# ETHANOLIC FERMENTATION OF D-XYLOSE AND PINE WOOD HYDROLYZATE BY THE YEAST *Pachysolen tannophilus*

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

**TZE SEN WONG**

**1984**

**ABSTRACT**

This thesis reports a study of the ethanolic fermentation of D-xylose and wood hydrolyzate to ethanol by the yeast Pachysolen tannophilus with a view to developing an effective use of renewable hemi-cellulose hydrolysis products from New Zealand forest biomass residues.

Initial work briefly addressed the problem of finding a suitable yeast from natural habitats suitable for the fermentation. Soon after that work commenced literature reports suggested that preliminary conversion of pentoses by enzymatic means was a possibility. Consequently, this aspect of conversion was considered and rejected. One reason for this was that literature was drawing attention to the pentose fermenting characteristics of Pachysolen tannophilus.

Laboratory scale studies demonstrated the yeast Pachysolen tannophilus to be capable of fermenting the hexose and pentose sugars present in the hydrolyzate. The yeast's specific growth rate in the hydrolyzate could be improved by neutralizing the inhibitory substances with 2 g/l of anhydrous sodium sulphite. Ethanol has an inhibitory effect on growth but can also be readily assimilated by the yeast.

Fermentation studies with gyration speeds of 50, 100 and 200 r.p.m. showed that oxygen was a critical parameter affecting growth and ethanol production. Batch fermentation experiments were pursued to examine this oxygen phenomenon more closely. Cell growth, substrate uptake rate and culture pH responded strongly to the supply of oxygen. However, production of ethanol accompanied cell growth only in late "exponential" phase.

Fermentation characteristics were established under continuous culture at an aeration rate of 0.37 l/l.min and values obtained were as follows; maximum specific growth rate, 0.046 $h^{-1}$; biomass yield, 0.04 g/g; ethanol yield, 0.17 g/g; Ks value, 13 g/l and Ki values, 0.5 g/l.

A redox potential controlled chemostat study revealed that steady-state culture poised at -50 mV exhibited a 55% increased ethanol concentration and 43% decreased xylitol concentration over the value observed without redox control.

With a knowledge of D-xylose fermentation as established in these batch and chemostat experiments, it was possible to consider more detailed aspects of the fermentation which would be applicable to process development. Questions addressed included which strain of Pachysolen tannophilus should be used, what quantity of inoculum was necessary, what interactions existed between fermentation variables. Statistically designed experiments were employed to answer these questions. Empirical models so developed revealed that ethanol yield has a linear relationship with initial substrate concentration. These models have given some insight into how environmental factors affect the ethanolic fermentation by this yeast and have also indicated the optimal conditions required for an effective fermentation of wood pentoses.

These important fermentation process variables were established and are expected to be useful in moving the process from laboratory scale as carried out here to a pilot plant scale of operations. The values established were temperature, 28° or lower; initial medium pH for ethanol production, 5.6 to 5.8; substrate concentration used can be up to 80 g/l of pentoses; minimum inoculum density, 5.5 g/l dry weight cells and NRRL Y-2461 was recommended as the best strain to achieve the fermentation. The pre-treatment of the prehydrolyzate by 2 g/l of anhydrous sodium sulphite was highly desirable in order to enhance growth and fermentation rates.

The research has shown that Pachysolen tannophilus is capable of fermenting pentose fraction of wood hydrolyzate and that the optimal conditions for this fermentation will lead to significant utilization of wood sugar. However, in the completely mixed reactor systems used in these experiments, the ethanol yields obtained were not as attractive as those observed for hexose fermentations under similar conditions. This, it is felt, points to the greater difficulty the yeast experiences in fermenting pentoses and it also suggests the need to investigate the value of other reactor formats at some future date.

17 December , 1984.

This is to certify that the work on which the thesis, *ETHANOLIC FERMENTATION OF D-XYLOSE AND PINE WOOD HYDROLYZATE BY THE YEAST Pachysolen tannophilus,* is based has not been accepted in whole or in part for any other degree or diploma and all the help and assistance received in the research on which the thesis is based, are fully acknowledged.

Tze Sen Wong.
Ph. D. Candidate.

**ACKNOWLEDGEMENTS**

I wish to acknowledge the following :

- my supervisors Dr. G.J. Manderson, Dr. V.F. Larsen and Dr. R.H. Archer for their kind supervision and guidance.
- Dr. J. Butcher, Dr. K. Mackie and Dr. T.A. Clark, Forest Research Institute for their advice and provision of the redox potential electrodes, the supply of acid hydrolyzate and prehydrolyzate and other materials and for the analyses of xylitol in culture supernatants.
- Dr. I.F. Boag for advice on the statistical experimental designs.
- Mrs. D. Steward for analyzing the sugar, xylitol.
- Mrs. K. Deverell for kind advice and assistance during my visit to the Forest Research Institute, Rotorua.
- Mr. J.T. Alger, Mr. P. Shaw, Mr. D.W. Couling and Mr. M.Stevens for their assistance in building and maintaining equipment and technical advice.
- Miss Fiona Choy for typing this thesis.
- My wife, Kin , for her kind and moral support throughout the entire course of study.
- finally, to my family and many other people who helped during the preparation of this thesis.

This research was supported by a grant from the New Zealand Forest Research Institute, Rotorua, New Zealand as Contract Number 149 between the New Zealand Forest Service and Massey University. This support is sincerely acknowledged.

TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| g | gram or gravitational force under centrifugation |
| h | hour |
| kg | kilogram |
| l | litre |
| M | molar or mole |
| mg | milli gram |
| min | minute |
| ml | milli litre |
| mM | milli mole |
| mV | milli volt |
| ppb | part per billion (i.e. μg/l) |
| s | second |
| r.p.m. | revolution per minute |
| vvm | gas volume/medium volume/min (l/l.min) |
| v/v | volume/volume (ml/l) |
| w/v | weight/volume (g/l) |

| | |
|---|---|
| A.R. | analytical reagent |
| ATP | adenosine-5'-triphosphate |
| AW-DMCS | acid washed - dimethyldichlorosilane |
| BOD | biochemical oxygen demand (g/l) |
| COD | chemical oxygen demend (g/l) |
| D | dilution rate ($h^{-1}$) |
| DEAE- | diethylaminoethyl- |
| DF | degrees of freedom |
| DOT | dissolved oxygen tension |
| dP/dt | rate of production formation (g/l.h) |
| dS/dt | rate of substrate consumption (g/l.h) |
| E | ethanol concentration (g/l) |
| F | F-ratio of mean sums of squares |
| FFAP | free fatty acid phase |
| $F_{1of}$ | F-ratio of MSLF to MSPE |
| Ki | ethanol inhibition constant for growth (g/l) |
| Km | the Michaelis constant |

| | |
|---|---|
| Ks | saturation constant |
| log | logarithm to base 10 |
| ln | logarithm to base e |
| m | maintenance energy coefficient (g substrate/g dry cell.h) |
| MRS | de Man, Rogosa, Sharpe agar or broth |
| MS | mean sum of squares |
| MSLF | mean sum of squares due to lack of fit |
| MSPE | mean sum of squares due to pure error |
| MSRG | mean sum of squares due to regression |
| MSRS | mean sum of squares due to residual |
| N | number of replicates |
| NADP | nicotinamide adenine dinucleotide phosphate |
| $N_c$ | number of centre points in experimental design |
| $N_f$ | number of factorial points in experimental design |
| $N_s$ | number of `star' points in experimental design |
| Qp | specific product formation rate (g/g.h) |
| Qs | specific substrate consumption rate (g/g.h) |
| So | initial sugar (D-xylose) concentration (g/l) |
| Sx | steady state value of D-xylose concentration (g/l) |
| Sxo | xylitol concentration (g/l) |
| SS | sum of square |
| STDEV | standard deviation |
| TCA | tricarboxylic acid cycle |
| W | dry weight of cells (g/l) |
| X | biomass concentration (g/l) |
| Y | response variable or<br>observed growth yield (g cells/g substrate) |
| Yg | true growth yield (g cells/g substrate) |
| $\hat{Y}$ | predicted value of response variable being modelled |
| Y p/s | product yield |
| Y p/x | product yield |
| Y x/s | biomass yield |
| Y xo/s | xylitol yield |
| Y xo/x | xylitol yield |

| | |
|---|---|
| $>$ | is greater than |
| $<$ | is less than |
| $\alpha$ | coded distance from origin of `star' or axial point in a central composite or central composite rotatable design |
| $\beta$ | represents the coefficient in the experimental design |
| $\beta_o$ | is the Y-intercept or constant term |
| $\mu$ | spcific growth rate $(1/X.\ dX/dt)$ $(h^{-1})$ |
| $\mu_{max}$ | spcific growth rate $(1/X.\ dX/dt)$ $(h^{-1})$ |
| $^o$ | represents degrees temperature expressed on Celsius scale |

oooOOooo

# CHAPTER ONE : INTRODUCTION

In New Zealand, the largest quantity of crop residue comes from the forestry sector. In 1977, about 3 million cubic metres of collected wood residues were used for wood-chip export, domestic fuel and pulp production (Harris et al, 1979). It has been predicted that an additional 6 million cubic metres of sawmill residue from a surplus supply of 10 million cubic metres of timber will be available per annum by the year 2000 (Mackie, 1982b).

A parallel issue to that of the potential of rapidly accumulating forest residues is that of New Zealand's need to become self-sufficient in energy production.

There are compelling economic and strategic reasons for New Zealand to develop her forest biomass as a renewable feedstock and alternative fuel resource.

These reasons are summarized as follows :

Extensive land availability for forest development.
An existing and efficient forest development and utilization programme.
An inexhaustible supply of wood and forest residues.
Satisfactory soil types and plentiful water supplies.
Intensive consumer demands for liquid fuels.
Increasingly unfavourable oil prices coupled with liquid fuel dependent economy.
The sense of technical feasibility to provide alternative fuels to replace those currently derived from imported oil.
The none renewability of traditional energy supplies (coal , gas and oil).
Vulnerability of the long-haul sea routes to disruption by political or other forces.

Therefore, wood materials must be considered as an effective renewable resource for producing liquid fuels and a means whereby New Zealand may move towards an independence from imported liquid fuels thus alleviating her payment debts incurred in buying imported oil. Appropriate technology must be developed to convert this resource to liquid fuel materials such as ethanol and/or butanol.

In considering wood as a renewable energy resource, a variety of methodologies exist by which energy, locked into the carbon skeleton of the macromolecules comprising "wood", can be extracted. Of particular interest in this discussion will be wood's carbohydrate fraction and its exploitation as a raw material convertible to liquid fuel.

Pentose is one of the major carbohydrate fractions derived from hemicellulose (chiefly xylan), which may constitute up to 40% dry weight of the total composition of the plant biomass, wood and agricultural residues. Hemicelluloses of the plant cell-walls are, by definition, short branched-chain heteropolysaccharides being a mixture of hexosans and pentosans. They are relatively easily hydrolyzed in the laboratory. D-xylose and L-arabinose are the major constituents of the pentosans while D-glucose, D-mannose and D-galactose are the significant constituents of hexosans (Timell, 1964 and 1965).

In wood , the amount, structure and composition of hemicellulose varies widely, depending on the type of tissue and on plant variety. Table 1.1 summarizes information on the composition of the carbohydrate present in different woody plant materials, such as hardwoods and softwoods with high and low xylan content respectively.

Table 1.1 Carbohydrate Composition of Hardwoods and Softwoods
Source: Tarkow et al. (1963); Mackie (1982b) *

| Species | Major Components ( % Oven-dry weight) | | |
|---|---|---|---|
| | 1 Glucan | 2 Xylan | 3 Arabinan |
| Aspen *Populus tremuloides* | 57.3 | 16.0 | 0.4 |
| Beech *Fagus grandifolia* | 47.5 | 17.5 | 0.5 |
| White birch *Betula papyrifera* | 44.7 | 24.6 | 0.5 |
| Yellow birch *Betula lutea* | 46.7 | 20.1 | 0.6 |
| Eucalypts * *Eucalyptus regnans* | 49.6 | 18.1 | 0.3 |
| White cedar *Thuja occidentalis* | 45.2 | 7.5 | 1.3 |
| White pine *Pinus strobus* | 44.5 | 6.3 | 1.2 |
| White spruce *Picea glauca* | 45.0 | 6.8 | 1.6 |
| Monterey pine * *Pinus radiata* | 43.2 | 7.3 | 2.8 |

1 Glucan : Polysaccharide composed of condensed glucose units.
2 Xylan : A pentosan composed either entirely or almost entirely of D-xylose units.
3 Arabinan : A pentosan composed of condensed L-arabinose units. (Whistler and Corbett, 1957)

Hydrolysis of hemicellulose by chemical methods, which has been recently reviewed by Mackie (1982b), produces primarily D-xylose, L-arabinose and D-glucose as the major sugar components. Softwoods, particularly *Pinus radiata*, constitute the major proportion of the New Zealand commercial forests. Softwood hydrolyzate contains soluble carbohydrate of which 12 to 15% are aldopentoses (Whitworth, 1976 and 1977) (see Table 1.2). These sugar constituents can be used as fermentable substrates by many micro-organisms for fermentative bioconversion to liquid fuel products.

Table 1.2 Sugar Compositions of Softwood (Pinus radiata) Acid Hydrolyzate Liquors
Mackie (1982b)

| Sugar | Pre-hydrolyzate | | Hydrolyzate | |
|---|---|---|---|---|
| | g/l | % of total sugar | g/l | % of total sugar |
| Arabinose | 2.5 | 7 - 9 | 0 | Negligible |
| Xylose | 5.9 | 18 - 20 | 0.9 | 3 - 5 |
| Mannose | 12.0 | 38 - 42 | 1.3 | 5 - 7 |
| Galactose | 3.4 | 10 - 12 | 0.3 | 1 - 2 |
| Glucose | 6.8 | 21 - 23 | 20.0 | 87 - 90 |
| Total | 30.6 | 100 | 22.5 | 100 |

Within 20 years New Zealand may be producing annually as much as 9 million cubic metres of forest residues suitable for acid hydrolysis to carbohydrate material. From softwoods, it may be expected that approximately 50% of the dry biomass can be recovered as fermentable carbohydrate. It can be calculated that 0.45 million tonnes per annum of carbohydrate may be available for conversion to liquid fuels by micro-organisms. If, for example, yeast is used to produce ethanol, and if wood sugars could be converted to ethanol with the same efficiency as brewer's yeasts convert malt sugars to ethanol, then New Zealand might produce 0.18 million tonnes of ethanol per year. The question arises though, can it be done ?

In this thesis, the microbiological conversion of wood pentose, particularly D-xylose, to ethanol is the principal interest of the study. Through these studies it is hoped that this important question can be answered, or alternatively, that these studies will provide details of the nature of the fermentation which will help in formulating an answer to this above question.

# CHAPTER TWO : REVIEW OF LITERATURE

## 2.1 INTRODUCTION

The commercial production of ethanol by yeast fermentation of wood waste or sulfite waste liquor received attention in the 1940s (Ericsson, 1947; Harris et al., 1948; Leonard and Hajny, 1945). The interest diminished during the 1950's, as cheap and readily available sources of liquid fuels and other organic compounds became available from the rapidly expanding petrochemical industries. However, after the petroleum price increases in 1973, the production of chemical feedstocks and liquid fuels by fermentation from renewable resources has received considerable interest.

Early fermentation studies on the conversion of woody biomass to ethanol considered only the cellulose fraction, and the hemicellulose fraction was wasted (Harris et al., 1948; Leonard and Hajny, 1945). In order to make the biomass conversion economically feasible, it is essential that the hemicellulose fraction also be converted efficiently into ethanol (Rosenberg, 1980). In this review , attention is paid to the hemicellulose fraction which is composed of pentose sugars. This has until recently been regarded as nonfermentable by yeasts (Barnett, 1976; Prescott and Dunn, 1959). Attention will also be drawn to the general concepts of metabolism of pentoses by micro-organisms. This review also attempts to muster all the information concerning the current advances in the area of bioconversion of pentose sugars, directly or indirectly, by yeast(s) to ethanol during the past five years (mid 1979 -mid 1984). In particular, emphasis is placed on the yeast, Pachysolen tannophilus, called "odd" by two French Scientists twenty seven years ago (Boidin and Adzet, 1957).

## 2.2 MICROBIAL PENTOSE CONVERSIONS

The overall metabolic events and biochemical details relating to the microbial conversion of pentoses to ethanol have been intensively reviewed by Jeffries (1983a); Kosaric et al.(1983); Rosenberg (1980)

and Wood (1961). Generally, the microbiological conversion of pentoses can be conceived as proceeding in three clearly define stages:

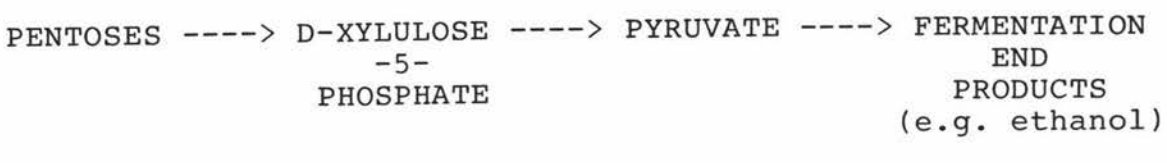
Stage 1 : The conversion of the pentose to the phosphorylated keto sugar intermediate (pentulose-5-phosphate).

Stage 2 : The conversion of the phosphorylated keto sugar to pyruvate.

Stage 3 : The conversion of pyruvate to fermentation end-products of interest as liquid fuels such as ethanol.

This process is summarized in Figure 2.1 which in simplified form depicts the metabolic stages for the conversion of pentoses to a neutral end-products, e.g. ethanol. Unfortunately, the conversion is not quite as simple as this figure would indicate as there are characteristic problems associated with the various types of micro-organism (for example bacteria, fungi or yeasts) which one may wish to employ for the fermentation.

Figure 2.1 The Conversion of Pentoses to End-products



2.2.1 Bacteria

This discussion is restricted to the conversion of pentoses to ethanol by bacteria. The reader is referred to Wiegel (1980) for information concerning ethanol production from other substrates. Most of the pentose-fermenting, ethanol-producing bacteria convert pentoses to pyruvate via a combination of pentose-phosphate and Embden-Meyerhof pathways (Horecker, 1962) (i.e. through metabolic Stages 1 and 2 of the scheme as described above). This pyruvate is then converted by anaerobic metabolism to multiple end-products (stage 3). End-products include alcohols (e.g. butanol, ethanol, isopropanol and 2,3-butanediol), organic acids (e.g. acetic, butyric, formic and lactic acid), ketones (e.g. acetone), and gases (e.g. hydrogen and carbon dioxide). This variety of end-product results from the activities of numerous bacteria. Pathways for formation of pyruvate are illustrated in Figure 2.2. Those bacteria fermenting glucose and/or xylose to ethanol may also produce acetone, 2,3-butanediol, butanol and a variety of organic acids as shown in Table 2.1. Recently, the bacterial conversion of pentose sugars to acetone and n-butanol (Volesky and Szczesny, 1983), and 2,3-butanediol (Jansen and Tsao, 1983; Yu and Saddler, 1982 a and b; Yu and Saddler, 1983; Yu, et al., 1982) and to a mixture of ethanol, lactate and acetate (Flickinger, 1980) by clostridia has been described and can be cited as indicative of the widening and renewed interest in pentose fermentations.

From Table 2.1, it is clear that bacterial fermentations of pentoses seldom yield a clean end-product. It would seem that for the present moment at least, no known naturally occurring bacterium is suited immediately for the pentose to ethanol fermentation. More recently, Weimer et al.(1984) studied the anaerobic conversion of 6-deoxyhexoses (L-rhamnose, L-fucose and D-fucose) to 1,2-propanediol, acetone, ethanol, hydrogen and carbon dioxide by Bacillus macerans. Esser and Karsch (1984) also listed out the advantages and disadvantages of the ethanol producing bacteria in the fermentation industry. They concluded that the formation of relatively high amounts of organic acids by the thermophilic bacteria was one of the main

Table 2.1

End Products Formed During the Conversion of Glucose and/or Xylose by Selected Bacteria

| Bacterium | Fermentation Products (moles produced per mole carbohydrate consumed) | | | | |
|---|---|---|---|---|---|
| | Acetone | 2,3-butanediol | Butanol | Ethanol | Acids |
| *Aeromonas hydrophila* | | | | | |
| glucose | 0 | 0.5 | 0 | 0.5 | 0.05 |
| xylose | 0 | 0.4 | 0 | 0.5 | - |
| *Bacillus macerans* | | | | | |
| glucose | 0.3 | 0 | 0 | 0.9 | 0 |
| xylose | 0.1 | - | - | 0.6 | 0 |
| *Bacillus polymyxa* | | | | | |
| glucose | 0 | 0.7 | 0 | 0.7 | 0.02 |
| xylose | 0 | 0.4 | 0 | 0.6 | 0.08 |
| *Clostridium acetobutylicum* | | | | | |
| glucose | 0.22 | - | 0.56 | 0.07 | 0.18 |
| *Clostridium thermohydro-sulfuricum* | | | | | |
| glucose | 0 | 0 | 0 | 1.0 | 1.0 |
| *Spirocheata litoralis* | | | | | |
| glucose | 0 | 0 | 0 | 1.1 | 0.4 |
| *Zymomonas mobilis* | | | | | |
| glucose | 0 | 0 | 0 | 1.8 | 0 |
| xylose | 0 | 0 | 0 | 0 | 0 |

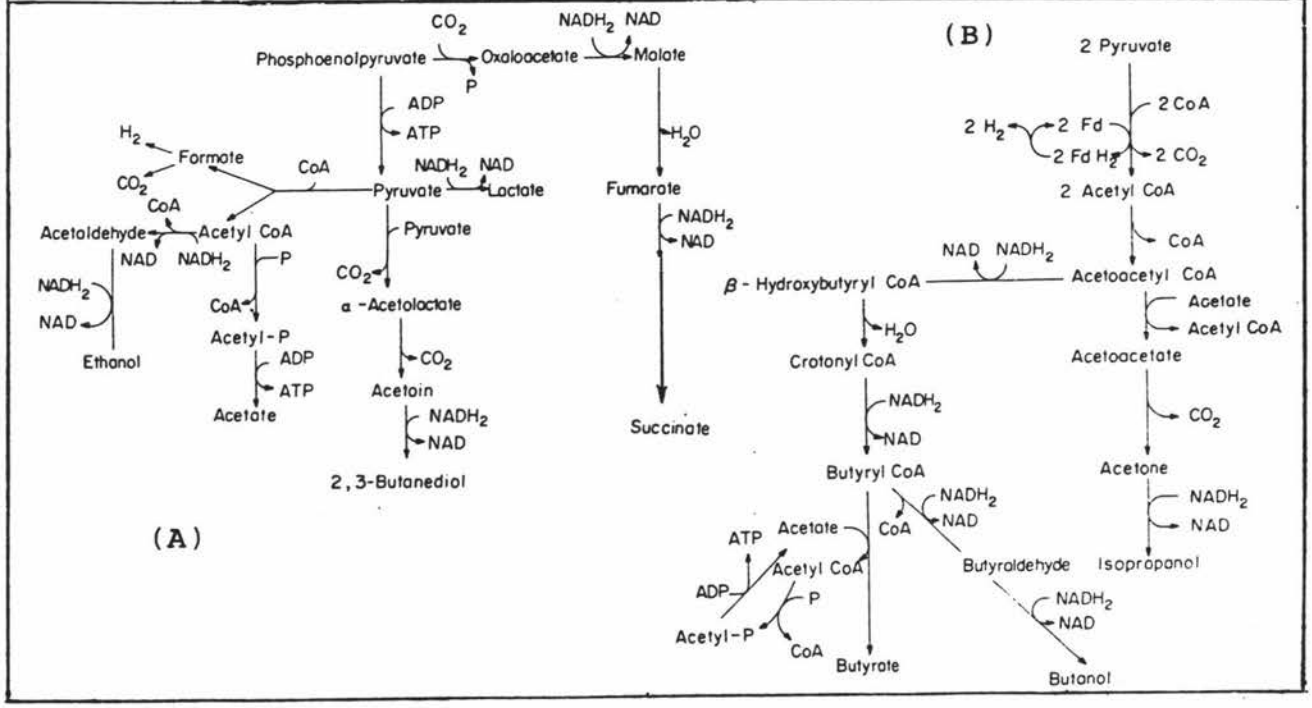
a Strainer and Adams, 1944
b Northrop *et al.*, 1919
c Adams *et al.*, 1945
d Wood, 1961
e Weigel *et al.*, 1979
f Hespell and Canale-Parole, 1970
g Swings and de Ley, 1977

disadvantages in bacterial ethanol production. While these mixed product fermentations may appear problematic to the industrial sector, there could be conceivably some value in having a number of products of industrial importance arising from the single fermentation.

### 2.2.2 Mycelial Fungi

Several filamentous fungi belonging to the genera Fusarium (Batter and Wilke, 1977; Gibbs et al., 1954; Suihko and Enari, 1981; White and Willaman 1929 a and b), Mucor, Rhizopus (Gleason, 1971; Perlman, 1950; Ueng and Gong, 1982) and Monilia (Gong et al., 1981d) are known to ferment D-xylose to ethanol. A number of these genera produce ethanol together with acetic acid and traces of lactic acid. However, the main drawback in utilizing these moulds is their slow fermentation rate (Batter and Wilke, 1977; Suihko and Enari, 1981; Ueng and Gong, 1982). Furthermore, Fusarium is known to be inhibited by lignins and phenolic compounds found in the wood hydrolyzate (Wiley et al., 1941).

Figure 2.2 Pathways Used by Bacteria for the Formation of End-products
(A) the mixed acids and 2,3-butanediol
(B) acetone, butanol, butyric acid and isopropanol.
Source : Rosenberg, 1980.



2.2.3 Yeasts

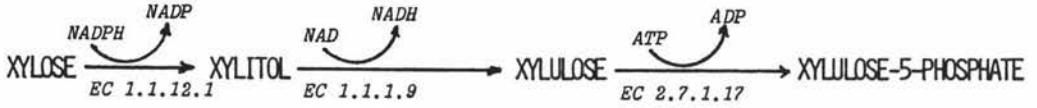
In those yeasts capable of converting pentose to ethanol the mechanism generally follows the three previously defined stages ( Figure 2.1). Details of these stages can now be discussed. Yeasts and other prokaryotic micro-organisms exhibit fundamentally different metabolic mechanisms in the early stages of pentose assimilation. Yeasts (e.g. Candida utilis, Pichia quercuum) metabolize the pentose through an oxidation-reduction reaction whereas, in most bacteria, an isomerization of pentose to keto sugar occurs (Barnett, 1976; Charkravorty et al., 1962; Suzuki and Onishi, 1973). Furthermore, in yeasts, D-xylose can be reduced to xylitol by an NADPH-dependent D-xylose reductase (E.C. 1.1.12.1., aldose reductase). Xylitol may either accumulate (Debus et al., 1983) or be catalyzed further, being oxidized to xylulose by an NAD-dependent D-xylitol dehydrogenase (E.C. 1.1.1.9., D-xylulose reductase) as described in Figure 2.3. Both of these enzymes are active in Pachysolen tannophilus (Smiley and Bolen, 1982). Phosphorylation of xylulose to xylulose-5-phosphate follows and the pentose phosphate is then converted to pyruvate through both the pentose-phosphate and Embden-Meyerhof metabolic pathways. Thus, in pentose-fermenting yeasts, these sugars are ultimately converted to xylitol, ethanol, glycerol, acetic acid and carbon dioxide (Barnett, 1976; Gong, 1983; Gong et al., 1981a). This process is summarized in Figure 2.4.

A general comparison of the pentose metabolism by yeasts with that of bacteria and mycelial fungi is shown in Table 2.2. It is apparent that the initial stages of prokaryote and eukaryote catabolism are profoundly different regardless of the end-products formed. Only a few exceptions to this generalization have been reported amongst the yeasts. (Hofer et al., 1971; Tomoyeda and Horitsu, 1964). The two step oxidation-reduction reaction which converts D-xylose to D-xylulose has been recognized as an essential mechanism for D-xylose assimilation in yeasts (Barnett, 1968; Onishi and Suzuki, 1969; Jeffries, 1983a). Although this generalized route for D-xylose metabolism by yeasts (as described in Figure 2.4 and Table 2.2) has been demonstrated to proceed under aerobic conditions, there had been no reports of yeast fermenting D-xylose (Barnett, 1976).

FIGURE 2.3 PATHWAYS OF PENTOSE CONVERSION TO D-XYLULOSE-5-PHOSPHATE USING ISOMERIZATION OR OXIDATION-REDUCTION STEP

**Isomerization : (Bacteria)**


**Oxidation-Reduction : (Yeasts and Mycelial Fungi)**




*EC 5.3.1.5* *xylose isomerase*

*EC 2.7.1.17* *xylulose kinase*

*EC 1.1.12.1* *aldose reductase (NADPH- dependent D-xylose reductase)*

*EC 1.1.1.9* *xylulose reductase (NAD - dependent D-xylitol dehydrogenase)*

Table 2.2 General Comparison of Pentose ( D-Xylose ) Metabolism of Bacteria, Yeasts and Mycelial Fungi

| Organisms | Stage 1 | Stage 2 | Stage 3 |
|---|---|---|---|
| | Xylose → Xylulose-5-P | Xylulose-5-P → Pyruvate | Pyruvate → End-Product(s) |
| Bacteria | Isomerization [c] | Pentose Phosphate + Embden-Meyerhof | Ethanol + Mixed Acids [a]<br>Ethanol + 2,3-butandiol<br>Ethanol + Acetone + Butanol |
| Yeasts | Oxidation-Reduction [c] | Pentose Phosphate + Embden-Meyerhof | Ethanol [b] |
| Mycelial Fungi | Oxidation-Reduction [c] | Pentose Phosphate + Embden-Meyerhof | Ethanol + Acetic and Lactic acids [b] |

a as described Figure 2.2

b as described Figure 2.4

c as described Figure 2.3

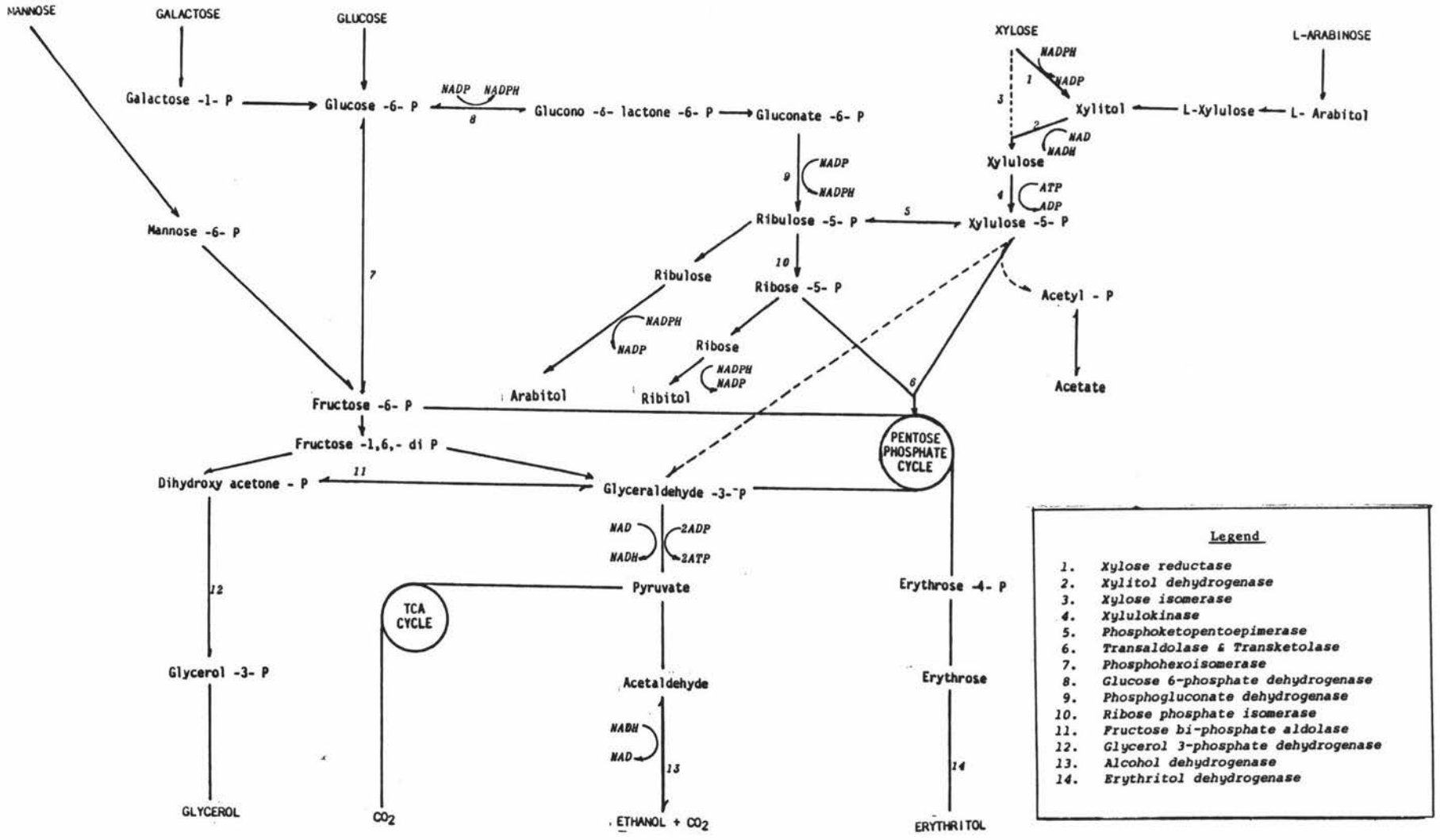



Figure 2.4 Wood Sugar Metabolism by Yeasts
(Data modified from Barnett,1976 & Gong,1983)

## 2.3 INDIRECT ALDOPENTOSE FERMENTATION : ENZYME LINKED PENTULOSE FERMENTATION

The evidence from literature suggests that D-xylose fermentation is not a common phenomenon among the yeasts. Barnett (1976) investigated D-xylose utilization in 434 strains of yeast capable of ethanolic fermentation of glucose and/or sucrose to find that only 214 strains utilized the pentose aerobically and that none did so anaerobically. As described in Figure 2.3 the metabolism of pentoses involves the formation of the intermediate D-xylulose using the enzymatic mechanisms outlined in Table 2.2. This knowledge has stimulated research into the evaluation of D-xylulose as a possible substrate for ethanolic fermentation. The rationale proposed by Wang et al. (1980a) was that D-xylose would be converted through an enzymatic process to D-xylulose which would then be fermented by a yeast culture to ethanol. This proposal has much merit. However, if the vast pentose resources of waste wood are to be utilized effectively, then the chosen technology for such a conversion of D-xylose to D-xylulose to ethanol must be appropriate to the magnitude of the task.

