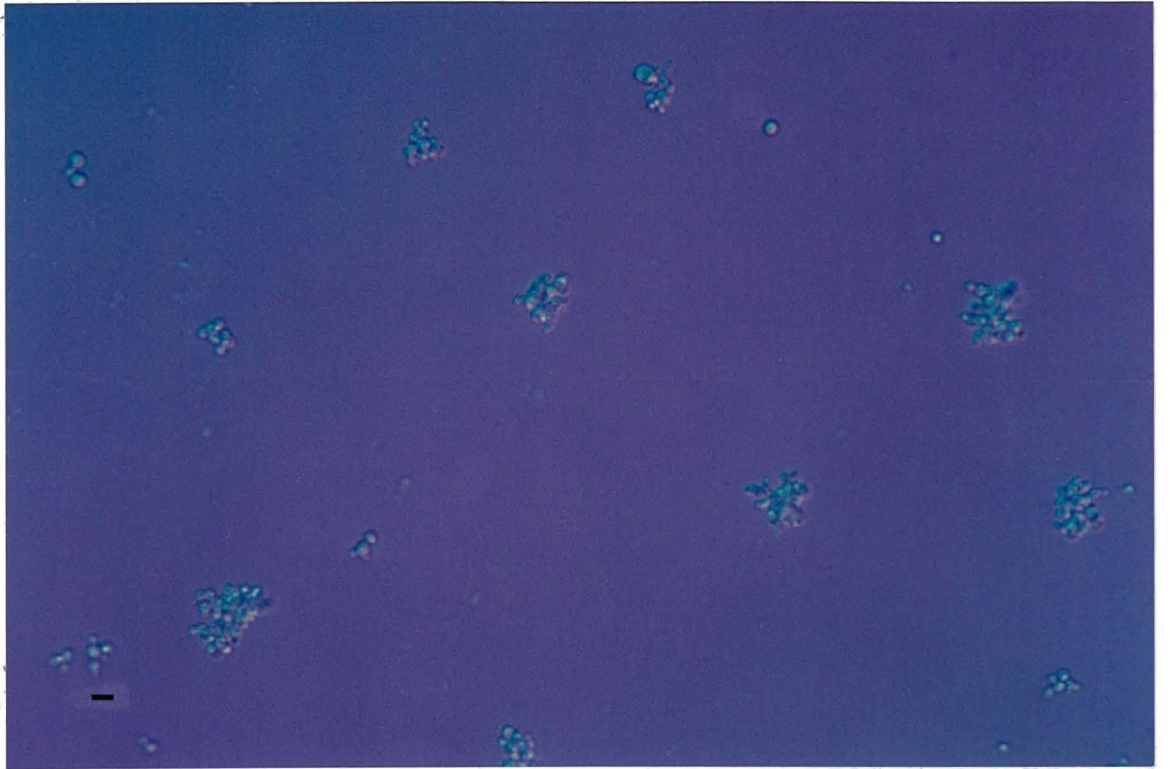


Plate 5. *Pichia stipitis* mutant 77 grown for five days in MMG at 25°C. The cells grow in large clumps. Photographed with Nomarski Optics plus blue colour and polarising filters. Bar is ten microns long.

Plate 6. *Pichia stipitis* Y7124 grown for three days at 25°C on Gorodkova Medium (per litre: 1 g D-glucose, 10 g bacto peptone, 5 g NaCl, 20 g agar) underneath a coverslip and then suspended in water. Large structures are pseudohyphae. Bright inclusions within cells are crystals of uric acid in the vacuole (commonly termed 'dancing bodies'). Photographed with Nomarski Optics plus blue colour and polarising filters. Bar is five microns long.



## 2.7 Anaerobic utilisation of sugars

The Durham method (Barnett *et al.* 1983) was used to test the ability of yeasts to grow anaerobically on glucose and xylose. This method was used with the following modifications: The test tubes were 150 mm in length and 15 mm in diameter. The Durham tubes were 50 mm in length and 15 mm in diameter. The tubes were incubated for two weeks.

## 2.8 UV Mutagenesis and screening by indicator plate

A two day culture of *Pichia stipitis* Y7124 containing  $4 \times 10^7$  cells / ml was diluted 1000-fold in sterile distilled water and 5 ml were exposed to 40 seconds of UV radiation in a sterile glass petri dish. A kill of approximately 90% was obtained. Samples (0.1 ml) of the exposed culture were spread onto 45 plates of Indicator MG and incubated in the dark for six days at 30°C.

*Pachysolen tannophilus* 2530 was treated similarly. The culture initially contained  $9 \times 10^8$  cells / ml and was diluted 100-fold before exposure to UV radiation. A kill of between 90 - 99% was achieved. Samples (0.1 ml) of the exposed culture were spread onto 160 plates of Indicator MG (modified) and incubated in the dark for a period of four days at 30°C.

## 2.9 UV mutagenesis and 2-deoxyglucose selection

A two day culture of *Pichia stipitis* Y7124 was grown in MMG. A 5 ml sample was placed undiluted into a glass petri dish and exposed to 40 seconds of UV radiation. The culture contained  $1.54 \times 10^8$  cells / ml before exposure. A kill of 72% was achieved. Samples (0.1 ml) of the exposed culture were spread,

undiluted, onto plates of YNB-malate supplemented with 6 mM 2-deoxyglucose. These plates were incubated in the dark for six days.

#### 2.10 Estimation of dry cell mass

Cell number and colony forming units were found to be unsuitable methods for measuring the amount of yeast present. The two species studied are both bipolar budding yeast and in the case of *Pichia stipitis* grew in MMG in the form of branched chains of five to ten cells extending out into all three planes (Plate 2). Accurate counting of cells was difficult and, in addition to this, certain mutant strains of *Pichia stipitis* formed dense spheres containing very many cells (Plate 5). Thus, dry cell mass was used as the standard method of measurement. Dry cell mass was related to the absorbance of light at 600 nm (Abs<sub>600</sub>) for the wild-type strains of *Pachysolen tannophilus* and *Pichia stipitis*.

A two day culture of the appropriate species was grown in MMG and counted using an Improved Neubauer Haemocytometer. Six 250 ml Erlenmeyer flasks containing 100 ml of MMG (pH 3.0) topped with fermentation traps were prepared. Aliquots of culture were added to each of the flasks to give a cell number of  $1 \times 10^5$  cells / ml.

The flasks were incubated at 25°C for five days while shaken at 100 rpm. A 25 ml sample of each flask was vacuum aspirated onto a 47 mm diameter nitrocellulose filter of 0.45 µm pore size. Each filter had been pre-dried and pre-weighed. In the case of *Pachysolen tannophilus* the culture was first washed twice to remove mucoid material (polysaccharide). Washing was by spinning down the culture at 7649 x g for ten minutes, followed by resuspension. The filters were dried under vacuum, at 65°C and 20 mm of mercury, to a constant weight; a process which took 64 hours.

At the time of aspirating the cultures onto filters an additional aliquot was taken from each flask and diluted with MMG to produce 10 dilutions from 1-in-10 to 1-in-100. The absorbance of light at 600 nm (Abs<sub>600</sub>) of each dilution was measured. The cell number of each dilution was determined by counting cells using an Improved Neubauer Haemocytometer.

For each dilution, Abs<sub>600</sub> was plotted against dry cell mass. For each flask, regression analysis of the data was performed by the software package: Cricket Graph, version 1.2. The correlation coefficient (R<sup>2</sup>) was typically 0.99 for *Pachysolen tannophilus* and 1.00 for *Pichia stipitis*. (Appendix 5.2 provides an example). To find the increase in dry cell mass represented by an increase of Abs<sub>600</sub> of 1.0, the linear equations were solved for y. The mean of the six values was calculated and the sample standard deviation found using the equation:

$$\sigma_{n-1} = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1}}$$

The relationship between cell number and Abs<sub>600</sub> was also found using this method.

### 2.11 Performance of semi-anaerobic fermentations

To study the uptake of organic acids and sugars under semi-anaerobic conditions the following protocol was followed:

Cultures were set up using standard procedures (Sections 2.3 & 2.5) and were performed in duplicate. A 10 ml sample was taken from each flask once per 12 or 24 hours and the Abs<sub>600</sub> measured. The sample was spun at 3910 x g for 30 minutes in a Heraeus Christ Labofuge GL bench top centrifuge to remove the yeast

cells. The supernatant was filtered through a 0.45  $\mu\text{m}$  nitrocellulose filter after its pH had been measured and frozen to await HPLC analysis.

### 2.12 HPLC methods

The major separation necessary for this study was between glucose and malic acid. The separation was achieved by minimising dead volumes in the HPLC and by using a cation exchange column followed by a reverse phase column.

The work was performed on a Shimadzu LC-4A High Performance Liquid Chromatograph. The samples were injected using a SIL-2AS Autosampler. The columns were protected with a Biorad Microguard® Cation H guard cartridge (dimensions 40 mm long with an internal diameter of 4.6 mm). Samples were separated using two columns in series - a Brownlee PPH-224 cation exchange column (Polypore H, dimensions: 220 mm long with a 4.6 mm internal diameter), followed by a Brownlee OSS-MP reverse phase column (Spheri 5, dimensions: 100 mm long with an internal diameter of 4.6 mm). The columns were maintained at the appropriate temperature by a CTO-2AS column oven. Peaks were detected on a SPD-2AS Ultraviolet (UV) Detector or a RID-2AS Refractive Index (RI) Detector. Chromatograms were recorded on a C-R3A Chromatopac.

The chromatopac was calibrated using low and high combined standards of malic acid and glucose. All standards were made in 250 ml amounts, dispensed into 3.5 ml amounts, and then frozen until required. The high standard contained 9.008% (0.5 M) glucose and 2.0115% (0.15 M) malic acid. Calibration of the Chromatopac for succinic acid and glucose was performed in an identical manner, except the high standard contained 1.7715% succinic acid (0.15 M). The low standard was prepared by diluting the high standard 10-fold volumetrically. The RI calibration was a two point calibration. The UV measurements were made

using a Chromatopac program that used one and two point calibrations together to give a three point calibration. It was shown that malic acid and glucose gave a linear response over differing concentrations. (Appendices 6.3 & 6.4).

The operating conditions for the separation were: solvent 0.013 N H<sub>2</sub>SO<sub>4</sub>, flow rate 0.3 ml per minute and oven temperature of 30°C, the UV detector was set to a wavelength of 210 nm and full range. The RI detector was set to range 4.

Malic and succinic acid concentrations were measured using the UV detector. Glucose concentrations were measured using the RI detector. Samples were run 44 at a time. Low and high standards preceded and followed each set of experimental samples and analysed to check consistency of analysis.

#### 2.13 Preparation of cell free extracts

A *Pichia stipitis* culture was prepared by the procedure described in Section 2.5 and allowed to grow for 72 hours (to mid exponential phase). A 300 ml aliquot of the culture was harvested and spun down at 2560 x g for ten minutes, washed twice in chilled (4°C) sterile distilled water before being finally resuspended in 10 ml of 0.1 M Tris pH 7.6. The cells were broken by passing the suspension through a French Press three times. Breakage was estimated by counting broken and unbroken cells under the microscope, and was estimated to be about 50% in all cases. Cell debris was removed by centrifuging at 7796 x g for 20 minutes and the supernatant recovered.

#### 2.14 Hexokinase assays

Hexokinase assays were carried out according to the protocol of Joshi and Jagannathan (1966) and were performed at 30°C in a water jacketed Gilford 260

Spectrophotometer. These assays were carried out on both fresh and frozen cell free extracts.

### 2.15 Protein assays

Total protein concentrations were determined by the method of Ehresmann *et al.* (1973) using a Gilford 260 Spectrophotometer. The standard was bovine serum albumin (BSA). Appendix 5.5 shows the relationship of BSA concentration to absorbance.

### 2.16 Nomarski optics microscopic photography

Cultures of *Pachysolen tannophilus* and *Pichia stipitis* were grown on solid media and in liquid media as per Sections 2.2 and 2.3 with the following modifications: all incubation was at 25°C, and some cultures were grown on solid media underneath a cover slip (i.e. anaerobically).

Photography was performed using a Zeiss Axiophot photographic microscope utilising Nomarski Optics, and a variety of coloured and polarising filters. Images were recorded on Kodak Ektachrome colour slide film (35 mm, ASA 50).

### 3.0 RESULTS

In order to isolate and characterise mutants of *Pachysolen tannophilus* and *Pichia stipitis* that utilise organic acids at higher rates in the presence of glucose, the physiology of the wild type strains of these species was studied. Mutants were generated by UV irradiation and were selected or screened for by a variety of methods. A number of mutants, both from this study and from elsewhere (Table 3), were further characterised.

#### 3.1 Growth of *Pachysolen tannophilus* and *Pichia stipitis* wild type strains on different organic acids

The ability of *Pachysolen tannophilus* 2530 and *Pichia stipitis* Y7124 to grow on citrate, fumarate and malate over a pH range of 2.5 to 6.5 was determined (Tables 4 & 5). The degree of growth was scored on the basis of colony size. It was found that the best growth occurred at a pH where the majority of acid molecules were in the uncharged state. An exception was *Pichia stipitis* grown on citrate, where best growth occurred on plates containing citrate in the singly deprotonated state. Poor growth of both species at pH 6.5 was specifically associated with organic acids as a carbon source, because both species grew well on YNB-xylose at this pH. The inability of *Pachysolen tannophilus* to grow on citrate agrees with results given in standard taxonomic texts (Barnett *et al.* 1983, Kurtzman 1984a).

#### 3.2 Anaerobic utilisation of sugars

The wild-type strains of *Pachysolen tannophilus* and *Pichia stipitis* were tested for the ability to utilise glucose and xylose anaerobically (Table 6).

Table 4. *Pachysolen tannophilus* 2530 grown on solid media plus organic acids

YNB-Organic acid plate				
pH	Citrate	Succinate	Fumarate <sup>a</sup>	Malate
2.5	-	+++	+++	+++
3.0	-	+++	+++	++
3.8	-	+++	+++	+++
4.0	-	++	++	++
5.0	-	+	+	+
6.5	-	-	-	-

+++ : Good Growth; ++ : Reduced growth; + : Slow growth (not apparent until 3 days); +/- : Weak growth (not apparent until 6 days); - : No growth at 6 days.

a: Concentration and pH is approximate.

Table 5. *Pichia stipitis* Y7124 grown on solid media plus organic acids

YNB-Organic acid plate				
pH	Citrate	Succinate	Fumarate <sup>a</sup>	Malate
2.5	+/-	+++	++	+++
3.0	+/-	+++	++	+++
3.8	+++	+++	+++	+++
4.0	+++	+++	+++	++
5.0	+++	+++	++	++
6.5	++	+	+	+

+++ : Good Growth; ++ : Reduced growth; + : Slow growth (not apparent until 3 days); +/- : Weak growth (not apparent until 6 days); - : No growth at 6 days.

a: Concentration and pH is approximate.

Plate 7. Comparison of response of *Pichia stipitis* strains on Indicator MG medium.

A = Y7124 (wild type).      B = mutant 411.      C = mutant 77.

Yeast was streaked out and the plates incubated at 30°C for six days.

Plate 8. Behaviour of *Pachysolen tannophilus* mutant F/G 2 on different media.

A = Indicator MG.      B = Indicator M.      C = Indicator MG supplemented with 2 g/l xylose.

Yeast was streaked out and the plates incubated at 30°C for six days.

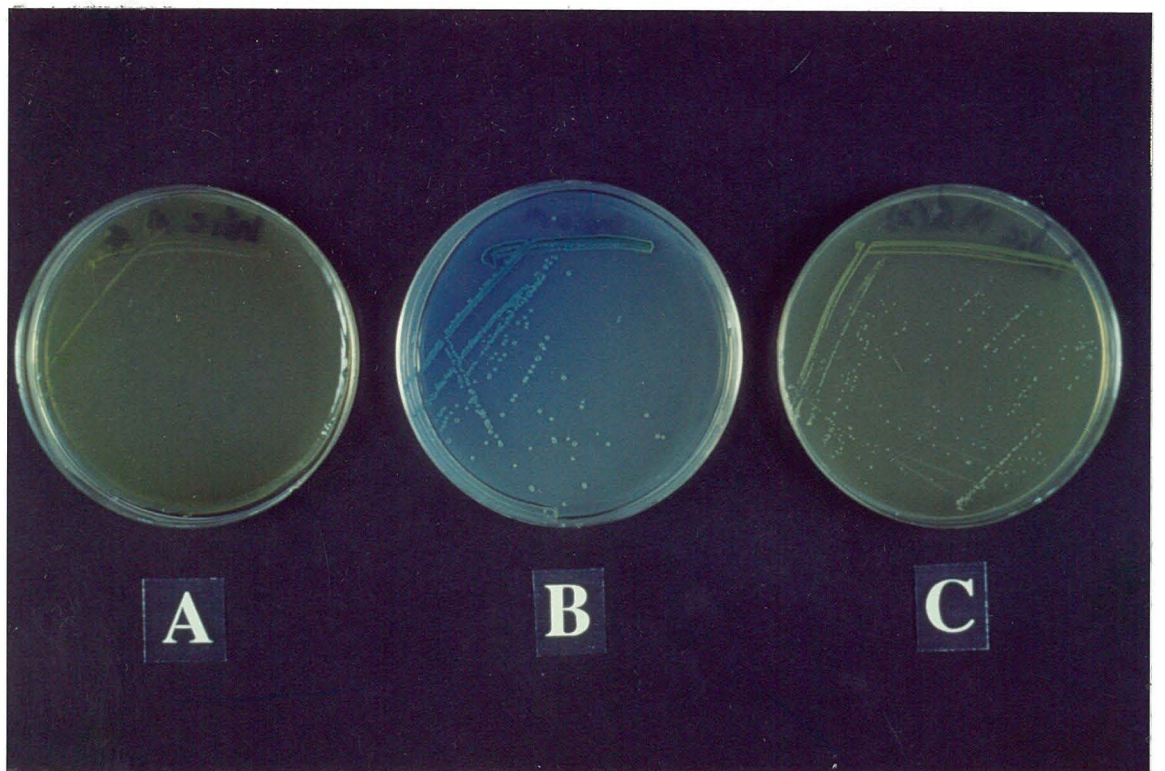
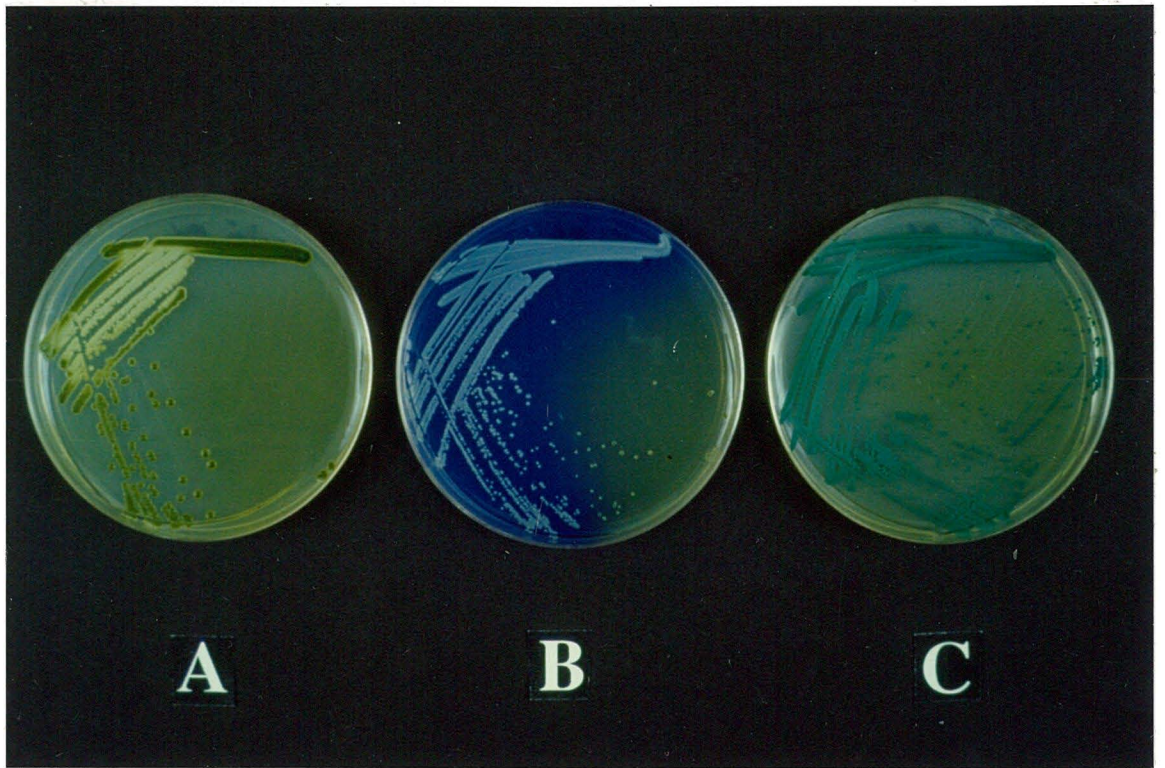


Table 6. Ability of strains to utilise sugars anaerobically

Yeast	Strain	Glucose	Xylose
<i>Pachysolen</i>	2530	+	+/-
<i>Pichia</i>	Y7124	+	+

+: gas formed; +/-: gas formed in one tube; -: no gas formed.

