

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

INVESTIGATIONS ON THE HEXOSE-PHOSPHORYLATING ENZYMES
IN THE PENTOSE-FERMENTING YEAST, *PACHYSOLEN TANNOPHILUS*

A thesis presented in partial fulfilment
of the requirements for the degree
in Doctor of Philosophy
in Microbiology
at Massey University

DAVID NEIL WEDLOCK

1988

Massey University Library. Thesis Copyright Form

Title of thesis:

Investigations on the Hexose-phosphorylating
enzymes in the pentose-fermenting yeast;
Pachysolen tannophilus

- (1) (a) I give permission for my thesis to be made available to readers in the Massey University Library under conditions determined by the Librarian.
- (b) I do not wish my thesis to be made available to readers without my written consent for 12 months.
- (2) (a) I agree that my thesis, or a copy, may be sent to another institution under conditions determined by the Librarian.
- (b) I do not wish my thesis, or a copy, to be sent to another institution without my written consent for 12 months.
- (3) (a) I agree that my thesis may be copied for Library use.
- (b) I do not wish my thesis to be copied for Library use for 12 months.

Signed

JN Wedlock

Wedlock

Date

31st May 1988

The copyright of this thesis belongs to the author. Readers must sign their name in the space below to show that they recognise this. They are asked to add their permanent address.

NAME AND ADDRESS

DATE

David Neil Wedlock

31st May 1988

82A Russell St. P. North

ABSTRACT

Mutants of *Pachysolen tannophilus*, resistant to 2-deoxyglucose, the toxic analogue of D-glucose, have been isolated and characterised. Their growth characteristics on hexose and pentose sugars, resistance to 2-deoxyglucose and cellular hexose-phosphorylating activities were investigated. Loss of hexose-ATP-kinase activity was found to correlate with loss of ability to grow on hexose sugars and increased resistance to 2-deoxyglucose. The growth of these mutants on D-xylose was not affected.

A further series of fructose-negative and glucose-negative mutants were isolated by selecting for increased resistance to 2-deoxyglucose and by UV mutagenesis. Mutants, defective in each of the three hexose-phosphorylating enzymes found to be present in this yeast, were completely negative for growth on D-glucose, but could slowly convert this sugar to D-fructose. The conversion of D-glucose to D-fructose was hypothesised to be catalysed by the enzymes xylose reductase and xylitol dehydrogenase and experiments were conducted to investigate this possibility.

Cell-free extracts from the wild type strain and several of the glucose-negative mutants were chromatographed on DEAE-cellulose. The results of hexokinase assays and anion-exchange chromatography confirmed the existence of three hexose-phosphorylating enzymes in *P. tannophilus*. Two hexokinases which phosphorylated both D-glucose and D-fructose, exhibited F/G ratios of 1.3/1.0 and 3.0/1.0,

while a glucokinase specific for D-glucose was also present. These enzymes were referred to as hexokinase A and B and glucokinase.

Examination of the hexose-ATP-kinase profiles on DEAE-cellulose of the wild type extract from cells grown on D-glucose, D-xylose and glycerol indicated that the glucokinase and hexokinase B were constitutive, while hexokinase A was inducible.

Glucose repression of xylose reductase and xylitol dehydrogenase was found to require an active hexokinase A enzyme. This enzyme was purified from a glucokinase defective mutant by DEAE-cellulose chromatography, followed by affinity chromatography on Cibacron Blue F3G-A Sepharose (Blue Sepharose) and examined further. The K_m values for D-glucose and D-fructose were 0.36 and 2.28 mM respectively. An estimated V_{max} fructose/ V_{max} glucose was 1.5/1.0. When incubated with D-xylose in the presence of $MgCl_2$ and ATP, the enzyme was inactivated.

A strain of *Pachysolen tannophilus*, defective in all three hexose-phosphorylating enzymes, was transformed with a plasmid carrying the cloned PII hexokinase gene from *Saccharomyces cerevisiae*. The gene was expressed and the presence of the enzyme within the cells was demonstrated by DEAE-cellulose chromatography of a cell-free extract.