### 2.3.1 Conversion of D-xylose to D-xylulose

Xylose isomerase, (i.e. D-xylose keto-isomerase, E.C. 5.3.1.5, also known as glucose isomerase), catalyzes the reversible isomerization of both D-xylose to D-xylulose and D-glucose to D-fructose. This enzyme has been reviewed extensively in recent years particularly in the context of high fructose syrups (Bucke, 1977; Chen, 1980a, b).

D-xylose isomerase is produced in many bacteria in response to the presence of D-xylose. Mitsuhashi and Lampen (1953) first demonstrated that cell-free extracts of Lactobacillus pentosus (later called Lactobacillus plantarum) grown on D-xylose contained an enzyme capable of converting D-xylose to D-xylulose. Later, this same enzyme was demonstrated in Pseudomonas, isomerizing D-glucose to D-fructose (Marshall and Kooi, 1957). Subsequently, it was successfully employed

in a full industrial process to produce sweeteners (e.g. fructose, in the form of high fructose corn syrup) as a sucrose replacement (Abbott, 1978; Antrim et al., 1979; Bucke, 1983 ; Fullbrook and Vabo, 1977; Mermelstein, 1975).

D-xylose isomerase is an intra-cellular enzyme. Factors which influence its stability, reaction rate and the equilibrium are temperature, pH, dissolved oxygen and the presence of cofactors such as $Co^{++}$, $Mn^{++}$ and $Mg^{++}$. The optimal pH and temperature for the isomerization depend on the source of the enzyme. Generally, however, isomerization of xylose to xylulose by D-xylose isomerase is carried out at 50-70° under slightly alkaline conditions in the presence of divalent cations such as $Mn^{++}$, $Co^{++}$ and $Mg^{++}$ (Chen, 1980 a,b). These optimum reaction conditions must be considered when designing techniques to link isomerization with fermentation. In considering any large scale isomerase application, two further factors must be considered;

Firstly, the concentration of D-xylulose in the equilibrium mixture which is available for fermentation.

Secondly, the availability of the isomerase enzyme, in quantity, its cost, stability and if it can be recovered.

With regard to xylulose concentration, the isomerization does not favour xylulose and at equilibrium some 75-80% of the xylose will remain unconverted (Ueng et al., 1981). However, the presence of borate in the reaction mixture shifts the equilibrium to favour D-xylulose. Mitsuhashi and Lampen (1953) observed that the presence of borate (2 micro-moles per ml) in the isomerization mixture made possible an increase in the ketose content to 40-45% of the total sugar. Similar results were claimed by Slein (1955). There, the presence of borate (0.02 moles per ml) shifted the equilibrium to yield 60-65% xylulose as compared with 16% xylulose in its absence. Recently, Hsiao et al.(1982) used 0.3 moles per ml of sodium tetraborate to achieve a maximum conversion yield of 80% to xylulose

from xylose. Because of problems with borate recovery there is some doubt as to the commercial acceptability of such a system (Gong, 1983). A non-chemical xylulose preparation technique involving isomerization, precipitation and microbial utilization of xylose has allowed the preparation of pure xylulose syrups (Chiang, et al., 1981b).

With regard to the availability, cost and stability of the isomerase, both soluble and immobilized forms of the enzyme are available in commercial quantities from NOVO Industry, Inc., Denmark as Sweetzyme A and Q and from Miles Laboratories, Inc., U.S.A. as Takasweet. These isomerases are currently produced for the production of high fructose syrups. No detailed costing has been found to date in literature. However, a tentative evaluation in terms of additional cost per kg ethanol attributable to use of enzyme had been prepared by Larsen (1981) and is collected in Appendix 1.

The additional cost of 10 to 30 cents per kg of ethanol produced was calculated. These costs, although tentative, indicate that enzyme conversion of xylose to xylulose may be an economic proposition if moderate activities and enzyme usage were to be obtained. Furthermore, little information on the durability of the enzyme in wood hydrolyzate or in the presence ethanol seems available. These are further topics needing investigation.

### 2.3.2 D-Xylulose Fermentation

Wang's investigation of D-xylose metabolism by yeasts has shown that nine out of eleven cultures tested grew aerobically utilizing a catabolic pathway involving D-xylulose (Wang and Schneider, 1980; Wang et al., 1980b). This knowledge has stimulated interest in using D-xylulose as a fermentation substrate. Several yeasts were shown to ferment D-xylulose and produce ethanol. (Gong et al., 1981b; Jeffries, 1981a and Ueng et al., 1981; Suihko and Poutanen, 1984). This phenomenon suggested to Jeffries (1983a) that the reductive/oxidative conversion of D-xylulose may be the regulating or rate-limiting metabolic stage in yeasts. Pretreatment with the enzyme xylose isomerase, may overcome this metabolic defect by converting

D-xylose to D-xylulose extra-cellularly. The subsequent uptake and fermentation to ethanol of the isomerization product is metabolically uncomplicated. Much interest has been expressed in this possible approach for the commercial application of pentose fermentation.

The success of an isomerase linked fermentation will also depend upon the ability of the chosen yeast to ferment xylulose. An early report on D-xylulose utilization by yeast was presented by Onishi and Suzuki (1969) who named 128 strains of yeast which would do so. However, the first reports of an ethanolic fermentation using D-xylulose were provided by Wang et al. (1980b) and Wang and Schneider (1980). e.g. Schizosaccharomyces pombii NCYC 132 when grown on D-xylulose, produced ethanol in amounts similar to that produced when grown on glucose. Further details on the topic soon followed (Gong et al., 1981b; Jeffries, 1981a; Maleszka and Schneider, 1982a; Suihko and Drazic, 1983; Ueng et al., 1981) and data for 7 strains of Saccharomyces species, 8 strains of Schizosaccharomyces species, 6 strains of Candida species and of species of a further 7 genera became available (Gong et al., 1983).

The optimum conditions for xylulose fermentation need to be considered if the fermentation is to be linked to isomerization of xylose. Simultaneous isomerization and fermentation may be impractical as the two processes have different optima, viz: pH 7.0 to 8.0, 27-30° for the xylulose fermentation (Suihko and Drazic, 1983; Jeffries, 1981a) and 60-65° and pH >7.0 depending on the enzyme (Fullbrook and Vabo, 1977; Gong, 1983) for the isomerization step. Gong (1983) has listed characteristics that govern the conversion of D-xylulose to ethanol by yeast.

### 2.3.3 Isomerization Linked Fermentation

With the idea of the incorporation of the xylose isomerase to isomerize aldopentose (i.e. D-xylose) to pentulose (i.e. D-xylulose) and further convert to ethanol by a numbers of yeasts, like Saccharomyces, a number of researchers' work has been briefly summarized in below Table 2.3.

Table 2.3 Ethanol Production from Xylulose by Yeasts Grown in Batch Culture

| Species/ Strains | Ethanol Produced ( g/l ) | | Reference |
|---|---|---|---|
| | D-Xylulose | D-Xylose | |
| Saccharomyces | * (30) | | a |
| carlsbergensis 26602 | 13.9 | 0 | Gong et al. (1981b) |
| Saccharomyces cerevisiae 24860 | 10.8 | 0 | |
| Saccharomyces cerevisiae (Baker's yeast) | 10.0 | 0 | |
| Saccharomyces | * (50) | | b |
| cerevisiae Hensen 2.399 | 16.6 | 0 | Chen (1983) |
| Saccharomyces cerevisiae (Baker's yeast) | 20.5 | 0 | |
| Saccharomyces cerevisiae K.W. No.1 | 17.0 | 0 | |
| Torula utilis Heneb 2.281 | 17.2 | 0 | |
| Candida | * (13) | | c |
| tropicalis ATCC 1639 | 4.6 | - | Jeffries (1981a) |
| Candida tropicalis Mac 6 | 4.4 | - | |
| Schizosaccharomyces pombe ATCC 2473 | 4.1 | - | |

* Initial substrate concentration ( g/l )

a 28°/2 days

b 30°/2 days

c 27°/3 days

## 2.4 DIRECT ALDOPENTOSE FERMENTATION

### 2.4.1 Using Naturally Occurring Yeasts

In the early 80's a study by Wang et al. (1980b) of yeasts capable of fermenting ketopentose to ethanol aroused many researchers' interest in the possibility of direct fermentation of aldopentoses instead of ketopentoses to ethanol. Later, the same group at the Research Council of Canada, reported having discovered a species of naturally occurring yeast that could convert D-xylose directly into ethanol (Schneider, 1981a). This yeast was identified as Pachysolen tannophilus. Simultaneously with this report, Jeffries (1981b) reported that Candida tropicalis ATCC 1369 produced ethanol from D-xylose under aerobic conditions. Maleszka and Schneider (1982a) screened 15 D-xylose-fermenting yeasts for their ability to convert D-xylose to ethanol under "semi-aerobic" conditions. They further demonstrated differences in ethanol productivity between yeasts. Pachysolen tannophilus produced the highest ethanol concentration (9.7 g/l), followed by Candida guilliermondii (4.5 g/l) and Candida terebra (3.0 g/l). The ethanol concentation in these cultures was unaffected by nitrogenous supplementation of the medium. Gong et al.(1983) tested 20 strains of Candida belonging to 11 species, 21 strains of Saccharomyces belonging to 8 species and 8 strains of Schizosaccharomyces pombe. The results showed that the majority of the Schizosaccharomyces pombe strains tested produced ethanol at concentrations ranging from 1 to 5 g/l and also xylitol from D-xylose. Particular strains of Candida blankii and Candida tropicalis produced ethanol in the highest concentrations. Margaritis and Bajpai (1982) described eight strains of Kluyveromyces marxianus, capable of fermenting the aldopentose directly to ethanol under aerobic conditions. du Preez and van der Walt (1983) also reported a strain of Candida shehatae able to ferment 90 g/l D-xylose directly to ethanol within 40 hours under aerated conditions. Sakano and Mikata (1983) screened 4 strains of Pichia stipitis, a strain of Candida shehatae and Candida steatolytica. They found that static cultures of Pichia stipitis and Candida shehatae produced 11 to 18 g/l of ethanol after 5 days of incubation, but the recycled cells could produce 18 g/l of

ethanol from 50 g/l of D-xylose after 2 days of incubation.

### 2.4.2 Using a Yeast Mutant

The development of a yeast mutant capable of fermenting pentose to ethanol has been reported (Gong et al., 1981e). A strain of Candida tropicalis was used to prepare the mutant. To the author's knowledge, no similar yeast mutant has been described in the literature. However, the screening of both wild types and mutant yeasts continue.

### 2.4.3 Using Yeast Strains Improved by Recombinant DNA Methodology

Recent advances in molecular genetic techniques should facilitate the construction of a suitable yeast capable of the rapid and efficient conversion of D-xylose to ethanol. The incorporation of, for example, the D-xylose isomerase genes from a bacterium into one or several species of Saccharomyces species (which are capable of fermenting D-xylulose) is one approach (Wang et al., 1980b). To date, the isolation and characterization of an Escherichia coli plasmid bearing the D-xylose isomerase gene has been completed (Ho et al., 1983; Lawlis et al., 1984; Maleszka et al.,1982a; Wocha et al., 1983). The next stage will be to introduce this plasmid into appropriate yeasts and to test the replicability, stability and expression of the gene. Beyond this stage of development is the possibility of an artificial (synthesized) D-xylose isomerase gene. With yeasts, success has already been achieved in building linear plasmids for incorporation into the yeast chromosome (Murray and Szostak, 1983). These techniques may possibly be applicable to the isomerase gene now that it has been characterized and Saccharomyces cerevisiae has been suggested as a suitable recipient (Suihko and Drazic, 1983). Protoplast fusion has been attempted between Candida utilis which may contain isomerase activity and Saccharomyces cerevisiae but the fusion product was unstable and segregated into parental types (reported by Suihko and Drazic, 1983). Another approach has been the reconstruction of the present haploid Pachysolen tannophilus to polyploid and aneuploids. Maleszka et al.(1983a) , James and Zahab (1983) recently obtained a number of triploid and a tetraploid strains and claimed that increasing

chromosome number improved aspects of ethanol production from D-xylose.

## 2.5 BIOLOGY OF *Pachysolen tannophilus*

The yeast *Pachysolen tannophilus* was originally isolated from concentrated tanning liquors extracted from broad-leafed trees (*Castanea vesa* and *Acacia mollissima*) and was first designated by Boidin and Adzet (1957). The yeast remained somewhat obscure and attracted little interest until the early 1980's. Only a limited description of the yeast's biology will be presented in this work as a recent review is available (Kurtzman, 1983).

### 2.5.1 Morphological Description of *Pachysolen tannophilus*

When *Pachysolen tannophilus* was grown at 25° on Yeast Morphology Agar for 3 days (Wickerham, 1970), the cells were ellipsoidal to spherical, measured (1.5 -5.0) X (2.0 -7.0) μm and produce one or occasionally two buds per cell. The colonies are gray-white, glistening, round, low convex with margin entire, mucoid becoming butyrous with age.

### 2.5.2 Reproduction of *Pachysolen tannophilus*

#### 2.5.2.1 Asexual (Vegetative) Reproduction

Vegetative reproduction of *Pachysolen tannophilus* occurs by budding of yeast cells with one or two buds. Pseudohyphae were usually present and may be undifferentiated or highly branched (Kurtzman, 1983).

#### 2.5.2.2 Sexual Reproduction (Ascus Formation)

The *Pachysolen* ascus is formed when a vegetative cell produces a thick, straight or curved, refractile tube, the ascophore. The tube may be short or long, up to 50 μm in length. At its tip the tube enlarges to form the ascus which contains a maximum of four

hemispheroidal ascospores. When the ascus ruptures, releasing ascospores, the tube reveals a V-shaped notch at its end. Wickerham (1970) and Kurtzman et al. (1982) claimed that the Pachysolen asci may be conjugated or unconjugated depending upon the strain. The strain NRRL Y-2460 (CBS 4044) forms an unconjugated diploid ascus while other strains (NRRL Y-2461; Y-2462; Y-2463 and Y-6704) are conjugated haploid forms. However, other investigators have claimed that the four strains of Pachysolen tannophilus examined in their laboratories are predominately haploid (James and Zahab, 1982). All strains of Pachysolen tannophilus are reported to be homothallic (Wickerham, 1970; James and Zahab, 1982).

### 2.5.3 Phylogenetic Relationships of Pachysolen tannophilus with other Yeast Genera

The similarity of the biochemical and physiological characteristics of Pachysolen and Hensenula species (Wickerham and Burton, 1961) and also the common capability of producing an extracellular phosphomannan (Slodki et al., 1961) provided enough evidence for Wickerham (1970) to prepare a phylogenetic scheme to illustrate the relationships existing between the genera Hensenula and Pachysolen. Wickerham suggested that the genus Pachysolen has a close evolutionary kinship with certain "primitive" Hensenula species. This closeness has been emphasized by Yamada and his group (1973) who analyzed the co-enzyme Q system of 128 strains of yeasts belonging to the genera Hensenula, Pichia, Citeromyces, Pachysolen and Wingea. These authors concluded that two species of "primitive" Hensenula, Hensenula capsulata and Hensenula holstii, together with Pachysolen tannophilus, Citeromyces matritensis and Pichia pastoris, possesed the same co-enzyme Q8 system. This closeness was also recognised by Campbell (1973) who suggested that the genus Pachysolen be incorporated into the genus Hensenula. This suggestion has never been accepted.

## 2.6 GROWTH AND FERMENTATION CHARACTERISTICS OF *Pachysolen tannophilus*

Prior to this current decade, yeasts had been considered as unable to produce ethanol from pentoses (Barnett, 1976). This view changed with the discovery of a number of naturally occurring yeasts and a yeast mutant (as referred to in Sections 2.4.1 and 2.4.2) capable of converting D-xylose directly to ethanol. Among the naturally occurring yeasts, *Pachysolen tannophilus* has been identified as a possible yeast for the conversion for D-xylose to ethanol (Schneider *et al.*, 1983). Therefore, the following Sections will focus attention on growth and fermentation characteristics of this yeast in a D-xylose medium or in wood hydrolyzate.

### 2.6.1 Effects of pH on Growth and Fermentation

Following the report from Schneider and collaborators (1981a) that *Pachysolen tannophilus* directly converts D-xylose to ethanol under "anoxic" and "semi-aerobic" conditions, Slininger *et al.*(1982b) demonstrated that both growth and ethanol fermentation were maximal when the initial pH was 2.5. Wayman (1982) also claimed that pH 2.5 to 3.0 was the favourable pH range for ethanol production by *Pachysolen tannophilus*. On the other hand, Dekker (1982) found *Pachysolen tannophilus* unable to grow at an initial pH value lower than 3.0. Debus *et al.*(1983) concluded that the pH optimum was pH 4.8 for *Pachysolen tannophilus* grown on 30 g/l of D-xylose medium, a result again contradicting some previous reports (Slininger *et al.*, 1982b; Wayman, 1982). In the case of bacteria, initial pH is important in establishing the batch fermentation's trajectory (Nishio *et al.*, 1983). It would appear that this also true for the yeasts (Rainbow and Rose, 1963).

### 2.6.2 Temperature Effects

The influence of temperature on yeast ethanol formation has been studied by Krouwel and Braber (1979), especially at supraoptimal temperatures, ranging from 30-40°. They demonstrated that *Saccharomyces cerevisiae* was able to produce ethanol at 39.6°, but no

ethanol or cells were produced at or above 39.8°. Here, dead cells were predominant. Slininger et al.(1982b) found Pachysolen tannophilus to exhibit maximal ethanol yield at 32° and maximum growth temperature range was between 37-40°. Debus et al. (1983) reported that the optimum growth temperature for their Pachysolen strain IfG 0101 growing in either glucose or a xylose medium was 31°.

### 2.6.3 Fermentation Substrates

It is desirable that the micro-organisms employed for the fermentation of biomass derived polysaccharide hydrolyzates should be able to convert a mixture of diverse sugars to ethanol. Dekker (1982) claimed that, besides D-xylose, Pachysolen tannophilus can ferment L-arabinose, D-glucose, D-fructose, D-cellobiose and D-glucuronic acid, but not sucrose. Maleszka et al. (1982c) reported that Pachysolen tannophilus can convert D-galactose and glycerol to ethanol but that the fermentation of galactose required a longer lag phase of 7 days. A similar result was reported by Debus et al.(1983) with galactose as the sole source of carbon, a long adaption period of about 2 days was required. For D-xylose fermentation, Slininger et al. (1982b) claimed that xylose concentrations exceeding 50 g/l interfered with growth and ethanol production. Dekker (1982) revealed a similar effect on cell growth. The final cell concentration was maximal at 20 g/l D-xylose but decreased at xylose concentrations over 100 g/l xylose. However, ethanol concentration was maximum for 100 g/l xylose. With its capability for fermenting a variety of sugar substrates, Pachysolen tannophilus has been suggested by Neirinck et al.(1982), as being a useful starting point for detailed strain improvement programmes to obtain organisms that will function well on spent sulphite liquor.

### 2.6.4. Aeration Effects

The supply of trace amounts of oxygen in otherwise anaerobic ethanol fermentation accelerates and enhances the yeasts' specific ethanol production (Cysewki and Wilke, 1976). These authors have also shown that the optimal oxygen concentration for Saccharomyces ethanolic fermentation is in the part per billion range. This was later

confirmed by Nishizawa and his group (1978) using Saccharomyces cerevisiae CBS 1200. Recently, Haraldson and Rosen (1982) demonstrated that ethanol production decreases almost linearly with an increase in oxygen tension up to 1000 parts per billion. Maric and Einsele (1981) have studied the effect of the low dissolved oxygen concentrations on ethanol production using Candida utilis ATCC 32113. They have also claimed that the lower the oxygen partial pressure, the higher the amount of ethanol produced.

An oxygen residual in the fermentation medium for Saccharomyces is considered necessary for the synthesis of unsaturated membrane lipids; if fully anaerobic conditions prevail, supplementation of the medium with ergosterol and/or oleic acid seems necessary (Longley et al., 1978; Watson and Rose, 1980). However, with Pachysolen tannophilus, the supplementation of D-xylose medium with lipids was ineffective (Maleszka et al. 1982c). Thus, the role of oxygen in Pachysolen fermentation remains unclear. Slininger et al. (1982a) considered that oxygen was not required for converting xylose to ethanol although it is required for growth. Debus et al. (1983) found that xylitol and ethanol are excreted in variable ratios under anaerobic and semi-aerobic conditions (i.e. 0.002 vvm of air). Mutz and Wandrey (1983), using a chemostat, observed that with increasing oxygen limitation, the yield of ethanol increased while the cell yield decreased. The influence of oxygen, especially during "semi-aerobic" conditions, on the xylose fermentation by Pachysolen tannophilus seems to be critical (Schneider and Wang, 1981).

Although the ethanol production appears to be maximal under semi-aerobic conditions, an understanding of oxygen's role on yeast metabolism is restricted by the unavailability of suitable measurement devices for very low dissolved oxygen concentrations (less than 1% of air saturation value). Therefore, it is important to have a tool that can provide information about the degree of oxygen limitation experienced in a culture (Pirt, 1957 and 1975). Several investigators have attempted to use redox potential measurements to generate more information about the oxidative status in aerobic and semi-aerobic cultures. Shibai and his group (1974) successfully demonstrated

microbial production of inosine by controlled redox potential. However, they failed, using a commercial dissolved oxygen probe, in detecting very low concentrations of dissolved oxygen. Kjaergaard (1976); Kjaergaard and Joergensen (1981) employed redox potential control on a microbial culture and showed enhanced acetic acid production by Bacillus licheniformis under low redox potential readings. More recently, Bruinenberg et al. (1983) have emphasized the important role of redox balances in the anaerobic fermentation of xylose by different yeasts. These authors speculated that at the appropriate redox potential, the intracellular pool for the NAD+ were maintained at a level sufficient for the D-xylose catabolism.

### 2.6.5 Inoculum Concentration

The value of high yeast concentrations to initiate fermentation is well known (Chaing et al.,1981; Gong et al., 1981). Strehaiano et al. (1983) studied the effect of inoculum level on cell and product yields and specific growth rate using three Saccharomyces strains. They noted that fermentation times, maximum specific growth rates and cell yields decreased as inoculum size increased, but the ethanol yield remained constant. In the case of fungal micro-organisms, Brown and Zainudeen (1978) demonstrated that differing inoculum concentrations resulted in distinct dissolved oxygen demand profiles. The advantages of using increased inoculum concentrations of Pachysolen tannophilus NRRL Y-2460 were explored by Maleszka et al. (1981). They found that the time needed for the culture supernatant to reach maximum concentration of product was shorter with increased inoculum concentration. Deverell and Whitworth (1980) have postulated that the effect of growth inhibitors such as furfurals in the wood hydrolyzate may be overcome if a high concentration of inoculum is used.

### 2.6.6 Effect of Inhibitors on Growth and Fermentation

#### 2.6.6.1 Inhibitory Substances in Hydrolyzates

During acid hydrolysis of wood, many potentially toxic compounds are formed. Furfural, 5-hydroxymethyl furfural, organic acids (such as

levulinic acid, formic acid) and phenolic compounds are frequently encounted in the hydrolyzate. Leonard and Hajny (1945) investigated yeast fermentation of wood sugars and concluded that some pretreatment of the wood hydrolyzate was necessary to remove these inhibitors. Fermentability of wood hydrolyzates from pine sources can be improved by treatment with hot sodium sulphite (Deverell and Whitworth, 1980). Detroy et al. (1982 a and b) also emphasized that the failure of yeasts to ferment wheat straw hydrolyzates was attributable to the presence of furfural and lignin derivatives. Furfural at 2.5 to 3.0 g/l was found lethal to Pachysolen tannophilus (Detroy et al., 1982b). For Saccharomyces, 3.0 g/l furfural decreased growth rate and this yeast was found to be more sensitive to furfural then to 5-hydroxymethyl furfural (Azhar et al., 1981). Banerjee and his group (Banerjee et al.,1981) revealed that furfural restricted Saccharomyces growth and ethanol production. The mechanism of these effects was associated with the inhibition of triosephosphate dehydrogenase and alcohol dehydrogenase by furfural.

2.6.6.2 Ethanol Inhibition

The inhibitory effect of the ethanol on both the growth and fermentation by yeast cells has been studied (Aiba et al., 1968; Bazua and Wilke, 1977; Brown et al., 1981; Hoppe and Hansford, 1982; Navarro, 1980; Novak et al., 1981). Slininger et al. (1982b) observed that severe inhibition of fermentation occurred by Pachysolen tannophilus at ethanol concentrations exceeding 20 g/l. Recently, Roman and his co-workers (1984) studied the end-products inhibition on D-xylulose fermentation by Schizosaccharomyces pombe and reported that the maximum ethanol production rate decreased linearly with increasing initial ethanol concentration. They reported that the inhibition constant for maximum specific ethanol production rate was 79 g/l. A number of recent studies have shown that yeast cells accumulate ethanol internally (Stucley and Pamment, 1982). Ethanol produced intracellularly by the yeast itself is more lethal than ethanol added extracellularly to the medium (Egamberdiev and Ierusalimskii, 1968; Nagodawithana and Steinkrauss, 1976; Navarro and Durand, 1978). Novak et al. (1981) reported that inhibition constant value (Ki) of 105.2

g/l for added ethanol compared with 3.8 g/l for ethanol produced during batch fermentation. Table 2.4 summarizes the kinetic constant data of other workers on various ethanol inhibition studies.

The data show that low values of the ethanol inhibition constant for growth are obtained with autogenous ethanol (i.e. ethanol produced intracellularly) compared with considerably higher values with exogenous ethanol. The data of Righelato et al. (1981) are an exception. It would appear that autogenous ethanol is more inhibitory than ethanol added to the medium. In a recent study of the role of intracellular ethanol in the yeast cells, (Stucley and Pamment,1982 ; Dasari et al (1983) observed that the intracellular ethanol content of mid-exponential phase cells of Saccharomyces cerevisiae was significantly higher than the external concentration. They cited Thomas and Rose (1979) as having reported similar results.

The biochemistry of ethanol inhibition is not yet fully understood. A number of different inhibitory mechanisms appear to be involved. The presence of a high level of intracellular ethanol in yeast cells has been found to coincide with marked inhibition of hexokinase in glucose fermentation (Nagodawithana et al., 1977; Navarro and Finck, 1982) and of many membrane-associated enzymes (Ingram, 1976). The denaturating effect of ethanol on enzymes was suggested by Stucley and Pamment (1982) as an explanation. Ethanol is also reported to exert a primary toxic effect on the plasma membrane (White, 1978) and to show an inhibitory effect on the uptake of solutes by yeasts (Thomas and Rose, 1979).

Table 2.4 Comparison of Kinetic Constants of Various Ethanol Inhibition Studies of Saccharomyces Strains

| References / Kinetic Constants | Egamberdiev Ierusalimskii 1968 | Aiba and Shoda 1969 | Pironti 1971 | Cysewski 1976 | Bazua and Wilke 1977 | Azhar et. al. 1981 | Brown et. al. 1981 | Novak et. al. 1981 | Righelato et. al. 1981 | Hoppe and Hansford 1982 |
|---|---|---|---|---|---|---|---|---|---|---|
| °C | 28 | 30 | 30 | 35 | 35 | 26 | 23 | 30 | 30 | 30 |
| $\mu_{max}$ $h^{-1}$ | 0.31 | 0.43 | 0.26 | 0.58 | 0.64 | 0.31 | 0.28 | 0.31 | 0.25 | 0.64 |
| $K_s$ g/L | - | - | 15.5 | 4.9 | 0.24 | 12.6 | - | - | - | 3.3 |
| $K_i$ g/L | 26 | 55 | 13.7 | 5.0 | 40 | 2.97 | 20.1 | 3.8 | 57 | 5.2 |
| $Y_{p/s}$ g/g | 0.39 | 0.35 | 0.47 | 0.44 | 0.52 | 0.40 | - | - | 0.46 | 0.43 |
| Source of Ethanol Fermentation | Added Batch | Added Batch | Autogenous Continuous | Autogenous Continuous | Added Continuous | Autogenous Batch | Added Batch | Autogenous Batch | Autogenous Continuous | Autogenous Continuous |

$\mu_{max}$ : Maximum Specific Growth Rate
$K_s$ : Substrate Saturation Constant
$K_i$ : Ethanol Inhibition Constant
$Y_{p/s}$ : Product Yield Coefficient

## 2.7 CONCLUSIONS

Perhaps it could be said that the western world has not willingly taken up the study of pentoses fermentations to ethanol. Rather, the topic has been thrust upon it by virtue of the fact that fuel resources are limited and that alternatives must be found. Also, because the renewable forest resource with its fundamental cellulosic and hemi-cellulosic molecular structure represents an essential component. The successful utilization of such a massive resource as waste woods will be tempered by an ability to successfully utilize each monomeric sub-unit of the polymers that comprise that resource. Thus the fermentation of pentoses, in particular xylose must be seen as part of that scheme.

The theoretical basis for a rational understanding of pentose exploitation has been presented above. The struggles of biochemists to understand the biological degradation of pentoses can now be seen in a prespective which emphasizes the metabolic diversity found in nature. Surely this diversity can be exploited to ensure that any desired conversion product can be obtained free of contaminating by-products and, as well, at maximum yield. It has been demonstrated in this review that yeasts, above all, may provide the most immediate solution to the problem of converting pentoses to ethanol in appropriate yields and purity. Naturally occurring bacteria and fungi were eliminated in the discussion, essentially on the grounds that these organisms generate a variety of products. However, as detailed knowledge of biochemical and genetic systems progress, it is possible that laboratory manipulated bacteria may prove, in the distant future, superior to yeasts.

Nonetheless, the amount of information pertaining to the ethanolic fermentation by Saccharomyces cerevisiae utilizing hexoses makes an admirable data base for any comparative work, be it with naturally occurring microorganisms or genetically manipulated ones capable of ethanolic fermentation of pentose. The final choice of microorganism

is probably still to be made and it is hoped that the data developed in this thesis will contribute to a realistic decision.

In the last few years information has accumulated concerning the conversion of pentoses directly to ethanol using naturally occurring yeasts. It is probably true to say that screening work continues in various laboratories. It is also true that genetic manipulation of yeasts, particularly Saccharomyces and Candida, may produce strains with superior characteristics for pentose fermentation. Such a direct industrial fermentation would be simpler to develop and maintain than a two stage process which would first demand the conversion of the unfermentable pentose to the easily fermentable pentulose by enzymatic steps using a pentose isomerase followed by fermentation.

Furthermore, such an enzymatic step will increase the cost per litre of the final product. This is currently undesirable as any gasoline programme must maintain ethanol costs per litre at values below petrol costs. The investor therefore is going to demand, in all likelihood, a single step, low cost process represented by a direct fermentation of pentose to ethanol.

To enhance yields and minimize costs, the fermentation must be run under optimal conditions with the yeast encouraged to produce to its maximum. Conditions influencing such yields have been given attention in the above review as well as those factors likely to inhibit product formation.

The data in the literature are not complete. A fuller understanding of the details of the biochemical mechanisms leading to the conversion of pentoses to ethanol remains the goal of many investigators. The importance of this knowledge will be that a rational and effective approach to the problem of converting renewable biomass material to liquid fuels will be possible. The bioconversion of pentoses will than occupy an important position in the total energy cycle.

# CHAPTER THREE : MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 Microbiological Media

Supplies of Bacto Yeast Morphological Agar, Bacto Yeast Nitrogen Base (No. 0392) and Bacto Yeast Extract were obtained from Difco Laboratories, Detroit, Michigan, U.S.A. Microbiological Agar was obtained from Davis Gelatin Limited, Christchurch, New Zealand. de Man, Rogosa, Sharpe (M.R.S.) Agar (CM 361) and Broth (CM 359) and Tryptone Soya Agar (CM 131) was purchased from Oxoid Australia Pty. Limited, Hurstville, New South Wales, Australia.

### 3.1.2 Chromatographic Materials

For column chromatography, Diethylaminoethyl (DEAE)- Sephadex A-50 and Gel Sephadex G-200 (particle size 40-120 μm) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. For gas chromatography, the packing material, 10% Free Fatty Acid Phase (FFAP) on Chromosorb G (AW-DMCS), 80-100 mesh, was obtained from Supelco Inc., Bellefonte, Pennsylvania, U.S.A.

### 3.1.3 Gases

Oxygen-free nitrogen (certified grade) and carbon dioxide (food grade) were supplied by New Zealand Industrial Gases Limited, Palmerston North, New Zealand.