Tubes scored after 14 days.

The inability of *Pachysolen tannophilus* 2530 to grow on xylose at low oxygen levels confirmed previous observations (Schneider *et al.* 1981, Maleszka & Schneider 1982, Watson *et al.* 1984). Under the same conditions, *Pichia stipitis* Y7124 grew well.

*Pichia stipitis* Y7124 showed a significant increase in cell density in the control tubes, which contained yeast extract and no sugars. No gas bubbles were produced. Yeast extract from some sources is rich in  $\alpha,\alpha$ -trehalose (Barnett *et al.* 1983). Some strains of *Pichia stipitis* can utilise this sugar. This result suggests that  $\alpha,\alpha$ -trehalose was present and was utilised by *Pichia stipitis* Y7124.

### 3.3 Mutants isolated on the indicator plate

*Pichia stipitis* Y7124 was UV mutagenised and the survivors screened on Indicator MG. Ninety colonies, from a total of 30 000 were selected for further study on the basis of their unusual colony colouration. Wild-type colouration was olive green (Plate 7). Mutants varied from dark green through lime green to turquoise. Theoretically, mutants capable of utilising malate in the presence of glucose would lower the pH of the medium and the colony would turn blue. One

mutant was selected for further characterisation: mutant 77, a turquoise colony forming isolate (Plate 7).

#### 3.4 Mutants isolated on the modified indicator plate

*Pachysolen tannophilus* 2530 was UV mutagenised and the survivors screened on Indicator MG (modified). A total of 374 white colonies from 160 000 survivors were selected for further study. The white colonies were patched onto plates of Indicator MG (modified) medium, 32 per plate and incubated at 30°C for four days. Fourteen colonies retained the white colour and these were individually streaked onto plates of Indicator MG medium and incubated at 30°C for six days. Only one isolate turned this medium blue: mutant 335. This mutant was chosen for further characterisation.

#### 3.5 Mutants isolated by selection on malic acid and 2-deoxyglucose

*Pichia stipitis* Y7124 was UV mutagenised and the survivors screened on plates containing malic acid (20 g/l) and the lethal glucose analog 2-deoxyglucose at concentrations of 6 mM and 8 mM. Approximately 1 000 colonies grew on each plate. A total of 103 of the largest colonies were picked from plates of each of the two concentrations of 2-deoxyglucose. Fifty-two colonies were patched per plate, onto YNB plates containing malate and 6 mM 2-deoxyglucose. After five days incubation at 30°C, they were replica plated onto the following plates: YNB-malic acid, YNB-malic acid plus 20 mM 2-deoxyglucose, YNB-xylose, YNB-glucose, YNB-mannose, YNB-fructose and incubated at 30°C for three days. The isolates were divided into three phenotypic classes on the basis of growth on YNB-mannose and YNB-fructose: mannose<sup>+</sup> fructose<sup>w</sup>, mannose<sup>w</sup> fructose<sup>w</sup>, and mannose<sup>w</sup> fructose<sup>-</sup> (where + = growth, w = weak growth, and - = no growth). One isolate was chosen from each phenotypic group for further study.

These were mutants 287, 238 and 411, respectively. It was observed that isolates resistant to 6 mM 2-deoxyglucose were also resistant to 20 mM 2-deoxyglucose. Mutants 238 and 287 were initially isolated on 6 mM 2-deoxyglucose. Mutant 411 was isolated on 8 mM 2-deoxyglucose.

### 3.6 Relationship of dry weight and cell number to optical density

Cell number and colony forming units were found to be unsuitable methods for measuring the amount of yeast present (Section 2.10).

The relationship between the absorbance of light at 600 nm ( $Abs_{600}$ ) and dry cell mass was determined,

for *Pachysolen tannophilus* as:

$$\Delta Abs_{600} \text{ of } 1.0 = 0.58 \pm 0.05 \text{ mg/ml dry cell mass.}$$

and for *Pichia stipitis* as:

$$\Delta Abs_{600} \text{ of } 1.0 = 0.61 \pm 0.06 \text{ mg/ml dry cell mass.}$$

The relationship between the  $Abs_{600}$  and cell number was also determined,

for *Pachysolen tannophilus* as:

$$\Delta Abs_{600} \text{ of } 1.0 = 1.87 \times 10^8 \pm 7.69 \times 10^7 \text{ cells per ml.}$$

and for *Pichia stipitis* as:

$$\Delta Abs_{600} \text{ of } 1.0 = 5.80 \times 10^7 \pm 1.24 \times 10^7 \text{ cells per ml.}$$

The error given is one sample standard deviation ( $\sigma_{n-1}$ ).

### 3.7 Growth of strains on solid media plus sugars

Increased malate utilisation in the presence of glucose might be expected in strains that were carbon catabolite derepressed. Carbon catabolite repression has been linked to the action of hexokinases and the loss of repression to the loss of these enzymes (Section 1.4). Individual hexokinases may vary in the ability to phosphorylate a variety of sugars, and different hexokinases within one species of yeast may differ from each other. Thus, the loss of one or more hexokinases can alter the ability of a strain to grow on a variety of sugars. The strains of interest were tested in this manner (Tables 7 & 8).

The results for *Pachysolen tannophilus* strains P510-5A, P509-3C, P509-1B and D/X A agreed with previously reported results (Wedlock *et al.* 1989), except that growth of strain P509-3C on some sugars was slightly slower. The ability of *Pachysolen tannophilus* to grow on maltose was unexpected and disagreed with results cited in commonly used taxonomic texts (Barnett *et al.* 1983, Kurtzman 1984a). However, it has been observed that yeasts may become able to utilise maltose if passaged on this carbon source (Kreger-Van Rij 1969).

### 3.8 Growth of strains on malic acid plus 2-deoxyglucose

Resistance to 2-deoxyglucose has been related to the absence of hexokinase enzymes (Lobo & Maitra 1977b). Strains lacking one or more hexokinases show greater resistance to 2-deoxyglucose than the wild-type strain. Strains with more hexokinases missing show higher levels of resistance than the wild-type strain or those lacking fewer hexokinases. The strains of interest were grown on solid medium containing malate as a carbon source and concentrations of 2-deoxyglucose between 2 mM and 200 mM.

Table 7. *Pachysolen tannophilus* strains on solid media plus sugars

YNB-Sugar plate					
Strain	Xylose	Glucose	Mannose	Fructose	Maltose
2530	+++	+++	+++	+++	+
P510-5A	+++	+++	+++	+++	+/-
P509-3C	+++	++	+/-	++	+
335	+++	+++	+++	+++	+
P509-1B	+++	-	-	+	-
D/X A	+++	-	-	-	-
F/G 2	+++	-	-	-	-

+++ : good growth; ++ : reduced growth; + : slow growth (not apparent until 3 days); +/- : very slow growth (not apparent until 6 days); - : no growth at 6 days.

Table 8. *Pichia stipitis* strains on solid media plus sugars

YNB-Sugar plate					
Strain	Xylose	Glucose	Mannose	Fructose	Maltose
Y7124	+++	+++	+++	+++	+++
77	ND	ND	ND	ND	ND
238	+++	+++	+	+	+++
287	+++	+++	+	+	+++
411	+++	++	+	++	+++

+++ : good growth; ++ : reduced growth; + : slow growth (not apparent until 3 days); +/- : very slow growth (not apparent until 6 days); - : no growth at 6 days; ND: not determined.

*Pachysolen tannophilus* 2530, P510-5A, P509-3C, 335 and *Pichia stipitis* Y7124 did not grow on 2-deoxyglucose at a concentration of 2 mM. *Pachysolen tannophilus* D/X A and *Pichia stipitis* 77 were not tested on this medium. *Pichia stipitis* 238, 287 and 411 were resistant to 2-deoxyglucose at 200 mM concentrations. The pattern of the remaining strains is given in Table 9.

### 3.9 Growth of strains on xylose plus 2-deoxyglucose

Since the above results shed no light on the nature of the lesion in *Pachysolen tannophilus* mutant 335, the experiment was repeated using plates containing xylose as the carbon source supplemented with varying levels of 2-deoxyglucose (Table 10).

### 3.10 Response of strains to indicator plus organic acids and glucose

Blue colouration of the indicator plate has been used as a sign of malate utilisation in the presence of glucose (Section 4.4). By substituting other acids for malate, the yeast strains under study were further characterised (Tables 11 & 12).

Most of the *Pachysolen tannophilus* strains fell into two groups (Table 11). The first group included strains known to have none or one of the hexokinases missing, i.e. strains 2530, P510-5A, and P509-3C. These strains did not colour the plate blue, so either they did not utilise organic acids in the presence of glucose, or the acid by-products of glucose metabolism masked utilisation. *Pachysolen tannophilus* cannot utilise citrate. Indicator CG plates turned yellow-green as the products of glucose metabolism were excreted into the medium, and thereby lowered the pH.

Table 9. *Pachysolen tannophilus* strains on solid media plus malate and 2-deoxyglucose

YNB-Malate plate plus 2-deoxyglucose						
Strain	2 mM	4 mM	10 mM	20 mM	40 mM	80 mM
P509-1B	++	(+)	(+)	(+)	(+)	-
F/G 2	++	++	++	(+)	(+)	(+)

+: good growth observed within 1 - 3 days; (+): inhibition of growth;

-: no growth.

Table 10. *Pachysolen tannophilus* strains on solid media plus xylose and 2-deoxyglucose

YNB-Xylose plate plus 2-deoxyglucose					
Strain	1 mM	2 mM	4 mM	8 mM	16 mM
2530	+	+	(+)	-	-
P509-1B	+	+	+	+	+
335	+	+	+	+	(+)

+: good growth observed within 1 - 3 days; (+): inhibition of growth;

-: no growth.

The second group included strains known to have lost two or three hexokinases: P509-1B and D/X A. These strains could not grow on Indicator CG as they could not efficiently utilise either glucose or citrate. These strains utilised malate, succinate and fumarate in the presence of glucose.

Mutant 335, showed an intermediate phenotype, utilising glucose on Indicator CG and turned other plates blue. Strain F/G 2 did not grow on these plates as it could

Table 11. *Pachysolen tannophilus* strains on indicator plus organic acids and glucose (plate colour)

Indicator plate plus organic acids and glucose				
Strain	Indic CG	Indic SG	Indic FG	Indic MG
2530	Y-G	G	G	G
P510-5A	Y-G	G	G	G
P509-3C	Y-G	G	G	G
335	Y-G	B	B	B
P509-1B	-	B	B	B
D/X A	-	B	B	B
F/G 2	-	+/-	+/-	-

Table 12. *Pichia stipitis* strains on indicator plus organic acids and glucose (plate colour)

Indicator plate plus organic acids and glucose				
Strain	Indic CG	Indic SG	Indic FG	Indic MG
Y7124	Y-G	G	G	G
77	Y-G	G	G	G
238	B	B	B	B
287	B	B	B	B
411	B	B	B	B

CG: citrate, glucose; SG: succinate, glucose; FG: fumarate, glucose; MG: malate, glucose; Y-G: yellow green; G: green; B: blue; +/-: weak growth; -: no growth.

Plates were scored after six days at 30°C.

not utilise glucose, nor grow on organic acids in the presence of glucose. However, it did grow on malate alone (Plate 8).

*Pichia stipitis* strains fell into two groups: those that utilised organic acids in the presence of glucose and those that did not (Table 12). This latter group consisted of Y7124 and mutant 77; the former consisted of the mutants selected on malate and 2-deoxyglucose: i.e. 238, 287 and 411. The lesion in 238, 287 and 411 which allowed them to utilise malate, also permitted them to utilise the other acids.

Mutant 77 did not discolour the plate which remains either yellow-green or green, however, the colonies were turquoise coloured on Indicator CG, Indicator FG and Indicator MG (Plate 7) and green on Indicator SG .

### 3.11 Response of strains to indicator plus organic acids and xylose

The effect of xylose on the utilisation of organic acids was examined (Tables 13 & 14), because some of the mutants being characterised in this study were isolated in solutions of xylose in the presence of 2-deoxyglucose (Section 2.6).

*Pachysolen tannophilus* strains 2530, P510-5A and 335 did not grow on xylose in the presence of citrate. *Pachysolen tannophilus* strains P509-3C and D/X A, did grow on xylose in the presence of citrate and weak growth of strain F/G 2 was observed, which did not modify the colour of the medium. Generally , organic acids were either not utilised in the presence of xylose or products of xylose metabolism masked this utilisation.

*Pichia stipitis* mutant 287, however, was clearly able to utilise fumarate and succinate in the presence of xylose. The colonies of *Pichia stipitis* 77 were coloured turquoise but the colour of the plate was not modified.

Table 13. *Pachysolen tannophilus* strains on indicator plus organic acids and xylose (plate colour)

Indicator plate plus organic acids and xylose				
Strain	Indic CX	Indic SX	Indic FX	Indic MX
2530	-	G	G	G
P510-5A	-	G	G	G
P509-3C	Y-G	G	G	G
335	-	G	G	G
P509-1B	+/-	G	G	G
D/XA	Y-G	G	G	G
F/G 2	-	G	G	G

Table 14. *Pichia stipitis* strains on indicator plus organic acids and xylose (plate colour)

Indicator plate plus organic acids and xylose				
Strain	Indic CX	Indic SX	Indic FX	Indic MX
Y7124	Y-G	G	G	G
77	ND	ND	ND	G
238	Y-G	G	G	G
287	Y-G	B	B	G
411	Y-G	G	G	G

CX: citrate, xylose; SX: succinate, xylose; FX: fumarate, xylose; MX: malate, xylose; Y-G: yellow green; G: green; B: blue; +/-: weak growth; -: no growth.

Plates were scored after six days at 30°C.

Figure 1. Fermentation of *Pachysolen tannophilus* 2530 (wild-type) on MMG.