As part of the overall plan to attempt genetic improvement in *P. tannophilus*, two superior ethanol producing mutants were hybridised and the segregants made available for fermentation trials at the Forest Research Institute.

Hexose-negative mutants able to ferment D-xylose in the presence of D-glucose were selected for and subjected to fermentation trials. Several of these mutants produced promising concentrations and yields of ethanol from the fermentation of D-xylose, both as a sole carbon source and in a mixture of D-glucose and D-xylose.

ACKNOWLEDGEMENTS

I wish to thank my supervisors, Dr Roy J. Thornton and Dr Eric A. Terzaghi for their constant guidance and encouragement.

The Department of Microbiology and Genetics for providing the space and equipment for this work.

The Forest Research Institute, Rotorua for providing funds and a Research Grant for the duration of this project.

The New Zealand Energy Research and Development Council for providing a Research Grant during this study.

I would also like to thank:

Dr Allen P. James for initial advice on the micro-manipulation of *Pachysolen tannophilus* and for his work on isolating glucose-negative strains of this yeast.

Dr K.-D. Entian, Federal Republic of Germany for his kind gift of the YRp7 plasmid carrying the cloned hexokinase PII gene.

Dr Graham Pritchard and Mrs Carole Flyger (Biochemistry Department) for demonstration of polyacrylamide disc gel electrophoresis.

Dr Susan B. Rodriguez for suggesting the use of Blue Sepharose and Dr Graham Midwinter for providing the method and materials for manufacture.

Juliana Mansvelt for her patience, love and encouragement.

To my Mother
and in memory of my Father

TABLE OF CONTENTS

	Page
Abstract	ii
Acknowledgments	v
Table of Contents	vii
List of Figures	xiv
List of Tables	xvii
List of Plates	xviii

HISTORICAL REVIEW

1. <i>Pachysolen tannophilus</i>	1
1.1 General Description	1
1.2 Utilisation and Fermentation of Carbon Compounds	4
1.3 Fermentation of Hydrolysates	13
2. Improvement of D-xylose Fermentation	15
2.1 Process Control	15
2.2 Genetic Manipulation	17
3. Biochemistry of <i>P. tannophilus</i>	21

INTRODUCTION

1. Hexose-Phosphorylating Enzymes in Yeast	25
2. The Use of 2-Deoxyglucose in Obtaining Hexose-ATP-Kinase Mutants	26
3. Aims of This Investigation	29

TABLE OF CONTENTS CONT'D

	Page
<u>MATERIALS AND METHODS</u>	
1. MICROBIOLOGICAL METHODS	31
1.1 Microbial Strains and Maintenance	31
1.2 Media and Cultivation	31
1.21 Yeast extract peptone medium (YEP)	31
1.22 Malt extract yeast extract peptone glucose (MYGP)	31
1.23 Yeast nitrogen base medium (YNB)	31
1.24 Malt extract yeast extract (YM)	34
1.25 Luria broth (LB)	34
1.26 Preparation of media	34
1.27 Culture growth and purity	36
2. GROWTH EXPERIMENTS	36
2.1 Materials	36
2.11 Replica-plating velveteens	36
2.2 Growth on Solid Media	36
2.3 Growth in Liquid Media	36
2.31 Screening trials and general vegetative growth	36
2.32 Growth and sugar utilisation	37
2.4 Measurement of Growth	37
2.5 Preparation of Standard Curve	38
3. SUGAR UTILISATION AND FERMENTATION TRIALS	38
3.1 Culture Conditions and Sampling	38
3.2 High Performance Liquid Chromatography (HPLC)	39
3.21 Preparation of samples	39
3.22 Analysis of sugar and end-products	39