### 3.1.4 Chemicals

#### 3.1.4.1 Sugars

D-xylose was obtained from either B.D.H. Chemicals Limited, Palmerston North, New Zealand; Ajax Chemicals, Sydney, Australia or Sigma Chemical Company, St. Louis, Missouri, U.S.A. Analytical grade (A.R.) D-glucose and D-mannose came from B.D.H. Chemicals Limited, Poole,

England while L-arabinose and D-galactose were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

3.1.4.2 Enzymes

D-xylose isomerase of Lactobacillus fermentum was partially purified from the experimental studies as described in Chapter 4. Sweetzyme (Type Q) was a gift from Novo Industrias, Copenhagen, Denmark. Taka-Sweet was a gift from Miles Laboratories Inc., Elkhart, Indiana, U.S.A.

3.1.4.3 Other Chemicals

Any other chemicals used were analytical reagent or laboratory reagent grade and were purchased from B.D.H. or May and Baker (New Zealand) Limited, Lower Hutt, New Zealand.

3.1.5 Solvents

Absolute ethanol (99.5% v/v assayed) was purchased from Ajax Chemicals, Sydney, Australia. Other solvents used as standards for gas chromatography analysis were B.D.H. analytical reagent grade (B.D.H. Chemicals Limited, Palmerston North, New Zealand).

3.1.6 Wood Hydrolyzate

Wood hydrolyzate was prepared and supplied by the Forest Research Institute, Rotorua, New Zealand. The process used for its preparation was by dilute sulphuric percolation at 180°, a modification of the Madison Process (Mackie, 1982a). The hydrolyzate generated from the softwood, Pinus radiata, was made available with the composition shown in Table 3.1, i.e. as prehydrolyzate, which is to say, the acidic percolate collected during the initial stages of hydrolyzation and was the breakdown products of the hemicellulosic fraction. This material was used for the experiments described in Chapter 7, after neutralization with sodium hydroxide and filtration. A single large batch, frozen until required, was utilized throughout.

Table : 3.1 Composition of Wood Hydrolyzate Fractions
(Mackie, 1982a)

| Carbohydrate | Concentrations (g/l) | |
|---|---|---|
| | Prehydrolyzate | Main Hydrolyzate |
| Arabinose | 2.5 | 0 |
| Xylose | 5.9 | 0.9 |
| Mannose | 12.0 | 1.3 |
| Galactose | 3.4 | 0.3 |
| Glucose | 6.8 | 20.0 |
| Total | 30.6 | 22.5 |
| Suspended Solids | 1.4 | 3.0 |
| Total Solids | 35 | 26 |

(1) see Section 3.1.6 for description of prehydrolyzate and main hydrolyzate

Table 3.2 : Composition of Distillation Residues Following the Ethanolic Fermentation by Saccharomyces cerevisiae of Lime Neutralized Wood Hydrolyzate (Mackie, 1982a)

| Types | Concentration (g/l) |
|---|---|
| pH | 4.5-5.0 |
| Acidity | 1.25 $CaCO_3$ (pH 8.3) |
| Sulphate | 1.5 |
| Nitrogen | 0.018 (Kjeldahl) |
| Phosphorous | 0.003 |
| Calcium | 1.4 |
| Phenols | 0.0005 |
| Metals* | 0.075 |
| Total Solids | 21.9 |
| Suspended Solids | 1.6 |
| COD | 22 |
| BOD | 13 |
| Colour | 2400 (chloroplatinate units) |
| Xylose | 4.3 |
| Arabinose | 2.2 |

* include Cu, Cr, Fe, Mn and Ni.

### 3.1.7 Micro-organisms Used

#### 3.1.7.1 Bacteria

The bacteria used in the enzyme studies in Chapter 4 were :

| | |
|---|---|
| *Bacillus coagulans* | NCIB 8870 |
| *Lactobacillus fermentum* | ATCC 9338 |
| *Streptomyces griseus* | NCTC 7807 |

#### 3.1.7.2 Yeasts

| | |
|---|---|
| *Candida macedoniensis* | UCD,FST 49-27 |
| *Candida pseudotropicalis* | CBS 2234 |
| *Candida tropicalis* | CBS 2321 |
| *Candida tropicalis* | FD 0261 |
| *Candida utilis* | Local isolate |
| *Hensenula anomola* | IFO 0135 |
| *Hensenula capsulata* | IFO 0721 |
| *Hensenula holstii* | IFO 1479 |
| *Hensenula saturnus* | IFO 1466 |
| *Hensenula silvicola* | IFO 0807 |
| *Hensenula wickerhamii* | IFO 1706 |
| *Kluyveromyces marxianus* | Y 42 |
| *Pachysolen tannophilus* | IFO 1007 |
| *Pachysolen tannophilus* | NRRL Y-2460 |
| *Pachysolen tannophilus* | NRRL Y-2461 |

ATCC American Type Culture Collection, Rochville, Maryland, U.S.A.

CBS Central Bureau for Schimmelcultures, Baarn, Holland.

FD Food Technology Department, Massey University, Local isolated

IFO Institute Fermentation of Osaka, Osaka, Japan.

NCIB National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K.

NCTC National Collection of Type Cultures, Colindale Avenue, London, U.K.

NRRL Northern Regional Research Laboratories, Peoria, Illinois, U.S.A.

UCD FST University of California at Davis, Faculty of Food Science and Technology, California, U.S.A.

Y 42 obtained from Department of Microbiology, University of Maryland, College Park, Maryland, U.S.A.

Cultures were obtained from the institutions named above (3.1.7.1). Bacteria were maintained on either M.R.S. Agar (Oxoid) (Lactobacillus fermentum) or Tryptone Soya Agar (Oxoid) (Bacillus coagulans and Streptomyces griseus), while the yeasts were maintained on Bacto Yeast Morphological Agar (Difco).

## 3.2 MEDIA STERILIZATION

### 3.2.1 Membrane Filtration

Solutions of xylose, Yeast Nitrogen Base and prehydrolyzate which had been pre-adjusted to the desired pH and supplemented with Bacto Yeast Nitrogen Base (6.7 g/l) for shake-flask studies were sterilized by passing through a Millipore filter (0.45 μm) obtained from Millipore Corporation, Bedford, Massachusetts, U.S.A.

### 3.2.2 Autoclave

All remaining heat stable media for culture maintenance or inoculum preparation were autoclaved for 15 min at 121°.

## 3.3 CLEANING OF GLASSWARE

All glassware were routinely washed in hot tap water containing "Teepol" (Liquid detergent) obtained from Shell Oil New Zealand Limited, Wellington, New Zealand, thoroughly rinsed in warm tap water and distilled water and then air dried at about 70-80°. Glassware used for enzyme studies were soaked in chromic acid overnight prior to washing, then rinsed several times with tap water and distilled water.

## 3.4 ANALYTICAL METHODS

### 3.4.1 Microbial Cell Counts

Yeast cells in an aliquot of cell suspension from the fermenter or from a shake-flask culture were counted directly using a haemocytometer counting chamber under 400X magnification with an Olympus BH binocular microscope.

### 3.4.2 Dry Weight Determination

An accurately known volume of fermenter culture or of a shake-flask culture (10 ml) was pipetted into a centrifuge tube then centrifuged under 2,000 g at ambient temperature. The supernatant was then discarded and the cells washed twice with 10 ml volumes of cold sterile distilled water before careful transfer into a pre-weighed aluminium moisture dish. The dish was then dried at 105 $\pm$ 2° overnight, cooled in a desiccator and then re-weighed. Heating and cooling were repeated until constant weight was reached. A calibration curve of cell counts (X) ($x10^6$ cells/ml) versus dry weight (W) (g/l) is shown in Figure 3.1 which was prepared from "exponential" phase cells. Such a curve was prepared for each experimental run.

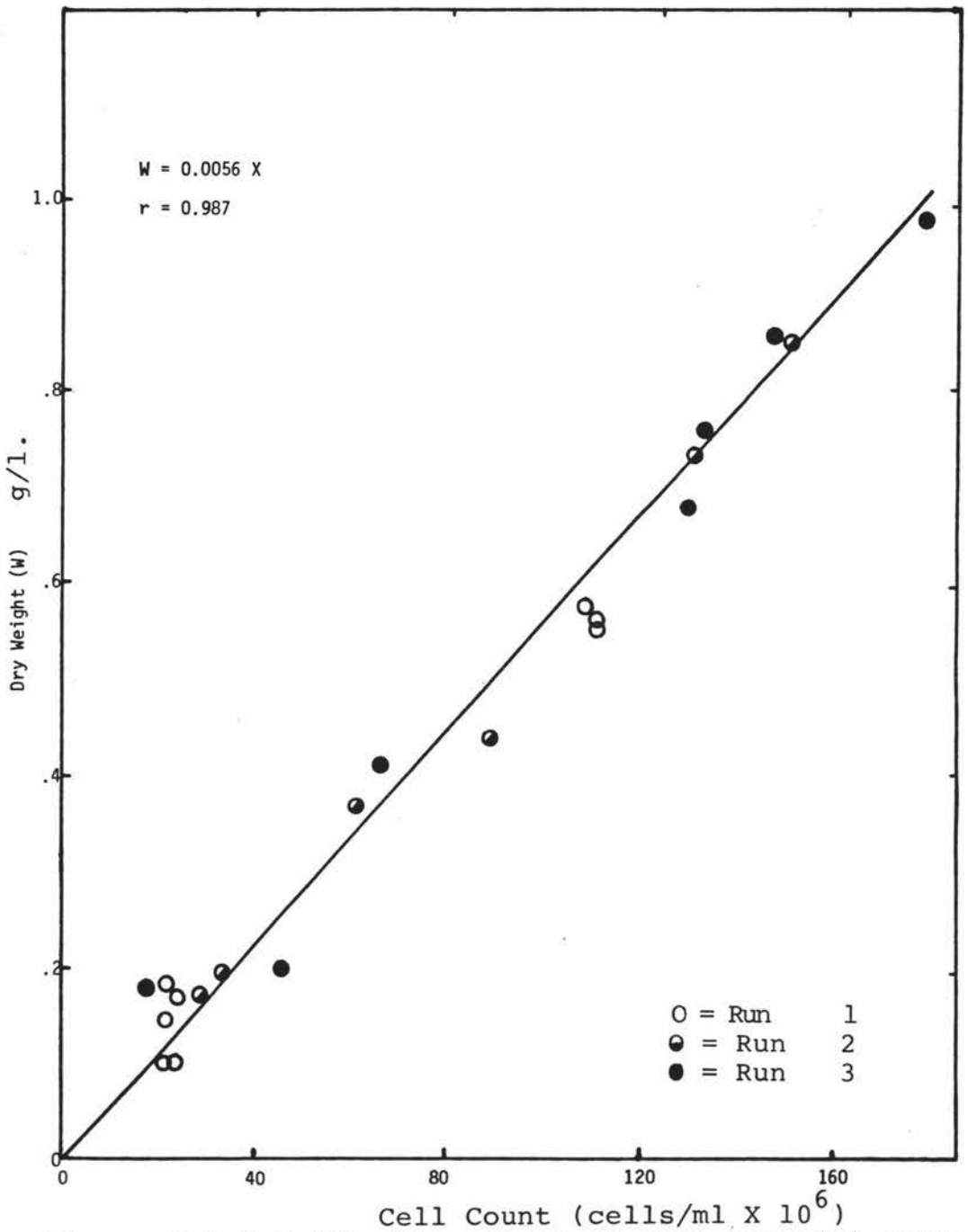



Figure 3.1 A Calibration Curve of Cell Count(X) versus Cell Dry Weight(W) for *Pachysolen tannophilus*.

### 3.4.3 Analysis of Sugars

Total reducing sugars in wood hydrolyzate or D-xylose of fermentation samples were determined by a modification of the Somogyi-Nelson Colorimetric Method as described by Mackie (1982a). This method was used for all the D-xylose analyses throughout these studies.

Reagents :

(A) Low-alkalinity copper reagent -

Potassium sodium tartrate (12 g) and anhydrous sodium carbonate (24 g) were dissolved in about 250 ml of distilled water. A solution of 4 g of cupric sulphate pentahydrate in distilled water was added with stirring, followed by 16 g of sodium hydrogen carbonate. A solution of 180 g of anhydrous sodium sulphate in 500 ml of distilled water was boiled to expel air; then the two solutions were combined and diluted to 1 litre. After 3-7 days of standing, the clear supernatant solution was used. This reagent was stored in a warm place.

(B) Arseno-molybdate reagent -

25 g of A.R. ammonium molybdate was dissolved in 450 ml of distilled water to which was added 21 ml of 96% sulphuric acid, followed by 3 g of di-sodium hydrogen arsenate heptahydrate dissolved in 25 ml of distilled water.
The mixed solution was incubated for 24 h at 37° and stored in a glass-stoppered brown bottle.

Procedure :

Samples from shake-flask or fermentation culture were diluted with distilled water so as to contain between 50 to 250 µg sugar per ml of solution. Low-alkalinity copper reagent (2 ml) was added to 2 ml of the diluted sample and also to 2 ml distilled water as a reagent blank.

The mixed sample was heated in a boiling water bath for 10 min and then cooled with running water. Arseno-molybdate reagent (1 ml) was added and the samples were mixed thoroughly. After mixing, the samples were diluted with 20 ml of distilled water, then mixed again by inverting tubes several times. All tubes were allowed to stand for 5 to 10 min. The absorbances of the samples were read at 500 nm against a reagent blank using a Shimadzu Spectrophotometer UV-120-02 (Shimadzu Corporation, Kyoto, Japan). The absorbance of each sample was then calculated from a standard curve prepared on the day. All analyses were performed in duplicate. The composite of standard curves for D-xylose analyses by the modified Somogyi-Nelson Method is presented in Figure 3.2

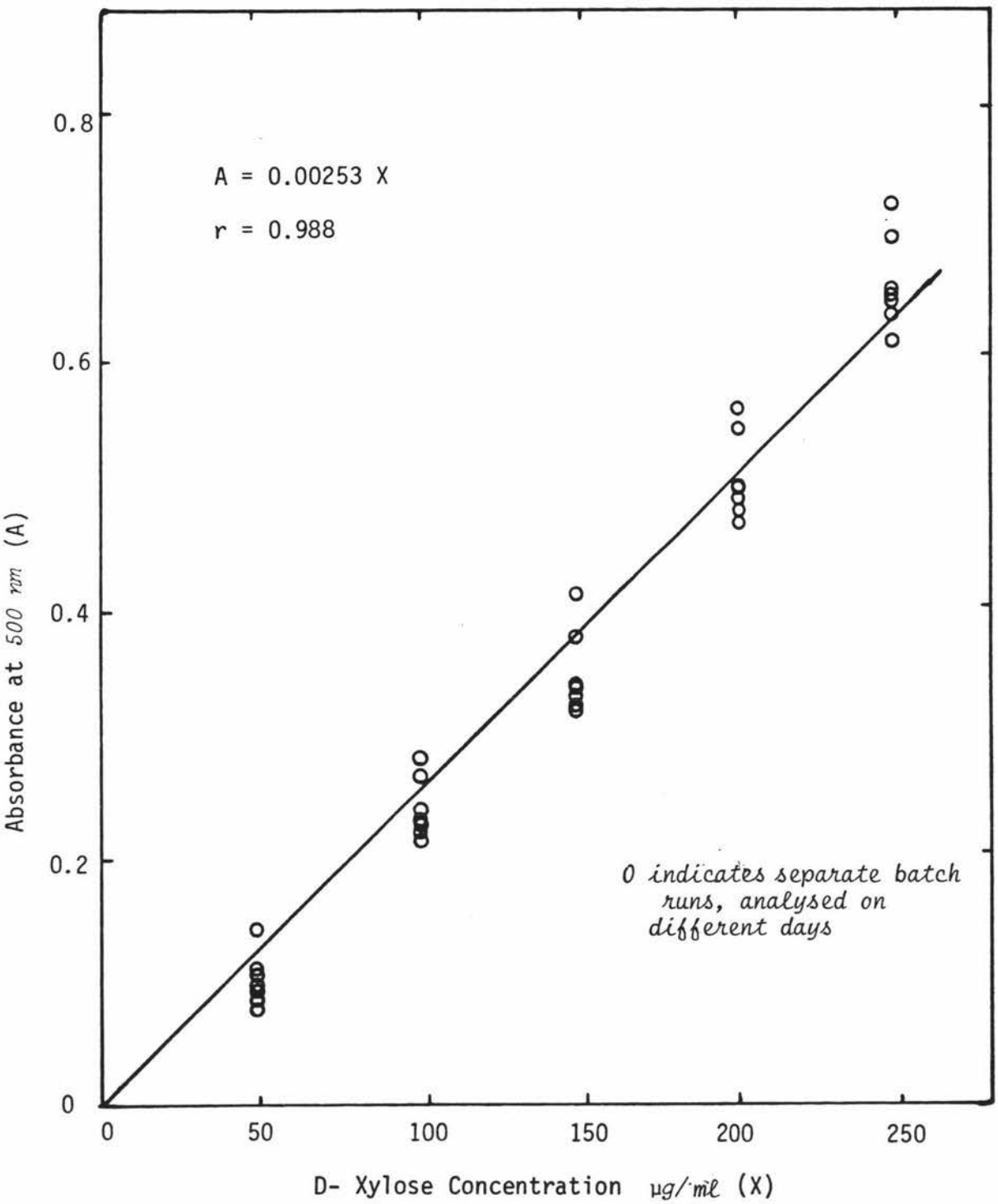



Figure 3.2 Standard Curve for Total Reducing Sugar (as D-Xylose) Using the Somogyi & Nelson Method.

### 3.4.4 Column Chromatography

#### 3.4.4.1 Ion Exchange Chromatography

A Sephadex ion exchanger, DEAE - Sephadex A50 (Pharmacia Fine Chemicals, Uppsala, Sweden), was used in enzyme purification (see Chapter 4). DEAE - Sephadex A50 was allowed to swell in distilled water and then washed repeatedly with 0.5N NaOH on a Buchner funnel until free of chloride ions. The NaOH was then removed by rinsing with distilled water and followed by 0.5N HCl treatment. This acid was then removed by rinsing with distilled water until the resin gave a neutral pH reaction. Finally it was equilibrated with 0.02M phosphate buffer at pH 7.0. The ion exchanger was then poured into the column (internal diameter 2.5 X 30 cm). The packed column was connected to the eluent reservoir which was allowed to flow through for 16 h at 5 ml per min. The active column was stored at 4°. A known volume of dialyzed enzyme solution was then applied to the column. After the column was washed with 75 ml of the same buffer, the enzyme was eluted by linear gradient of 0 to 0.25M KCl. Fractions ( 7.5 ml each ) were collected using an LKB UltraRac Fraction Collector (LKB Produkter AB, Stockholm, Sweden). Collected fractions were then measured for their absorbance at 280 nm and their enzyme activity.

#### 3.4.4.2 Gel Filtration

Sephadex G-200 (particle size 40-120 μm) from Pharmacia Fine Chemicals AB, Uppsala, Sweden, was used in the gel filtration study (Chapter 4). The preparation of the Gel Sephadex G-200, was according to the Pharmacia's Gel Filtration handbook (Pharmacia, 1976). The slurry was packed in the column (internal diameter 1.0 X 60 cm) which had been previously equilibrated with 0.02M phosphate buffer, pH 7.0. The enzyme was eluted with the same buffer. Eluent volumes of 3.0 ml were collected using an LKB UltraRac Fraction Collector (LKB Produkter AB, Stockholm, Sweden). These fractions were measured for their protein absorbance at 280 nm and the presence of enzyme activity.

### 3.4.5 Gas Liquid Chromatography

Ethanol assays were carried out using a Shimadzu GC5 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) using a flame ionization detector. Hydrogen gas and air flow rates for flame ionization detector were 55 ml per min and 900 ml per min respectively. The instrument had a U- shaped glass column (1.85 m X 2 mm internal diameter) which was packed with 10 % Free Fatty Acid Phase (FFAP) on Chromosorb G (AW-DMCS) 80-100 mesh (Supelco Inc., Bellefonte, Pennsylvania, U.S.A.). Injection port, oven and detector were operated at 185 , 95 and 225° respectively. Nitrogen gas was ed as the carrier, with a flow rate of 80 ml per min. The recorder chart speed was 2.0 mm per min. Sample volumes of 1.0 µl were injected using a Hamilton microlitre syringe No.801 RN (Hamilton Company, Reno, Nevada, U.S.A.). An external standard was used throughout the studies. The calibration curve for ethanol analysis is shown in Figure 3.3

### 3.4.6 High Performance Liquid Chromatography

Xylitol analysis was carried out at the New Zealand Forest Research Institute using a High Performance Liquid Chromatography (Model HPLC 200) comprising a U6K septumless loop injector and a pump (Model 6000A) and R401 differential refractometer (Waters Associates Inc., Milford, Maryland, U.S.A.). A Biorad (HPX-87H) Organic Acid Column with 0.157% w/v phosphoric acid, flow rate 0.6 ml/min, was used for separations.

### 3.4.7 Polyacrylamide Disc Gel Electrophoresis

The partially purified enzyme (see Chapter 4) was subjected to polyacrylamide disc gel electrophoresis (Davis, 1964) for purposes of establishing the relative purity of the fractions at each stage of the purification. Polyacrylamide was prepared as a single batch and the protein applied to each gel was standardized to 10 µg. All gels were treated identically and run concurrently. Coomassie Blue staining was used (Yamanaka and Takahara, 1977).

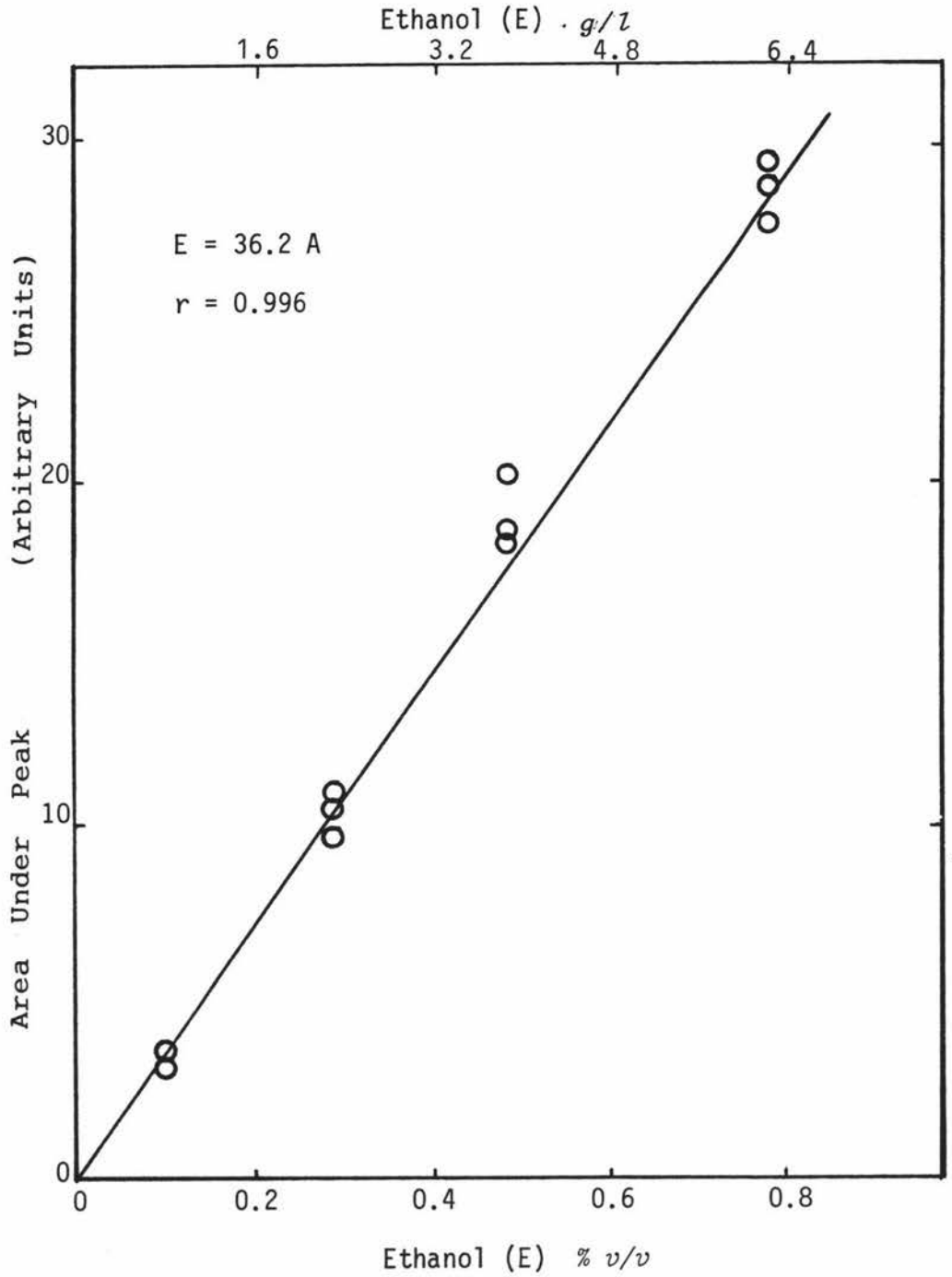



Figure 3.3 Standard Curve for Ethanol Analysis.

## 3.5 ENZYME ASSAY

### 3.5.1 Preparation of Cell-Free Extract

For the purpose of D-xylose isomerase purification, bacterial cells were harvested after 20 h of incubation at 30° in MRS medium with 20 g/l D-xylose substituted for glucose in the recipe. Cells were harvested using a Sharples (Model No.T313A49A24B8R) centrifuge (Sharples France, Rueil-Malmaison, France) at 10,000g and at 20° then washed twice with 0.02M phosphate buffer at pH 7.0. The washed cells were resuspended in 0.1% w/v of cationic detergent and then incubated at 37° for 1 hour, prior to a 30 seconds disruption using a Braun Cell Homogenizer MSK (B. Braun, Melsungen, West Germany). The disruption capsule was constantly cooled with liquid carbon dioxide to prevent any heat denaturization of cell proteins.

### 3.5.2 Enzyme Assay

The activity of D-xylose isomerase was determined by measuring the rate of D-xylulose formation from D-xylose according to the method of Yamanaka (1966), with a slight modification. A reaction mixture was prepared which contained 1.0 ml of 0.01M (i.e. 1500 µg per ml) of D-xylose, plus 1.0 ml of 0.02M phosphate buffer at pH 7.0 containing 0.01 mM $MnCl_2$ and 0.01 mM $CoCl_2$ and 1.0 ml of crude cell-free extract or appropriately diluted enzyme solution. The blank contained 1.0 ml of distilled water instead of enzyme extract together with all other components. The total reaction mixture was then mixed and incubated in a water bath at 40° for 10 min. 0.3 ml of the total reaction mixture was drawn off and the reaction stopped with 0.7 ml of 0.5N HClO . The formation of D-xylulose was then measured using Cysteine-Carbazole Method (Dische and Borenfreund, 1951). The absorbance was read at 540 nm by using Cecil CE 272 Linear Readout UV-Spectrophotometer (Cecil Instrument, Cambridge, England).

### 3.5.3 Cysteine-Carbazole Test

Cysteine-Carbazole Test (Dische and Borenfreund, 1951) was used to determine the equilibrium concentration of D-xylulose produced by D-xylose isomerase. 1 ml of the ketopentose (D-xylulose) or 1 ml of standard reaction mixture (i.e. 0.3 ml of total mixture plus 0.7 ml of 0.5N HClO) were mixed with 0.2 ml of 1.5% (v/v) cysteine hydrochloride. To this mixture was added 6 ml of 70% (v/v) concentrated sulphuric acid and then 0.2 ml of 0.12% (w/v) carbazole (in alcoholic solution). The mixture was shaken well and then held at room temperature for 15 min. A reddish tint was formed and was measured as an absorbance at 540nm. The standard curve of D-xylulose and D-xylose (from 10 to 200 μM) using this Cysteine-Carbazole assay is depicted in Figure 3.4.

### 3.5.4 Enzyme Units (D-Xylose Isomerase)

One unit of enzyme (D-xylose isomerase) was defined (Yamanaka, 1966) as that activity needed to produce 1 μM of D-xylulose per min in the standard reaction mixture (see section 3.5.2 and 3.5.3).

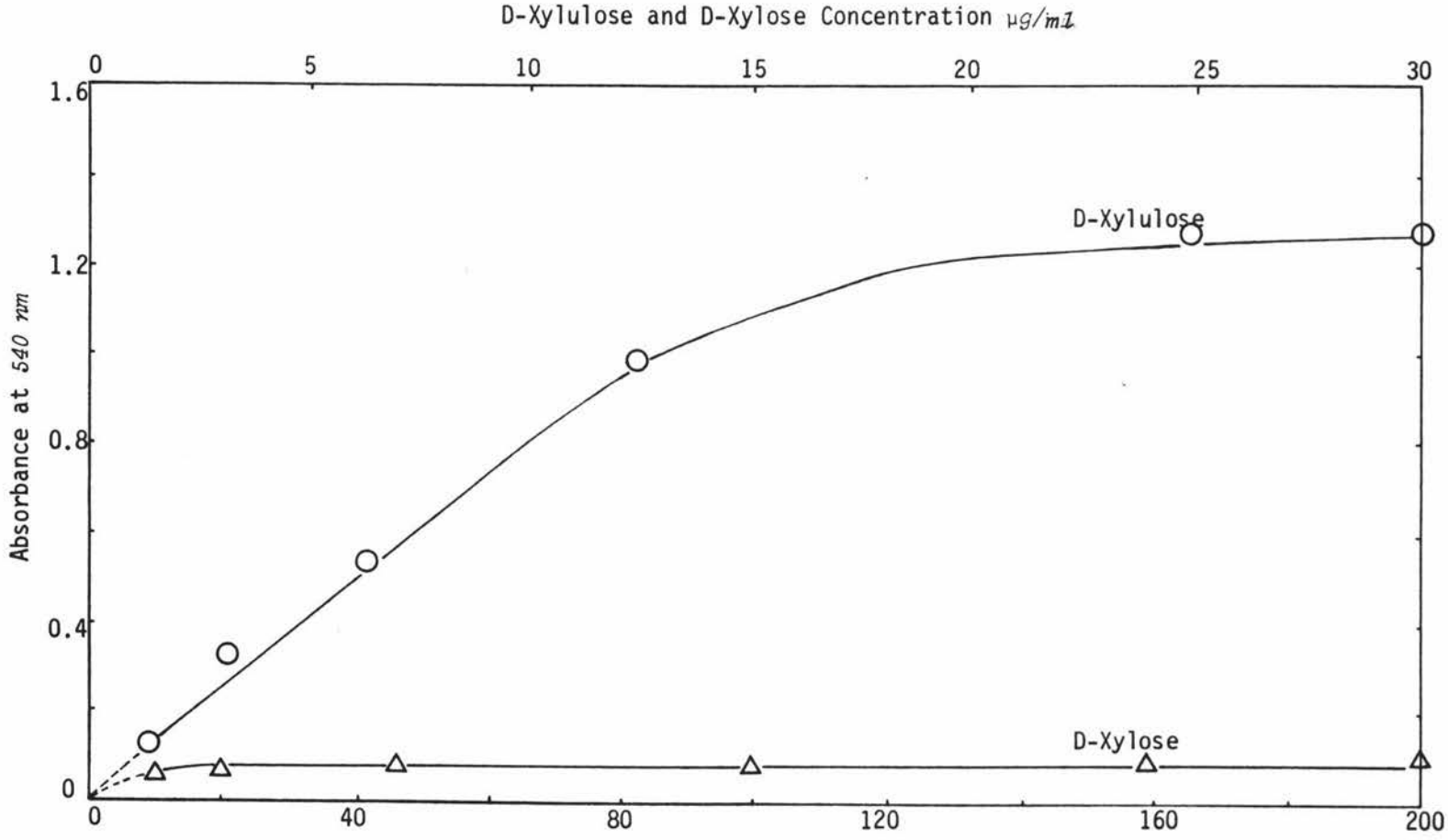



Figure 3.4 Cystein Carbazole Method for Assay of D-xylose & D-xylulose

Standard Curve for Assay

## 3.6 CULTURE GROWTH CONDITIONS

### 3.6.1 Shake - Flask Cultures

All the experimental studies in Chapters 6 and 7 were performed on shaken flask cultures in 50 ml of synthetic medium containing Difco Yeast Nitrogen Base (No. 0392) plus either 50 ml of pre-hydrolyzate or 20 g/l of D-xylose. Varying concentrations of Yeast Nitrogen Base were used (e.g. 1, 4 and 7 g/l) in the statistically designed experiments, but unless otherwise stated, the concentration was 6.7 g/l. Acidity was pre-adjusted to the desired pH values (i.e. 2.5, 4.5 and 6.5). All media were sterilized using membrane filtration (0.45 μm, Millipore; refer section 3.2.1). After inoculation, flasks were incubated at 30° (or other stated temperatures) with constant shaking at 100 r.p.m. in an Orbit Water Bath Shaker (No. 3535) or Environ Shaker (No.3597) (Lab-line Instruments Inc., Melrose Park, Illinois, U.S.A.).