○ Glucose      ■ Malate      ▲ Cell mass      ◇ pH

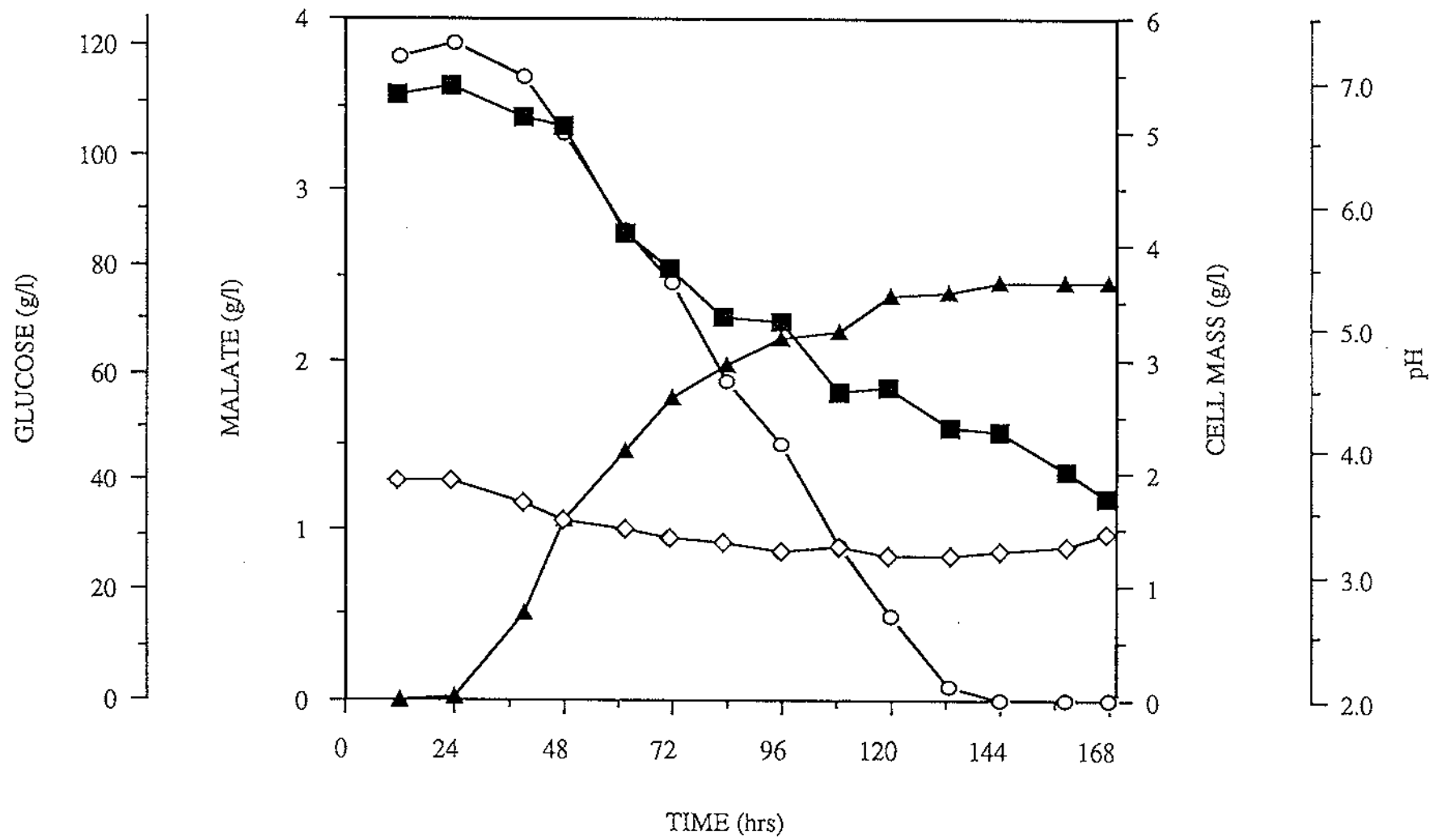


Figure 2. Fermentation of *Pachysolen tannophilus* P510-5A on MMG

○ Glucose      ■ Malate      ▲ Cell mass      ◇ pH

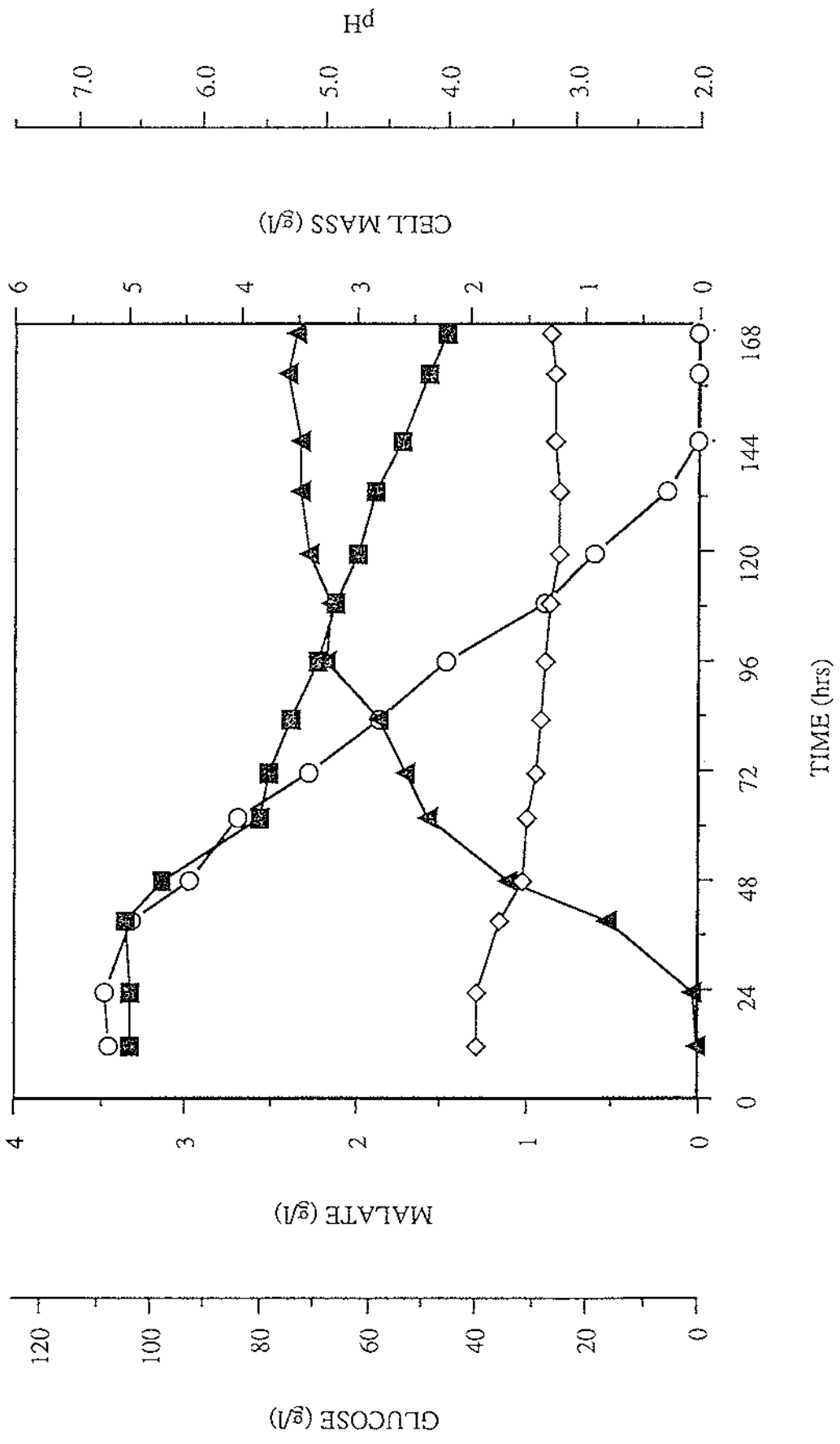


Figure 3. Fermentation of *Pachysolen tannophilus* P509-3C on MMG

○ Glucose      ■ Malate      ▲ Cell mass      ◇ pH

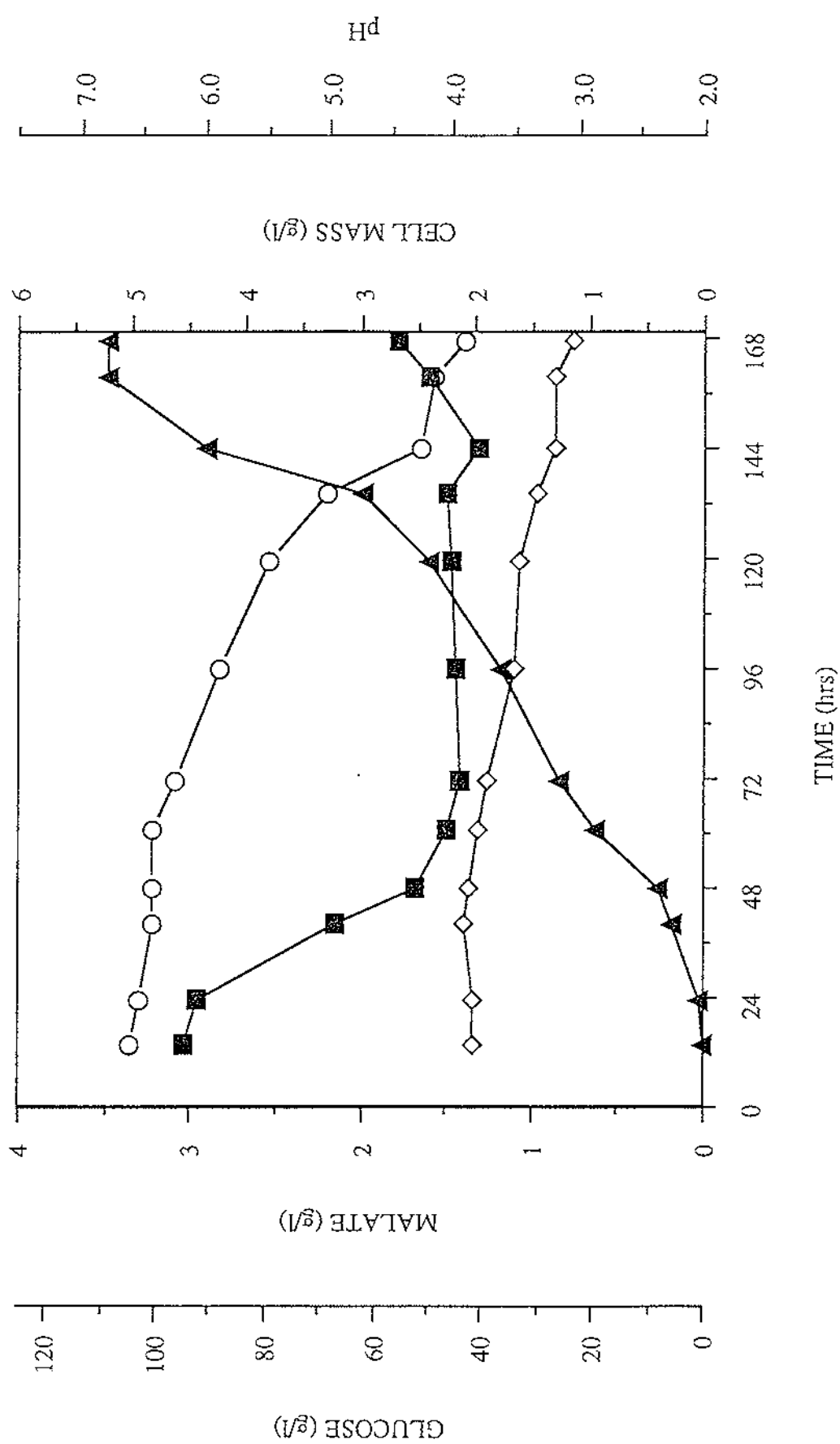


Figure 4. Fermentation of *Pachysolen tannophilus* 335 on MMG

○ Glucose    ■ Malate    ▲ Cell mass    ◇ pH

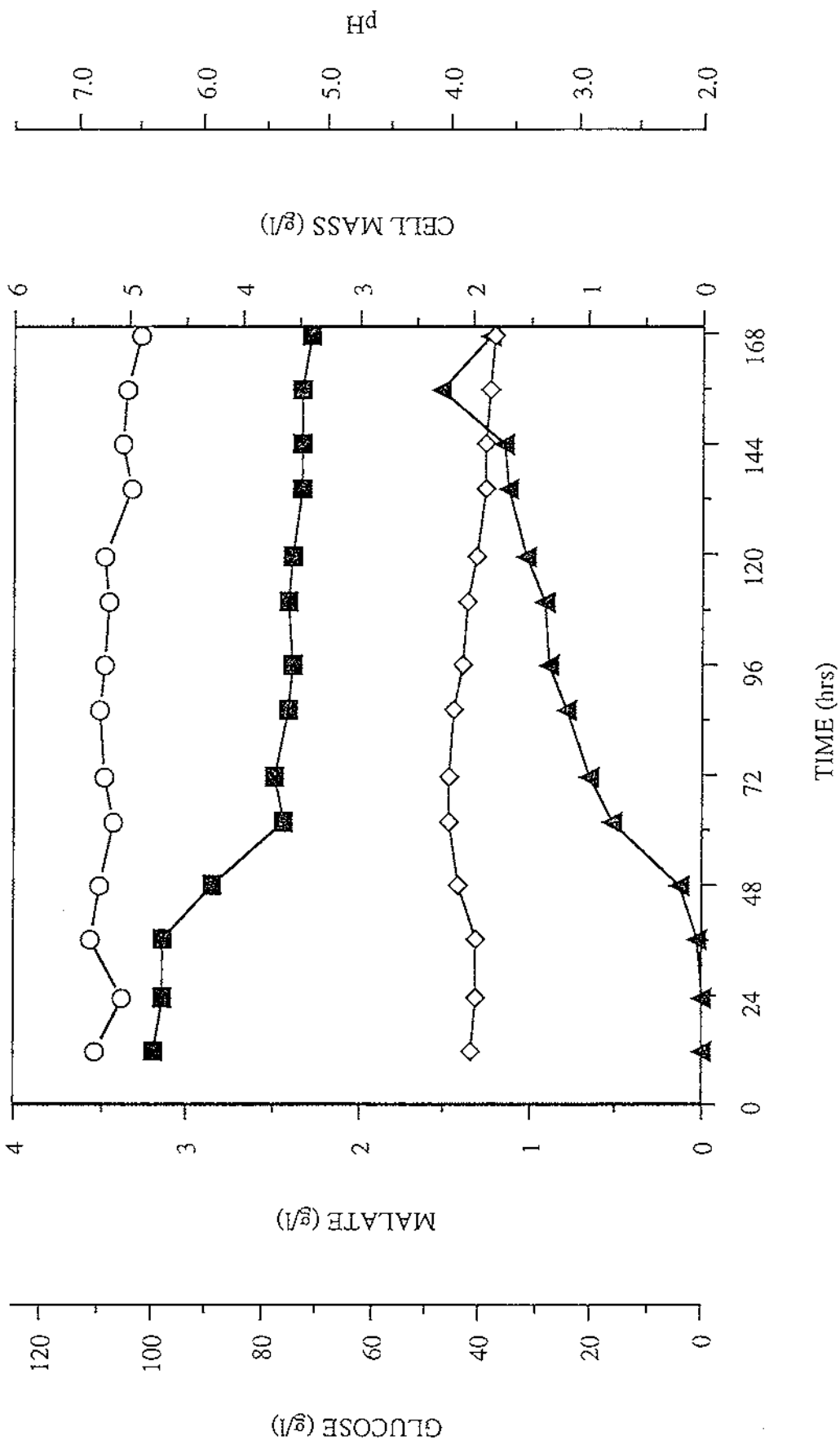


Figure 5. Fermentation of *Pachysolen tannophilus* P509-1B on MMG

○ Glucose    ■ Malate    ▲ Cell mass    ◇ pH

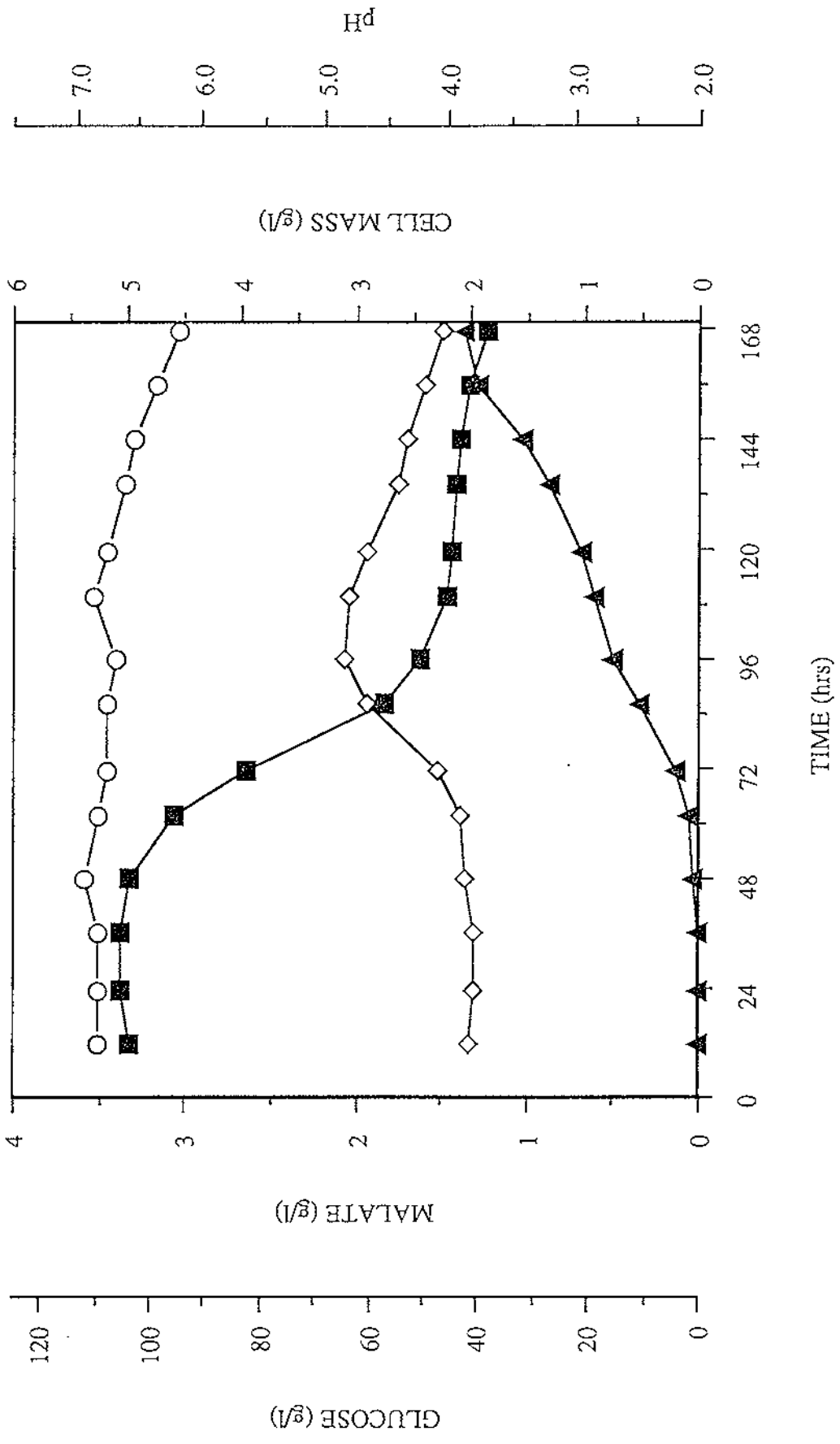


Figure 6. Fermentation of *Pachysolen tannophilus* D/X A on MMG

○ Glucose      ■ Malate      ▲ Cell mass      ◇ pH

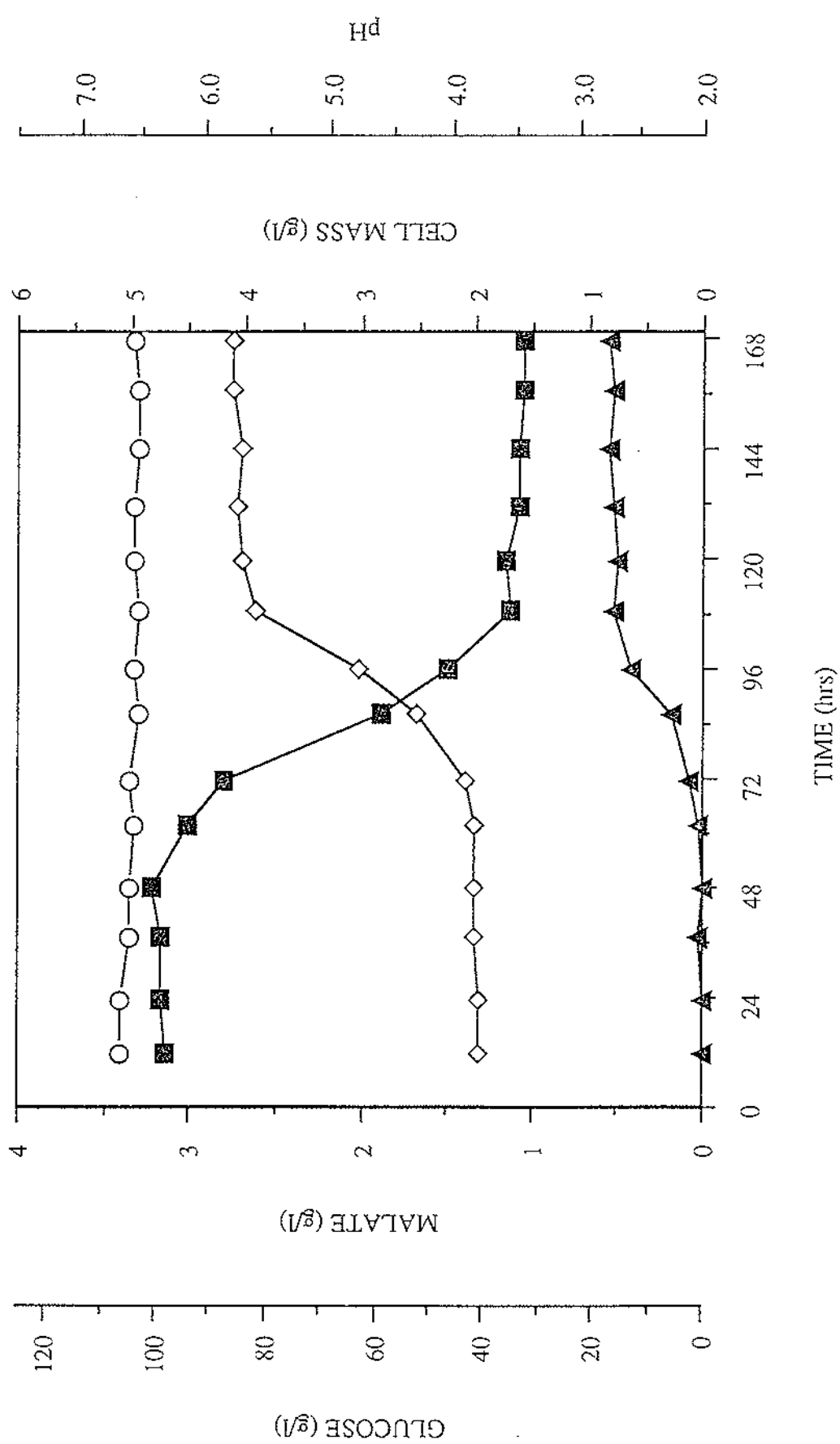