TABLES OF CONTENTS CONT'D

	Page
3.23 Regeneration of columns	41
4. MEASUREMENT OF ENZYME ACTIVITIES	41
4.1 Materials	41
4.11 Buffers	41
4.111 Tris-HCl	41
4.112 Potassium phosphate buffer (KPB)	41
4.113 Triethanolamine buffer	42
4.12 Phenylmethanesulphonyl fluoride (PMSF)	42
4.13 Cofactors	42
4.14 Enzymes for coupled reactions	42
4.15 Adenosine-5'-triphosphate (ATP)	42
4.2 Preparation of Cell-Free Enzyme Extracts	43
4.3 Enzyme Assays	43
4.31 Measurement of specific activities	43
4.32 Reaction mixtures	44
4.321 Hexose-ATP-kinase	44
4.322 Phosphoglucose isomerase	45
4.323 Glucose-6-phosphate dehydrogenase	45
4.324 Xylose reductase	45
4.325 Xylitol dehydrogenase	46
4.326 Fructokinase	46
4.33 Determination of protein concentration	47
5. CHROMATOGRAPHY OF CELL-FREE EXTRACTS	47
5.1 Materials	47
5.11 Potassium phosphate buffer (KPB)	47
5.12 Diethylaminoethyl cellulose (DEAE-cellulose)	48
5.2 Hexose-ATP-Kinase Spot Test	48

TABLE OF CONTENTS CONT'D	Page
5.3 Anion-Exchange Chromatography	49
6. KINETIC STUDIES ON HEXOKINASE A	50
6.1 Preparation of Cibacron Blue F3G-A	50
6.2 Enzyme Purification	52
6.3 Polyacrylamide Disc Gel Electrophoresis	54
6.31 Hexose-ATP-kinase activity stain	54
6.32 Coomassie Brilliant Blue stain for protein	54
6.33 Disc gel electrophoresis	55
6.331 Preparation of stock solutions	55
6.332 Preparation of gels	56
6.333 Electrophoresis	57
6.4 Determination of Km values	58
6.5 Inactivation by D-xylose	58
7. PREPARATION OF DNA	59
7.1 Materials	59
7.11 Ethylene diamine tetraacetic acid (EDTA)	59
7.12 Ampicillin	59
7.13 Lysozyme	59
7.14 Protease K	59
7.15 Ribonuclease A	59
7.16 Sodium dodecyl sulphate (SDS)	60
7.17 STET buffer	60
7.18 Tris-saturated phenol	60
7.19 Sodium acetate	60
7.2 Large Scale Preparation of Plasmid DNA	61
7.3 Rapid Preparation of Plasmid DNA	62
7.4 Restriction of Plasmid DNA	63

TABLE OF CONTENTS CONT'D	Page
7.5 Electrophoresis of DNA	64
7.51 Tris-acetate buffer	64
7.52 Sucrose-bromophenol blue loading buffer	64
7.53 Gel electrophoresis	64
8. TRANSFORMATION WITH PLASMID DNA	65
8.1 Transformation of <i>Escherichia coli</i>	65
8.2 Transformation of <i>Pachysoles tannophilus</i>	65
9. DEMONSTRATION OF GLUCOSE CONVERSION IN CELL-FREE EXTRACTS	66
9.1 Reaction Mixtures	66
9.2 HPLC Analysis of Samples	67
10. GENETIC TECHNIQUES	67
10.1 Ultraviolet Light (UV) Mutagenesis	67
10.2 Hybridisation of Haploid Strains	68
10.3 Sporulation of Diploid Strains	68
10.4 Micromanipulation	68
10.5 Tetrad Analysis	70
11. SELECTION FOR HEXOSE-NEGATIVE MUTANTS	72
11.2 Selection for Fructose-Negative Mutants	72
11.3 Selection for Glucose-Negative Mutants	72
12. STRAIN IMPROVEMENT IN <i>PACHYSOLEN TANNOPHILUS</i>	72
12.1 Selection for Xylose Utilisation in the Presence of Glucose	72
12.2 Elimination of Ethanol Utilisation	73
12.3 Hybridisation of the <i>eth2-1</i> strain with NO_3^- - NO_3^- -4	73