### 3.6.2 Fermenter Culture

#### 3.6.2.1 Equipment - Fermenters and Electrodes Used

Either a Multigen F2000 or a Biogen Laboratory Fermenters (New Brunswick Scientific Company Limited, New Brunswick, New Jersey, U.S.A.) with the 2-litre vessel (1.5-litre or 1.35-litre working volume) was used for batch and continuous cultures. The assembled fermenters in continuous culture operations are depicted in Figures 3.5 and 3.6. Figure 3.7 shows a schematic diagram of the fermenter and its ancillary equipment. Either air or oxygen-free nitrogen gas was supplied to the fermenter through a (internal diameter 2 x 20 cm) sterilizing glass-wool filter. Gas entered the vessel through numerous holes in the base of the impeller shaft. The gas flowrate was measured with a flow-meter (Platon Flow Control Limited, BasingStoke, Hampshire, England), with a flow range of 50-60 ml per min. The agitation speed of the dual (four-bladed disc-turbine) impellers was 100 r.p.m., driven by magnetic motor at the base of the vessel (A built-in revolution

counter was available on the instrument). Temperature throughout the studies was controlled by a thermistor sensor at 28 ± 0.5° inserted through the head plate and submerged into culture. The exhaust air from the culture vessel was passed through a water cooled condensor and then via a splash-head into a concentrated sulphuric acid catch-pot. This absorbed all the water vapour before passing the gases through a silica gel drying tube and then on to a Taylor Servomex Oxygen Analyzer, Type OA272 (Taylor Servomex Limited, Crowborough, Sussex, U.K.). The analyzer was calibrated according to the manufacturer's specification. A galvanic dissolved oxygen (DO) probe, Type M1016-0208 (New Brunswick Scientific Company Inc., New Brunswick, New Jersey, U.S.A.) was used to measure the dissolved oxygen tension (DOT) in the culture. The probe was connected to Dissolved Oxygen Analyzer (DO-40) (New Brunswick Scientific Company Inc., New Brunswick, New Jersey, U.S.A.) and its output was recorded on the recorder chart. The calibration of DO probe was conducted in situ by sparging oxygen-free nitrogen gas through the medium to strip off any dissolved oxygen. This gave zero oxygen saturation. The culture was then aerated with air to give 100% of saturation. Redox potential control was carried out using the same method adopted by Clark (1982) for dissolved oxygen control.

3.6.2.2 pH Measurement

The routine pH measurement was performed with either a Metrohm Herisau pH meter E520 (Metrohm Herisau, AG, Herisau, Switzerland) or a New Brunswick pH meter (New Brunswick Scientific Company Inc., New Brunswick, New Jersey, U.S.A.).

3.6.2.3 Dissolved Oxygen Tension (DOT) Measurement

Dissolved oxygen tension was constantly monitored by using either a Beckman Fieldlab Oxygen Analyzer (No.100800) in combination with its Polarographic Oxygen Sensor (No. 39550) (Beckman Instruments Inc., Fullerton, California, U.S.A.) or a New Brunswick Dissolved Analyzer (DO-40) (New Brunswick Scientific Company Inc., Edison, New Jersey, U.S.A.)

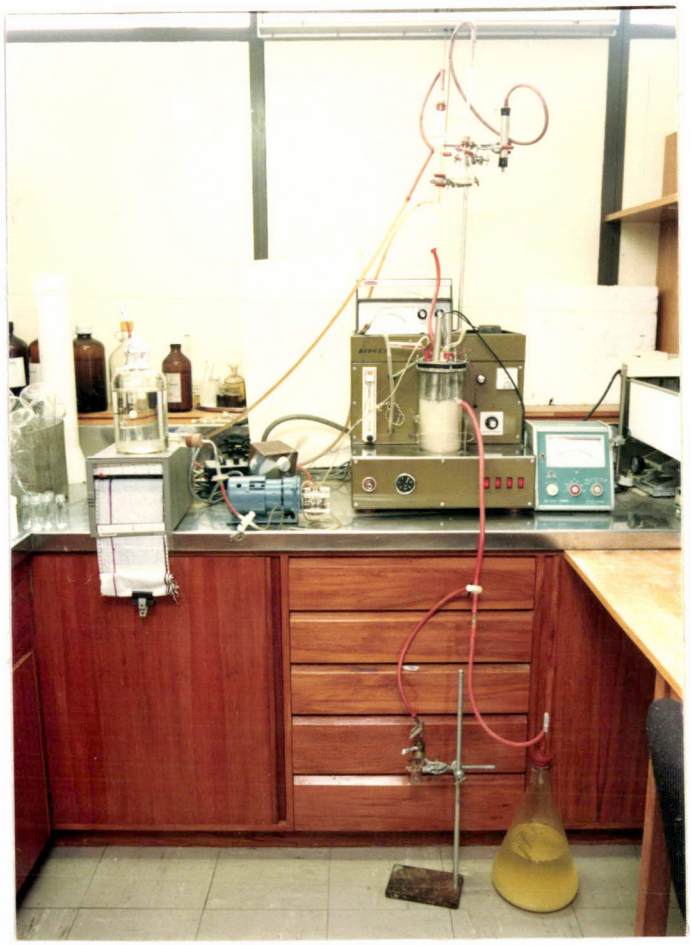


Figure 3.5 The Assembled Fermenter in Continuous Aerated Chemostat

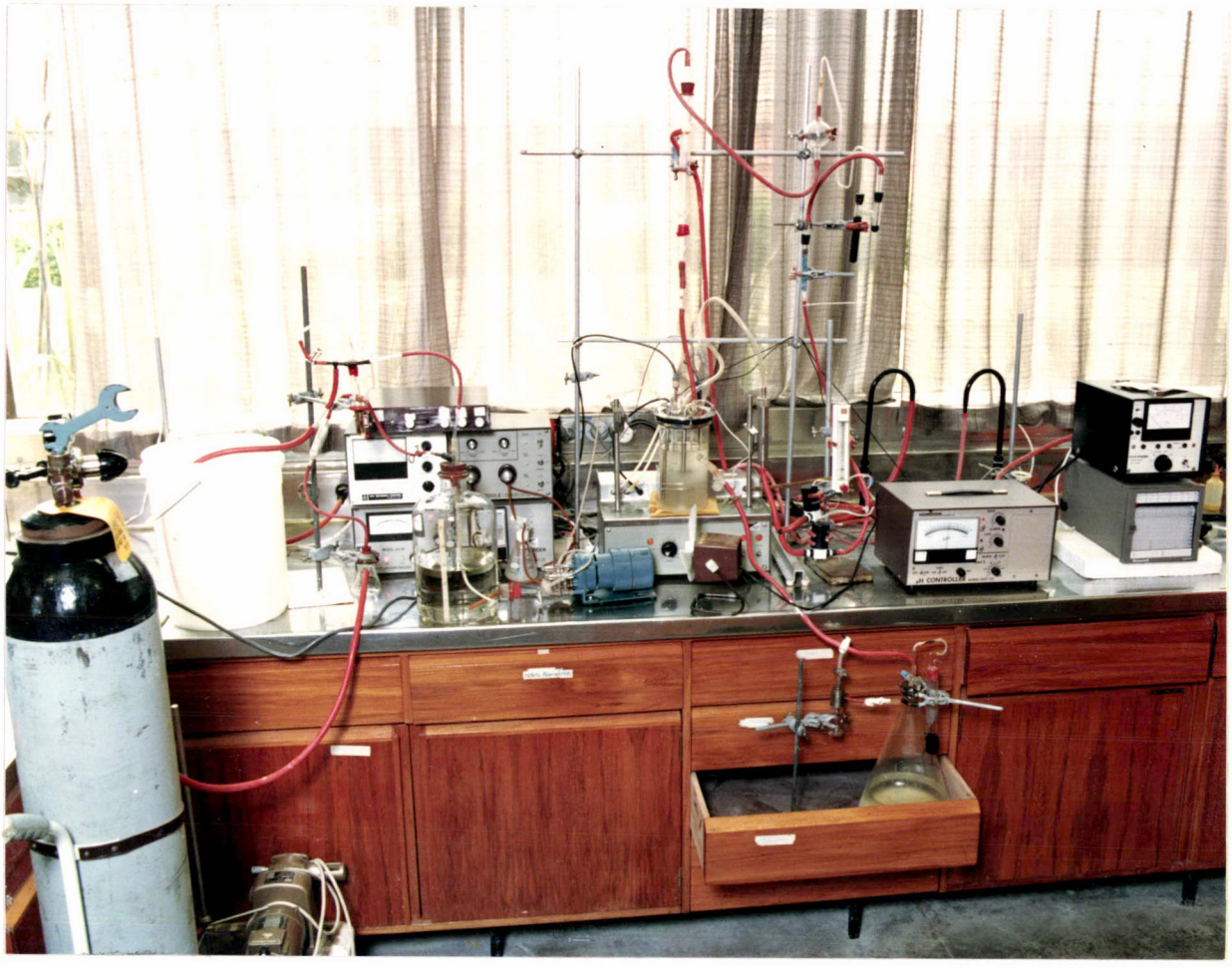


Figure 3.6 The Assembled Fermenter in Continuous Flow System with Redox Controller (see Line Drawing for Full Explanation)

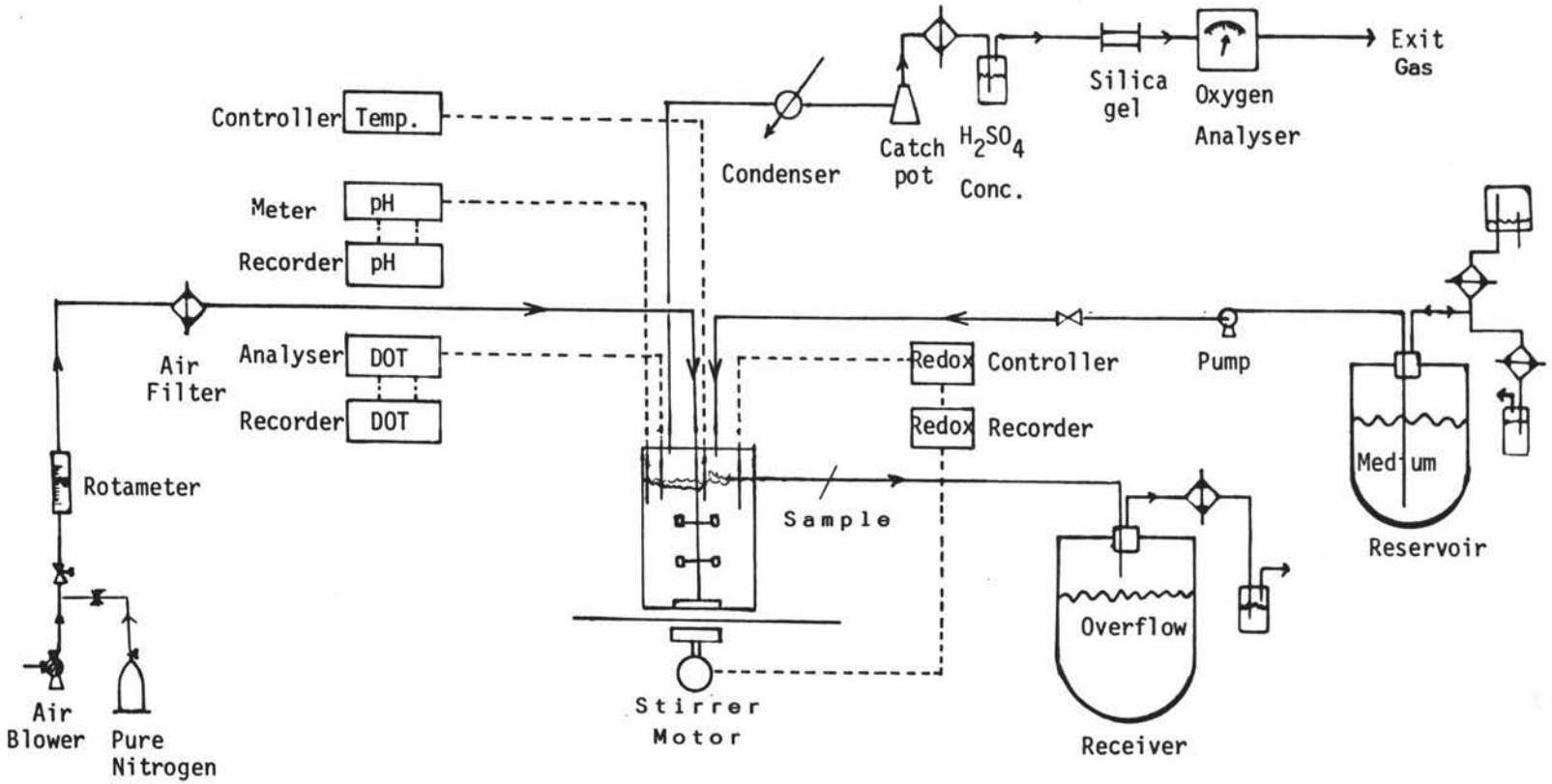



Figure 3.7 A Schematic Diagram of the Fermenter and its Ancillary Equipment as used in Continuous Culture Studies

3.6.2.4 Redox Potential Measurement

A Calomel Reference Electrode (K401) in combination with a Platinum (P101) Electrode were obtained from Radiometer, Copenhagen, Denmark, and was used to control and monitor redox potential in chemostat studies (8.3.4).

## 3.7 DISCUSSION OF METHODS

The calibration curve of the total yeast cell count versus dry weight as shown in Figure 3.1, was checked by a linear regression for all experimental data. The relationship was always linear and the regression data are presented in Appendix 2, the F-ratio of mean square due to regression and to mean square due to residual was highly significant at the level of 0.1%. This meant that the dry biomass could therefore be estimated with a high degree of confidence from the calibration curve if the observed cells number were counted using a haemocytometer. This was a point of convenience in the experiments.

The standard curve for the modified sugar analysis by the Somogyi and Nelson (Mackie, 1982a) method showed poor day to day reproducibility. This is apparent from Figure 3.2 which is a plot of eight such standard curves collected over a two month period. Appendix 3 contains the result of a two way analysis of variance which showed a significant variation at to 1% level. The high day to day variability could be due to the great sensitivity of this analysis to the time and temperature of heating in water-bath (Mackie, 1982a). To circumvent this problem, a fresh standard curve was prepared for each new set of analyses.

Errors associated with the gas chromatographic assays of ethanol were examined statistically by employing replicate injections of an external standard. Each of the four external standard concentrations was injected in triplicate each time the chromatograph was operated. A two way analysis of variance was performed on all the data and thus

revealed considerable day to day variations. One way analysis of variance performed on the data for each concentration showed that day to day variations were greatest at the highest concentration of standard. The results of these analyses of variance are presented in Appendix 4. The pooled standard deviations for each concentration provided a measure of the machine error arising at that particular concentration and allowed an estimation of 95% confidence limits for each ethanol concentration point on the standard curve (Figure 3.3).

# CHAPTER FOUR : PRELIMINARY STUDIES OF SCREENING FOR PENTOSE FERMENTING ORGANISMS AND ENZYME LINKED PENTULOSE FERMENTATION

This Chapter consists of two parts. It describes two totally independent approaches to the fermentation of D-xylose to ethanol. The first part describes the screening of organisms for their ability to ferment D-xylose while the second part considers the enzymatic conversion of D-xylose to D-xylulose using D-xylose isomease. This enzyme's activity in three bacterial types was measured and a partially purified enzyme was prepared from Lactobacillus fermentum ATCC 9338.

## 4.1 Part I : SCREENING FOR XYLOSE FERMENTING ORGANISMS BY USING ENRICHMENT CULTURE TECHNIQUES

### 4.1.1 INTRODUCTION

At the time of commencing this screening work, the state of knowledge concerning the bioconversion of hexoses and pentoses to ethanol and other related metabolic products by bacteria could be summarised according to Table 2.1. It was considered that further screening and culture enrichment studies could be used to find a novel pentose fermenting organism. The following series of experiments were designed with this objective in mind, that is, to isolate an organism with an ability to convert D-xylose primarily to ethanol and perhaps to other useful products (such as volatile acids).

### 4.1.2 EXPERIMENTAL METHODS

The enrichment method of Claus and Wilmanns (1974) was adcpted. The media used for all the enrichment cultures stated below contained 40 g/l of D-xylose with or without a supplement of Difco Yeast Extract

and were pre-adjusted to pH 6, 7 or 8. The enrichments were incubated at 30° for four days and then three subsequent transfers of the culture were carried out.

Anaerobic digester sludge microorganisms were established in an enrichment medium dispensed in 250 ml glass stoppered bottles.

Decaying wood fragments were taken from a compost heap. Test fermentations were run at pH 6, 7 and 8 with and without yeast extract supplements.

No nitrogen supplementation was used in this trial again set up at pH values of 6, 7 and 8. However, to select for spore-forming bacteria (such as Clostridium) a series of inoculated flasks were heat treated at 80° for 10 min prior to incubation.

### 4.1.3 RESULTS

#### 4.1.3.1 Enrichment Cultures from Anaerobic Digestion Sludge

Results for anaerobic sludge cultures are presented in Table 4.1. The main fermentation products yielded were acetic acid, butyric acid and ethanol. Best production of ethanol was at pH 8. Addition of yeast extract had little, if any affect. At all other pH values, that is, 6 and 7, supplementation of the fermentation with 1 g/l of yeast extract slightly enhanced the ethanol production. It was presumed that anaerobic sludge provided sufficient nitrogenous material for the developing cultures. Both cultures at pH 8, with and without nitrogen enrichment, produced acetic acid, butyric acid and ethanol in the approximate ratio of 3:3:2 (see Table 4.1).

#### 4.1.3.2 Enrichment Cultures from Decaying Wood

The analytical results for culture supernatants are set out in Table 4.2. These cultures were noteworthy for they produced more acetic acid than ethanol.

Table 4.1 Fermentation End-Products in Supernatants from Enrichement Cultures Developed from Anaerobic Digester Sludge

| Product | Initial pH of Culture | | | | | |
|---|---|---|---|---|---|---|
| | 6 | | 7 | | 8 | |
| g/l | A | B | A | B | A | B |
| Ethanol | 0 | 0.64 | 0.56 | 0.96 | 1.04 | 1.12 |
| Acetic Acid | 1.05 | 1.16 | 2.42 | 1.58 | 1.37 | 1.79 |
| Butyric Acid | 0.77 | 0.86 | 0.19 | 0.67 | 0.96 | 1.63 |
| Biomass Yield (g/l dry weight) | 1.54 | 1.24 | 1.47 | 3.87 | 1.47 | 2.45 |
| Cell Count ($x10^8$/ml) | ND | 8.75 | 7.60 | ND | 10.35 | 12.25 |

A = Difco Yeast Extract at 6.7 g/l included in medium.

B = Absence of Difco Yeast Extract from medium.

ND = Not Determined. Initial xylose was 40g/l.

Table 4.2 Fermentation End-Products in Supernatants from Enrichement Cultures Developed from an Inoculum of Rotted Wood Fragments

| Product | Initial pH of Culture | | | | | |
|---|---|---|---|---|---|---|
| | 6 | | 7 | | 8 | |
| g/l | A | B | A | B | A | B |
| Ethanol | 0.40 | 0.16 | 0.56 | 0.16 | 0.64 | 0.16 |
| Acetic Acid | 19.21 | 18.17 | 23.52 | 6.20 | 24.78 | 19.53 |
| Butyric Acid | 3.65 | 1.44 | 3.17 | 0.19 | 5.18 | 0.29 |

A = Difco Yeast Extract at 6.7 g/l included in medium.

B = Absence of Difco Yeast Extract from medium.

Initial xylose concentration was 40g/l.

4.1.3.3 Enrichment Cultures from Garden Compost plus Soil

Table 4.3 presents the results from trials using a garden compost inoculum. It was shown that once again acetic acid producing bacteria dominated the population and that relatively low concentrations of ethanol developed where spore-forming organisms were selected. Butyric acid was the next significant end-product after acetic acid.

Table 4.3 Fermentation End-Products in Supernatants from Enrichement Cultures Developed from an Inoculum of Garden Compost plus Soil

| Product | Initial pH of Culture | | | | | |
|---|---|---|---|---|---|---|
| | 6 | | 7 | | 8 | |
| g/l | C | D | C | D | C | D |
| Ethanol | 0.24 | 0.16 | 0.16 | 0.16 | 0.24 | 0.16 |
| Acetic Acid | 7.35 | 18.17 | 12.39 | 6.20 | 26.25 | 19.53 |
| Butyric Acid | 4.03 | 1.44 | 4.70 | 0.19 | 6.72 | 0.29 |

C = Heat Treatment given at 80° for 10 min

D = Heat Treatment not given

Initial xylose concentration was 40 g/l.

The concentration of ethanol developed in these mixed cultures was not particularly high. For a given enrichment culture to warrant further investigation, i.e. purification of its component organisms responsible for ethanol formation, a products ratio for ethanol, acetic, butyric was arbitrarily set at 2:1:1. As no enrichment exhibited this ratio, further experimental work was discontinued.

### 4.1.4 DISCUSSION

The enrichment culture technique is based on free competition among different organisms in liquid media (Nakayama, 1981). It is an effective technique to select or isolate a new organism from the natural environment. A number of researchers have adopted enrichment culture techniques to isolate the mesophilic bacteria, such as Sarcina maxima (Claus and Wilmanns, 1974); Sarcina ventriculi from soil (Finn et al., 1984), and also extreme thermophilic bacteria, such as Clostridium thermohydrosulfuricum (Wiegel et al., 1979); Thermoanaerobacter ethanolicus (Wiegel and Ljungdahl, 1979 and 1981); Thermoanaerobium brockii (Zeikus et al., 1979), for the fermentation of pentose(s). The results of three different enrichment programmes described above (Sections 4.1.3.1 to 4.1.3.3) showed that cultures from decaying wood fragments and garden compost plus soil developed relatively high acid and low ethanol concentrations. Each enrichment produced low ethanol concentrations of less that 5 g of ethanol per 100 g of D-xylose consumed whereas Finn et al. (1984) using a maltose enrichment technique at pH 2.2 followed by repeated plating, were able to obtain a pure culture of Sarcina ventriculi, which fermented 20 g/l arabinose and yielded up to 30 g of ethanol/100 g of arabinose utilized.

During the period of this work other investigators were considering another approach, i.e. the initial conversion of D-xylose to D-xylulose prior to fermentation. Such an approach appeared feasible and formed the topic of the next section of work.

### 4.1.5 CONCLUSIONS

Three types of enrichment culture were developed from anaerobic digester sludge, decaying wood fragments and garden compost plus soil. The enrichment culture developed from anaerobic digester sludge yielded considerable amounts of acetic and butyric acids as well as ethanol. The enrichment cultures developed from decaying wood and garden compost plus soil produced a high quantity of acetic acid as compared with

butyric acid and ethanol which was in excess of 5 g per 100 g of D-xylose utilized. No culture was able to produce ethanol as a dominant product free of other undesirable metabolic end-products such as acetic acid. Consequently, the enrichment culture programme for screening pentose-fermenting organisms was discontinued.

## 4.2 Part II : ENZYME-YEAST SYSTEMS STUDIES

### 4.2.1 INTRODUCTION

The conclusion reached at the end of the previous Sections 2.2.1 and 4.1.5 was that a yeast capable of fermenting a pentose to ethanol could not easily be found during early 1981. However, in view of the observations that many yeasts do ferment pentuloses (Wang and Schneider, 1980; Wang et al., 1980b; Jeffries, 1981; Gong et al., 1981; Ueng, et al., 1981) to ethanol, it seemed appropriate to consider the conversion of pentoses to pentuloses by enzymatic means. A longer term objective would be fermenting with yeast the pentulose so formed to ethanol thus avoiding other end products such as acetic acid. The purpose of this section is to describe the screening of promising D-xylose isomerase producing organisms and to extract and then purify this enzyme for use in the isomerization of D-xylose to D-xylulose. This isomerization product could then be further fermented to ethanol by an appropriate yeast system.

### 4.2.2 EXPERIMENTAL METHODS

#### 4.2.2.1 Culture and Extraction of D-xylose Isomerase

Bacteria were grown in 250ml of either M.R.S. or Tryptone Soya medium (described in Section 3.1.1) at 30° in 500 ml Erlenmeyer flasks for 20 h with agitation at 150 r.p.m. Cells were then harvested and cell free extracts prepared (Section 3.5.1) and assayed (Section 3.5.2) for protein and enzyme activity.

#### 4.2.2.2 Enzyme Purification

The bacterium, Lactobacillus fermentum ATCC 9338, was grown in 10 l M.R.S. broth with D-xylose as a carbon source instead of glucose at 30° for 20 h. Cell free extracts were prepared either by disintegration (Section 3.5.1) or by treatment with 1 g/l of cationic detergent

(cetyltrimethyl ammonium bromide) followed by centrifugation (MSE Hi-Span 21 Centrifuge; MSE Scientific Instruments, Manor Royal, Sussex, England) at 14,700 g for 10 min at 4°.

The enzyme assay procedure was described in Sections 3.5.2 to 3.5.4.

Enzyme purification was initially based upon the purification methods of Yamanaka (1968) and Yamanaka and Takahara (1977). All purification steps were conducted below 4° unless otherwise stated. The procedures were as follows :

Manganese Chloride Treatment : 8.25 ml of 1 M manganese chloride solution was added dropwise into the crude cell free extract with constant stirring. 1 N sodium hydroxide was used to raise the pH to 6.5-7.0. After allowing the extract to stand for 30 min, the precipitate was then recovered by centrifugation at 4° at 14,700 g for 10 min.

The supernatant was the manganese chloride treated extract.

Ammonium Sulphate Fractionation : Preliminary experiments established that the 70-80% saturation fractions contained the highest specific activity (see Table 4.5). Thereafter all extracts were fractionated by adding solid ammonium sulphate to give 70% to 80 % saturation. The precipitate was collected by centrifugation at 14,700 g for 10 min at 4° and then dissolved in 0.02M phosphate buffer, pH 7.0 and dialyzed overnight against the same buffer containing 1 mM manganese chloride.

Column Chromatography : The dialyzed preparation was then applied to a column of DEAE-Sephadex A50 (internal diameter 2.5 X 30 cm) as described in Section 3.4.4.1.

Gel Filtration : The pooled enzyme solution from DEAE-Sephadex A50 was applied to the column of Sephadex G-200 (internal diameter 1.0 X 60 cm) as was described in Section 3.4.4.2.

The purity of the enzyme fractions from each stage of the purification was demonstrated by using polyacrylamide disc gel electrophoresis (Section 3.4.7).

#### 4.2.2.3 Enzyme Stability

Isomerase assay reaction mixtures were prepared in the usual manner. Under test, hydrolyzate as described in Table 3.2 was substituted for distilled water in the buffers (see Section 3.5.2).

### 4.2.3 RESULTS

#### 4.2.3.1 Screening of D-xylose Isomerase Producing Organisms

Three bacteria, Bacillus coagulans NCIB 8870; Lactobacillus fermentum ATCC 9338 and Streptomyces griseus NCTC 7807, were screened for the D-xylose isomerase activity. Bacillus coagulans and Lactobacillus fermentum were grown in M.R.S. broth and Streptomyces griseus in Tryptone Soya broth with D-xylose as a carbon source instead of glucose. All three exhibited activity and Bacillus coagulans was chosen arbitrarily to establish a cell disruption procedure for the release of the enzyme from cells. This procedure was then used for purification studies.

4.2.3.2 Extraction of D-xylose Isomerase from Bacillus coagulans NCIB 8870

M.R.S. broth cultures of Bacillus coagulans were grown at 30° for various periods prior to harvesting, standardized (absorbance) suspensions were disintergrated for studies of enzyme activity.

The results (Figure 4.1) demonstrated that using the Braun Homogeniser (3.5.1) the highest enzyme activity was extracted from cells harvested 20 h after inoculation.

Figure 4.2 shows that in extracte from cells harvested from 18 to 24 h cultures, the reaction equilibrium (isomerization) was established in the reaction mixture some 10 min after substrate addition. It was noted also that the equilibrium was not even achieved after 20 min for cell free extracts from 16 and 24 h cultures.

A comparison of two disruption methods (3.5.1) was also made. The results shown in Figure 4.3(A) suggested that the highest enzyme activity was achieved one hour after the commencement of treatment with cationic detergent. Hence, a one hour incubation time with 1 g/l of cationic detergent was adopted in further purification (4.2.3.3) work. The results shown in Figure 4.3(B) indicated that the optimum treatment time with the Braun Homogenizer was 30 seconds. Further increase of the disintegration time caused enzyme denaturation.

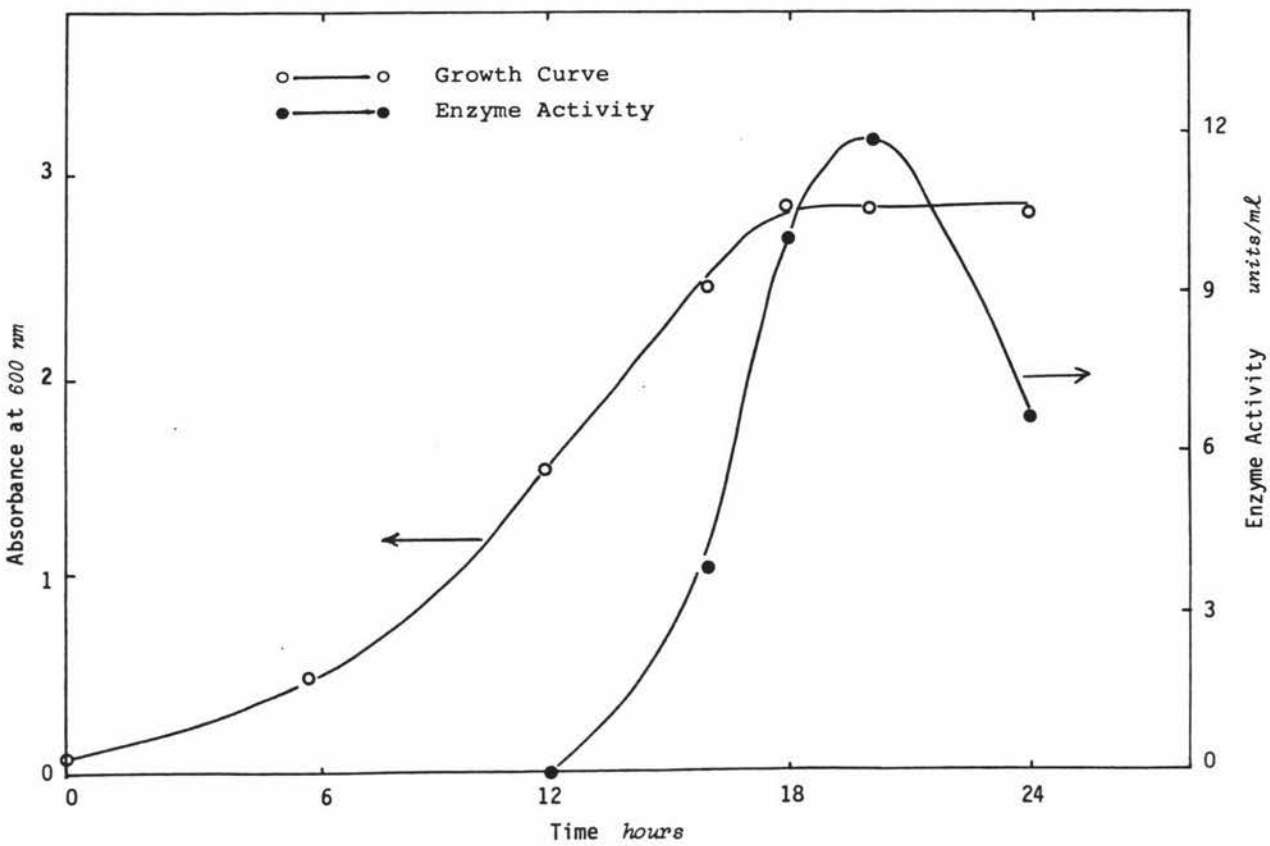
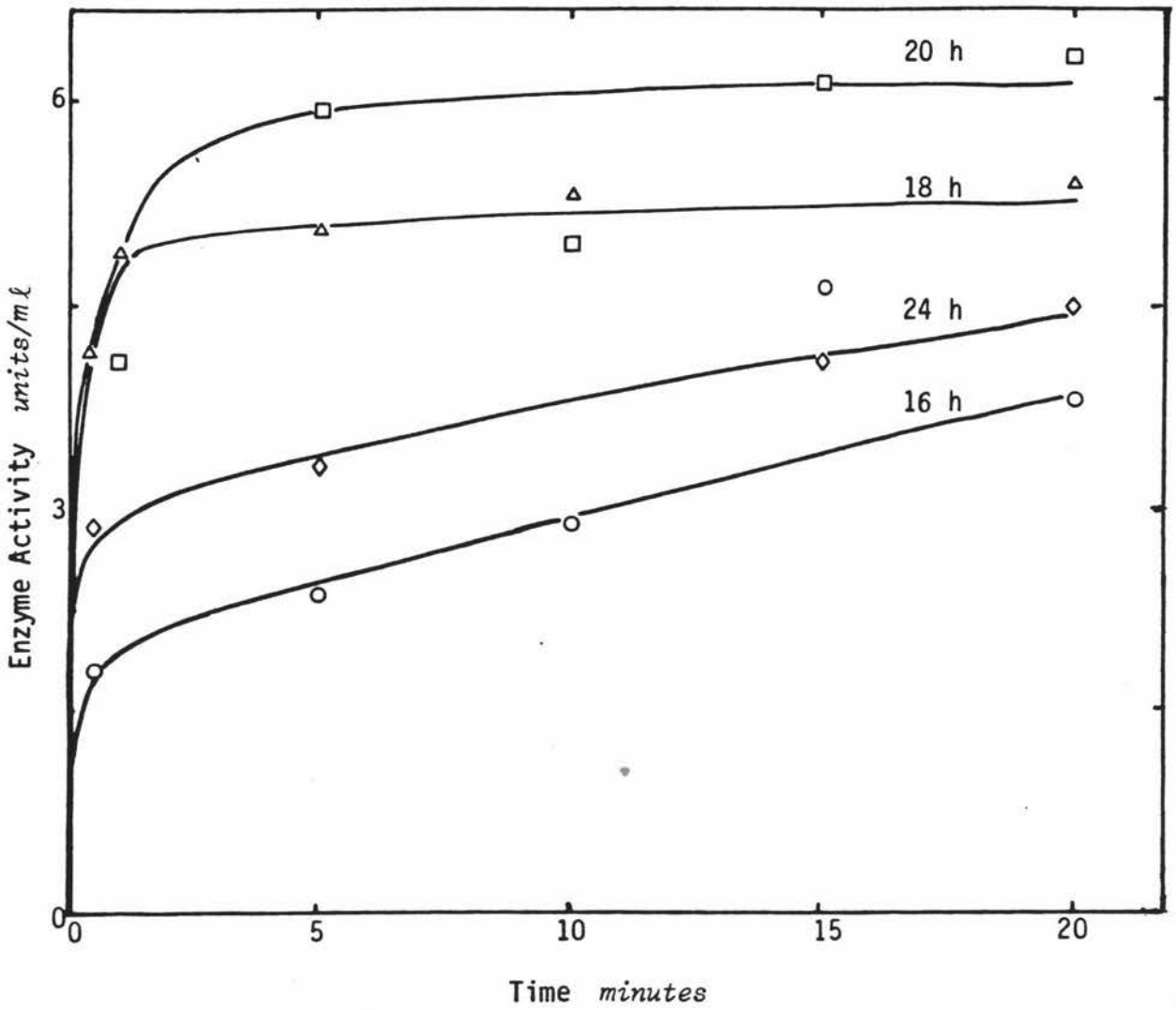
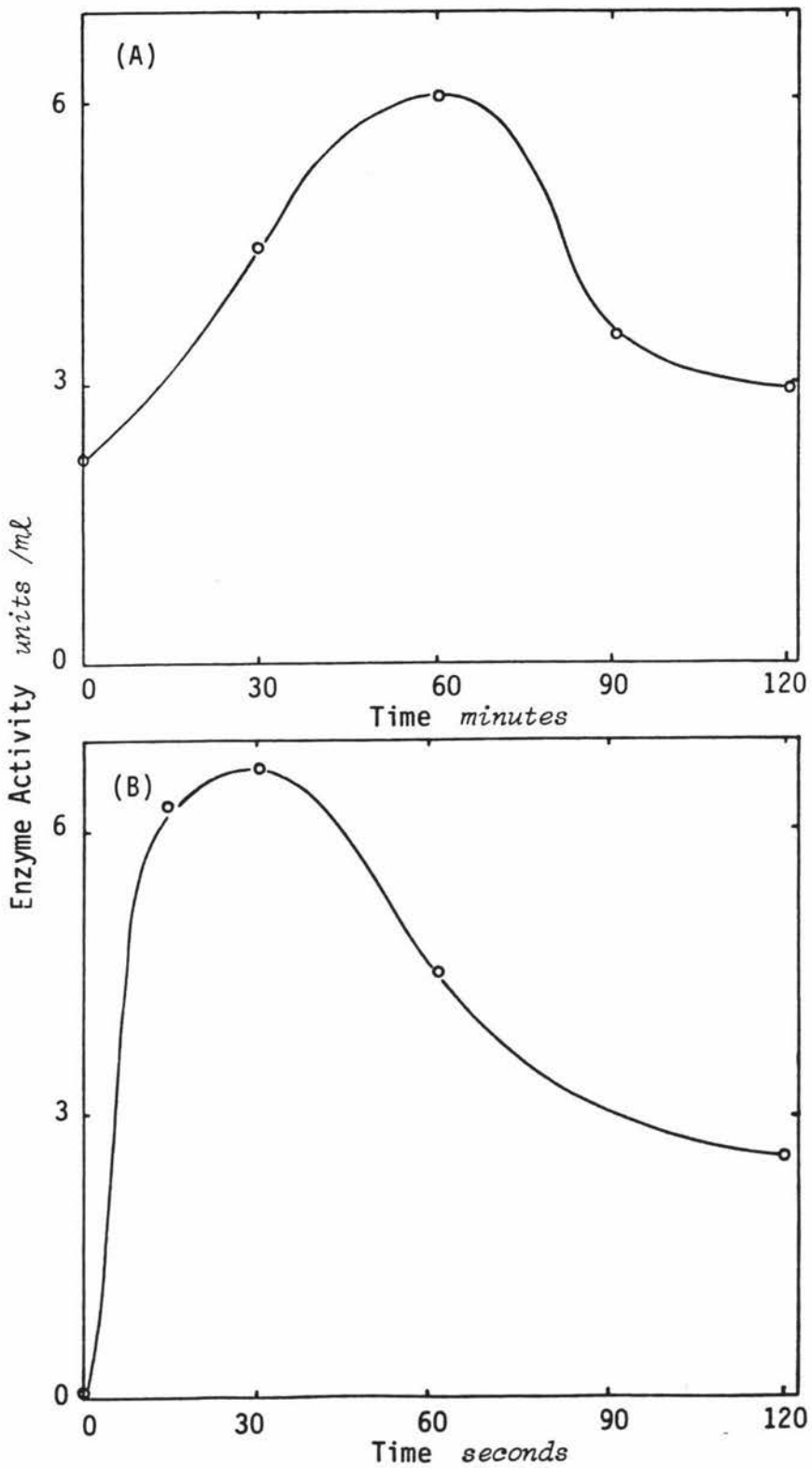