Figure 7. Fermentation of *Pichia stipitis* Y7124 (wild-type) on MMG

○ Glucose    ■ Malate    ▲ Cell mass    ◇ pH

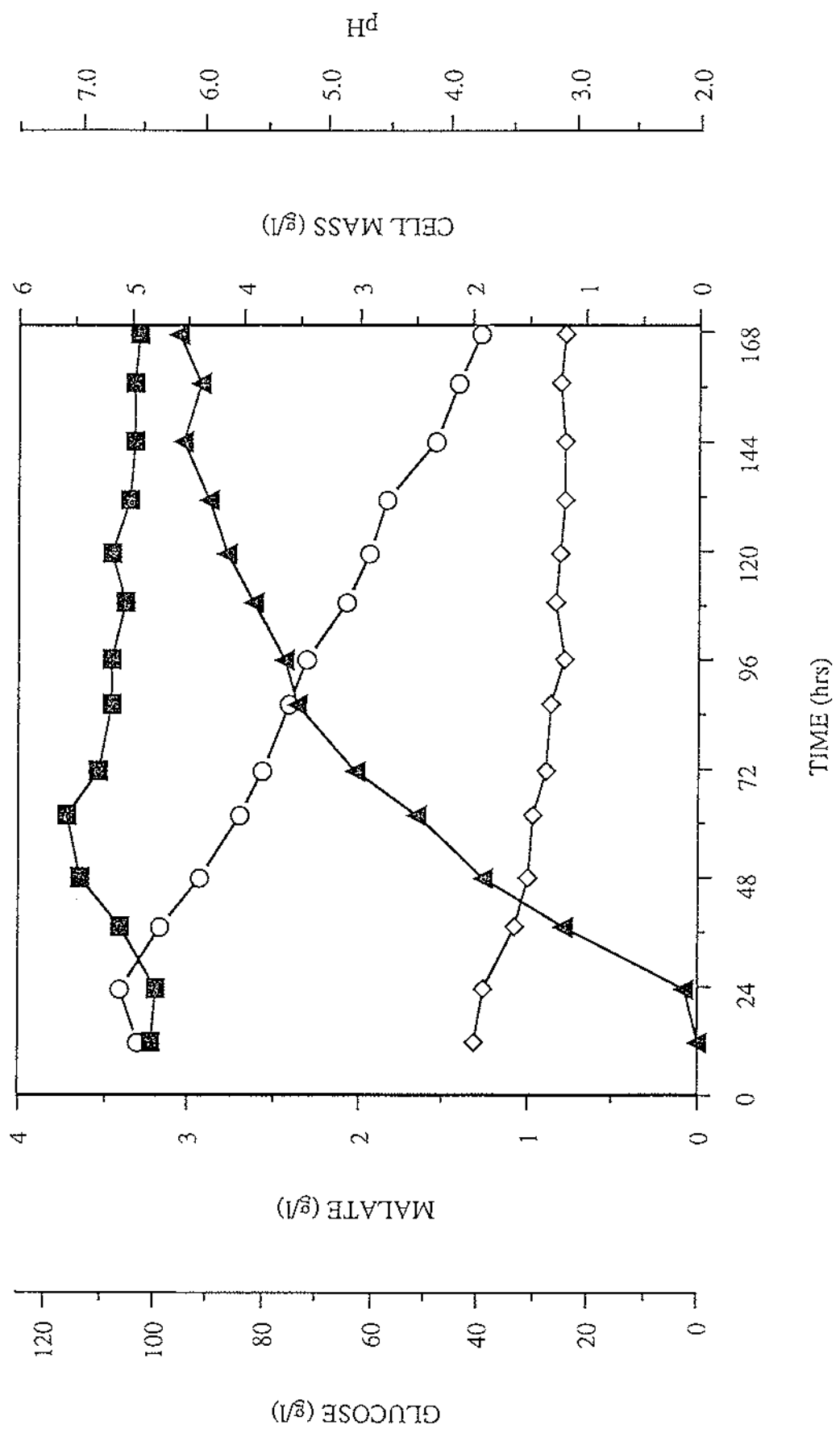


Figure 8. Fermentation of *Pichia stipitis* 77 on MMG

○ Glucose      ■ Malate      ▲ Cell mass      ◇ pH

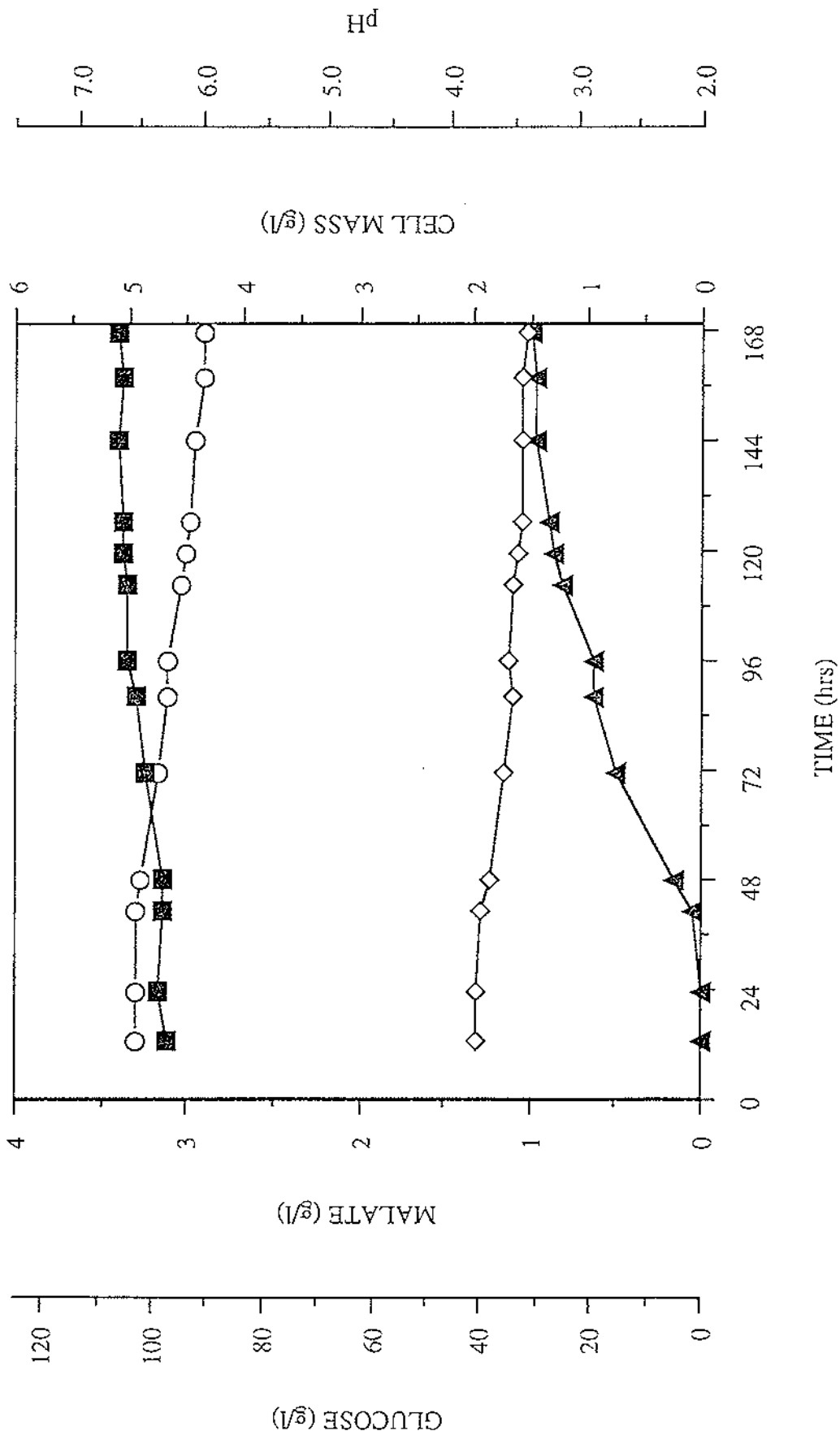


Figure 9. Fermentation of *Pichia stipitis* 238 on MMG

○ Glucose      ■ Malate      ▲ Cell mass      ◇ pH

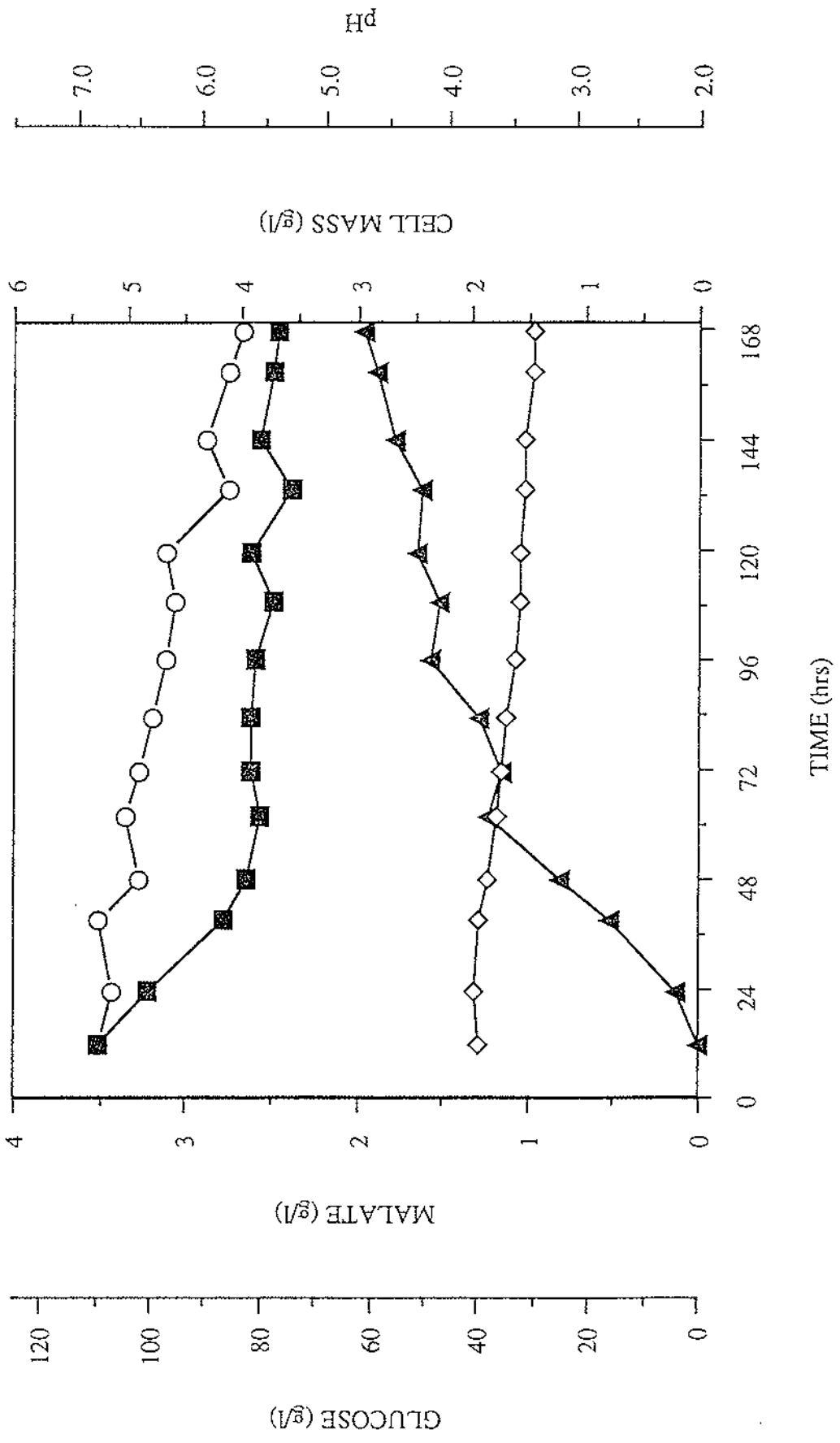


Figure 10. Fermentation of *Pichia stipitis* 287 on MMG

○ Glucose      ■ Malate      ▲ Cell mass      ◇ pH

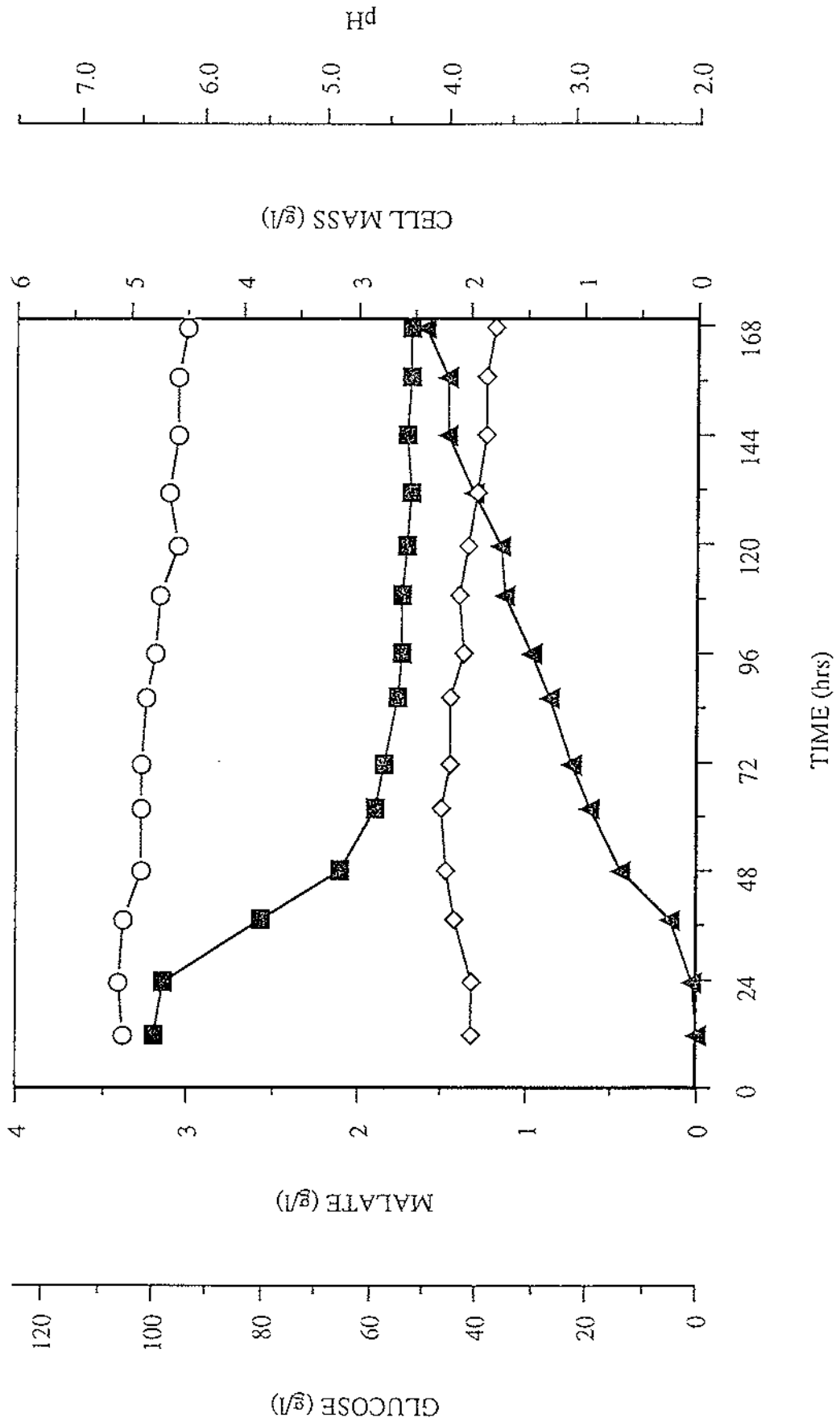


Figure 11. Fermentation of *Pichia stipitis* 411 on MMG

○ Glucose    ■ Malate    ▲ Cell mass    ◇ pH

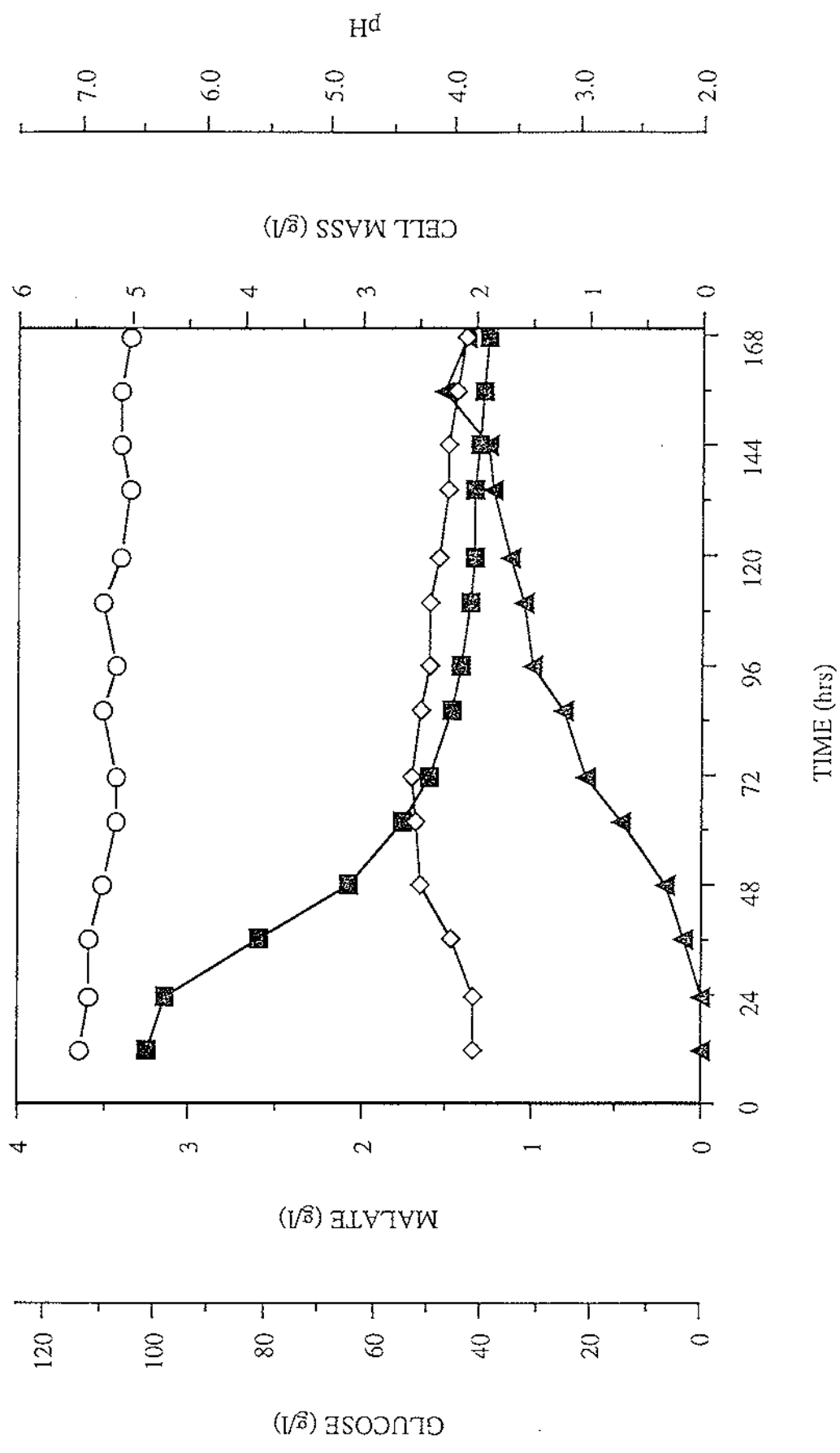


Figure 12. Fermentation of *Pachysolen tannophilus* 2530 (wild-type) on MM

■ Malate      ▲ Cell mass      ◇ pH