TABLE OF CONTENTS CONT'D PageRESULTS

1. Resistance to 2-Deoxyglucose	75
2. Growth and Sugar Utilisation	78
2.1 Growth on Solid Media	78
2.2 Growth in Liquid Media	80
2.3 Sugar Utilisation in Media Containing Two Carbon Sources	84
2.4 Glucose Utilisation at High Cell Density	90
3. Hexose-ATP-Kinase, Phosphoglucose Isomerase and Glucose-6-Phosphate Dehydrogenase Activities	94
4. Chromatography of Hexose-Phosphorylating enzymes on DEAE-cellulose	98
4.1 Chromatography of Wild Type and Hexose-ATP-Kinase Defective Mutants	98
4.2 Chromatography of Wild Type Grown on Different Carbon Sources	107
5. Xylose Reductase and Xylitol Dehydrogenase Activities	109
6. Kinetic Studies on Hexokinase A	113
6.1 Purification of Hexokinase A	113
6.2 Km values	115
6.3 Inactivation by D-xylose	119
7. Transformation of <i>P. tannophilus</i> with the YRp/HXK2-8 plasmid	119
8. Conversion of Glucose to Fructose	123
8.1 Growth of Strains on Sorbitol	123

TABLE OF CONTENTS CONT'D	Page
8.2 Conversion of Glucose by Cell-Free Extracts	125
9. Genetic Improvement of <i>Pachysolen tannophilus</i>	131
9.1 Hexose-Negative Mutants	131
9.2 Hybridisation of the <i>eth2-1</i> Strain with NO_3^- - NO_3^- -4	135
10. Fermentation Trials	135

DISCUSSION

1. Growth Characteristics, Enzyme Activities and Resistance to 2-Deoxyglucose of Mutants	142
2. Hexose-ATP-Kinase Enzymes in <i>P. tannophilus</i>	148
3. Kinetic Properties of Hexokinase A	156
4. Catabolite Repression in <i>P. tannophilus</i>	157
5. Transformation of <i>P. tannophilus</i>	162
6. Alternative Pathway for Glucose Utilisation	164
7. Strain improvement of <i>P. tannophilus</i> by Mutagenesis and Hybridisation	169
8. Final Conclusions	172

APPENDIX

Paper

Strain Improvement of the Xylose-Fermenting Yeast <i>Pachysolen tannophilus</i> by Hybridisation of Two Mutant Strains	175
--	-----

<u>BIBLIOGRAPHY</u>	180
---------------------	-----

LIST OF FIGURES

Figure		Page
1	Pathways of hexose and pentose metabolism in <i>P. tannophilus</i>	6
2	Purification of hexokinase A	53
3	Growth of strains P509-1B and D/X A in YNB-xylose (2% w/v) supplemented with 2-deoxyglucose	77
4	Utilisation of hexoses and D-xylose (2% w/v) in YNB media by wild type, P444-3D	82
5	Utilisation of hexoses and D-xylose (2% w/v) in YNB media by strain P510-5A	82
6	Utilisation of hexoses and D-xylose (2% w/v) in YNB media by strain P509-3C	83
7	Utilisation of hexoses and D-xylose (2% w/v) in YNB media by strain P509-1B	83
8	Growth and D-glucose utilisation in YNB-glucose (6% w/v) by wild type, P444-3D and mutant strains P510-5A, P509-3C and P509-1B	85
9	Utilisation of D-glucose and D-xylose (2%:2% w/v) in YNB by wild type, P444-3D	87
10	Utilisation of D-glucose and D-xylose (2%:2% w/v) in YNB by strain P509-1B	87
11	Utilisation of D-glucose and D-xylose (2%:2% w/v) in YNB by strain D/X A	89
12	Utilisation of D-glucose and glycerol (2%:2% w/v) in YNB by strain P509-1B	89
13	Time course of wild type, P444-3D, in YNB-glucose (2% w/v) at high cell density	91
14	Time course of strain P509-1B in YNB-glucose (2% w/v) at high cell density	91
15	Time course of strain D/X A in YNB-glucose (2% w/v) at high cell density	93