Figure 4.1 Effect of Culture Age on D-xylose Isomerase and Activity in Cell Free Extracts of Bacillus coagulans NCIB 8870 Harvested Throughout Growth Cycle




4.2 Time Course Study of D-xylose Isomerase Reaction Equilibrium in Cell Free Extracts of Bacillus coagulans NCIB 8870 Harvested between 16 and 24 hours of Growth




4.3 Time Course Study of the Release of D-xylose Isomerase from 20 hours Cells of Bacillus coagulans NCIB 8870 Using (A) 1 g/ℓ of Cationic Detergent and (B) Braun Homogenizer .

The results (Figures 4.3(A) and (B)) also revealed that cationic detergent alone released almost the same amount of enzyme as did mechanical disruption. A combination of both methods released as much enzyme as the cationic detergent method alone (Table 4.4). Thus, the disruption procedure chosen did not seem to be critical to enzyme release except that homogenization was faster.

Table 4.4 A Comparison of the Enzyme Activity Released into Supernatants from Bacillus coagulans by Different Cell Disruption Methods

| Methods | Enzyme Activity (Units/l) x1,000 |
|---|---|
| Cationic Detergent | 42.3 ± 10.6 |
| Detergent plus Homogenization | 42.8 ± 9.8 |

* 1 g/l Cetyltrimethyl ammonium bromide

The results of this work has given some indication of

a) the age of culture to use for maximal isomerase activity

b) the most appropriate reaction time for quantifying activity

c) the confirmation that disruption method was not critical.

This information was now used in the purification studies of the isomerase from Lactobacillus fermentum which appeared to demonstrate higher enzyme activity in cell free extracts then did the other two listed in 4.2.3.1.

### 4.2.3.3 Partial Purification of D-xylose Isomerase of *Lactobacillus fermentum* ATCC 9338

Having established a suitable cell disruption method it was possible to re-evaluate the isomerase activities of the three bacteria used in 3.2.3.1 and that of *Lactobacillus fermentum* was the highest. Thus, this bacterium's enzyme was used in purification studies. Cell free extracts were prepared for fractionation.

The results presented in Table 4.5 show that 70 to 80% ammonium sulphate fractions contained high D-xylose isomerase activities. Hence, 70 to 80% saturation ammonium sulphate was chosen to precipitate the manganese chloride treated cell free extract proteins.

Table 4.5 D-xylose Isomerase Activity and Protein Content of Various Fractions from Ammonium Sulphate Fractionation

| Fraction Number (% of saturation of $(NH)_2SO_4$ | Total Activity (Units/l) | Protein Content (g/l) | Specific Activity of Released Enzyme (Units/g protein) |
|---|---|---|---|
| 0 - 40% | 2,100 | 0.21 | 10,000 |
| 40 - 70% | 25,500 | 7.13 | 3,580 |
| 70 - 80% | 33,100 | 1.39 | 23,820 |
| Crude Extract of *L. fermentum* | 32,000 | 2.58 | 12,400 |

Figure 4.4 shows the single peak of enzyme activity corresponding to the protein peak.

The pooled enzyme solution from tubes 45 to 60 off the DEAE-Sephadex A50 was applied to the column of Sephadex Gel G-200 (internal diameter 1.0 X 60 cm) and the chromatogram of this gel filtration is shown in Figure 4.5. A rather symmetrical protein peak was observed.

An UV-spectrophotometer (Varian Series 634; Varian Techtron Pty. Limited, Mulgrane, Victoria, Australia) scan of the enzyme solutions from different steps of the purification procedure set out in Section 4.2.2.3, is shown in Figure 4.6. The crude L. fermentum cell free extract exhibited its peak at 264 nm whereas fraction number 15 of Sephadex G-200 showed its peak at 279 nm. These results suggested that an purer protein was eluted from the Sephadex G-200 column. Further study of the purity of fraction 15 was carried out using polyacrylamide disc gel electrophoresis, several bands were detected (Figure 4.7). It was concluded that the enzyme fraction obtained from Sephadex G-200 was a partially purified enzyme. The purification procedure is summarized in the Table 4.6 and a comparison of the protein purity at each stage of the purification using the electrophoresis gels is made in Figure 4.7.

The effect of D-xylose concentration on the partially purified D-xylose isomerase was studied by determination of the Michaelis constant (Km) for D-xylose. The Km value as calculated from Figure 4.8 as 10.8 mM D-xylose. This Km value is about twice that of D-xylose isomerase from different Lactobacillus species as reported by a number of researchers (see Table 4.7).

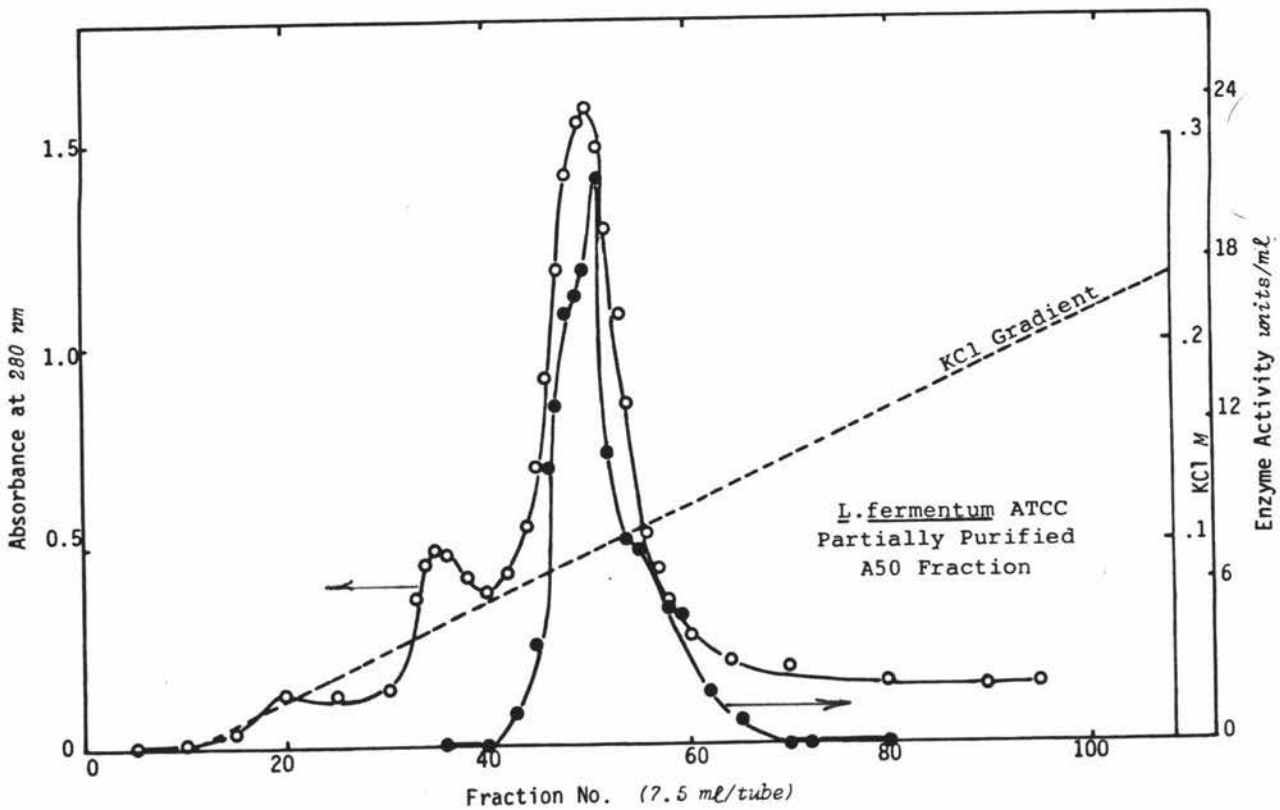



Figure 4.4 Chromatograph of D-xylose Isomerase on DEAE-Sephadex A50 Showing the Enzyme Peak and Protein Peaks

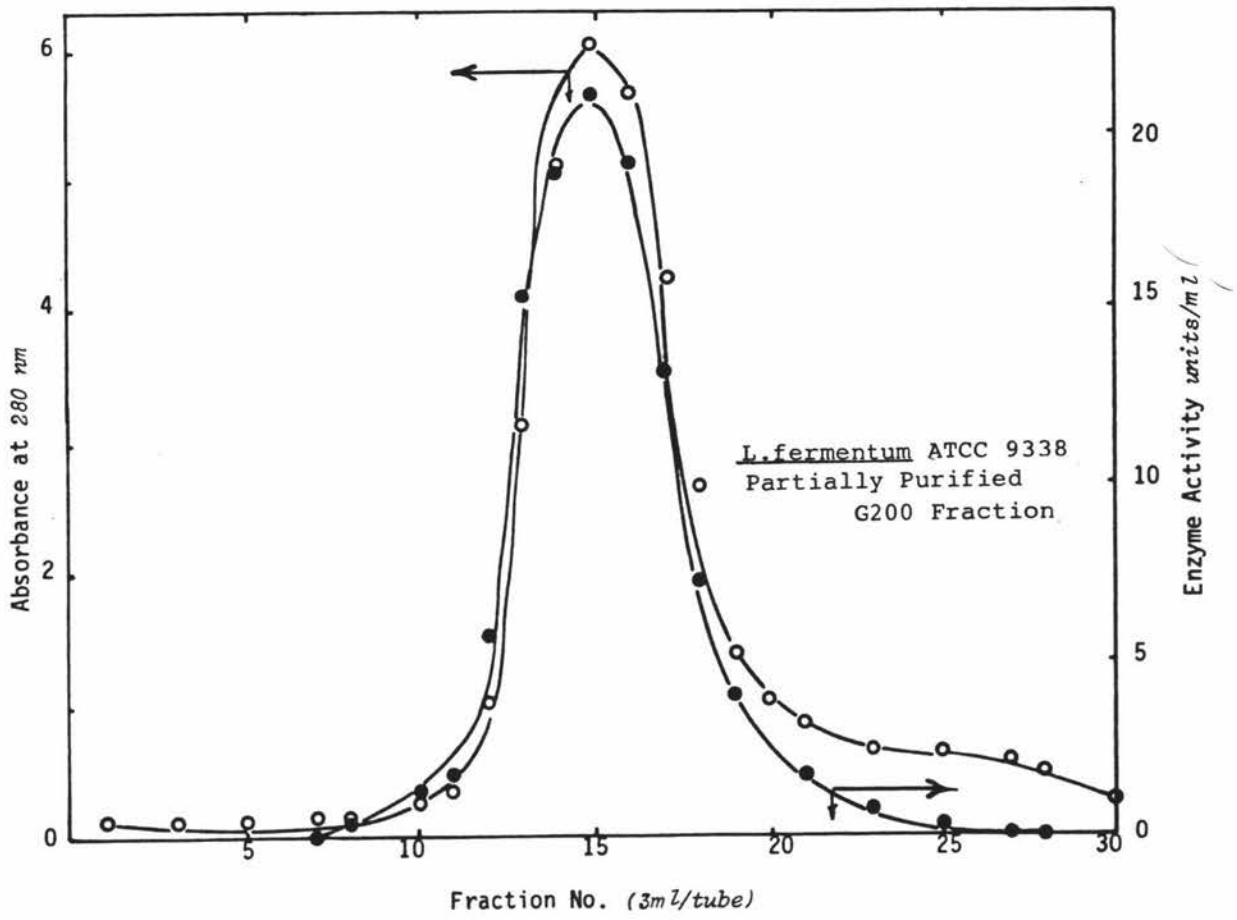



Figure 4.5 Gel Filtration of D-xylose Isomerase on Sephadex G-200
Showing a Single Enzyme Peak and a Protein Peak

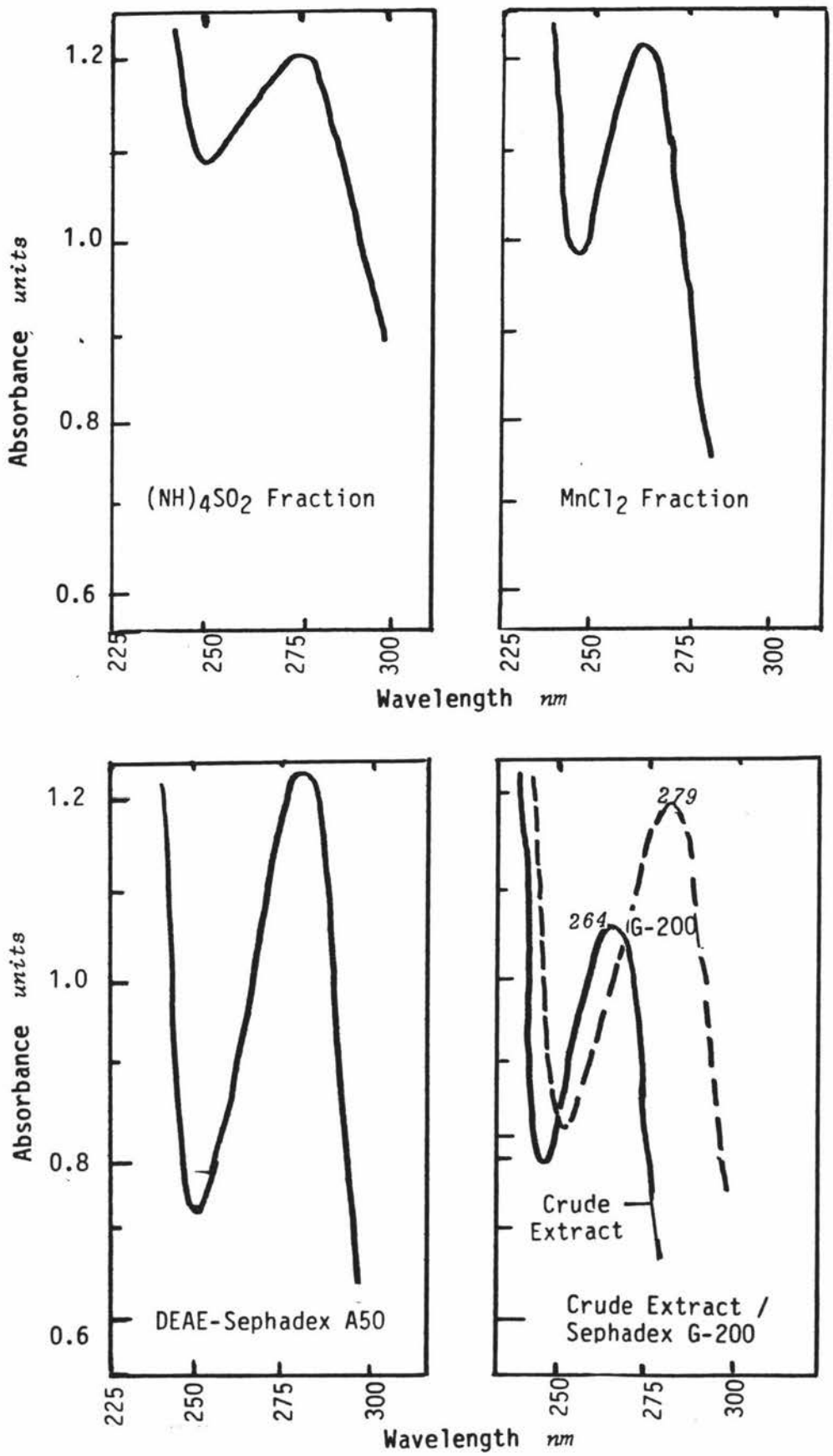



Figure 4.6 Absorption Spectra of Various Purification Fractions from Lactobacillus fermentum ATCC 9338

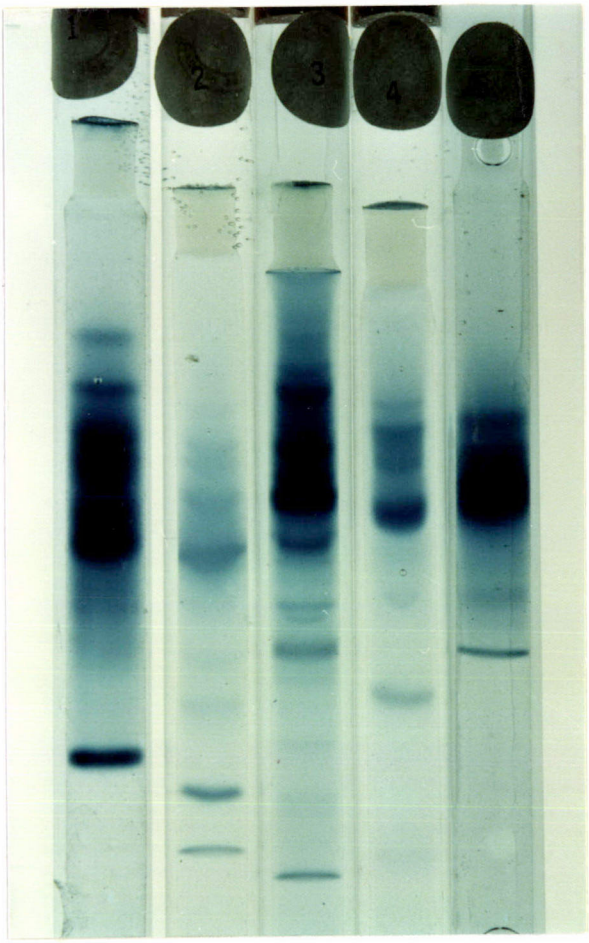
Table 4.6 Purification of D-xylose Isomerase of *Lactobacillus fermentum*

| Purification Step | Volume (l) | Activity (Units per l) x1,000 | Total Activity (Units) x1,000 | Total Protein (g) | Specific Activity (Units per g Protein) x1,000 | Recovery (%) | Relative Purification |
|---|---|---|---|---|---|---|---|
| Crude Extract | 0.169 | 17.14 | 2.90 | 0.88 | 3.30 | 100 | 1 |
| Manganese Chloride | 0.166 | 32.85 | 5.45 | 0.44 | 12.37 | 188 | 3.75 |
| Ammonium Sulphate | 0.076 | 29.44 | 2.24 | 0.38 | 5.89 | 77.2 | 1.78 |
| DEAE-Sephadex A50 | 0.115 | 19.00 | 2.19 | 0.14 | 15.64 | 75.5 | 4.74 |
| Sephadex G-200 | 0.051 | 26.73 | 1.36 | 0.11 | 12.36 | 46.9 | 3.75 |

Table 4.7 A Comparative Study of Km Values of D-Xylose Isomerase from *Lactobacillus* species

| Organisms | Purity | Substrate | Km (mM) | Reference |
|---|---|---|---|---|
| *Lactobacillus brevis* IFO 3960 | Pure | xylose | 5.0 | Yamanaka (1968) |
| *Lactobacillus xylosus* TUA 6-9 | Pure | xylose | 5.3 | Yamanaka and Takahara (1977) |
| *Lactobacillus brevis* NCDO-474 | Partially Purified | glucose | 0.8-1.6 | Kent and Emery (1973) |
| *Lactobacillus fermentum* ATCC 9338 | Partially Purified | xylose | 10.8 | Present work |

Figure 4.7 Polyacrylamide Gel Electrophoresis of D-xylose Isomerase of Various Purification Fractions




(1) Crude Extract
(2) Manganese Chloride Treatment
(3) Ammonium Sulphate Fractions (70-80%)
(4) DEAE-Sephadex A50 Fractions
(5) Sephadex G-200 Fraction No.15

L.fermentum ATCC 9338 was grown as a 10$\ell$ batch culture in MRS medium at 30° for 20 hours. After harvesting cytoplasmic material from disintegrated washed cells, gel preparations were standardized according to Section 3.4.7.

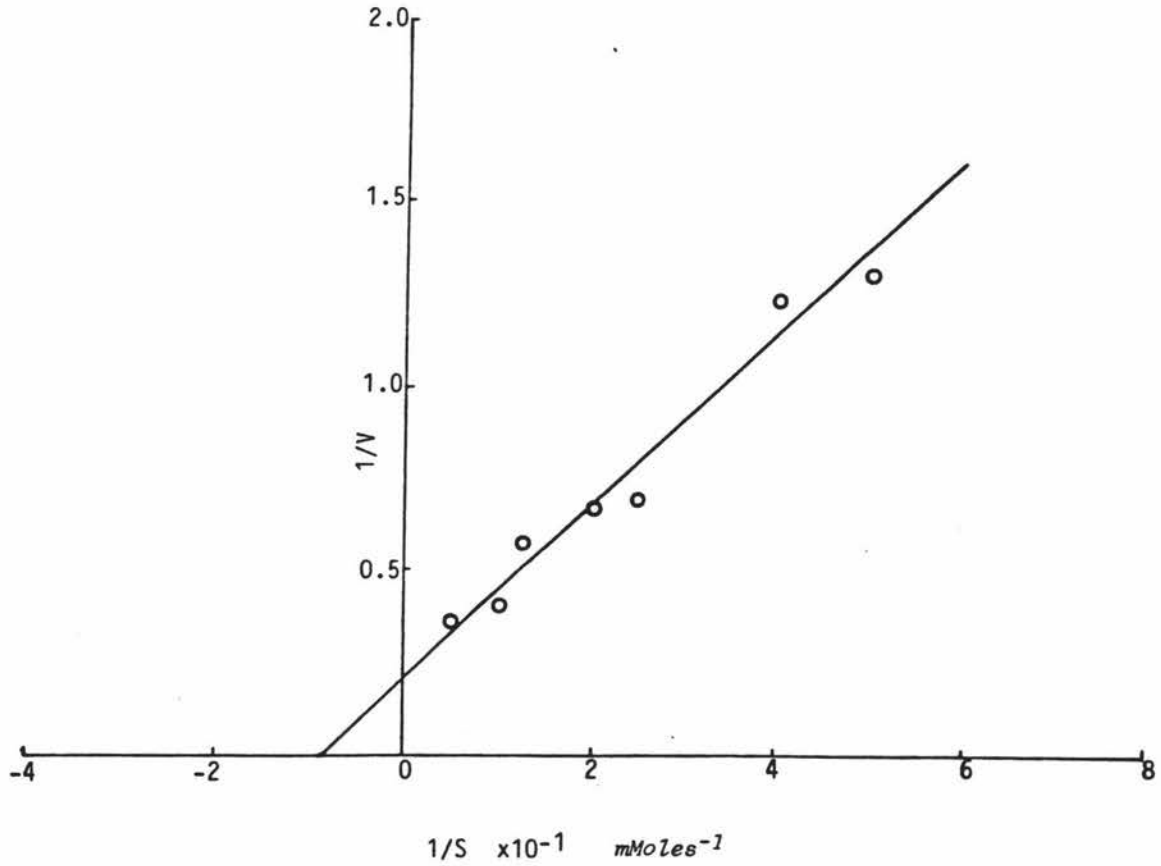



Figure 4.8 Lineweaver-Burk Plot of Data Collected from D-xylose Isomerase in Partially Purified Extract of *Lactobacillus fermentum ATCC 9338*

4.2.3.4 Study of D-xylose Isomerase Activity in Hydrolyzate

The loss of enzyme activity in an enzyme reaction mixture using hydrolyzate to replace distilled water is recorded in Table 4.8. The results indicated that the partially purified Lactobacillus D-xylose isomerase and the commercial `Sweetzyme' were completely inhibited by inhibitors in the hydrolyzate.

Table 4.8 A Study of Partially Purified Lactobacillus fermentum D-xylose Isomerase and `Sweetzyme' Activities in Pine Wood Acid Hydrolyzate

| Enzyme Used | D-xylose Isomerase Activity * (Units/l) x1,000 | |
|---|---|---|
| | Control (D-xylose Medium) | Test (Acid Hydrolyzate) |
| Lactobacillus fermentum (Partially Purified) | 21 | 0 |
| `Sweetzyme' Bacillus coagulans | 37 | 0 |

* All the experiments were carried out in duplicate.
`Sweetzyme' is the name given to the xylose isomerase marketed by Novo Industries, Copenhagen, Denmark.

4.2.4 DISCUSSION

Chen et al. (1980a and b) performed a comparative study of methods used to release D-xylose isomerase from Streptomyces flavogriseus. They found that both chemical (cationic detergent) and mechanical disruption methods released almost the same amount of enzyme. Kent and Emery (1973) compared heat autolysis and mechanical disruption methods. They claimed that heat autolysis was superior to mechanical methods for enzyme release. The present experiment compared chemical and mechanical methods and the combination of both. Cationic detergent alone released almost the same amount of enzyme as did mechanical disruption. Table 4.4 shows that the combination of both methods released the same amount of enzyme as the cationic detergent method. This research compared favourably with the results of Chen's study using Streptomyces flavogriseus.

The results suggested that the Lactobacillus fermentum D-xylose isomerase was a partially purified enzyme and had a higher Km value than the Km values of Lactobacillus brevis (Yamanaka, 1968) and Lactobacillus xylosus (Yamanaka and Takahara, 1977) isomerases using D-xylose as the substrate.

According to Mackie (1982a), lime neutralised hydrolyzate contains 1.4 g/l of calcium (Table 3.2). Calcium is an inhibitor of commercial Novo enzyme `Sweetzyme' and it was reported by Zittan et al. (1975) that as low as 35 mg/l of calcium concentration has an inhibitory effect. Fullbrook and Vabo (1977) also revealed that calcium is an effective inhibitor of the isomerization enzyme, `Sweetzyme'. They have strongly emphasized the importance of calcium removal to less than 1 mg/l in the production process for fructose syrups. Only after the removal of calcium ions from wood hydrolyzate will this raw material be suitable for treatment with D-xylose isomerase as a preliminary step to the ethanolic fermentation of the wood pentoses.

## 4.2.5 CONCLUSIONS

The extraction of D-xylose isomerase from the bacterial cells was performed using either chemical (cationic detergent), mechanical (cell homogenization) or a combination of both methods. Further purification of the Lactobacillus D-xylose isomerase by manganese chloride treatment, ammonium sulphate fractionation, followed by the column chromatography, ion exchange (DEAE-Sephadex A50) and gel filtration (Sephadex G-200) was carried out. This purification method yielded only a partially purified enzyme when examined by polyacrylamide disc gel electrophoresis. This partially purified D-xylose isomerase had a Km value of 10.8 mMole D-xylose.

Both the partially purified Lactobacillus D-xylose isomerase and commercially supplied `Sweetzyme' were completely inactivated by inhibitor(s) presumed to be calcium ions, present in the pine wood acid hydrolyzate. Hence, the study of the isomerization of D-xylose to D-xylulose by D-xylose isomerase and further conversion of the D-xylulose to ethanol by an appropriate yeast was discontinued.

# CHAPTER FIVE : PRELIMINARY SHAKEN FLASK STUDIES Pachysolen tannophilus IN D-XYLOSE SYNTHETIC MEDIUM AND WOOD HYDROLYZATE

## 5.1 INTRODUCTION

In Chapter Four, it was shown that pentose-fermenting bacteria produced a variety of end-products additional to ethanol, thus confirming reports in literature. The feasibility of an enzyme-yeast system was explored. This approach is a two stage process involving the enzymatic conversion of xylose to the easily fermented pentulose. Unfortunately, the enzyme (D-xylose isomerase E.C. 5.3.1.5.) was markedly inhibited, probably by the high concentration of calcium ions present in the wood hydrolyzate. This inhibition was seen as a possible problem in any commercial scale operation and requires further investigation. The first published reports of a D-xylose-fermenting yeast (Schneider et al., 1981a; Jeffries, 1981a) appeared at the time of these calcium ion inhibition studies. Consequently, work was re-directed towards a broad study of the growth and fermentation characteristics of Pachysolen tannophilus IFO 1007 in both a synthetic D-xylose medium and in prehydrolyzate.

## 5.2 EXPERIMENTAL METHODS

### 5.2.1 Shaken Flask Experiments

The shake flask studies in this programme were performed in 100 ml Erlenmeyer flasks containing Difco Yeast Nitrogen Base (No. 0392) at 6.7 g/l in either 50 ml prehydrolyzate or 50 ml synthetic medium with 20 g/l of one of the following sugars : D-xylose, D-glucose, D-mannose, D-galactose or L-arabinose. All flasks were incubated at 30° unless otherwise stated and with constant shaking at 50 r.p.m. (or 100, 150 or 200 r.p.m. where specified) in an Orbit Water- Bath Shaker (No.3535) or Environ Shaker (No.3597), Lab-line Instruments Inc.,

Melrose Park, Illinois, U.S.A. For ethanol assimilation studies, 16 g/l or 4 g/l absolute ethanol (B.D.H.) was used instead of 20 g/l of sugar as the growth substrate.

### 5.2.2 Yeast Cultures

Yeast cultures used are listed in Table 5.1. The cultures were obtained from different sources (see section 3.1.7). All the yeast cultures were maintained on Bacto Yeast Morphological Agar slopes.

## 5.3 RESULTS

### 5.3.1 Screening Yeasts for D-Xylose Fermentation

Pachysolen tannophilus is one of the few yeasts that can directly ferment D-xylose to ethanol. The genus Pachysolen is closely related, phylogenetically and physiologically, to the genus Hensenula (Wickerham and Burton, 1961; Wickerham, 1970; Yamada et al, 1973). Consequently, six species Hensenula, which are phylogenetically very close to Pachysolen tannophilus, four Candida species and a strain Kluyveromyces marxianus were tested for their ability to ferment D-xylose in synthetic media. Table 5.1 shows that Hensenula wickerhamii IFO 1706 and Hensenula capsulata IFO 0721 exhibited traces of ethanol in culture supernatants (ie. 0.4 g/l). Each strain Pachysolen tannophilus (NRRL Y-2460; NRRL Y-2461 and IFO 1007) produced 3 to 3.2 g/l ethanol from 20 g/l D-xylose. No further work was conducted using Hensenula, Candida or Kluyveromyces in view of the superiority of Pachysolen tannophilus strains.