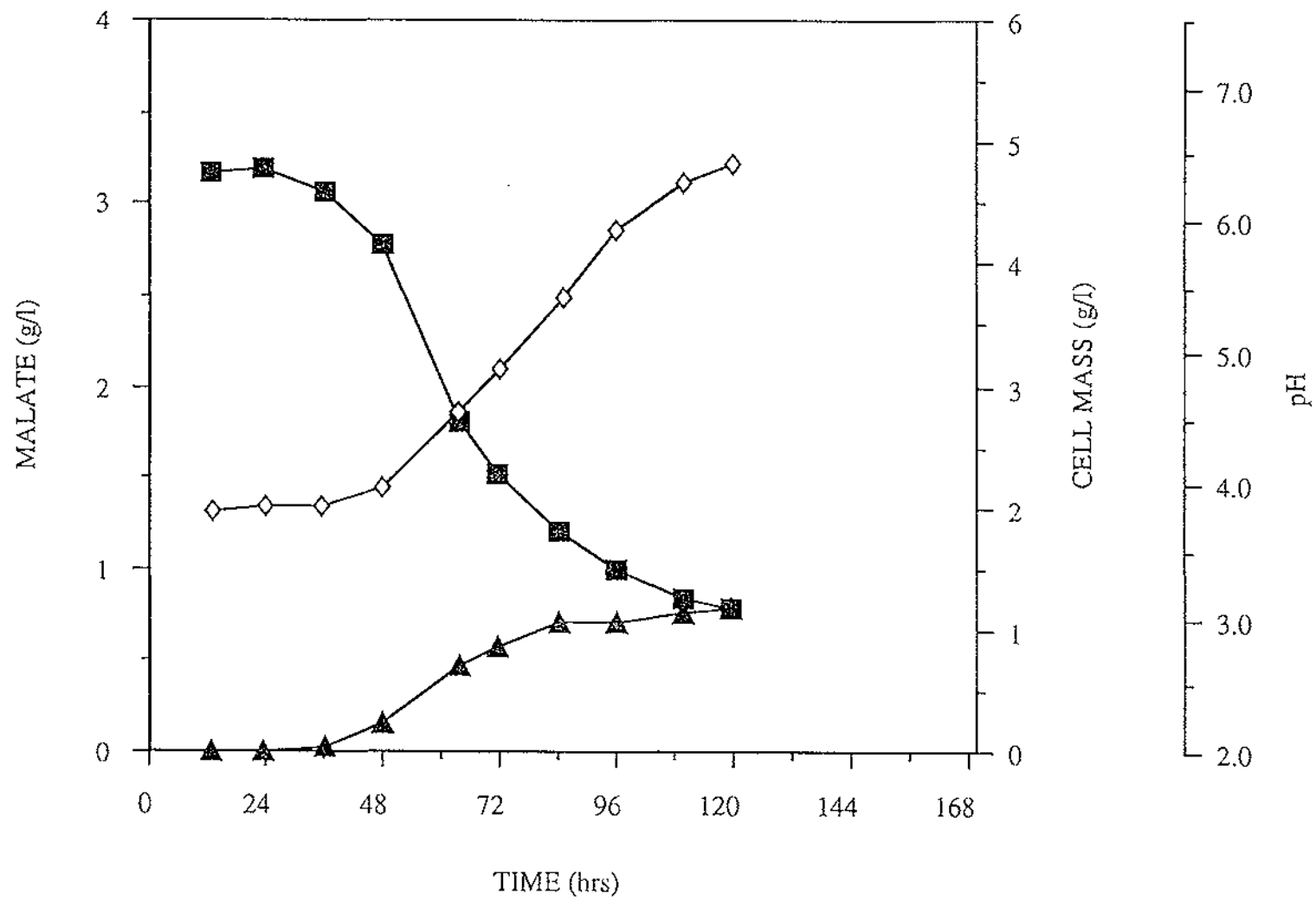


Figure 13. Fermentation of *Pachysolen tannophilus* P510-5A on MM

■ Malate      ▲ Cell mass      ◇ pH

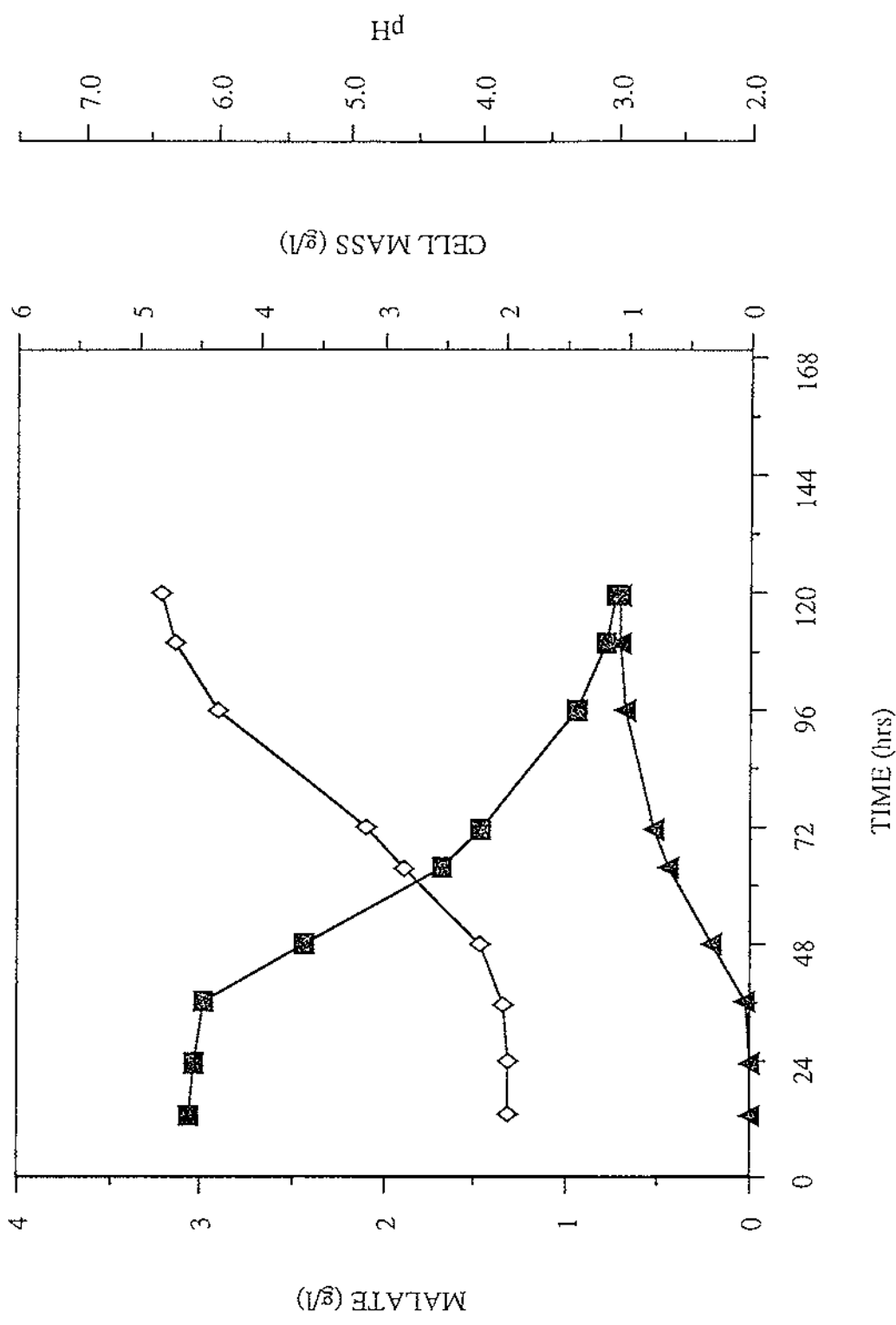


Figure 14. Fermentation of *Pichia stipitis* Y7124 (wild-type) on MM

■ Malate      ▲ Cell mass      ◇ pH

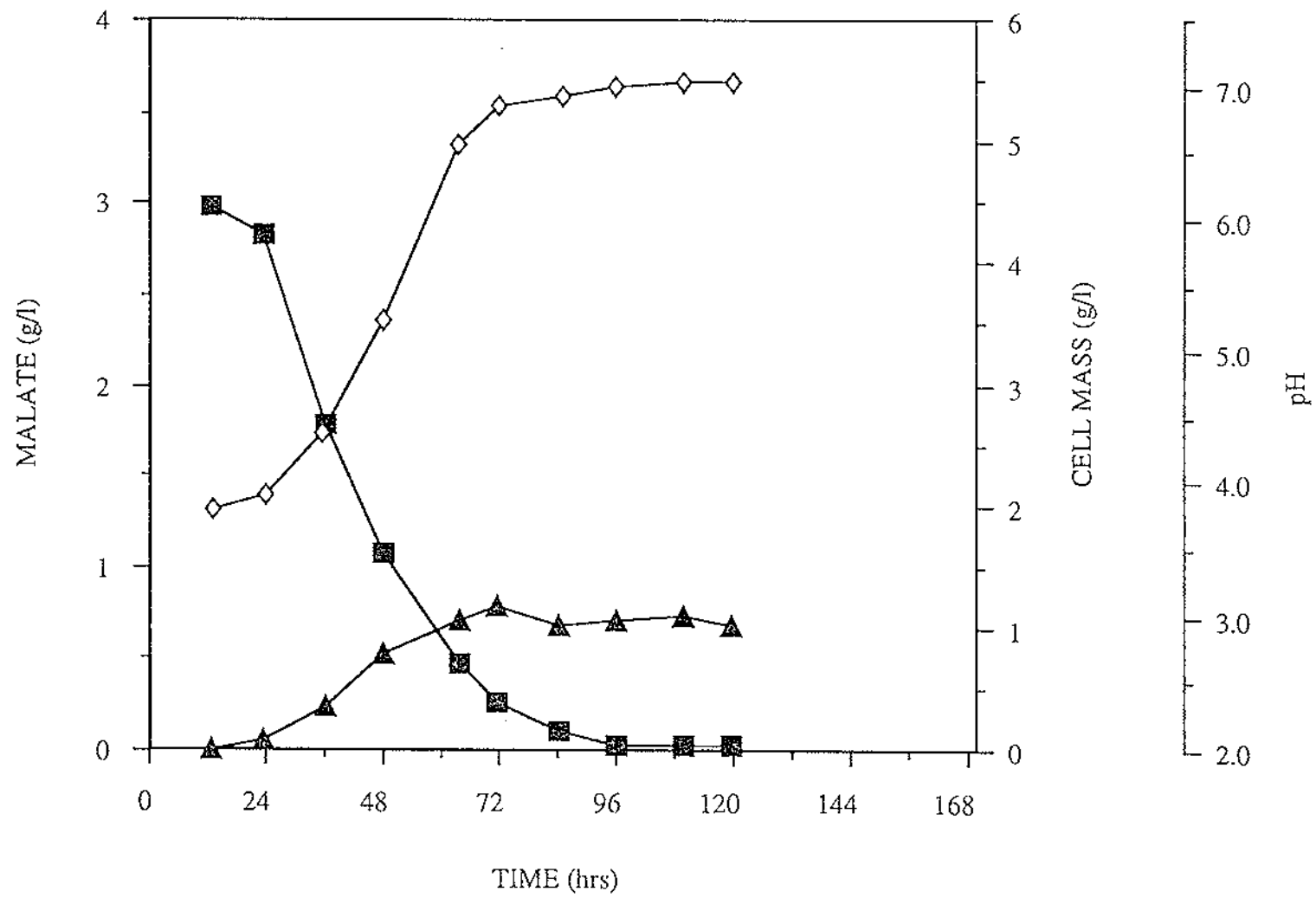


Figure 15. Fermentation of *Pichia stipitis* 77 on MM

■ Malate      ▲ Cell mass      ◇ pH

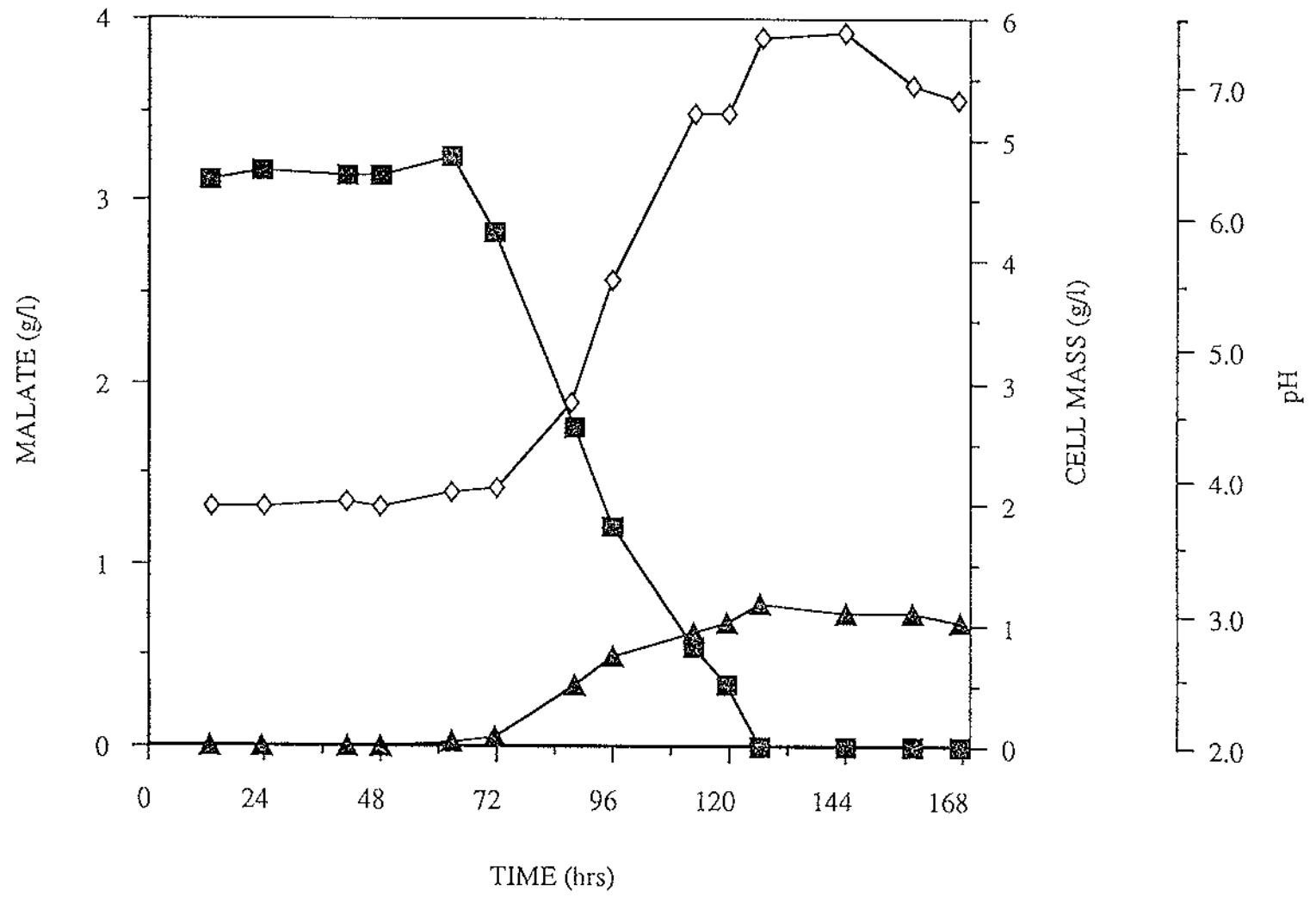


Figure 16. Fermentation of *Pichia stipitis* 238 on MM

■ Malate      ▲ Cell mass      ◇ pH

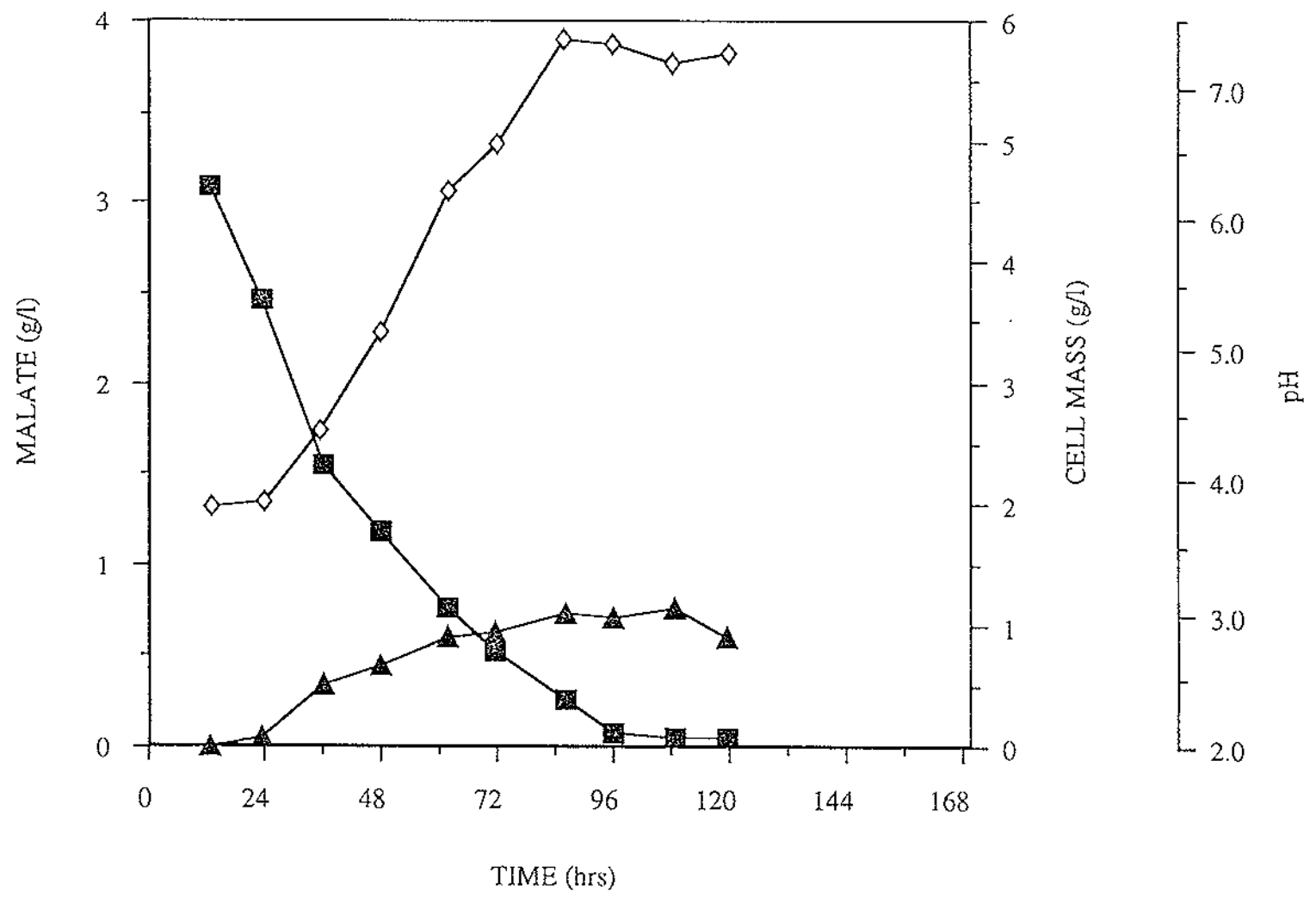


Figure 17. Fermentation of *Pichia stipitis* 287 on MM

■ Malate      ▲ Cell mass      ◇ pH

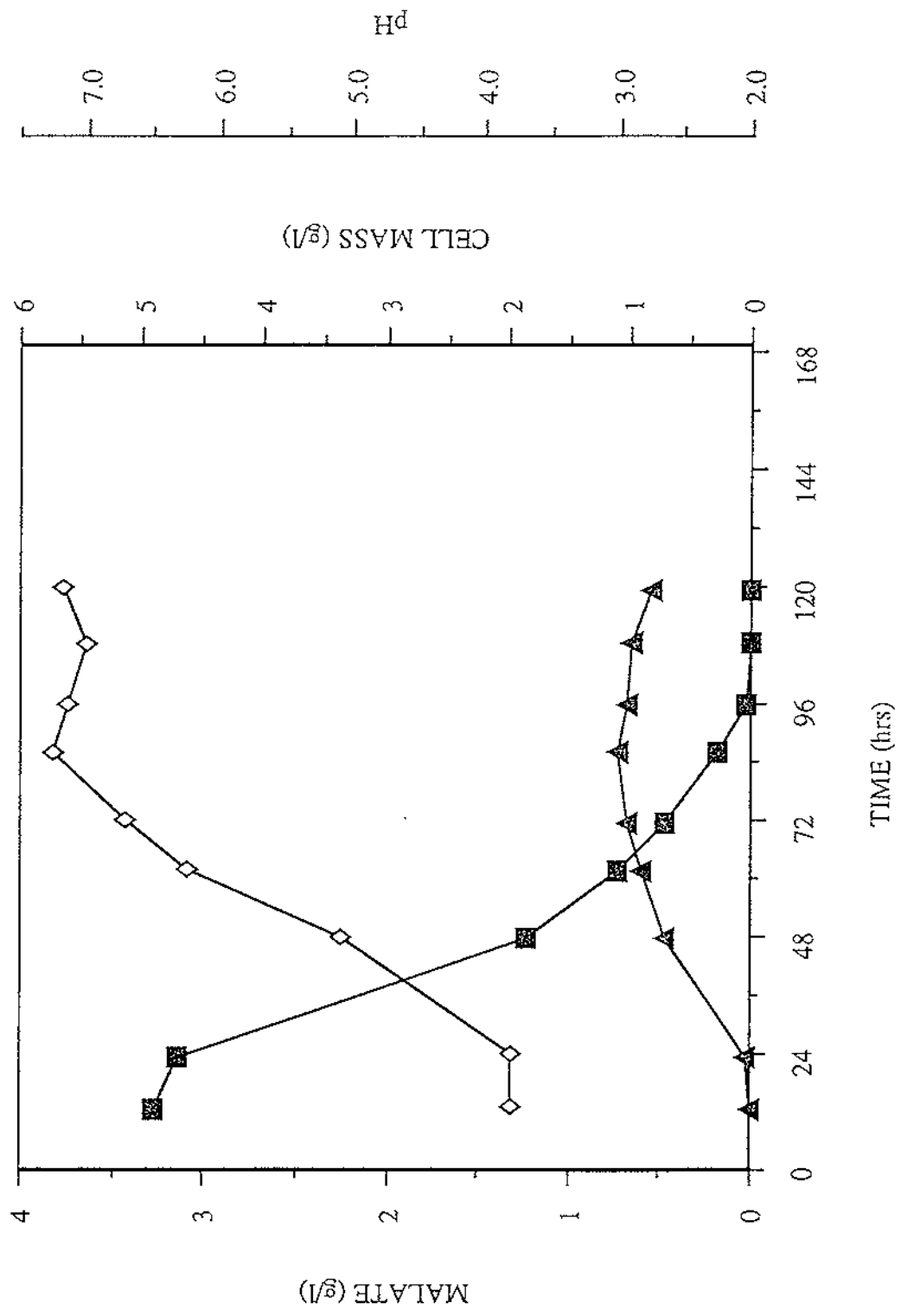


Figure 18. Fermentation of *Pichia stipitis* 411 on MM

■ Malate      ▲ Cell mass      ◇ pH