	LIST OF FIGURES CONT'D	Page
16	DEAE-cellulose chromatography of cell-free extract from wild type strain, P444-3D	100
17	DEAE-cellulose chromatography of cell-free extract from strain P510-5A	101
18	DEAE-cellulose chromatography of cell-free extract from strain P509-3C	103
19	DEAE-cellulose chromatography of cell-free extract from strain P509-1B	104
20	DEAE-cellulose chromatography of cell-free extract from strain P509-1B #6	105
21	DEAE-cellulose chromatography of cell-free extract from strain D/X A	106
22	DEAE-cellulose chromatography of cell-free extract from wild type, P444-3D; cells grown on D-glucose	108
23	DEAE-cellulose chromatography of cell-free extract from wild type, P444-3D; cells grown on D-xylose	110
24	DEAE-cellulose chromatography of cell-free extract from wild type, P444-3D; cells grown on glycerol	111
25	Double reciprocal plot of hexokinase A	118
26	Inactivation of hexokinase A by D-xylose	118
27	Growth of transformant T1 on D-glucose, D-xylose (2% w/v), D-glucose and D-xylose (2%:2% w/v) in YNB	121
28	Utilisation of D-glucose, D-xylose (2% w/v), D-glucose and D-xylose (2%:2% w/v) in YNB by transformant T1	121
29	DEAE-cellulose chromatography of cell-free extract from transformant T2	122
30	Time course of strain F/G 2 in YNB-glucose/xylose (2%:2% w/v)	134
31	Fermentation of D-glucose and D-xylose (2%:2% w/v) in YEP medium by wild type strain, P444-3D	139

LIST OF FIGURES CONT'D		Page
32	Fermentation of D-glucose and D-xylose (2%:2%w/v) in YEP medium by strain F/G 2	140
33	Proposed alternative pathway for D-glucose utilisation in <i>P. tannophilus</i>	166

APPENDIX FIGURES

A1	Standard curve relating optical density to viable cell numbers (wild type cells)	178
A2	Protein standard curve (Bovine Serum Albumin)	179

LIST OF TABLES

Table	Page
I Strains of yeast and bacteria used in this study	32
II Amino acid supplements for complete YNB medium	35
III Resistance of <i>Pachysoles tannophilus</i> strains to 2-deoxyglucose on solid media	76
IV Growth of wild type and mutants on different carbon sources on solid media	79
V Hexose-ATP-kinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase activities of wild type and mutants	95
VI D-Glucose and D-fructose-phosphorylating activities of wild type and mutants	96
VII Specific activities of xylose reductase and xylitol dehydrogenase by wild type and mutants grown on different carbon sources	112
VIII Summary of purification of hexokinase A from strain P510-5A	114
IX Kinetic properties of hexokinase A	117
X Growth of <i>S. cerevisiae</i> and <i>P. tannophilus</i> strains on D-sorbitol and other carbohydrates	124
XI Retention times of sugars and sugar alcohols on the Bio-Rad Aminex HPX-87H column	132
XII Summary of crosses between the <i>eth2-1</i> strain and NO ₃ -NO ₃ -4	136
XIII Maximum ethanol concentration and yields from the fermentation of YNB and YEP-xylose and YNB and YEP-xylose/glucose	137

LIST OF PLATES

Plate		Page
1	Haploid cells of <i>Pachysolen tannophilus</i>	2
2	Diploid cells of <i>Pachysolen tannophilus</i>	2
3	Shimadzu LC-4A high performance chromatograph	40
4	Schematic diagram for anion-exchange chromatography	51
5	Dissection chamber and agar slab for micromanipulation	69
6	Needle for dissection of asci	69
7	Micromanipulator and microscope	71
8	Dissected asci on agar slab	71
9	Polyacrylamide disc gel electrophoresis of partially purified hexokinase A	116
10	Agarose gel electrophoresis of YRp/HXK2-8 and plasmid DNA extracted from <i>E. coli</i>	116
11	HPLC chromatograph: conversion of D-glucose to D-sorbitol and D-fructose by cell-free extract from D/X A (NADPH)	127
12	HPLC chromatograph: conversion of D-glucose to D-sorbitol by cell-free extract from D/X A (NADPH and NAD)	128
13	HPLC chromatograph: conversion of D-sorbitol to D-fructose by cell-free extract from D/X A (NAD)	129
14	HPLC chromatograph: conversion of D-xylose to xylitol by cell-free extract from D/X A (NADPH)	130