Table 5.1 A Screening of Yeasts for D-Xylose Fermentation Capabilities

| Yeast Tested | | Ethanol Production (g/l) |
|---|---|---|
| *Candida macedoniensis* | UCD,FST 49-27 | nil |
| *Candida pseudotropicalis* | CBS 2234 | nil |
| *Candida tropicalis* | CBS 2321 | nil |
| *Candida tropicalis* | FD 0261 | nil |
| *Candida utilis* | Local isolated | nil |
| *Hensenula anomola* | IFO 0135 | nil |
| *Hensenula capsulata* | IFO 0721 | trace |
| *Hensenula holstii* | IFO 1479 | nil |
| *Hensenula saturnus* | IFO 1466 | nil |
| *Hensenula silvicola* | IFO 0807 | nil |
| *Hensenula wickerhamii* | IFO 1706 | trace |
| *Pachysolen tannophilus* | IFO 1007 | 3.0 |
| *Pachysolen tannophilus* | NRRL Y-2460 | 3.2 |
| *Pachysolen tannophilus* | NRRL Y-2461 | 3.2 |
| *Kluyveromyces marxianus* | Y 42 | nil |

Yeasts were grown in 20 g/l D-xylose plus 6.7 g/l Yeast Nitrogen Base medium at 30° for 7 days with agitation at 50 r.p.m.

### 5.3.2 Effect of Agitation Rates on Cell Growth and Ethanol Production

Four groups of flasks (group I to IV) containing 6.7 g/l Yeast Nitrogen Base plus 20 g/l D-xylose were prepared and cultures of Pachysolen tannophilus IFO 1007 were grown at 30°.

Group I, Static Flask Cultures (i.e. no agitation)

After 9 days of fermentation there was no significant increase in cell population and the observed ethanol yield 1 g/l was considered low.

Groups II and III , Shaken Flask Cultures

Flask agitation speeds were set at 50 (Group II) and 100 (Group III) r.p.m. on the Orbital Shakers. Results in Figure 5.1 show that by the 9th day, cells under slow agitation (50 r.p.m.) had yielded 4.8 g/l ethanol whereas those at 100 r.p.m. had produced 4.1 g/l. Both flask groups had formed comparably high cell densities by this time (9.2 and 9.8 $x10^7$ cells/ml) respectively and high ethanol concentrations.

Group IV, Magnetic Stirred Cultures

Flask cultures in this group were agitated using a magnetic stirrer set at 150 r.p.m. A rubber bung sealed each flask to prevent the entrainment of oxygen into the growth medium. Headspace air volume, estimated to be initially 258 ml, was available to the culture.

Cell population increased rapidly to 3.8 $x10^7$ cells/ml during the first two days. After this time, however, the growth pattern become quite irregular (Figure 5.1,IV) with the population diminishing and then increasing once more towards the 8th day.

The pattern of ethanol formation was similar to that seen in shaken flasks Group III (shake at 100 r.p.m.). The ethanol concentration had reached 4.1 g/l by the 9th day.

These studies demonstrated clearly the necessity to provide some degree of agitation (and aeration) to the fermentation. The rate of ethanol production (g/l.h) was very similar for all treatments and it seemed that agitation at 50 and/or 100 r.p.m. was appropriate for future shaken flask experiments.

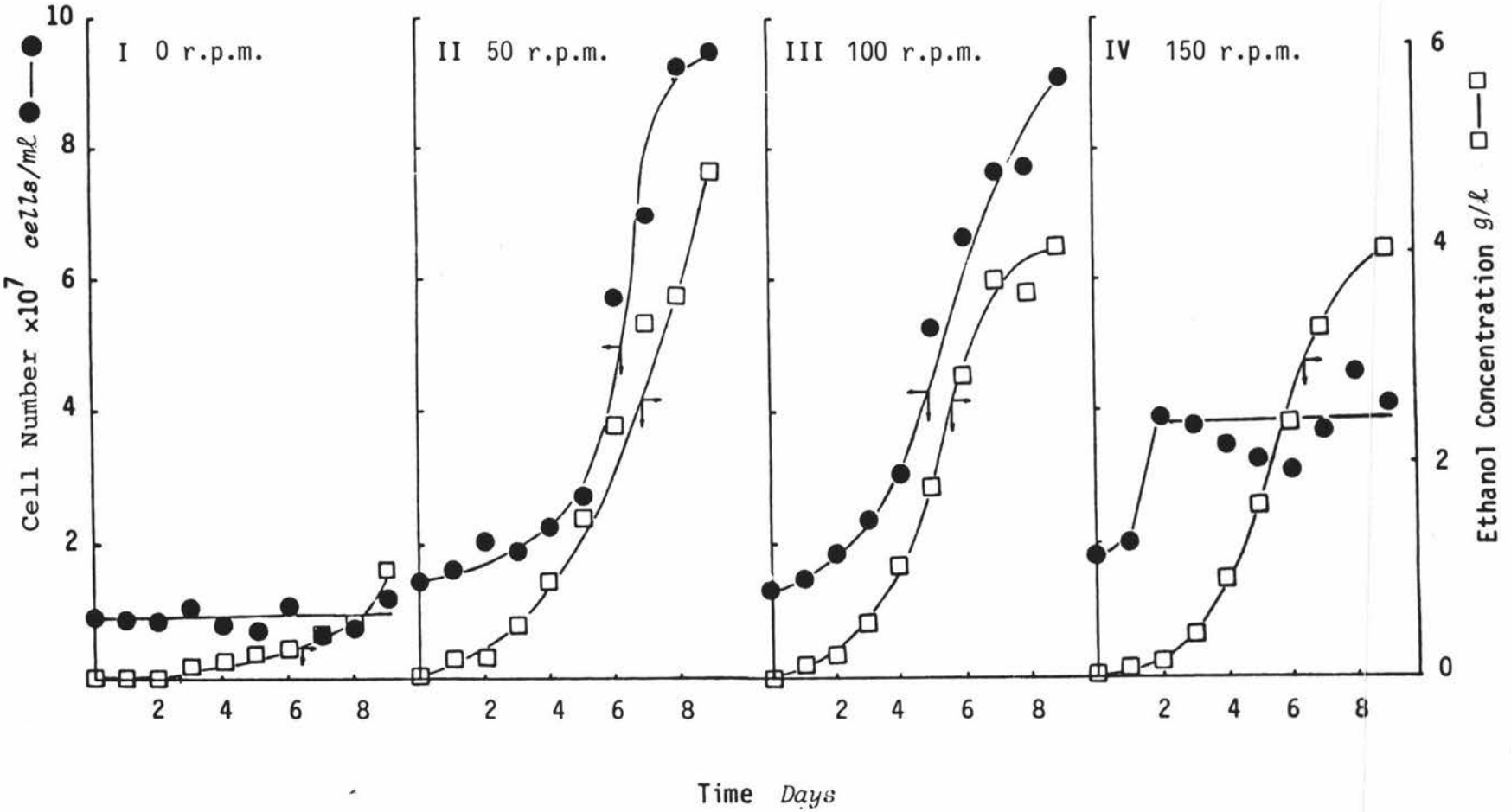



Figure 5.1 Effect of Agitation Speeds on Cell Growth and Ethanol Production in Shaken Flask Cultures of *Pachysolen tannophilus* IFO 1007 at an initial sugar concentration of 20 g/l (xylose).

### 5.3.3 Study of the Fermentation of Wood Sugars to Ethanol by Pachysolen tannophilus

It was desirable to test the ability of Pachysolen tannophilus IFO 1007 to ferment to ethanol the major sugar fractions of prehydrolyzate. An inoculum was prepared by pre-culturing the yeast in 20 g/l D-xylose containing 6.7 g/l Difco Yeast Nitrogen Base (No. 0392). "Exponential" growth phase cells were harvested, washed twice with sterile 0.02M phosphate buffer at pH 7.0 and then added to five groups of test flasks, each containing 20 g/l of a particular test sugar (i.e. D-glucose, D-mannose, D-xylose, D-galactose or L-arabinose) plus 6.7 g/l Difco Yeast Nitrogen Base (No. 0392) at 30°. Cultures were grown at 50 r.p.m.on an Orbit Water-Bath Shaker.

The results of these studies are shown in Figure 5.2. A rapid growth of cells to approximately $3.5 \times 10^8$ cells per ml was observed in both glucose and mannose substrates but poor growth developed in xylose, galactose and arabinose media. Galactose was utilized to completion as the culture moved into its stationary phase whereas arabinose and xylose demanded long adaption times (2 days) before growth commenced. At the termination of the experiment, growth was still proceeding in the arabinose and xylose flasks . The graphs illustrate clearly the slower utilization of these pentoses which contrasts with the more rapid uptake of the hexoses. The yeast's ability to ferment these wood sugars is also summarized in Table 5.2 where it is shown that glucose, mannose and xylose yield better than 3 g/l ethanol from 20 g/l sugars, even though the xylose and mannose fermentations were incomplete (see growth curves). The L-arabinose produced only traces ethanol at the 7th day of fermentation. Again, this particular fermentation was probably incomplete.

Table 5.2 Wood Sugars as Substrates for Growth and Fermentation using Pachysolen tannophilus in Shaken Flasks (50 r.p.m.) at 30° for 7 days

| Initial Sugar Concentration (20 g/l) | Cell Growth * ( x $10^8$ cells/ml) | Ethanol Production (g/l) |
|---|---|---|
| Arabinose | 2.54 ± 0.6 | 0.51 |
| Xylose | 2.14 ± 0.3 | 3.32 |
| Galactose | 2.04 ± 0.2 | 1.33 |
| Glucose | 3.52 ± 0.3 | 3.95 |
| Mannose | 3.63 ± 0.5 | 3.53 |

* Initial inoculum concentration = 1.36 ± 0.2 x $10^8$ cells/ml

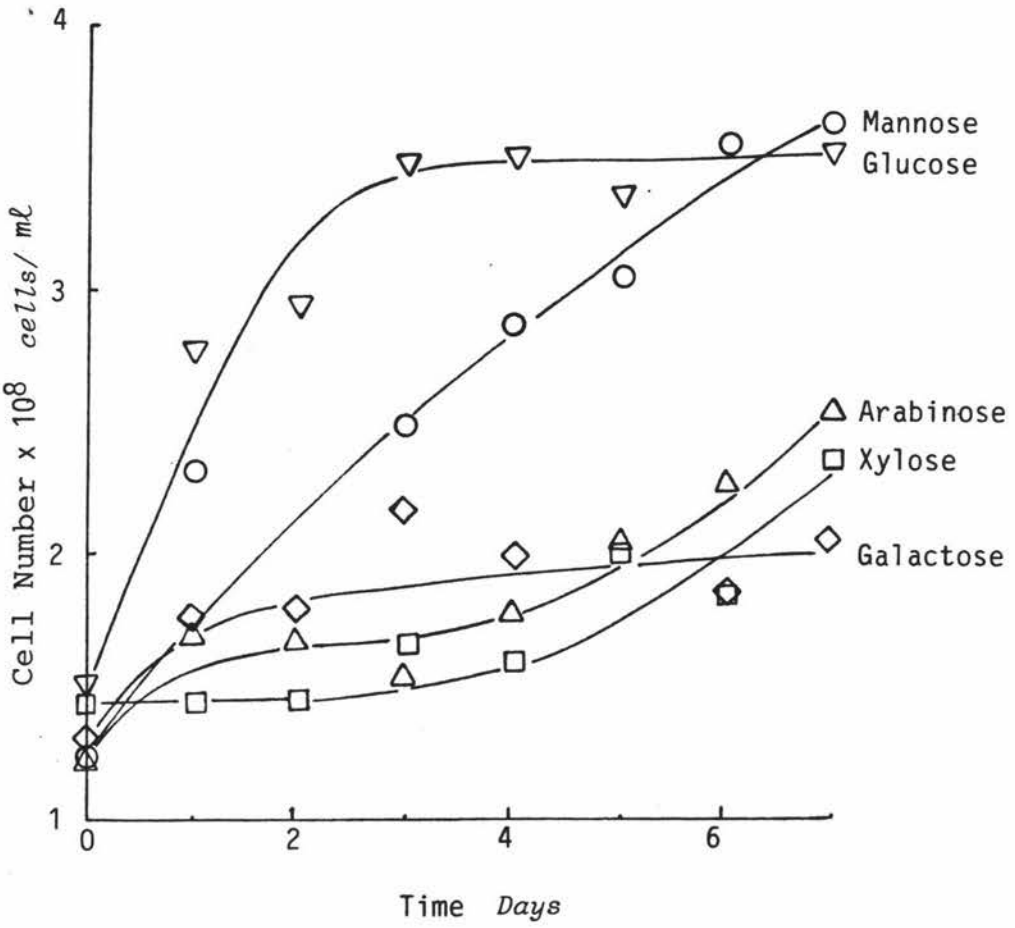



Figure 5.2 Fermentation of Wood Sugars to Ethanol by *Pachysolen tannophilus* with the Initial Sugar Concentration of 20 g/ℓ.

## 5.3.4 Effect of Inoculum Sizes on Cell Growth, Fermentation Time, Ethanol Production and Specific Growth Rate

Four inocula concentrations of Pachysolen tannophilus IFO 1007 were tested viz: 0.2%; 1.0%; 2.0%; and 20% by volume per volume medium. The medium used was 20 g/l of D-xylose and 6.7 g/l Yeast Nitrogen Base. Flasks were agitated at 50 r.p.m. Inocula were prepared as in 5.3.3. A washed suspension containing $4 \times 10^9$ cells was prepared and distributed into flasks to give the volumetric concentrations listed above. Cell counts were made on each inoculum.

The results (Figure 5.3A) show that the flasks with the highest inoculum (20% v/v) produced two-thirds of maximum ethanol (i.e. 3.4 g/l of ethanol) by the second day while all other flask cultures needed four to five days to achieve this same concentration. All flasks reached their peak yield within five to six days after the inoculation. Figure 5.3B illustrates that cultures with a low inoculum exhibited high specific growth rates and vice versa. At 2% and 20% inocula levels, the specific growth rates were 0.0087 $h^{-1}$ and 0.0067 $h^{-1}$ respectively. Interestingly, there was an inverse linear relationship between inoculum concentration and the fermentation time required to achieve two-thirds of maximum ethanol production (Figure 5.4)

Mathematical expressions were developed for the purpose of predicting the influence of inoculum concentration on both fermentation time and specific growth rate of an agitated flask culture with xylose as substrate.

Firstly, for Fermentation Time :

If Fermentation time, in hours, to two-thirds the final possible yield ethanol = T

Log cell numbers per ml in the inoculum = I

(Range from $10^7$ to $10^9$ cells per ml)

Then

$$T = 395 - 38.7\ I$$

This linear relationship suggests that using an inoculum value of $10^{10}$ cells per ml, fermentation time will approach its minimum value.

Secondly, for Specific Growth Rate :

If $\mu$ is specific growth rate ( $h^{-1}$ )

X is cell count ( x $10^7$ cells per ml)*

a is a constant

b is a constant

Then

$$\mu = 5.39 - aX + bX^2$$

where,

$a = 6.58 \times 10^{-8}$

$b = 8.20 \times 10^{-17}$

* (range from $10^7$ to $10^9$ cells/ml was used)

Second order reaction kinetics were followed in this case and population development was fastest when X was minimal.

These equations are illustrated in Figure 5.4 where it is demonstrated that increasing initial cell counts produced shorter fermentation times (graph A), lower specific growth rates (graph B) when the fermentaion was considered 66.7% complete.

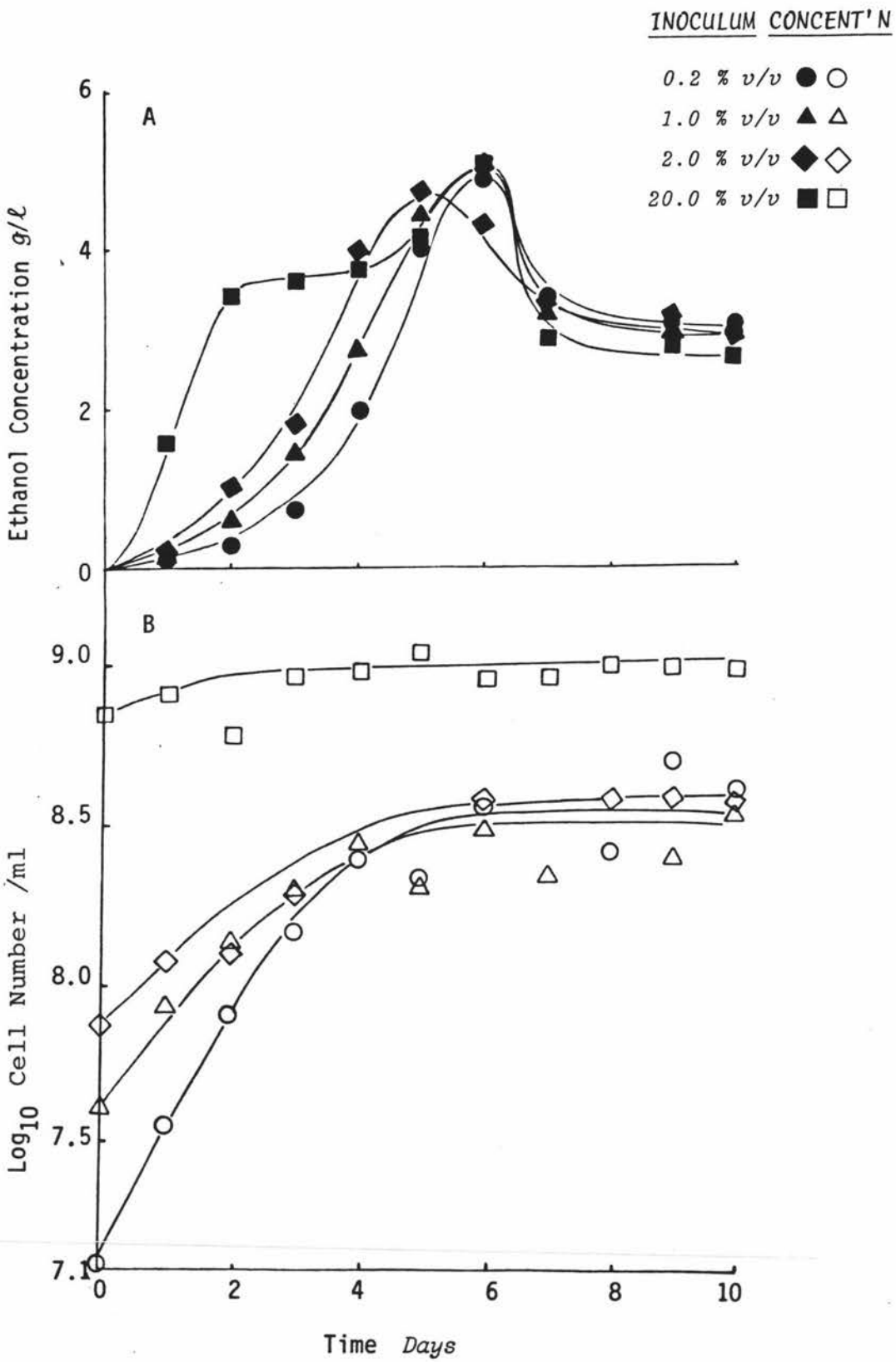



Figure 5.3 Effect of Inoculum Size on (A) Ethanol Production and (B) Cell Growth by *Pachysolen tannophilus* IFO 1007.

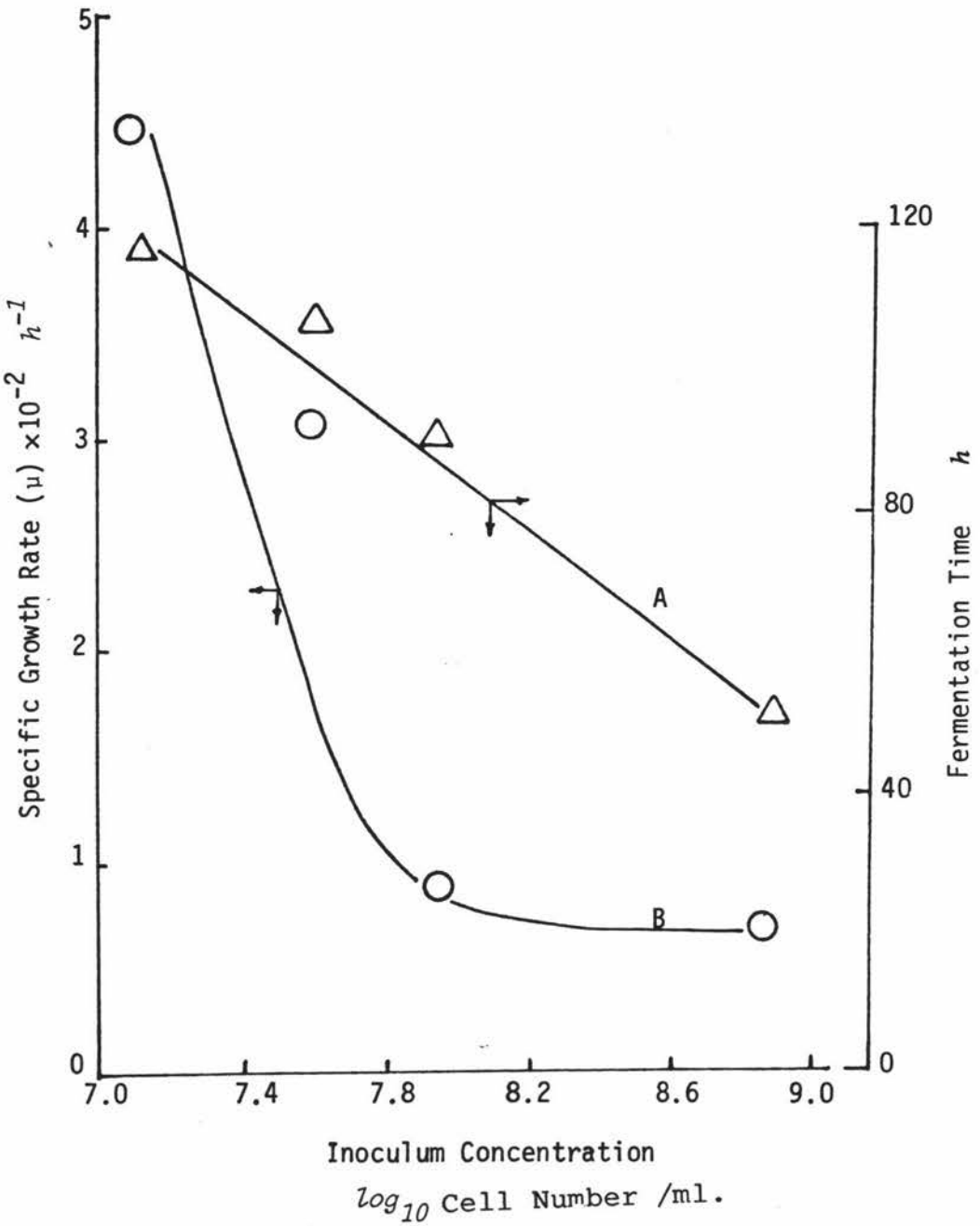



Figure 5.4 The Effect of Inoculum Concentration (cells/ml) on Specific Growth Rate and Fermentation Time (hours) to Two Thirds Maximum Ethanol Concentration.

### 5.3.5 Effect of Oxygen Supply on Cell Growth and Ethanol Production in the Shake-Flask Cultures

A series of shaken flasks studies were conducted by growing Pachysolen tannophilus IFO 1007 on 14 g/l D-xylose with 6.7 g/l Yeast Nitrogen Base in shaken flasks agitated at 200 and 100 r.p.m. respectively, with a heavy inoculum 6 x $10^8$ cells per ml.

The results showed that at 200 r.p.m. low ethanol concentration was produced on day one but rapidly disappeared afterward (Figure 5.5, block I). At 100 r.p.m. (Figure 5.5, Block II), lesser amounts of biomass were formed and nearly twice the amount of the ethanol was produced as compared with the 200 r.p.m. cultures. Ethanol rapidly disappeared from the culture supernatant through assimilation after the third day of incubation at 30°.

The other two experimental groups (see Figure 5.5, Block III and IV) were gyrated at 200 r.p.m. Group III was sparged with air and group IV with oxygen-free nitrogen at the 24th hour. Cell growth was reduced in both groups. However, higher ethanol concentrations were detected as compared with cultures in group I and II.

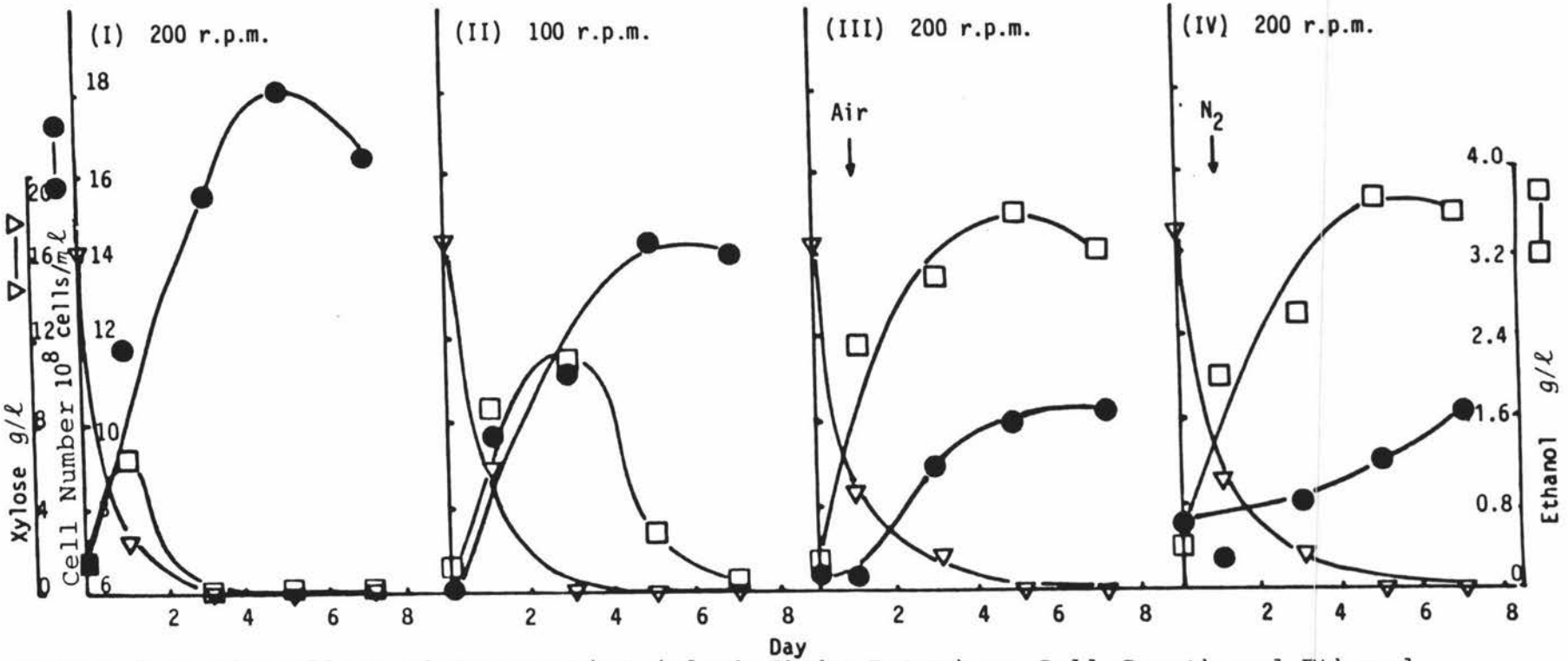



Figure 5.5 The Effect of Oxygenation (Flask Shake Rates) on Cell Growth and Ethanol Production in Shake Flask Cultures of *Pachysolen tannophilus* IFO 1007.

5.3.6 Assimilation of Ethanol by Pachysolen tannophilus

Pachysolen tannophilus IFO 1007 was grown in 20 g/l D-xylose with 6.7 g/l Yeast Nitrogen Base supplement for 24 hours with agitation at 50 r.p.m. Cells were harvested by centrifugation at 2,000×g and washed twice with sterile 0.02M phosphate buffer at pH 7.0. Then they were re-suspended in 50 ml 0.02M phosphate buffer at pH 7.0 for 6 hours before being used as an inoculum in a medium containing 0.5 or 2.0% v/v ethanol as growth substrate plus 6.7 g/l Yeast Nitrogen Base (Difco).

Figure 5.6(A) shows that the yeast grew well and assimilated 4 g/l ethanol. However, in 16 g/l ethanol (in Figure 5.6(B)), growth is initially inhibited, but a slight improvement was shownafter the 4th day of culture.

For the control flasks without ethanol (0 g/l), no growth was observed throughout the study period.

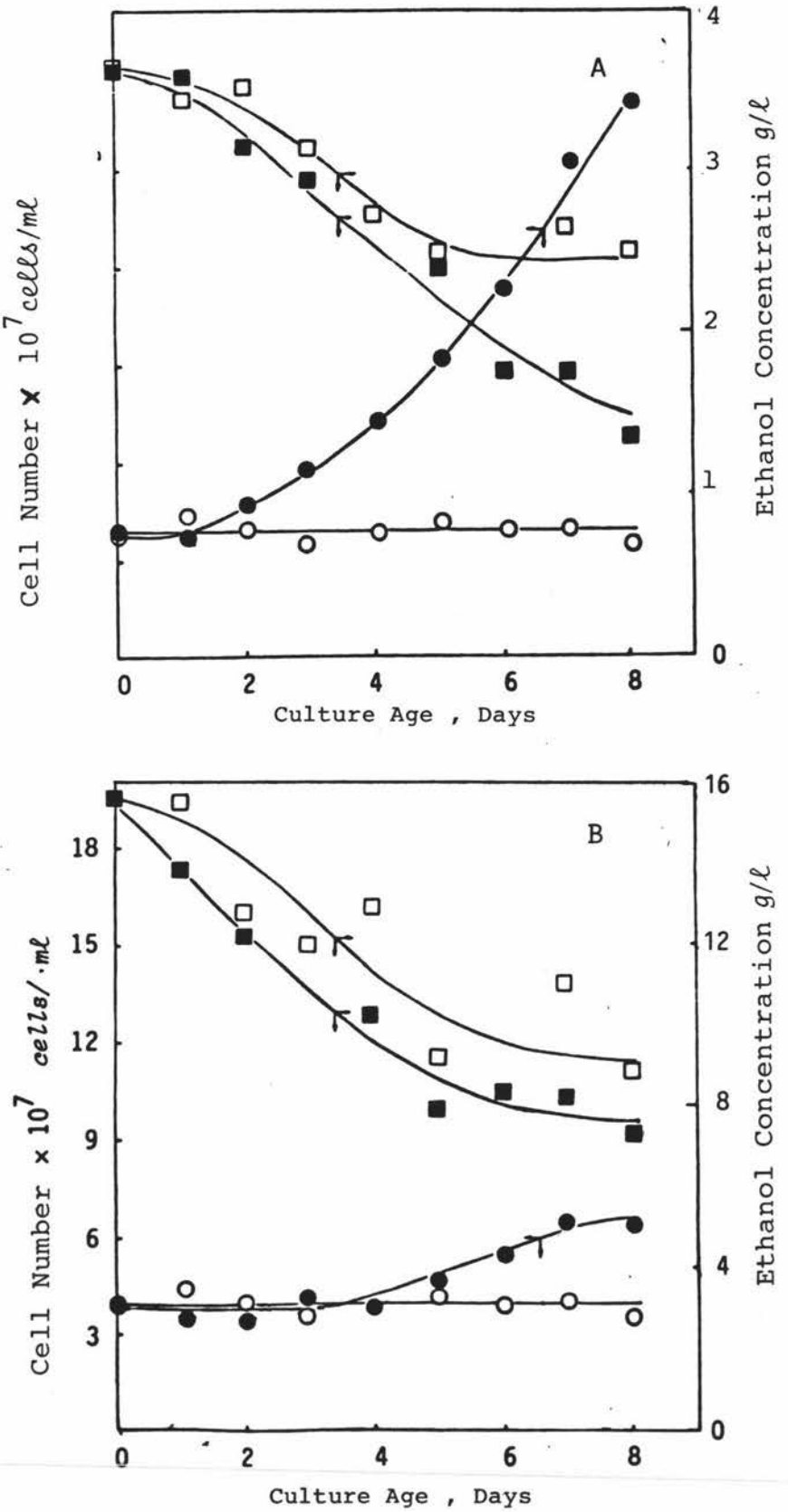



Figure 5.6 The Assimilation of Ethanol by *Pachysolen tannophilus* IFO 1007 in (A), 4 g/l Ethanol & (B) 16 g/l Ethanol added to a 6.7 g/l Yeast Nitrogen Base medium. o—o, is control; ●—● Test Flasks (Mean Cell Numbers); Square Symbols Indicate Duplicate Ethanol Assays.

### 5.3.7 Anhydrous Sodium Sulphite [$Na_2SO_3$] Treatment of the Prehydrolyzate

The use of sodium sulphite to overcome inhibitors in prehydrolyzate has been mentioned (2.6.6). Consequently, studies were initiated to determine the value of sulphite for improving growth rates of the test strains. Three different strains Pachysolen tannophilus were acclimatized to increasing concentrations of prehydrolyzate up to 50% v/v. After 48 hours of growth, 1 ml of inoculum was introduced into duplicate flasks of prehydrolyzate supplemented with 0.5, 1 and 2 g/l anhydrous sodium sulphite. The growth curve of each strain at each sulphite concentration was plotted and specific growth rates ($\mu$) of cultures were calculated. The collected results for strains NRRL Y-2460; 2461 and IFO 1007 are depicted in Figure 5.7.