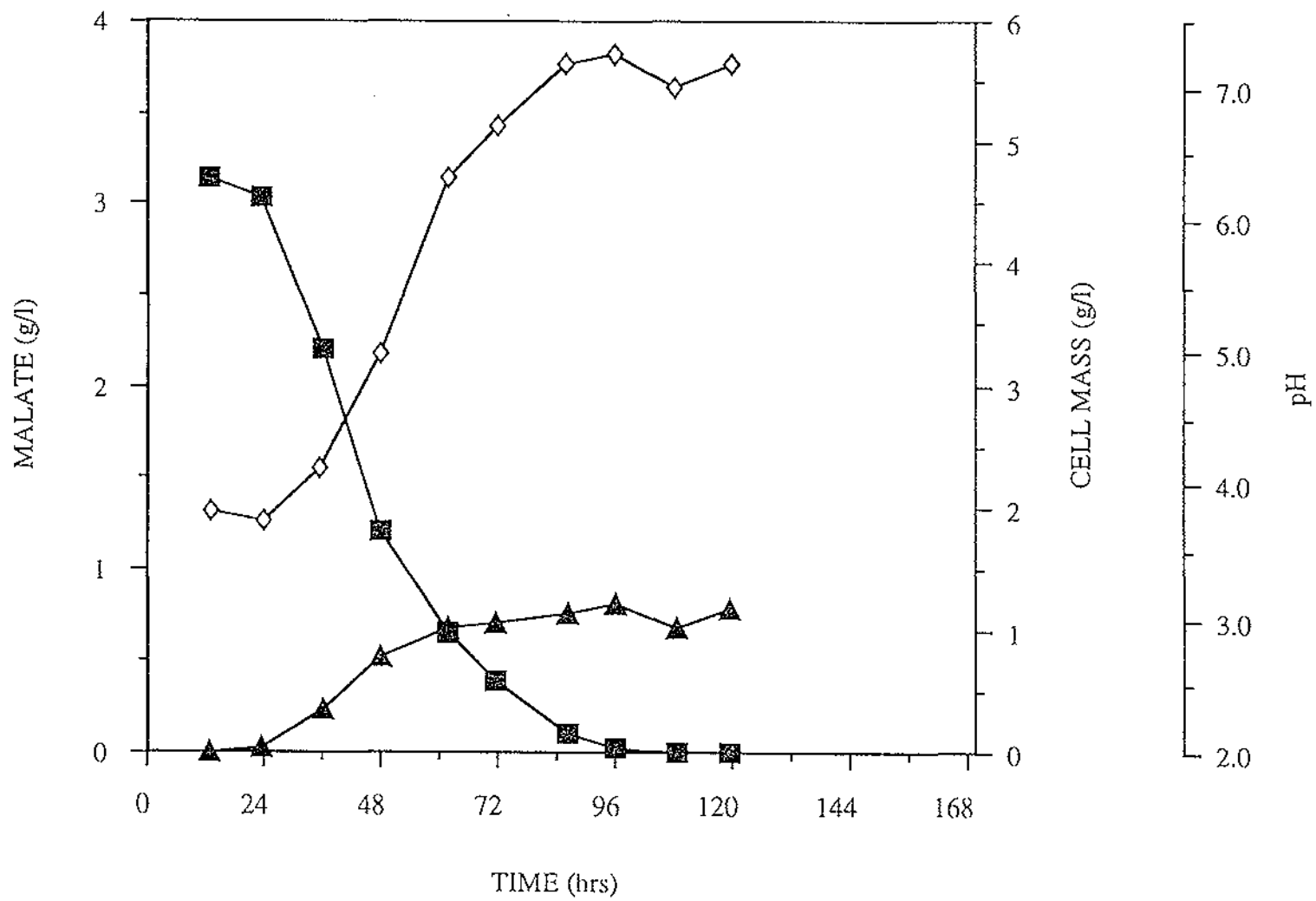


Figure 19. Fermentation of *Pachysolen tannophilus* 2530 (wild-type) on MSG

○ Glucose      ■ Succinate      ▲ Cell mass      ◇ pH

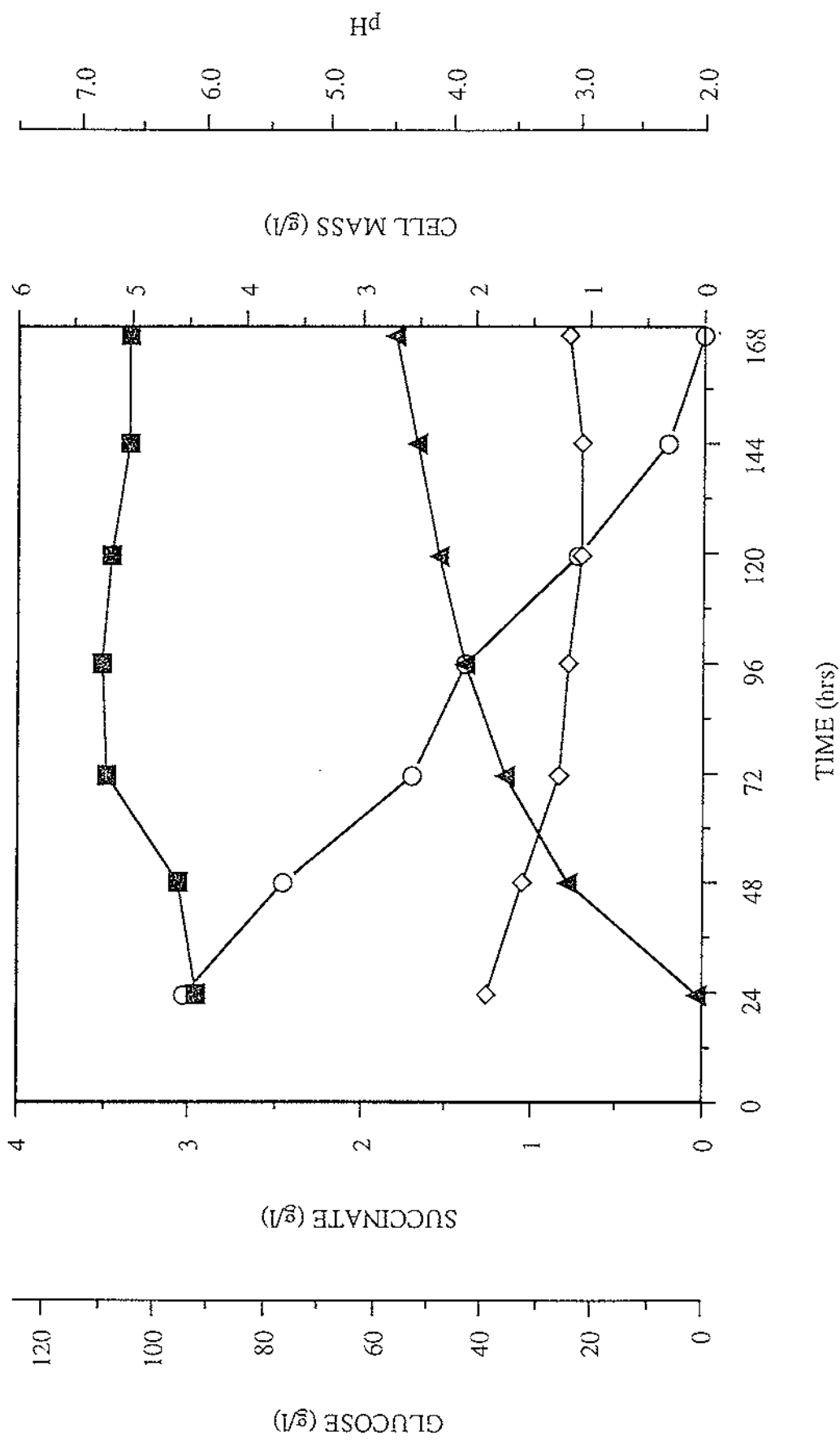


Figure 20. Fermentation of *Pachysolen tannophilus* P509-1B on MSG

○ Glucose      ■ Succinate      ▲ Cell mass      ◇ pH

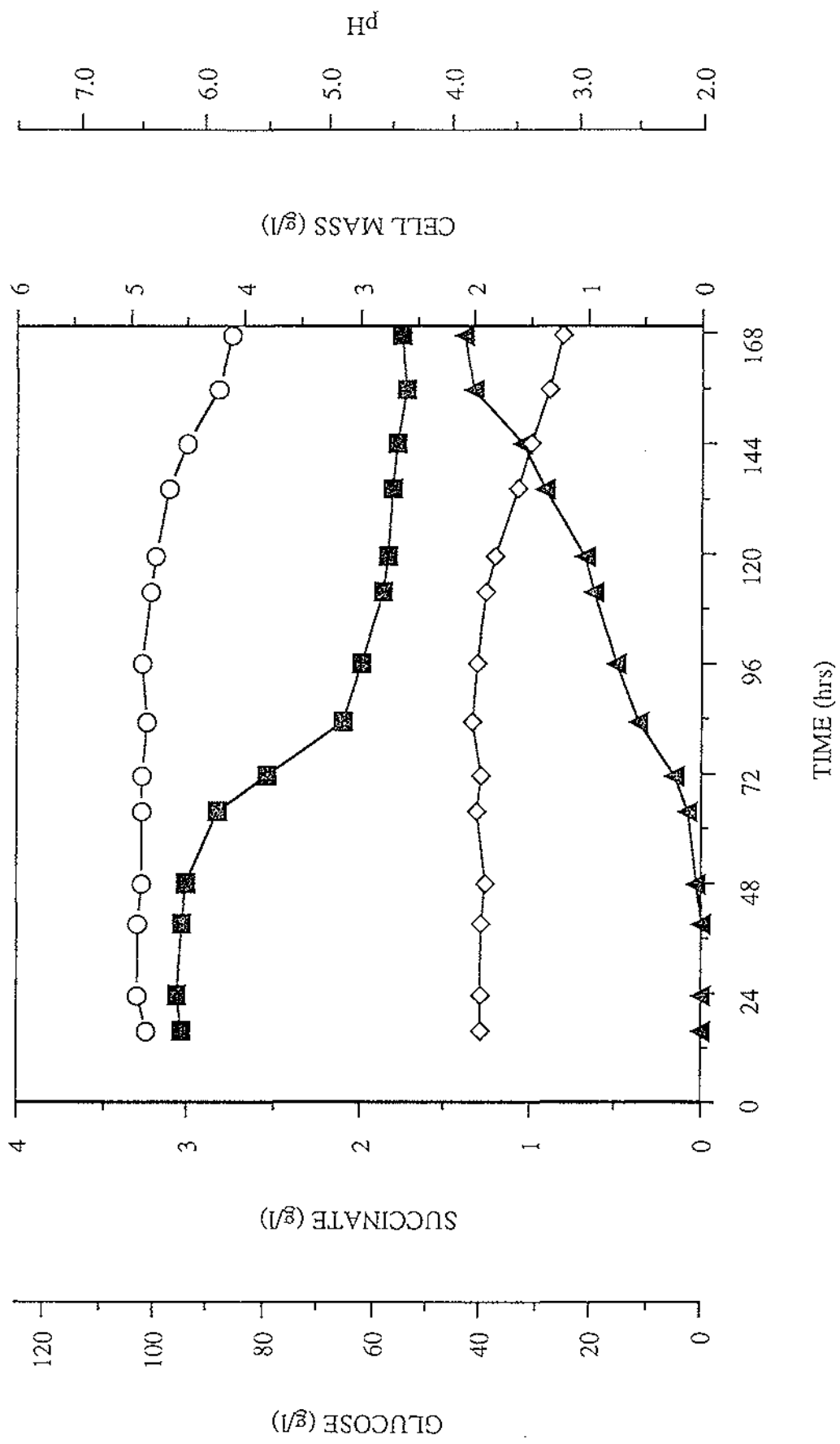


Figure 21. Fermentation of *Pichia stipitis* Y7124 (wild-type) on MSG

○ Glucose      ■ Succinate      ▲ Cell mass      ◇ pH

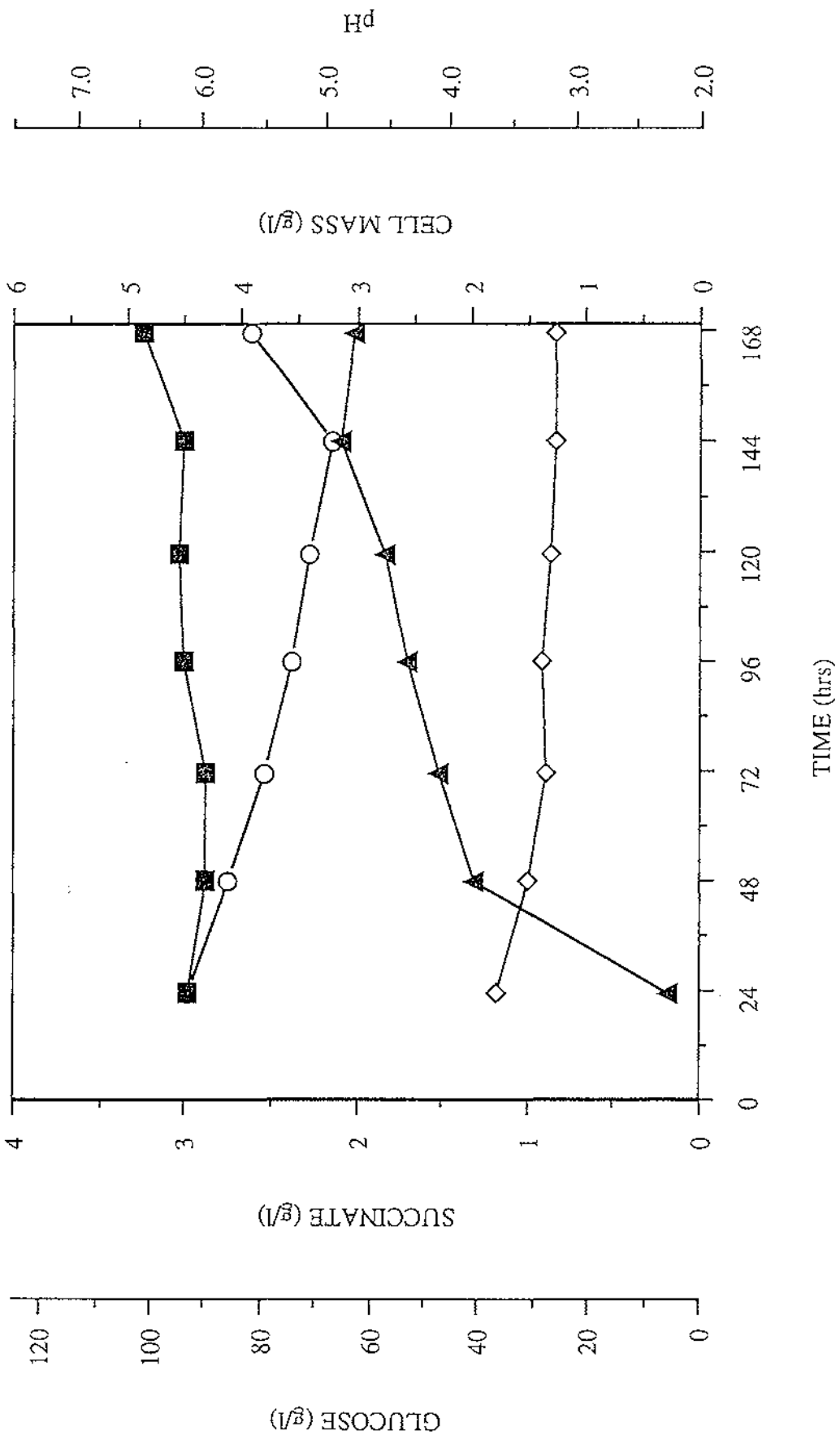
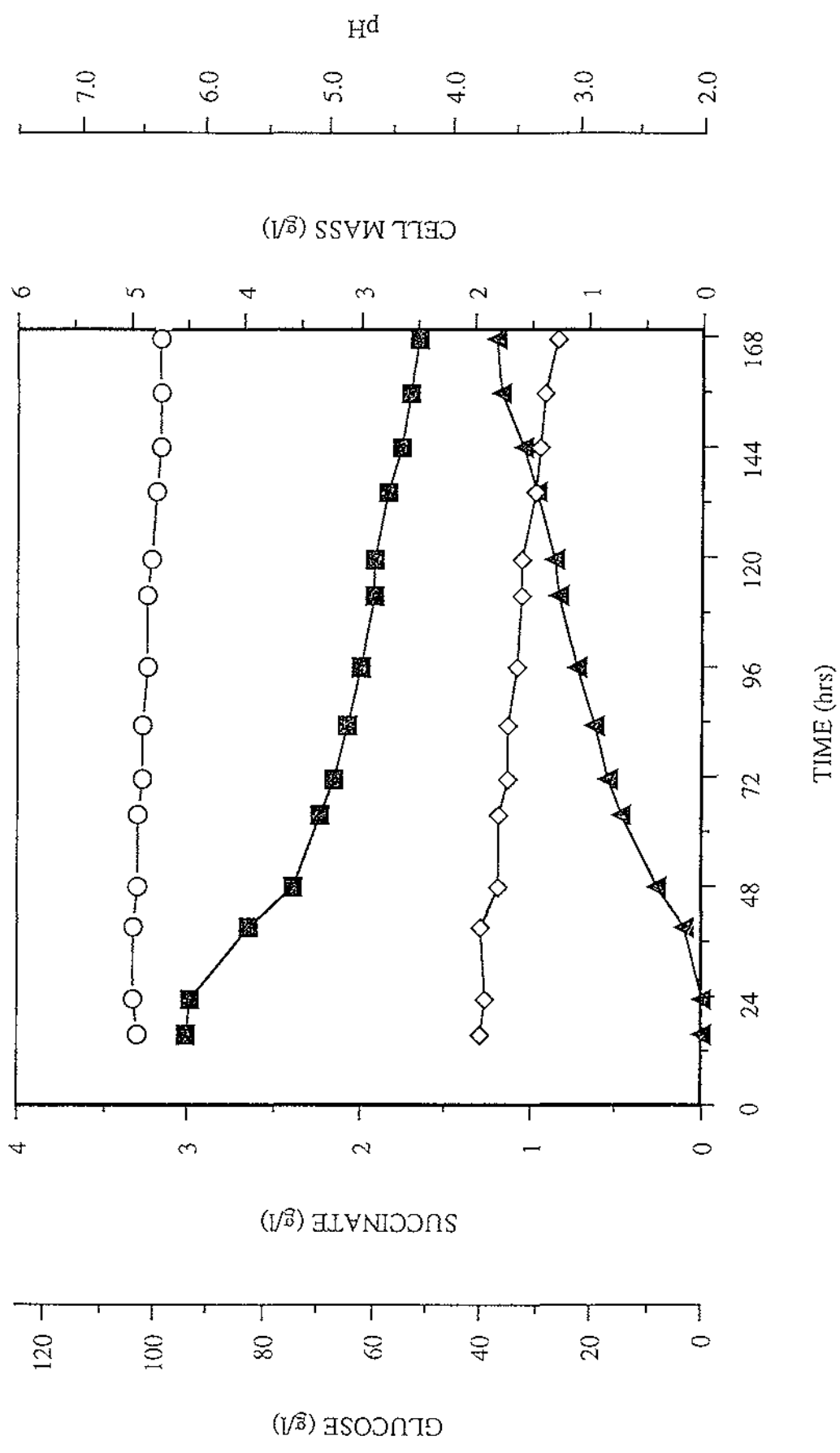


Figure 22. Fermentation of *Pichia stipitis* 411 on MSG

○ Glucose      ■ Succinate      ▲ Cell mass      ◇ pH



### 3.12 Semi-anaerobic fermentations

These fermentations have been recorded in the form of fermentograms - a standard format that allows all such results to be compared directly.