The results show that the higher the sulphite concentration then the higher was the specific growth rate obtained and that the strain NRRL Y-2461 grew faster than the other two strains at any given sulphite concentration.

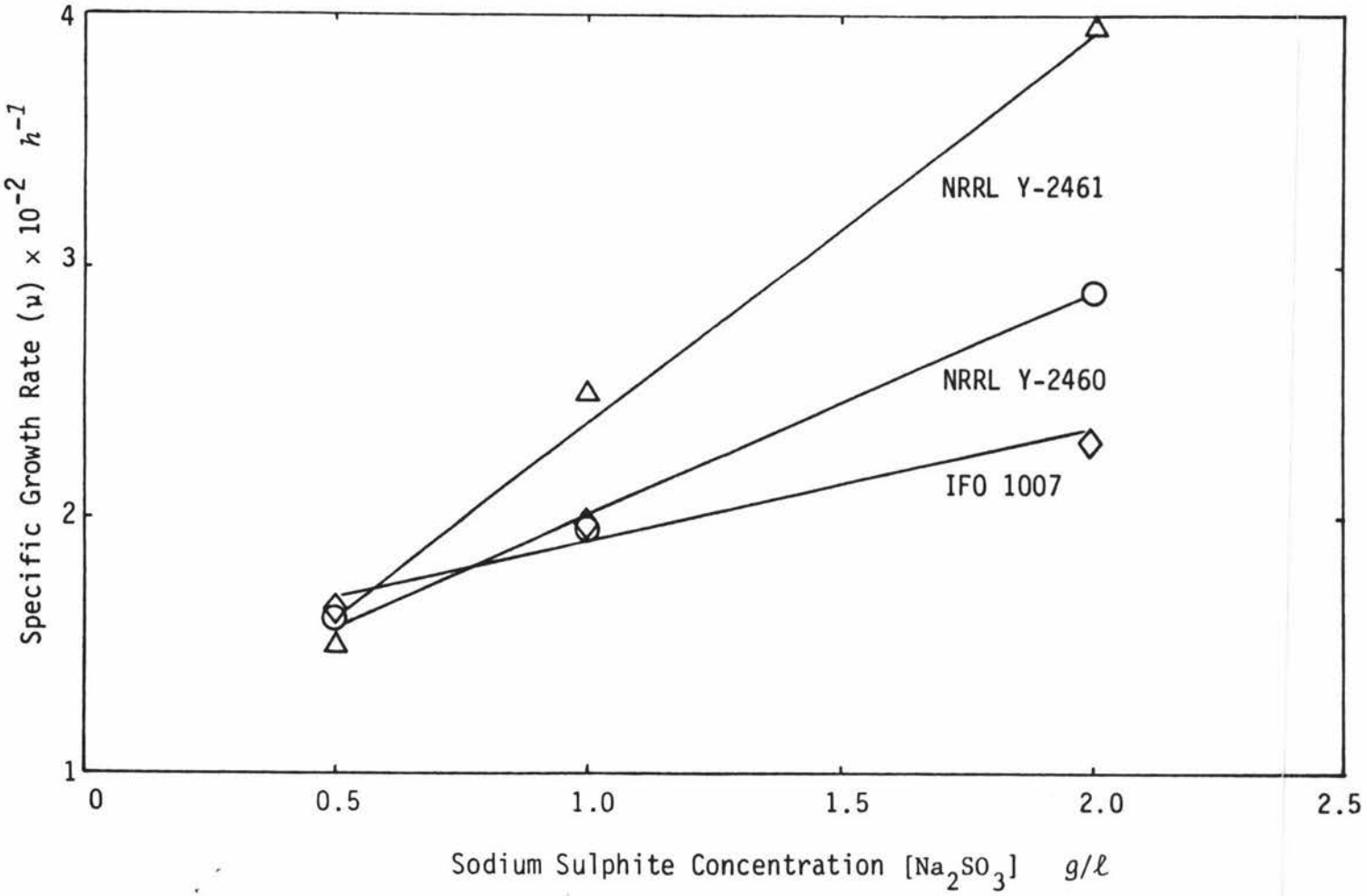



Figure 5.7 Effect of Sodium Suphite Concentration on the Specific Growth Rates of Three Different Strains of *Pachysolen tannophilus* in the Soft Wood Prehydrolyzate

## 5.4 DISCUSSION

The preliminary experimental work described in this Chapter has been pursued in parallel with that of other contemporary researchers elsewhere. With the discovery that the naturally occurring yeasts Pachysolen tannophilus (Schneider et al., 1981b) and Candida tropicalis (Jeffries, 1981b) would ferment D-xylose directly to ethanol, our efforts to screen D-xylose-fermenting yeasts were directed at those organisms which are closely related to Pachysolen tannophilus. Therefore, six species of Hensenula, four species of Candida and a strain of Kluyveromyces marxianus were screened. Regrettably, none of the yeasts tested at that time could produce an amount of ethanol that was superior to that produced by Pachysolen tannophilus. Hence, the screening programme was discontinued. Nonetheless there were a number of research groups in North America still screening for a better naturally occurring pentose-fermenting yeast. Maleszka and Schneider (1982a) reported that they had screened 15 different D-xylose-fermenting yeast strains and claimed that Candida guilliermondii and Candida terebra produced lower ethanol concentrations than Pachysolen tannophilus. Gong et al.(1983) tested 20 strains of Candida belonging to 11 species, 21 strains of Saccharomyces belonging to 8 species and 8 strains of Schizosaccharomyces pombe. Their results showed that the majority of the Schizosaccharomyces pombe strains tested produced ethanol at concentrations ranging from 1 to 5 g/l from D-xylose. Particular strains of Candida blankii and Candida tropicalis produced ethanol in highest concentrations. Margaritis and Bajpai (1982) reported eight of the strains of Kluyveromyces marxianus, capable of fermenting the aldopentose directly to ethanol under aerobic conditions. du Preez and van der Walt (1983) also reported a strain of Candida shehatae able to ferment 90 g/l D-xylose directly to ethanol within 40 hours under aerated conditions. Sakano and Mikata (1983) screened 4 strains of Pichia stipitis, a strain of Candida shehatae and Candida steatolytica. They found that static cultures of Pichia stipitis and Candida shehatae produced 11 to 18 g/l of ethanol after 5 days of incubation, but that recycled cells would produce 18 g/l of ethanol from 50 g/l of D-xylose after only 2 days of incubation. A large screening programme for

D-xylose-fermenting yeasts was carried out by Toivola et al. (1984). They tested the type strains of 200 species of yeasts capable of fermenting glucose and of growth on xylose. Brettanomyces naardenensis, Candida shehatae, Candida tenuis, Pachysolen tannophilus, Pichia segobiensis and Pichia stipitis all produced more than 1 g/l of ethanol from a 20 g/l initial D-xylose concentration.

Shaken flask studies described in this thesis demonstrated that Pachysolen tannophilus could ferment most wood sugars, namely D-glucose, D-xylose, D-mannose, D-galactose and L-arabinose, which are present in the prehydrolyzate, although the rapidity and completeness of pentose and hexose fermentation seemed to be variable. Dekker (1982) reported that his Pachysolen tannophilus could ferment D-xylose, L-arabinose, D-glucose, D-fructose, D-cellobiose and D-glucuronic acid while Malsezka et al. (1982b and c) and Debus et al. (1983) both reported that Pachysolen tannophilus could ferment D-galactose to ethanol but only after a relatively long adaptation period. Our preliminary shaken flask studies confirmed this phenomenon.

That Pachysolen tannophilus is capable of simultaneously producing and assimilating ethanol, even in the presence of available carbohydrate, has been reported by Maleszka and Schneider (1982b). Figures 5.6(a) and (b) indicated that Pachysolen tannophilus could assimilate ethanol at 4 and 16 g/l, although the latter concentration seemed to have an inhibitory effect on growth as evidenced by a lag period of four days. The assimilation of ethanol has bneen observed in Saccharomyces cerevisiae. Thus, Wills et al. (1982) claimed that cytoplasmic alcohol dehydrogenase I of Saccharomyces cerevisiae is primarily responsible for the formation of the ethanol in yeast and that mitochondrial alcohol dehydrogenase was involved in alcohol assimilation. The results reported by Maleszka and Schneider (1982b) and the present observation that Pachysolen tannophilus assimilates ethanol suggest the possibility that both cytoplasmic and mitochondrial alcohol dehydrogenases are present.

These enzymes are thus held responsible for both the assimilation and production of the ethanol from D-xylose.

The presence of toxic substances in wood hydrolyzates hindered yeast growth and pentose fermentation. Detroy et al. (1982a) demonstrated that furfural concentrations of only 2.5 to 3.0 g/l were lethal to Pachysolen tannophilus. The results in Section 5.3.7 indicated that 2 g/l of anhydrous sodium sulphite removed much of the inhibitory substances in the prehydrolyzate. With respect to yeast growth in the presence of sulphite, it was found that strain NRRL Y-2461 was much superior to the other two strains. Leonard and Hajny (1945) and Deverall and Whitworth (1980) also recommended the use of hot sodium sulphite to improve the fermentability of acid hydrolyzates. Recently, Lee and McCaskey (1983) also reported that the presence of the toxic substances could inhibit pentose fermentation by Pachysolen tannophilus. Most recently, Clark and Mackie (1984) who further investigated fermentation inhibitors in wood hydrolyzates derived from the softwood Pinus radiata claimed that the inhibitory substances were derived from carbohydrate degradation and additionally low molecular weight phenolics derived from lignin were toxic to the yeast Saccharomyces cerevisiae. Furthermore, the effects of these phenolic components were more lethal than the inhibitors derived from carbohydrate degradation.

High inoculum concentrations could overcome inhibitory compounds in the hydrolyzate but these increased inoculum levels decreased the specific growth rates. An inverse linear relationship between inoculum concentration and fermentation times was observed in the present studies.

Similar results have been reported by Strehaiano et al. (1983). These investigators used three different Saccharomyces yeasts. They observed that a decrease in the fermentation time, maximum specific growth rate and biomass yield occurred with increasing inoculum levels.

The influence of oxygen supply on the D-xylose fermentation in terms of aeration or agitation speed of the flask seemed to be critical for growth of Pachysolen tannophilus (Schneider, 1981b; Debus et al., 1983 and Mutz and Wandrey, 1983). Gong et al. (1981e) reported that a Candida mutant XF217 was capable of producing ethanol from D-xylose

under aerobic and anaerobic conditions and claimed that oxygen was essential for the uptake of D-xylose in the yeast. The data from these flask studies revealed that a static, non-agitated culture yielded only low cell populations and ethanol concentrations. Mild agitation of 50 and 100 r.p.m. would produce 4.8 and 4.1 g/l of ethanol respectively, whereas a heavy inoculum with a high agitation speed of 200 r.p.m. resulted in low ethanol and high cell yields. Under similar conditions, except that oxygen-free nitrogen gas was sparged into the culture after 24 hours of aerobic growth the previously observed yield patterns were reversed (see Figures 5.3.5) to give high ethanol and low cell yields. This fermentation pattern has been observed by Auling et al. (1984).

## 5.5 CONCLUSIONS

A study of D-xylose-fermenting yeasts which are phylogenetically and physiologically, related to Pachysolen tannophilus, such as Hensenula and Candida species, has not detected any that are superior to Pachysolen tannophilus with respect to an ability to produce ethanol from D-xylose.

Preliminary shaken flask studies focussed on the growth and fermentation characteristics of Pachysolen tannophilus. This yeast fermented all sugars in the prehydrolyzate, although the fermentation of pentoses was somewhat delayed and the yield of ethanol from L-arabinose as substrate seemed variable. Low amounts of ethanol (that is 4 g/l) could be easily assimilated by Pachysolen tannophilus when ethanol was used as a sole carbon source. A lag period as long as 4 days was observed when substrate ethanol was increased to 16 g/l.

Neutralization with anhydrous sodium sulphite of the inhibitory substances existing in the prehydrolyzate was demonstrated. Strain NRRL Y-2461 grew much better in prehydrolyzate with 20 g/l of anhydrous sodium sulphite than did the other two strains NRRL Y-2460 and IFO 1007. Large yeast inocula also overcame the inhibitors in prehydrolyzate. These increased concentrations of inocula lowered the

yeast's specific growth rate but shortened the fermentation time.

Agitation rates for shaken flask cultures of Pachysolen tannophilus produced quite significant growth and fermentation patterns depending on whether static, mild (50 and 100 r.p.m.) or rapid (200 r.p.m.) agitation was used. At the two extremes, static cultures produced traces of ethanol and low cell population while high agitation speeds produced low ethanol production but high cell populations. Mild agitation favoured maximum ethanol yields.

Consumption of ethanol occurred immediately after the depletion of D-xylose. Admitting to the growth vessel oxygen-free nitrogen gas 24 hours after inoculation drastically reduced the final cell yield but allowed the accumulation of high concentrations of ethanol. Thus, supply of oxygen into the culture seemed to be a critical characteristic of this fermentation as was an obligatory requirement to avoid total aerobiosis and total anaerobiosis.

# CHAPTER SIX : EXPERIMENT 1 - INTERACTIVE EFFECTS OF SUBSTRATE CONCENTRATION AND INOCULUM LEVEL ON PENTOSE FERMENTATION IN THE SYNTHETIC MEDIUM

## 6.1 INTRODUCTION

The preliminary shake flask studies described in Chapter 5 showed that at the initial sugar concentration of 20 g/l, increasing inoculum concentrations resulted in decreased specific growth rate. The purpose of the central composite statistically designed experiment described in this Chapter was to study the possible interactive effects of substrate and inoculum concentration on the cell yield and ethanol production by Pachysolen tannophilus IFO 1007 in the D-xylose synthetic medium.

## 6.2 EXPERIMENTAL DESIGN

Experimental design Number 1 was a two-factor, $2^2$, factorial central composite rotatable design experiment described by Eroshin et al. (1976); Mullen and Ennis (1979) was chosen to further investigate the main and interaction effects of inoculum size.

This experiment design was generated according to the rules outlined in Appendix 5 and can be represented as an octagon, shown in Figure 6.1. Table 6.1 shows the coded and uncoded values of the two independent variables, substrate concentration and inoculum size. These were encoded accord to the equations :

$$\text{Substrate Coded Value (S)} = \frac{\log s - 0.452}{0.319}$$

$$\text{Inoculum Coded Value (I)} = \frac{\log i - 0.801}{0.354}$$

where, s is the uncoded substrate concentration ( g/l )

i is the uncoded inoculum concentration ( ml/l )

The encoded variables S and I took the levels -1.414, -1, 0, +1 and +1.414.

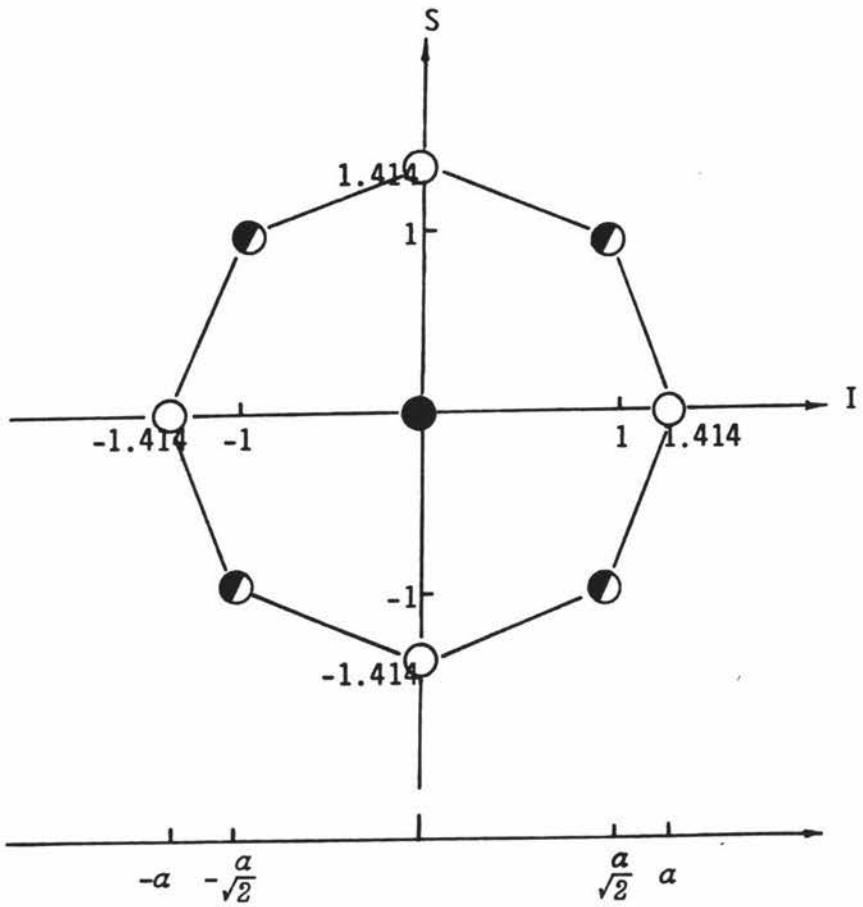



Figure 6.1 Two-factor Central Composite Rotatable Design with Axial 'Star' Points at Coded Distance $\alpha$ = 1.414 from the Origin. S and I represented the Two Independent Variables, Substrate and Inoculum Concentrations Respectively.

Table 6.1 A Central Composite Rotatable Statistically Design Experiment to Study the Interactions of Substrate Concentration and Inoculum Concentration on Ethanol Production and Cell Yields

| Flask number | Substrate Concentration (D-xylose) | | Inoculum Concentration (strain IFO 1007) | |
|---|---|---|---|---|
| | Coded (S) | Uncoded (s) g/l | Coded (I) | Uncoded (i) ml/l |
| 1 | -1 | 1.36 | -1 | 2.80 |
| 2 | 1 | 5.90 | -1 | 2.80 |
| 3 | -1 | 1.36 | 1 | 14.28 |
| 4 | 1 | 5.90 | 1 | 14.28 |
| 5 | $-\alpha$ | 1.00 | 0 | 6.32 |
| 6 | 0 | 2.83 | $-\alpha$ | 2.00 |
| 7 | $+\alpha$ | 8.00 | 0 | 6.32 |
| 8 | 0 | 2.83 | $+\alpha$ | 20.00 |
| 9 | 0 | 2.83 | 0 | 6.32 |
| 10 | 0 | 2.83 | 0 | 6.32 |
| 11 | 0 | 2.83 | 0 | 6.32 |
| 12 | 0 | 2.83 | 0 | 6.32 |
| 13 | 0 | 2.83 | 0 | 6.32 |
| 14 | 0 | 2.83 | 0 | 6.32 |
| 15 | 0 | 2.83 | 0 | 6.32 |
| 16 | 0 | 2.83 | 0 | 6.32 |

where, $\alpha = \pm 1.414$ (Coded Value)

The eight replicate flasks at centre point (coded at 0,0) and the remaining eight flasks at factorial and `star' points, were randomly arranged during incubation.

## 6.3 FERMENTATION CONDITIONS

Each experimental 100 ml flask contained 50ml of 6.7 g/l of Yeast Nitrogen Base plus D-xylose at a concentration described in Table 6.1. The inoculum of Pachysolen tannophilus IFO 1007 was prepared as follows. The yeast was adapted through three consecutive transfers in the above medium. Experimental flasks were inoculated using volumes as indicated in Table 6.1 with exponential phase cells from the final transfer. Incubation was at 30 °C with agitation at 100 r.p.m. using an Orbit Water-Bath Shaker (No. 3535) (Lab-Line Instruments Inc., Melrose Park, Illinois, U.S.A.). Fermentation was continued for 10 days, during which daily samples were collected for cell counts (Section 3.4.1) and ethanol analyses (Section 3.4.5).

## 6.4 RESULTS

For each experimental flask, cell yields and ethanol concentrations were plotted against fermentation time. Smooth curves were drawn to compute the specific ethanol production rates, maximum instantaneous ethanol production rates and average ethanol production rates (Figure 6.2). These derived results are listed in Appendix 6.

An empirical mathematical equation of the following form can be fitted to the data : (Box et al., 1978)

$$\hat{Y} = \beta_0 + \beta_S S + \beta_I I + \beta_{SI} SI + \beta_{SS} S^2 + \beta_{II} I^2$$

where, $\hat{Y}$ is the predicted value of the dependent variables

$\beta$ represents the coefficients

S and I are coded values of substrate concentration and inoculum size respectively

$\beta_0$ is the Y-intercept or constant term

The data in Appendix 6 were statistically analyzed using the multiple regression facility of the MINITAB computer package (MINITAB, version II, 1982, Pennsylvania State University).

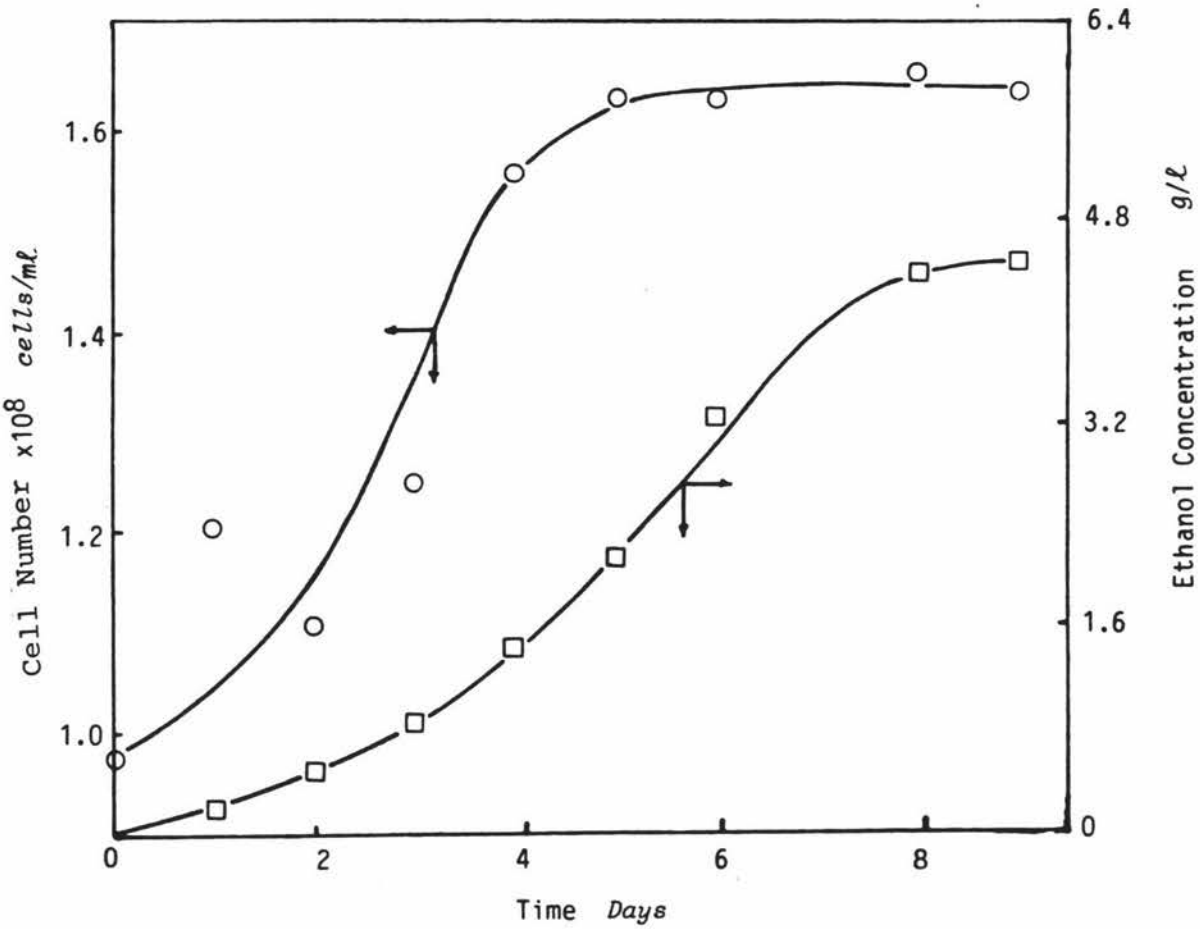



Figure 6.2 Plot of Cell Growth and Ethanol Production against Fermentation Time from One Flask at the Centre Point (0,0,0) of the Experimental Design 1. Pachysolen tannophilus IFO 1007 Used Throughout.

The full regression models are presented in Table 6.2. parsimonious models were derived from the full regression models by either a stepwise regression routine or by t-test.

These coefficients with asterisks, shown in Table 6.2, signify a certain level of statistically significance.

The assessment of the statistical significance of main and interaction effects, the estimation of response error (i.e. pure error) and test for lack of fit of the parsimonious models have been compiled in Appendix 7.

The pure error was calculated directly from the centre point replications. The test of lack of fit for each parsimonious model was evaluated by checking the ratio of lack of fit mean square (MSLF) to pure error mean square (MSPE) (Berenson et al., 1983).

$$F_{lof} = \frac{MSLF}{MSPE}$$

The adequacy of each parsimonious equation model was further checked by examining different residual plots.

(1) Standardized Residuals $(Y - \hat{Y})$ were plotted against predicted values $(\hat{Y})$ to check for the equality of residuals.
(2) Standardized Residuals were plotted against all the values of independent variables S and I to check for the homoscedasticity of residuals.
(3) Standardized Residuals were plotted against normal random numbers to check the normality of residuals.

Table 6.2 Full Regression Models for Experiment 1.

| COEFFICIENT | ETOH.YD g/l | M.ETOH.R g/l.h | 90%ETOHT h | 1/SpEtOH $_1$ (g/g.h) | CELL.YD cells/l | M.AVETOH g/l.h |
|---|---|---|---|---|---|---|
| $\beta_0$ | 4.84*** | 0.045*** | 170.63*** | 36.44*** | 1.78*** | 0.0255*** |
| $\beta_S S$ | 2.16*** | 0.051*** | 20.30*** | -30.45*** | 0.085 | 0.0092*** |
| $\beta_I I$ | -0.17 | -0.023*** | -16.26*** | 11.50** | 0.76*** | 0.0016 |
| $\beta_{SI} SI$ | 0.25 | -0.019** | 11.70*** | -8.58 | 0.26* | 0.00014 |
| $\beta_{SS} S^2$ | -0.086 | 0.022*** | -8.10*** | 4.33 | 0.22* | -0.00026 |
| $\beta_{II} I^2$ | -0.17 | 0.012** | -12.75*** | 0.091 | 0.45*** | 0.0013 |

*** Statistically significant at the 1 % level.

** Statistically significant at the 2 % level.

* Statistically significant at the 5 % level.

YD = Ethanol Yield, g/l.
M.ETOH.R = Maximum Ethanol Production Rate, g/l/h.
90%ETOH.T= Time (hours) to produce 90% of final ethanol concentration.
SpETOH = Specific Productivity of Ethanol, g EtOH/g Cells/hour.
M.ETOH.R = Maximum Ethanol Production Rate, g/l/h.
Cell YD = Yield (dry-weight) Cells per litre culture.
AV ETOH = Average production rate of Ethanol, g/l/h.

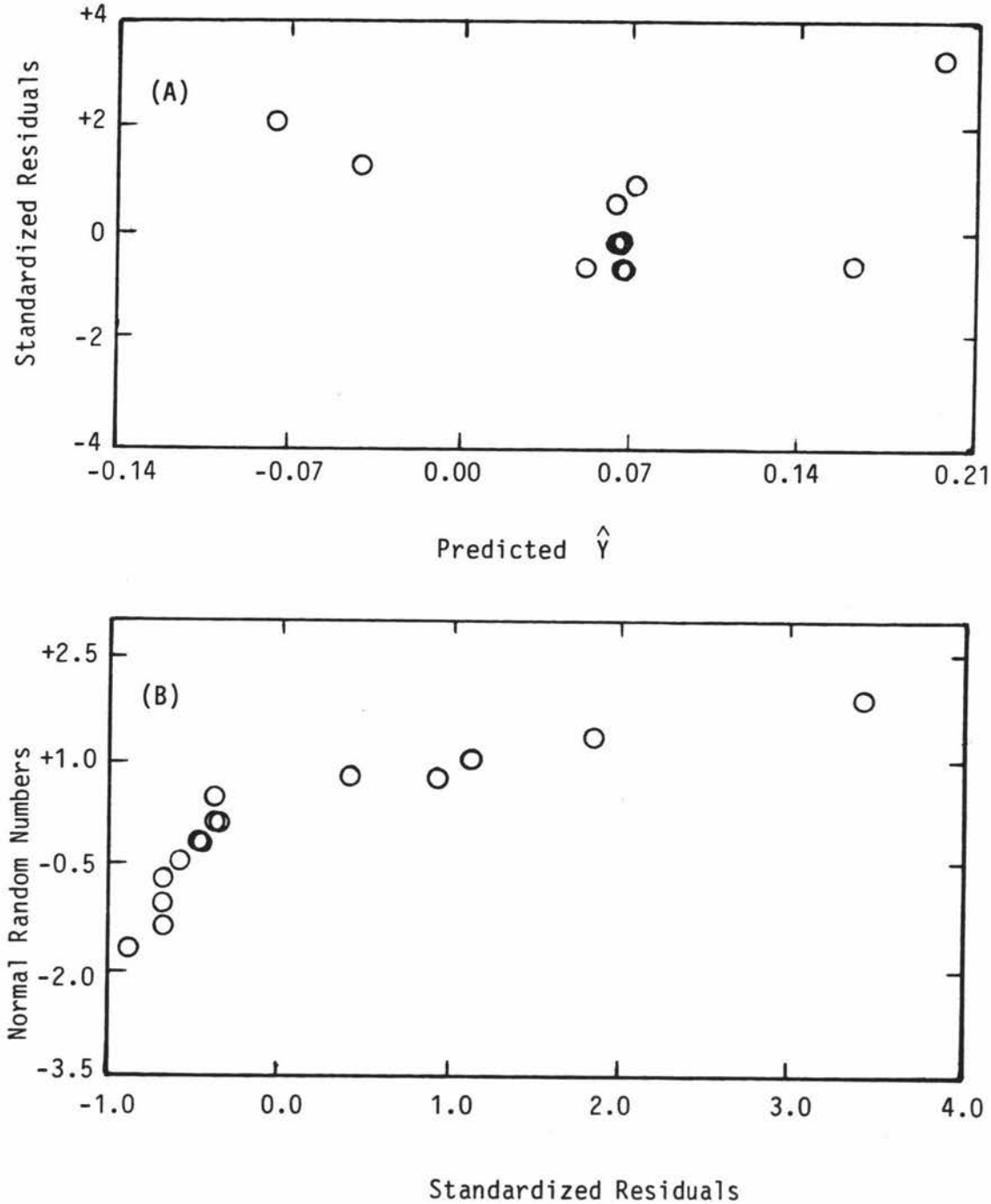



Figure 6.3 Residual Plots (A) Standardized Residuals versus Predicted Y (B) Standardized Residuals versus Normal Random Numbers for Parsimonious Equation for Specific Ethanol Production Rate of Experiment 1

The residual analysis and test of lack of fit for all the derived linear and parsimonious models listed in Table 6.2 were carefully evaluated. Only the model derived for specific ethanol production rate revealed a low R-squared value of 58.2% with a marked lack of fit and also demonstrated poor normality of the residual plots (Figures 6.3 A and B).

A new model was derived for reciprocal of specific ethanol production rate. This fitted the data adequately and gave acceptable residual plots.

$$1/\text{Specific Ethanol Rate} = 3.64 - 30.5\ S + 11.5\ I$$

To help locate the optimum conditions, the response surfaces described by the parsimonious models in Table 6.2 were plotted and contours were drawn. Figure 6.4 is such a plot for fermentation time. The marked asterisk in the contour plot represents the maximum predicted fermentation time of 183.8 hours. The minimum fermentation time is of more interest, occurring at low initial substrate concentration and high inoculum level. The opposite conditions gave maximum cell yield as shown in Figure 6.5. Maximum instantaneous ethanol production rate (Figure 6.6) was also predicted at high initial substrate level (80 g/l) but at the low inoculum level (i.e. 20 ml of standardized cell suspension per litre of culture medium).

It should be noted that in these graphs the axes are retained in the coded forms for convenience of presentation.