### 3.13 Semi-anaerobic fermentations in minimal malate glucose

Different patterns of glucose and malate utilisation were observed in fermentations by the *Pachysolen tannophilus* wild-type strain and its mutants (Figs. 1 - 6). The wild-type strain 2530 (Fig. 1) co-utilised both glucose and malate during both exponential growth and stationary phases. The mutants isolated by growth on 2-deoxyglucose (Figs. 2, 3, 5 & 6) are arranged in order of decreasing hexokinase activity (Wedlock *et al.* 1989, Table 16) and showed a trend of decreasing glucose utilisation. The mutants could be divided into two broad groups in terms of fermentation pattern. The first group contained P510-5A (Fig. 2) which was indistinguishable from the wild-type. The remainder, strains P509-3C, P509-1B and D/X A (Figs. 3, 5 & 6) showed an increase in the rate of malate utilisation. Strain F/G 2 did not grow in MMG because it cannot utilise glucose, and did not grow on malate in the presence of glucose (Table 11 & Plate 8). By contrast, mutant 335 (Fig. 4) isolated in this study by screening UV mutants directly for derepression of malate utilisation showed a different pattern. Its low glucose utilisation resembled that of the 2-deoxyglucose mutants, however, it used very little malate.

The *Pichia stipitis* wild-type strain Y7124 (Fig. 7) showed no utilisation of malate when glucose was present at the concentrations studied. *Pichia stipitis* mutant 77 (Fig. 8) isolated by UV mutagenesis used very little glucose and appeared to be unable to utilise malate in the presence of glucose. Synthesis of malate in the strain led to a net increase of extracellular malate. The mutants isolated by selection on

plates containing 2-deoxyglucose and malate are organised in order of decreasing glucose and increasing malate utilisation (Figs. 9 - 11). All of these mutants differed significantly from the wild-type in that they appear to have been released from glucose mediated control of malate utilisation.

Comparison of the malate utilisation of *Pachysolen tannophilus* and *Pichia stipitis* wild-type strains revealed, at the concentrations studied, that the former was a malate co-utiliser and the latter used no malate in the presence of glucose (Figs. 1 & 7). The *Pichia stipitis* wild-type strain was more efficient at converting catabolites into cell mass than the wild-type strain of *Pachysolen tannophilus* (Figs. 1 & 7). For strains which utilised malate, in both *Pachysolen tannophilus* and *Pichia stipitis*, most malate utilisation occurred during the exponential phase of cell growth (generally 24 - 72 hrs). Some malate utilisation occurred at high cell density during stationary phase (72 hrs onwards). No strain, wild-type or mutant, used more than two thirds of the malate initially present.

Both *Pachysolen tannophilus* and *Pichia stipitis* wild-type strains produced acid by-products. The evidence suggested that malate utilisation was sensitive to the pH of the media (Section 3.1). Observations made during fermentations in MMG supported this idea. In cases where glucose utilisation was markedly decreased, pH increased and malate uptake was halted (Fig. 5 at 84 hrs). Where no glucose was utilised, and hence no acid byproducts produced, a simple inverse relationship between malate concentration and pH was seen (Fig.6). Where no malate was utilised, pH decreased steadily as the glucose was utilised (Figs. 7 & 8).

### 3.14 Semi-anaerobic fermentation in minimal malate

*Pachysolen tannophilus* strains wild-type and P510-5A, fermented in minimal malate medium (MM) showed identical behavior. Both strains used most, but not all, of the malate present (Figs. 12 & 13).

By contrast, the five *Pichia stipitis* strains tested utilised all of the malate present (Figs. 14 - 18). With the exception of mutant 77 the pattern of response was identical, with all of the malate utilised by 96 hours. Mutant 77 (Fig. 15) had an initial lag in malate utilisation, but all malate was utilised by 126 hours. Mutant 77 was able to utilise malate in MM but not in MMG, suggesting that the presence of glucose inhibited malate utilisation. In all fermentations in MM most malate was used during exponential phase of cell growth and a little was used during stationary phase. A clear inverse relationship between malate concentration and pH was also evident.

During HPLC analysis of samples of MM in which any of these yeasts had grown, a major acid peak appeared. It was identified as tartaric acid by a study of HPLC retention times and by thin layer chromatography. Tartaric acid represented a major fermentation by product of both *Pachysolen tannophilus* and *Pichia stipitis* when grown on MM.

### 3.15 Semi-anaerobic fermentations in minimal succinate glucose

Results on solid media containing indicator, glucose and organic acids suggested that, if a lesion in a mutant strain released malate utilisation from glucose mediated control, other organic acids would also be released (Tables 11 & 12). To test this possibility, the wild-type and one mutant strain from each species was grown on MSG (Figs. 19 - 22). The *Pachysolen tannophilus* wild-type strain 2530 (Fig. 19)

showed a steady utilisation of glucose similar to that when grown in MMG (Fig. 1). However, the rate of growth was lower, presumably because in MMG malate was also consumed, while in MSG succinate was not. There was a net increase in the amount of succinate present in the medium during the exponential phase of cell growth, a situation analogous to that found in *Saccharomyces cerevisiae* (Heerde & Radler 1978). By contrast, the *Pachysolen tannophilus* mutant P509-1B grew equally well in MSG or MMG, utilising succinate or malate when glucose was present (Figs. 20 & 5).

The *Pichia stipitis* wild-type Y7124 appeared to use little glucose and no succinate and little succinate was accumulated (Fig. 21). The *Pichia stipitis* mutant 411 utilised succinate but not glucose (Fig. 22).

### 3.16 Hexokinase assays

Hexokinase assays were performed for *Pichia stipitis* Y7124 and the three mutants isolated on malate and 2-deoxyglucose. All 2-deoxyglucose mutants had reduced hexokinase activity (Table 15). These mutants are organised in order of decreasing glucose utilisation (see Figs. 9 - 11). It was observed that mutants with the highest glucose utilisation had the lowest hexokinase activity, and the poorest utilisers had hexokinase activities closest to the wild-type.

Table 15. Hexose-ATP-kinase activities of *Pichia stipitis* strains

Strain	Specific activity
Y7124	0.93
238	0.40
287	0.64
411	0.79

Specific Activity:  $\mu$ moles NADP reduced per minute per mg protein.

## 4.0 DISCUSSION

### 4.1 Sugar utilisation of *Pachysolen tannophilus* and *Pichia stipitis*

The *Pachysolen tannophilus* wild-type strain grew well on solid media containing any of the hexose and pentose sugars tested, including xylose (Table 7). As reported elsewhere (Wedlock *et al.* 1989), growth of *Pachysolen tannophilus* mutants on hexose sugars declined with decreasing hexokinase activity. (The hexokinases lost from each strain are given in Table 3 and the resulting hexokinase activities in Table 16). This organism does not grow on citrate (Table 4). Consequently, mutants with low hexokinase activities did not grow on solid medium containing citrate and glucose (Table 11). An unexpected observation was the inability of the wild-type strain to grow on mixtures of citrate and xylose (Table 13). A number of *Pachysolen tannophilus* mutants were able to grow on citrate plus xylose (Table 13). These mutants all lacked the enzyme hexokinase A (product of the HXK 2 gene), an enzyme associated with carbon catabolite repression (Wedlock & Thornton 1989), which implies that this is a regulatory phenomena. In support of this idea is the fact that *Pachysolen tannophilus* mutant F/G 2 failed to grow on citrate plus xylose. A presently uncharacterised lesion in this mutant has reinstated carbon catabolite repression of malate utilisation (Plate 8).

The *Pichia stipitis* wild-type strain grew well on solid media containing any of the hexose and pentose sugars tested (Table 8). The *Pichia stipitis* mutants isolated on 2-deoxyglucose showed decreased growth on hexose sugars (Table 8). However, unlike mutants of *Pachysolen tannophilus* (Tables 7 & 16), decreasing growth on hexoses did not correlate well with decreasing hexokinase activity (Tables 8 & 15).

Table 16. Hexose-ATP-kinase activities of *Pachysolen tannophilus* strains

Strain	Specific activity
P510-5A	0.33
P509-3C	0.38
P509-1B	0.042
D/X A	0.0061

Specific Activity:  $\mu$ moles NADP reduced per minute per mg protein.

(Source: Wedlock *et al.* 1989).

The wild-type strains of *Pachysolen tannophilus* and *Pichia stipitis* utilised glucose in liquid media under semi-anaerobic conditions in the presence of either malate or succinate (Figs. 1, 7, 19 & 21). *Pachysolen tannophilus* was more efficient than *Pichia stipitis*, utilising all the glucose present by 144 hours for mixtures of glucose and malate (Fig. 1), or 168 hours for mixtures of glucose and succinate (Fig. 19). Under these same conditions, *Pichia stipitis* had not utilised all of the glucose by 168 hours (Figs. 7 & 21). Hexokinase and mutants of both species used less glucose, depending on the number and type of kinases missing (Figs. 2 to 6 & 9 to 11). Neither *Pachysolen tannophilus* mutants D/X A (Fig. 6) or F/G 2 utilised glucose as they essentially lack hexose-ATP-kinase activity, but both could utilise xylose.

*Pachysolen tannophilus* mutant 335 was sensitive to 2 mM 2-deoxyglucose when grown on malate, but was resistant to 8 mM 2-deoxyglucose when grown on xylose (Table 10). A possible explanation for this observation, is that xylose has been shown to inactivate Hexokinase P II of *Saccharomyces cerevisiae* (Fernández *et al.* 1986) and the same mechanism may operate in *Pachysolen tannophilus*. Alternatively, metabolites from xylose catabolism flow through the EMP Pathway

and these may reduce the lethality of 2-deoxyglucose-6-phosphate present by diluting its metabolites, or by competition as enzyme substrates.

#### 4.2 Organic acid utilisation of *Pachysolen tannophilus* and *Pichia stipitis*

In the utilisation of organic acids, the behavior of these two species is very different. *Pachysolen tannophilus* utilised succinate, fumarate and L-malate but not citrate (Table 4). *Pichia stipitis* utilised all four TCA cycle intermediates (Table 5). Growth on organic acids was dependant on pH (Tables 4 & 5).

Mutants of *Pichia stipitis* that utilised malate in the presence of glucose, were easily obtained by selection on 2-deoxyglucose. They were all found to utilise the other TCA cycle intermediates in the presence of glucose both on solid and (where tested) in liquid media. Notably these mutations released citrate utilisation from glucose control, implying that both bi- and tri-carboxylic acid utilisation pathways have some regulatory elements in common.

For the most part, it was not clear from growth on solid media whether either species of yeast utilised organic acids in the presence of xylose. Such utilisation could well have been masked by the acid by-products of sugar metabolism in much the same way as when *Pachysolen tannophilus* 2530 was grown on Indicator MG medium (Table 11). However, *Pichia stipitis* 287, utilised succinate and fumarate in the presence of xylose.

In liquid culture, *Pachysolen tannophilus* 2530 co-utilised malate in the presence of glucose (Fig. 1), while in *Pichia stipitis* Y7124, utilisation of malate was under strict glucose control, and no malate was utilised (Fig. 7). However, some form of glucose control must affect the utilisation of malate by *Pachysolen tannophilus* 2530, as this yeast fermented malate more rapidly in the absence of glucose (Fig.

12) than in its presence (Fig. 1). For *Pichia stipitis* Y7124 the threshold at which malate utilisation is released from glucose control is somewhere below 40 g/l (222 mM). It has been reported elsewhere (Rodriguez & Thornton, 1990) that glucose control occurs at glucose concentrations as low as 5 g/l (28 mM) in *Pichia stipitis* Y7124.

The utilisation of succinate was under strict glucose control in the wild-type strains of both species (Figs. 19 & 21).

Results of fermentations in liquid media containing both glucose and malate indicated that malate utilisation was dependent on pH, presumably because this affects the ionic state of malic acid and its ability to be transported across the membrane. Other workers have noted that the transport of organic acids across membranes is dependant on pH (Côrte-Real & Leão 1990, Cássio *et al.* 1987).

In liquid culture containing malate and glucose, *Pachysolen tannophilus* mutants utilised malate less efficiently than the wild-type, probably due to slow rates of growth. Only mutant D/X A, which lacks all hexose-ATP-kinase activity, utilised malate more efficiently than the wild-type.

In fermentations in MM, *Pachysolen tannophilus* strains used two thirds of the malate initially present by 120 hours, but *Pichia stipitis* strains used it all by 96 hours, indicating a basic difference between the metabolism of these two yeasts.

Rodriguez & Thornton (1990) have reported that *Pachysolen tannophilus* 2530 utilises all of the malate present in MM medium by 72 hours, in apparent contradiction to the findings of this study. This may be explained, however, by the initial difference in pH of the medium in these two studies. Rodriguez & Thornton (1990) grew *Pachysolen tannophilus* 2530 in MM medium with an initial pH of

3.0. In this study, the initial pH of MM medium was 3.8. As has been shown, utilisation of malate is very dependant on the pH of the growth media (Table 4). Therefore, the higher starting pH used in this study led to a decreased rate of malate utilisation (Fig. 12).

*Pachysolen tannophilus* mutant F/G 2, carries a lesion that puts malate utilisation under tight glucose control. It did not grow on glucose, and in the presence of glucose did not utilise malate, but did grow on malate alone. In its malate utilisation, the behavior of F/G 2 reassembles that of *Pichia stipitis* mutant 77 (Section 3.12).

Mutations associated with the loss of hexose-ATP-kinase activity appeared to abolish glucose control of organic acid utilisation in these two yeasts. It has been reported that in the yeast *Hansenula anomala*, carbon catabolite derepression of malic acid utilisation is accompanied by inverse diauxy (Côte-Real *et al.* 1990). That is, rather than glucose being metabolised before malate (due to carbon catabolite repression), malate is utilised before glucose. This pattern of utilisation was not seen with mutants of *Pachysolen tannophilus* or *Pichia stipitis*. Instead, both substrates were co-utilised, with the rate of glucose utilisation being lower than that of the corresponding wild-type strain.

A simple model for glucose control of organic acid utilisation may be proposed:

In both the yeasts *Pachysolen tannophilus* and *Pichia stipitis* organic acids are utilised by the enzymes of the TCA cycle. In the presence of glucose these enzymes are under tight glucose control. *Pachysolen tannophilus* may also utilise malate via a second pathway which is not under such strict glucose control. A possible route for malate utilisation is by malic enzyme (Kuczynski & Radler 1982).

During some fermentations the concentration of organic acids were actually seen to increase (Fig. 7 at 60 hours, Figs. 8 & 19). In interpreting these results it is useful to remember that the glucose and malate concentrations in the medium are the net result of diffusion of catabolites into and out of the cells, as well as being the result of cellular catabolism and anabolism. Thus, within the medium, net malate or succinate levels may increase with time, if production and export of TCA cycle intermediates is greater than their utilisation. Such observations have been made before with *Saccharomyces cerevisiae* (Bhattacharjee et al 1968, Schwartz & Radler 1988).

#### 4.3 Hexokinases, 2-deoxyglucose resistance and gluconeogenesis

The metabolic pathways of gluconeogenesis and glycolysis (EMP pathway) are antagonistic. When one is in operation the other is essentially turned off. Otherwise cells would perform futile cycles - producing and catabolising glucose - until they ran out of energy and died. The mechanism by which this is regulated is not known.

In *Pachysolen tannophilus* grown on malate, the gluconeogenic pathway must be running to provide glucose for the cells needs. However, addition of 2-deoxyglucose is still lethal, suggesting that parts of the EMP pathway are still operating. Alternatively, 2-deoxyglucose may operate by some other mechanism, such as by inducing catabolite repression (Witt *et al.* 1966. However, see Section 1.5).

When yeasts are exposed to 2-deoxyglucose, a wide variety of mutant phenotypes are usually isolated (Zimmermann & Scheel 1977, Gancedo & Gancedo 1986). Notably, yeasts with elevated intracellular phosphatase may be isolated, which protect the cell by dephosphorylating 2-deoxyglucose-6-phosphate (Heredia & Sols

1964). In this work, 103 mutants of *Pichia stipitis* were isolated from plates containing malate and 6 mM to 8 mM 2-deoxyglucose (Section 3.5). Only one phenotype was seen. Of these 20 were tested at random on Indicator MG medium (data not shown). All discoloured the Indicator MG medium, indicating a loss of carbon catabolite repression and decrease in glucose utilisation (Section 4.4). Three were extensively characterised (238, 287 & 411). All had decreased hexose-ATP-kinase activity (Table 15). Resistance to 2-deoxyglucose was not due to inactivity of the high and low affinity glucose transport systems, because the mutants grew very well on 20 g/l glucose (approximately 110 mM. Table 8).

The nature of the lesion in the *Pichia stipitis* mutants, isolated in this study, is unknown. Rather than a simple lesion in the structural gene of a hexokinase, it may be in a regulatory gene. These mutants were isolated in the presence of a gluconeogenic carbon source (malate), whereas all other such studies have been done with glycolytic carbon sources such as fructose or raffinose. During gluconeogenic growth, the regulatory system of a yeast is configured differently than at other times. Consequently, it might be expected that different types of regulatory mutants arise.

Alternatively, the EMP pathway may be more tightly regulated in *Pichia stipitis* than in other yeasts. During gluconeogenesis, there may be no EMP activity in *Pichia stipitis*, and thus no phosphorylation of 2-deoxyglucose to its lethal form, 2-deoxyglucose-6-phosphate.

#### 4.4 Utility of the indicator plate

An indicator agar medium was developed to isolate mutants of the yeast *Schizosaccharomyces malidevorans* which utilised malate more completely (Rodriguez & Thornton 1989). Such mutants produced characteristic blue colonies

and turned the medium blue. Extensive use of this medium and its variants during this research has highlighted a number of its advantages and limitations.

The usefulness of the indicator plate methodology was with its rapidity and adaptability. Results could be obtained within six days of streaking yeast on indicator medium. By modifying the ingredients (CG, SG, M, FX etc.), different questions were addressed, and by modifying the relative amounts of the constituents e.g. with Indicator MG and Indicator MG (modified) the sensitivity of the plates could be altered. A wide range of phenotypes were observed because the plate exhibited colour changes, both of the medium and of the colonies, and the colony colour varied widely. Use of the indicator plate methodology in parallel with other forms of characterisation provided a tool for the rapid screening of a wide variety of mutants. *Pichia stipitis* mutant 77 was isolated through its altered phenotype on Indicator MG. Mutant 77 was not released from glucose control, but it did show altered regulation of malate utilisation (compare Figs. 7 & 8).

Screening mutants by colour of the indicator medium had some limitations. The operation of the indicator medium depended on a pH change within either the colonies or the medium. Both *Pachysolen tannophilus* and *Pichia stipitis* excreted acids into the medium during growth on glucose which masked the uptake of organic acids. Thus, while *Pachysolen tannophilus* 2530, P510-5A and P509-3C all co-utilised malate and glucose, no sign of malate utilisation was evident from the response of the plates (Table 11 & Figs. 1 - 6). Only when malate utilisation was accompanied by little or no glucose utilisation was a change observed, e.g. *Pichia stipitis* 411 (Table 12, Fig. 11 & Plate 7).

#### 4.5 Summary

The project objectives are detailed in Section 1.7. A summary of findings is given below:

- 1) In the wild type *Pachysolen tannophilus* strain 2530 the compounds malate and glucose are co-utilised. Succinate, on the other hand, is not co-utilised with glucose. In the wild type *Pichia stipitis* strain Y7124, glucose is utilised steadily but the utilisation of malate is under strict glucose control. The malate and glucose utilisation patterns of the mutants showed a tendency for increased malate utilisation accompanied by a decrease in glucose utilisation.
- 2) It was shown that of the four tricarboxylic acid cycle (TCA) intermediates used in this project, the yeast *Pachysolen tannophilus* could utilise succinic acid, fumaric acid and L-malic acid but could not use citric acid. The yeast *Pichia stipitis* could use all four. These results are in accordance with the published descriptions of these two species (Barnett *et al.* 1983, Kurtzman 1984a, 1984b).
- 3) Using the original indicator medium and a modified medium, attempts were made to isolate mutants of both species which were capable of increased malate utilisation in the presence of glucose. This was unsuccessful.
- 4) UV mutagenesis of *Pichia stipitis* Y7124, followed by selection on plates containing malate and 2-deoxyglucose, succeeded in producing mutants capable of increased malate utilisation in the presence of glucose. All those tested (103) turned the indicator medium blue. It was significant that such mutants were easy to isolate from a UV mutagenesis. Approximately  $1 \times 10^6$  cells were deposited on each plate and approximately  $1 \times 10^3$  2-

deoxyglucose resistant colonies grew. However, these mutants were not seen during screening on indicator medium. This must have been because of the masking effects of cells growing on glucose and excreting acid by-products into the medium. The study of *Pachysolen tannophilus* mutants, isolated by other workers on xylose and 2-deoxyglucose, revealed at least one mutant with increased malate utilisation in the presence of glucose (mutant D/X A).

- 5) Mutants of both species with altered rates of malate utilisation showed *similar* behaviour on indicator medium for the other TCA intermediates tested.
- 6) In liquid culture mutants of both *Pachysolen tannophilus* and *Pichia stipitis* utilised succinate in the presence of glucose. In liquid culture *Pichia stipitis* mutants utilised malate in the presence of glucose.

#### 4.6 Further work

A greater understanding of the malate and glucose utilisation of *Pachysolen tannophilus* and *Pichia stipitis* would be gained if glucose and malate transport assays were performed, on wild-type and mutant strains.

The wild-type and mutant strains of *Pichia stipitis* used in this work need to be further characterised, in terms of the number and types of hexokinase enzymes present.

The activity of TCA cycle enzymes need to be measured in the mutants and wild-type of *Pichia stipitis* when grown in MMG and MM medium, to determine if these enzymes are carbon catabolite derepressed in the mutants.

The lesion in *Pachysolen tannophilus* F/G 2 should be identified and fully characterised. Such work should aid in the understanding of the mechanisms by which carbon catabolite repression operates.

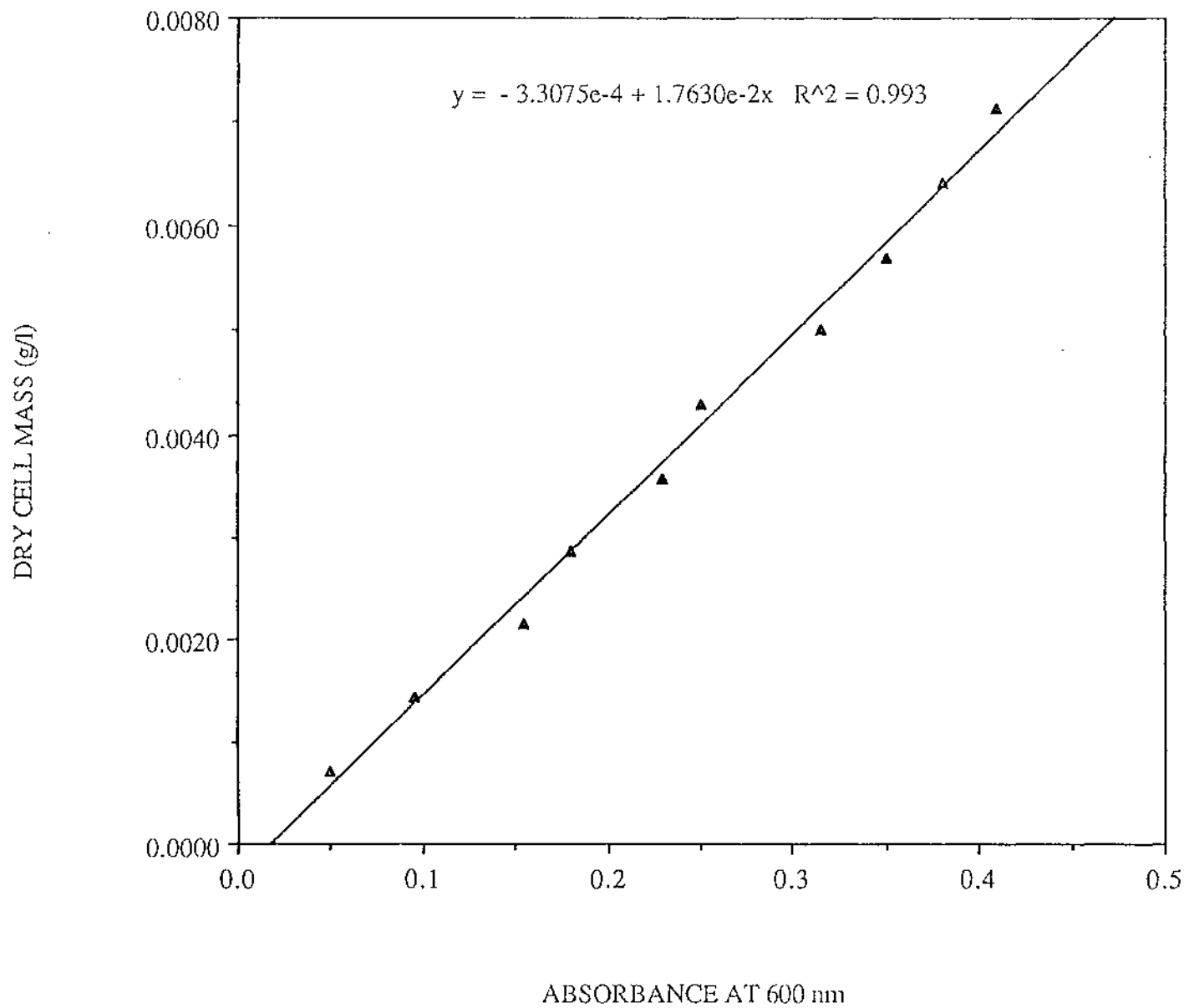
The reasons why *Pachysolen tannophilus* 2530 cannot grow on mixtures of citrate and xylose should be explored, as well as why the loss of hexokinase A alleviates this situation. It is hard to imagine how the enzymatic or regulatory functions of hexokinase A, an enzyme of the EMP pathway, could halt utilisation of xylose, in the presence of citrate.

## 5.0 APPENDICES

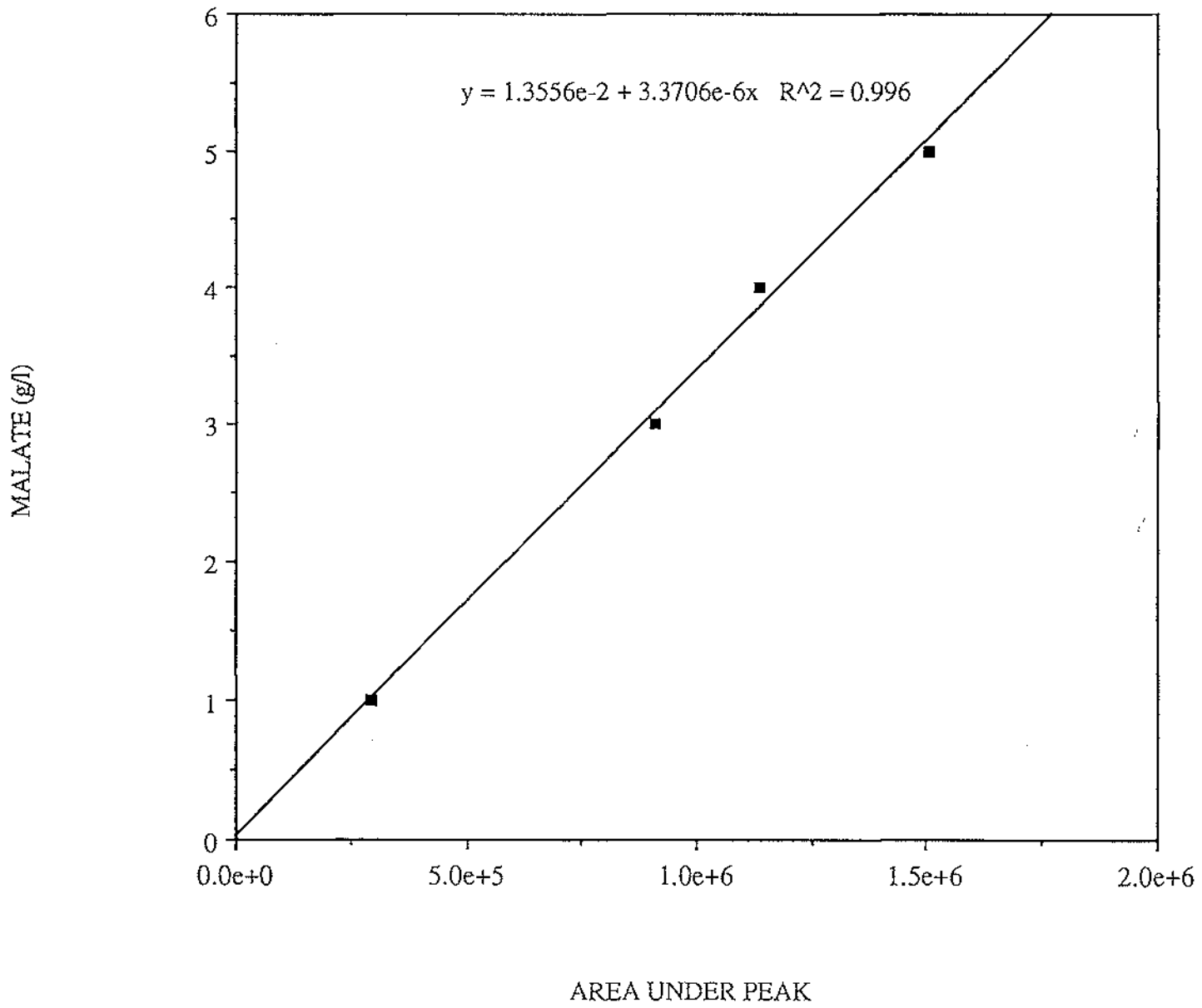
### 5.1 Method of regeneration of reverse phase columns

Over time the reverse phase column lost efficiency. This was presumably because lipids were produced by the yeasts and became bound to the column. Some efficiency may have been also lost by exposing the column continuously to an aqueous environment. To regenerate the reverse phase column the pump was set to a flow rate of 0.1 ml / minute, and a linear gradient was set up from water to tetrahydrofuran (THF) over 60 minutes and then back to water over the same time. This cycle was repeated for 24 hours. THF is a nonpolar solvent capable of dissolving lipids bound onto the column, and has the further advantage that it is fully miscible with water.

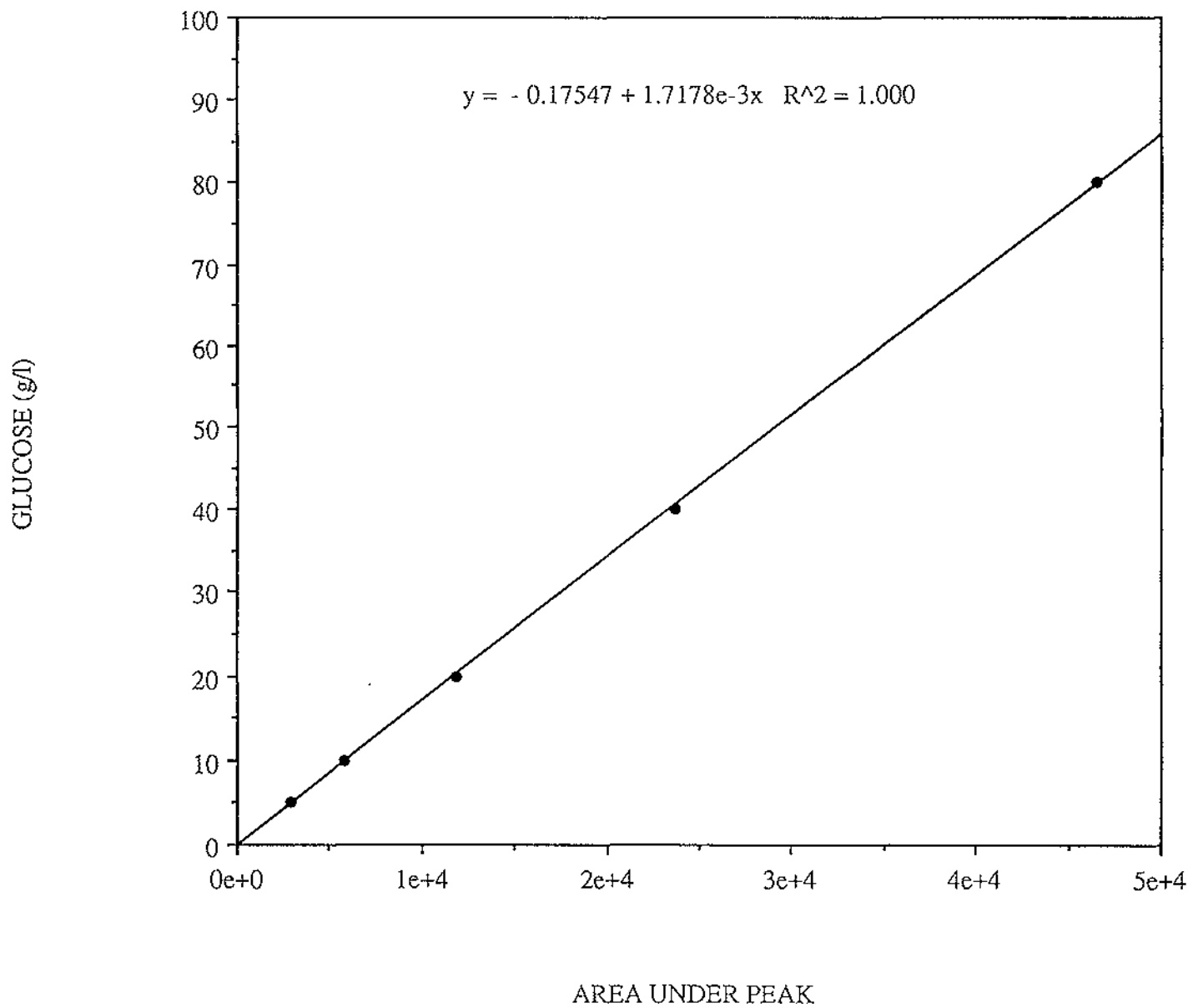
Appendix 5.2 Plot of  $\text{abs}_{600}$  vs dry cell mass for *Pichia stipitis* Y7124, flask 2.



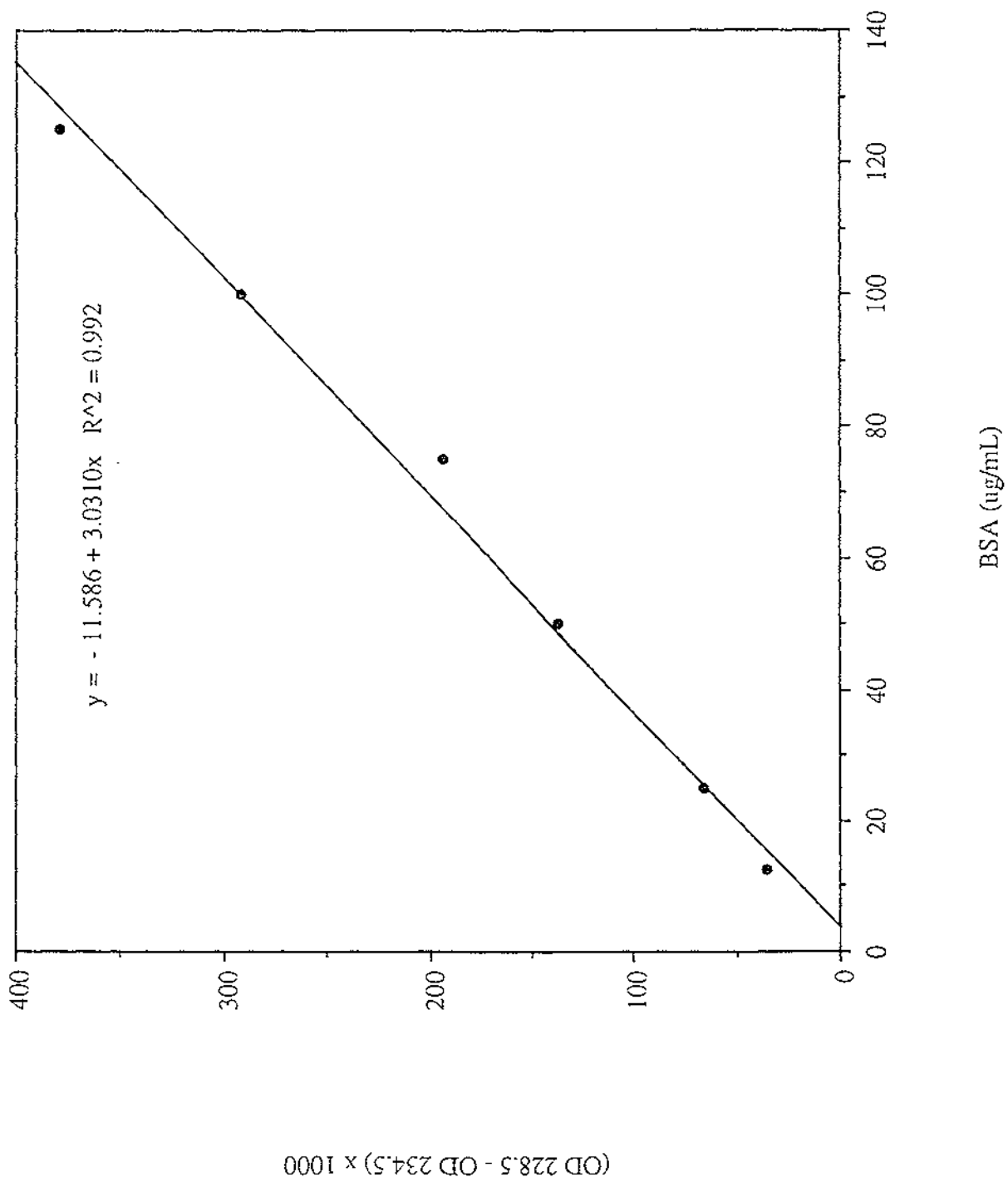
Appendix 5.3 Plot of malate concentration against HPLC response factors (UV detector).



Appendix 5.4 Plot of glucose concentration against HPLC response factors (RI detector).



Appendix 5.5 Relationship of protein concentration and absorbance for bovine serum albumin standard.



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