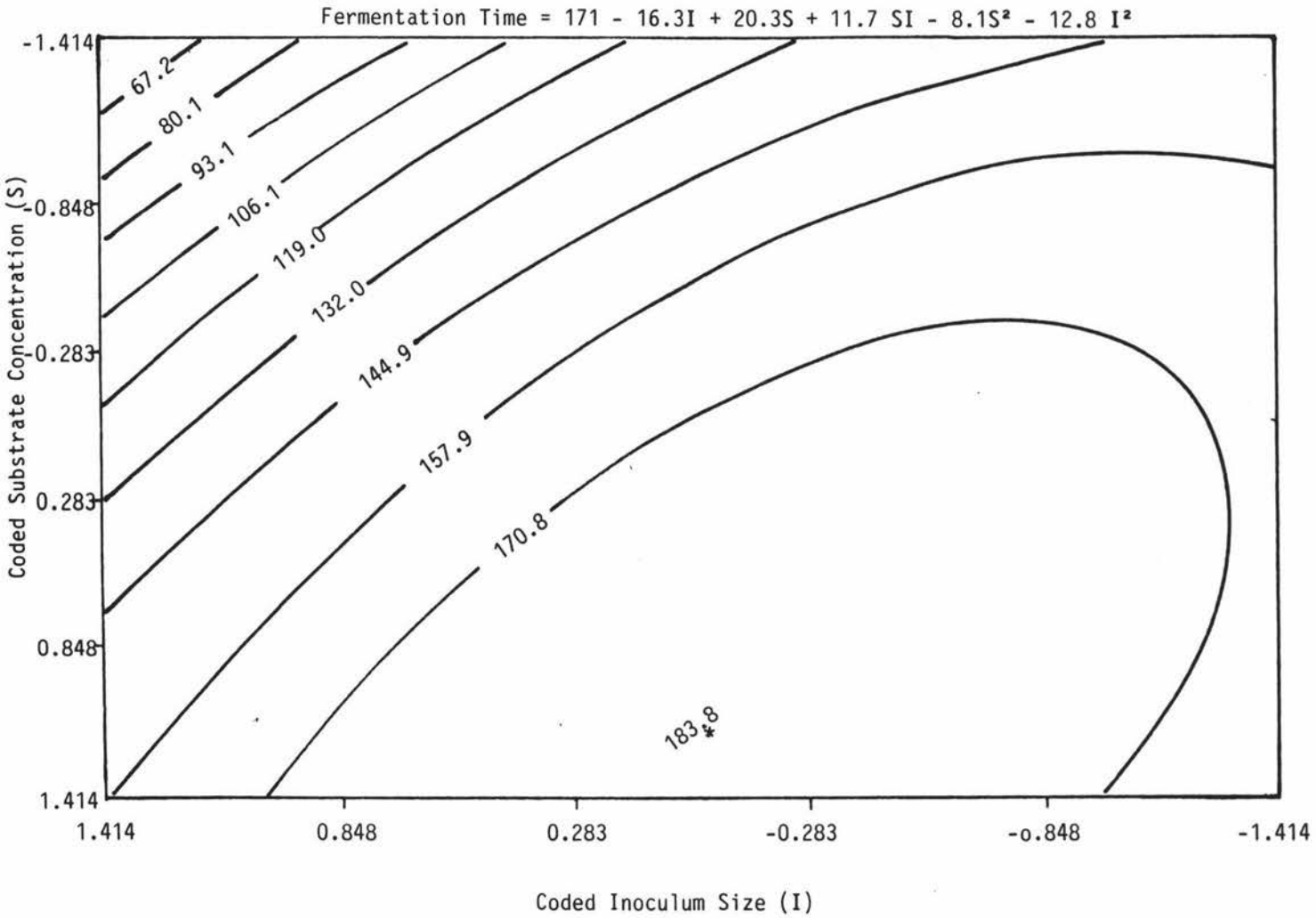



Figure 6.4 Contour Plots of Fermentation Time Observed in Shaken Flask Studies of *Pachysolen tannophilus* as a Function of Substrate Concentration and Inoculum Level in Synthetic Medium . (Strain IFO 1007 used throughout).

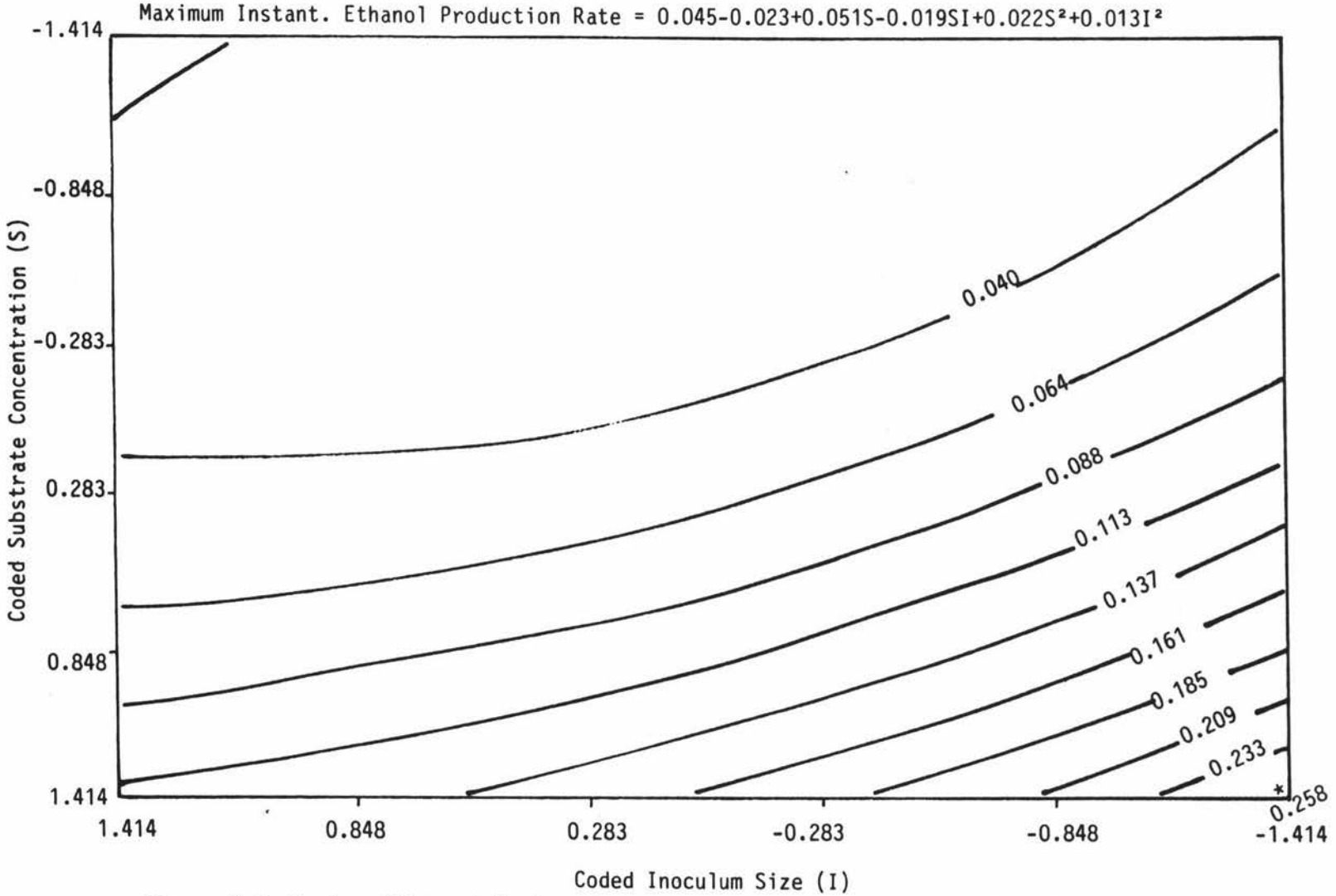



Figure 6.5 Contour Plots of Maximum Instantaneous Ethanol Production Rate in a Shaken Flask Studies of *Pachysolen tannophilus* as a Function of Substrate Concentration and Inoculum Level in Synthetic Medium (Strain IFO 1007 used throughout).

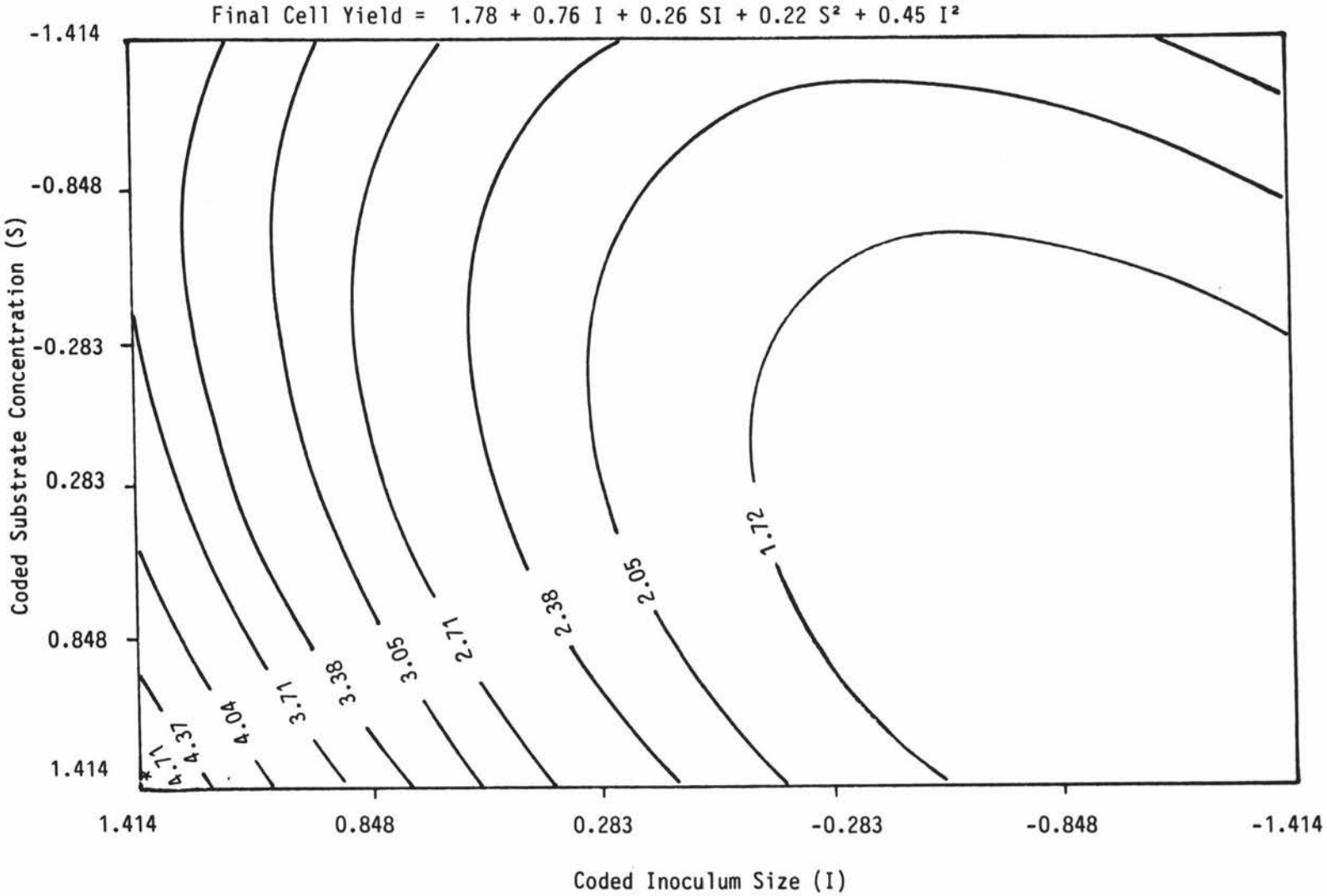



Figure 6.6 Contour Plots of Final Cell Yield in Shaken Flask Studies of *Pachysolen tannophilus* as a Function of Substrate Concentration and Inoculum Level in Synthetic medium (For strain IFO 1007).

## 6.5 DISCUSSION

The statistical analysis of the results of the experiment listed in Table 6.2, indicated that ethanol yield increased linearly with coded substrate concentration over the range investigated. A first-order additive model (that is no substrate-inoculum interaction term) described the reciprocal specific ethanol production rate. More complicated second-order models were required to describe fermentation time, cell yield and maximum instantaneous ethanol production rate.

Chen (1981) studied the ethanolic fermentation of glucose syrup by Saccharomyces cerevisiae and reported that increasing initial yeast concentration resulted in shorter fermentation time. Similar results were demonstrated by Strehaiano et al. (1983) for three strains of Saccharomyces yeast. In the current experiment, the minimum fermentation time was also observed at high inoculum and low substrate concentration.

In Section 5.3.4, a negative linear relationship between inoculum concentration and fermentation time was demonstrated. The effect of inoculum level upon fermentation time and other kinetic parameters may not be as simple as the results implied in Section 5.3.4. A number of researchers (Slininger et al., 1982b and Strehaiano et al., 1983) have ignored the importance of interactive effects of other variables.

This statistically designed experiment clearly indicated that with two variables, substrate concentration and inoculum size, a complicated interactive effect on fermentation time was evident.

Slininger et al. (1982b) demonstrated that the maximum specific ethanol production rate declined as the initial xylose concentration was increased. Specific ethanol production rate was similarly affected by initial xylose concentration in the current experiment and it further declined as inoculum size increased. These two variables, initial substrate concentration and inoculum size, acted independently here, having no interactive effect.

From Table 6.2, it was apparent that the interaction between substrate and inoculum size had an importance influence on fermentation time, ethanol production rate and cell yield. One might expect significant interactions between nutritional and environmental conditions in a system as complex as microbial fermentations although Moresi et al. (1979) used the factorial design approach to study whey fermentation and found such interactions to be of marginal importance.

The results reported in this chapter have indicated that, at least in the synthetic medium, simple fermentation variables can, singly and in combination, influence the progress and productivity of the xylose ethanol fermentation in a complex way. Furthermore, the statistically designed experimental approach can elucidate the nature of these influences. The next chapter reports on a major experiment involving a wider range of independent fermentation variables in a prehydrolyzate medium.

## 6.6 CONCLUSIONS

Greatest ethanol production rates occurred at greatest substrate and least inoculum concentrations. Increasing inoculum concentration resulted in shorter fermentation times. There was a linear relationship between the final ethanol yield and the coded value of initial substrate concentration. Interactions between initial substrate concentration and inoculum concentration were important influences on final cell yields and maximum instantaneous ethanol production rates.

# CHAPTER SEVEN : EXPERIMENT 2 - EFFECTS OF pH, TEMPERATURE AND NITROGEN SUPPLEMENTATION ON SOFTWOOD PREHYDROLYZATE FERMENTATION

## 7.1 INTRODUCTION

The preliminary shake flask studies described in Chapter 5 showed the need to identify the fermentation variables affecting cell yields and ethanol production. In Chapter 6 it was demonstrated that inoculum size and initial substrate concentration could affect the ethanol production rate and shorten the fermentation time. The literature as reviewed in Chapter 2, contains reports that imply that initial culture pH, temperature, inoculum size, substrate concentration and the strain of Pachysolen tannophilus used are important variables affecting the pentose fermentation.

The purpose of the experiments described in this Chapter was to determine which of these variables have significant effects, singly or in combination, on the pentose fermentation with a view to optimizing these parameters for an industrial process.

A statistically designed experimental approach was adopted for its efficiency, ability to resolve interactions and to assess the precision associated with the results achieved.

## 7.2 EXPERIMENTAL DESIGN

Experimental design Number 2, a three-factor, $2^3$, central composite design, was chosen to study the effect of pH, temperature and nitrogen supplementation on the cell yields and ethanolic fermentation. Nitrogen supplementation was achieved by adding Yeast Nitrogen Base (Difco Laboratories, Detroit, Michigan, U.S.A.) to the base medium.

The full experiment was employed three times, once with each of three strains of Pachysolen tannophilus, strain IFO 1007; NRRL Y-2460 and Y-2461.

This experimental design was generated according to the rules outlined in Appendix 5. The experimental design with coded factor-level combinations is presented as a cube in Figure 7.1 and in Table 7.1. The encoding procedure is described in Table 7.2.

Table 7.1 A three-factor Central Composite Statistical Design for Experiment 2

| | Temperature (T) | Nitrogen (N) | pH (H) |
|---|---|---|---|
| | ( Coded Value ) | | |
| Factorial points | -1 | -1 | -1 |
| | +1 | -1 | -1 |
| | -1 | +1 | -1 |
| | +1 | +1 | -1 |
| | -1 | -1 | +1 |
| | +1 | -1 | +1 |
| | -1 | +1 | +1 |
| | +1 | +1 | +1 |
| 'Star' Points | 0 | 0 | $-\alpha$ |
| | 0 | 0 | $+\alpha$ |
| | 0 | $-\alpha$ | 0 |
| | 0 | $+\alpha$ | 0 |
| | $-\alpha$ | 0 | 0 |
| | $+\alpha$ | 0 | 0 |
| Centre Point and Replicates | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 0 | 0 |

where, $\alpha = 1$ (for coded value) (Appendix 5)

Table 7.2 Level of Independent Variables in the Composite Design Experiment 2*

| Independent Variable | Symbol | Level | | | Unit |
|---|---|---|---|---|---|
| | | -1 | 0 | -1 | |
| pH | H | 2.5 | 4.5 | 6.5 | - |
| Temperature | T | 28 | 33 | 38 | °C |
| Nitrogen | N | 1.0 | 4.0 | 7.0 | g/l |

* The original values were coded into levels -1, 0, and +1 according to the following equations :

$$\text{Temperature coded level} = \frac{\text{Temp.} - 33°}{5}$$

$$\text{Yeast Nitrogen Coded Level} = \frac{\text{Nitrogen Concentration} - 4}{3}$$

$$\text{pH Coded Level} = \frac{\text{pH} - 4.5}{2}$$

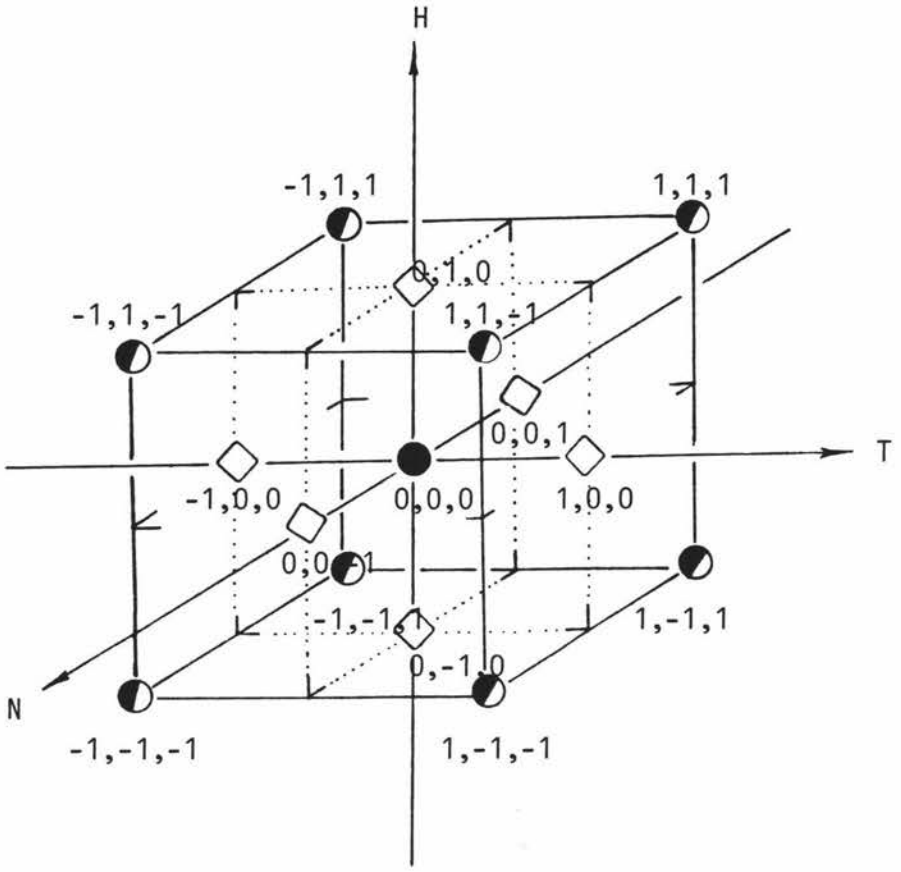



Figure 7.1 Three-factor Central Composite Design.
H, T and N represent the Three Independent Variables pH, Temperature and Nitrogenous Supplementation Respectively
The numbers are coded levels of the treatment combinations relative to the centre point.*

* see Appendix 5

## 7.3 FERMENTATION CONDITIONS

Each experimental flask (100 ml Erlenmeyer) contained 50 ml of prehydrolyzate (described in Table 3.1 and Section 3.6.1). Media were prepared and filter sterilized as described in Section 3.2. The media contained Yeast Nitrogen Base (Difco Laboratories, Detroit, Michigan, U.S.A.) at 1, 4 or 7 g/l as required by the experiment and were adjusted to pH 2.5, 4.5 and 6.5 with 1N NaOH prior to filter sterilization. Flasks were incubated at 28°, 33° or 38° with agitation at 100 r.p.m. using an Orbit Water-Bath Shaker (No. 3535) and Environ Shaker (No.3597) (Lab-Line Instruments Inc., Melrose Park, Illinois, U.S.A.). Fermentation was continued for 10 days, during which daily samples were collected for cell count (Section 3.4.1) and ethanol analysis (Section 3.4.5).

Three different strains of Pachysolen tannophilus, IFO 1007; NRRL Y-2460 and Y-2461, were initially acclimatized in the prehydrolyzate which had been previously treated with 2 g/l of sodium sulphite, supplemented with 4 g/l of Yeast Nitrogen Base and adjusted pH to 4.5, All cultures were kept at 33° for 24 hours. These parameters were the uncoded centre point values for the central composite experimental design (i.e. coded level for temperature, pH and Yeast Nitrogen Base supplementation was 0,0,0) as shown in Figure 7.1 and Tables 7.1 and 7.2.

## 7.4 RESULTS

The raw data concerning cell yield and ethanol concentration in each experimental flask were plotted against the incubation time (Figure 7.2). Smooth curves were drawn to calculate parameters such as specific ethanol production or growth rates, maximum instantaneous cell growth and ethanol production rates. Values of these parameters are listed in Appendix 8.

The full empirical mathematical equation for fitting the raw data in Appendix 5, is as follows : (Box et al., 1978)

$$\hat{Y} = \beta_0 + \beta_N N + \beta_H H + \beta_T T + \beta_{NH} NH + \beta_{NT} NT + \beta_{HT} HT + \beta_{NN} N^2 + \beta_{HH} H^2 + \beta_{TT} T^2 + \beta_{NNT} N^2 T + \beta_{HHT} H^2 T + \beta_{TTN} T^2 N + \beta_{NNH} N^2 H + \beta_{HHN} H^2 N + \beta_{TTH} T^2 H + \beta_{NNTT} N^2 T^2 + \beta_{NNHH} N^2 H^2 + \beta_{HHTT} H^2 T^2 + \beta_{NHT} NHT$$

where, $\hat{Y}$ is the predicted value of the dependent variables

$\beta$ represents the coefficients

N, H and T are coded values of Yeast Nitrogen Base, pH and Temperature respectively

$\beta_0$ is the Y-intercept or constant term

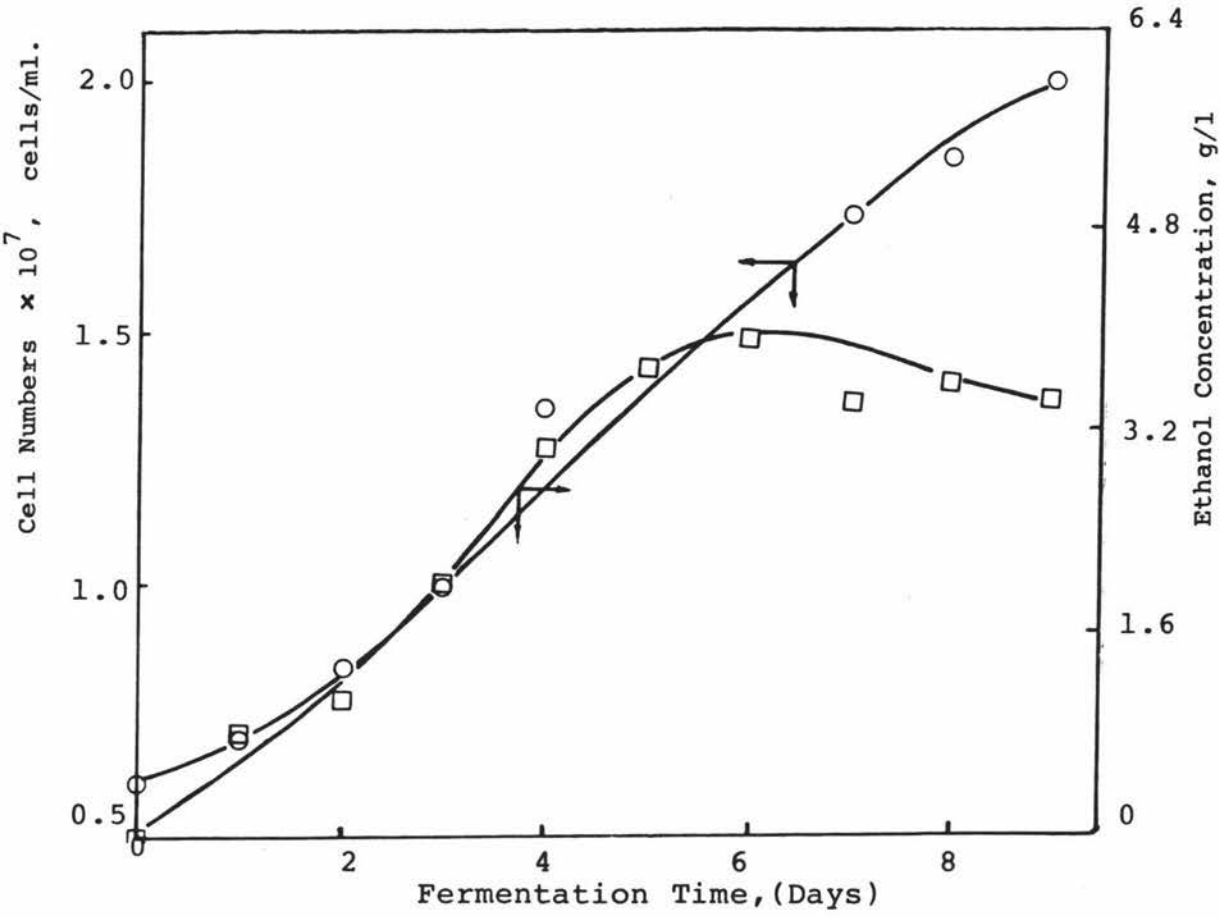



Figure 7.2 A Plot of Cell Growth (o-o) and Ethanol Production (square symbols) versus Fermentation Time from One of the Replicate Flasks Set Up Under Conditions Given by the Centre Point (0,0,0) for the Experimental Design Number 2. The Yeast Used was *Pachysolen tannophilus* NRRL Y2460.

Models derived by contraction of this equation were fitted to each of the six response variables chosen for each of the three strains of Pachysolen by using the stepwise multiple linear regression facility of the SPSS (Statistical Package for the Social Sciences, Version 9, 1982) Computer Package (SPSS Inc., Chicago, Illinois, U.S.A.). These models are presented in Tables 7.3 to 7.5.

For each of the parsimonious models derived, the analyses of residuals used in Section 6.3, were again employed to test for the appropriateness of the multiple regression models. These residual plots were used to check for equality, normality and the presence of the outliers. All the models tested showed adequate residual plots unless otherwise stated.

Lack of fit was tested for each parsimonious model by calculating the F-ratios of the mean square due to lack of fit to the mean square due to pure error. Tests for lack of fit of the parsimonious models have been compiled in Appendix 9.

The regression models derived from maximum instantaneous cell growth rate and maximum average ethanol production rate for the strain NRRLY-2461 revealed a significant lack of fit at the 5% level. Square-root transformations were employed with these response variables enabling adequate regression models to be developed. Table 7.6 is a comparison of the lack of fit criteria for the transformed and untransformed regression models.

The regression models for the specific ethanol production rates for all the three strains of Pachysolen tannophilus also exhibited a marked lack of fit at the 5% level. Only the natural logarithmic transformation of the response variables could eliminate these discrepancies.

Table 7.3 Derived Regression Models for the Strain IFO 1007***

| Coefficient | Cell Yield+ | Ethanol Yield | Specific Ethanol Production Rate++ | Specific Growth Rate | Maximum Cell Growth Rate | Maximum Average Ethanol Production |
|---|---|---|---|---|---|---|
| $\beta_o$ | 1.47 | 3.90 | -1.39 | 0.013 | 0.22 | 0.027 |
| $\beta_H$ | 0.33 | 1.07 | -0.84 | 0.006 | ns | 0.010 |
| $\beta_T$ | -0.34 | -2.12 | ns | -0.009 | -0.25 | 0.020 |
| $\beta_{HT}$ | -0.28 | -1.06 | ns | -0.006 | -0.24 | 0.009 |
| $\beta_{HH}H^2$ | ns | -1.90 | 0.68 | ns | ns | -0.015 |
| $\beta_{TT}T^2$ | -0.27 | -0.75 | -0.76 | -0.0032* | ns | ns |
| $\beta_{HHT}H^2T$ | ns | 1.07** | ns | ns | ns | 0.011* |
| $\beta_{TTN}T^2N$ | ns | ns | 0.54 | ns | ns | ns |
| $\beta_{NNH}N^2H$ | ns | ns | 0.72* | ns | 0.24 | ns |

++ Natural Logarithmic Transformation
+ Log Cell count per ml

*** All coefficients listed are statistically significant at the 1% level unless otherwise indicated
** Statistically significant at the 2.5% level
* Statistically significant at the 5% level
ns Terms correspond to these coefficients were discarded from the model by the stepwise regression routine.

Table 7.4 Derived Regression Models for the Strain NRRL Y-2460***

| Coefficient | Cell Yield+ | Ethanol Yield | Specific Ethanol Production Rate++ | Specific Growth Rate | Maximum Cell Growth Rate | Maximum Average Ethanol Production |
|---|---|---|---|---|---|---|
| $\beta_0$ | 1.52 | 4.32 | -1.06 | 0.013 | 0.30 | 0.029 |
| $\beta_H$ | 0.32 | 1.19 | 1.11 | 0.006 | ns | 0.009 |
| $\beta_T$ | -0.28 | -2.32 | ns | -0.007 | ns | -0.011 |
| $\beta_N$ | ns | ns | -0.54* | ns | ns | ns |
| $\beta_{HT}$ | -0.26 | -0.86 | -0.98 | -0.005 | -0.34 | 0.008** |
| $\beta_{HH}H^2$ | ns | -1.37 | ns | ns | ns | ns |
| $\beta_{TT}T^2$ | -0.26 | -1.60 | ns | -0.0034* | ns | -0.017 |
| $\beta_{NN}N^2$ | ns | ns | -0.98 | ns | ns | ns |
| $\beta_{HHT}H^2T$ | ns | 1.35* | ns | ns | -0.36 | ns |
| $\beta_{TTN}T^2N$ | ns | ns | 0.88 | ns | ns | ns |
| $\beta_{NNH}N^2H$ | ns | ns | ns | ns | 0.35 | ns |
| $\beta_{TTH}T^2H$ | ns | ns | 1.18 | ns | ns | ns |
| $\beta_{NNT}N^2T$ | ns | ns | -0.67 | ns | ns | ns |

++ Natural Logarithmic Transformation
+ Log Cell count per ml

*** All coefficients listed are statistically significant at the 1% level unless otherwise indicated
** Statistically significant at the 2.5% level
* Statistically significant at the 5% level
ns Terms correspond to these coefficients were discarded from the model by the stepwise regression routine

Table 7.5 Derived Regression Models for the Strain NRRL Y-2461***

| Coefficient | Cell Yield+ | Ethanol Yield | Specific Ethanol Production Rate++ | Specific Growth Rate | Maximum Cell Growth Rate+++ | Maximum Average Ethanol Production+++ |
|---|---|---|---|---|---|---|
| $\beta_0$ | 1.75 | 4.38 | -1.18 | 0.016 | 0.65 | 0.170 |
| $\beta_H$ | 0.32 | 1.20 | -0.71 | 0.008 | ns | 0.130 |
| $\beta_T$ | -0.55 | -2.82 | 0.99 | -0.011 | -0.47 | -0.130 |
| $\beta_N$ | ns | ns | 0.47 | ns | ns | ns |
| $\beta_{HT}$ | -0.31 | -1.13 | ns | -0.009 | -0.40 | 0.031 |
| $\beta_{HN}$ | ns | ns | 0.20 | ns | ns | ns |
| $\beta_{HH}H^2$ | ns | -1.79 | 0.50 | -0.004 | ns | -0.047 |
| $\beta_{TT}T^2$ | -0.51 | -1.01 | ns | ns | ns | -0.039 |
| $\beta_{NN}N^2$ | ns | ns | -0.58 | ns | ns | ns |
| $\beta_{HHT}H^2T$ | ns | 1.63 | ns | ns | ns | 0.083 |
| $\beta_{TTN}T^2N$ | ns | ns | -0.44 | ns | ns | ns |
| $\beta_{NNH}N^2H$ | ns | ns | ns | ns | 0.32 | -0.093 |
| $\beta_{TTH}T^2H$ | ns | ns | 0.73 | ns | ns | ns |
| $\beta_{NNT}N^2T$ | ns | ns | -0.45 | ns | ns | ns |
| $\beta_{NHT}$ | ns | ns | 0.52 | ns | ns | -0.025 |

+++ Square-root Transformation
++ Natural Logarithmic Transformation
+ Log Cell count per ml

*** All coefficients listed are statistically significant at the 1% level unless otherwise indicated
** Statistically significant at the 2.5% level
* Statistically significant at the 5% level
ns Terms correspond to these coefficients were discarded from the model by the stepwise regression routine.

Table 7.6 Comparison of the Tests of Lack of Fit with and without the Square-root Transformation of the Response Variables of Pachysolen tannophilus NRRL Y-2461 Growing on Prehydrolyzate

| | F - Ratios (Lack of Fit) | |
|---|---|---|
| | Original Response Variable (Y) | Transformed Response Variable ($\sqrt{Y}$) |
| dX/dt Maximum Cell Growth Rate | $F = 5.53$ $> 2.83$ $F_{.95}(11,11)$ | $F = 2.69^{*}$ $< 2.83$ $F_{.95}(11,11)$ |
| dP/dt Maximum Average Ethanol Production Rate | $F = 5.46$ $> 2.95$ $F_{.95}(8,11)$ | $F = 2.40^{*}$ $< 3.09$ $F_{.95}(6,11)$ |

* Not significant at the 5% level.

Contour plots of the response surface described by the final regression equations were drawn.

For all three strains of Pachysolen tannophilus, predicted ethanol yield increased as pH increased above pH 4.5 and as temperature decreased to 28°. For the strains NRRL Y-2460 and Y-2461, higher ethanol concentrations were observed at 28° and pH 5.8 whereas strain IFO 1007 showed peak yield at pH 5.6 (see Figure 7.4).

The contour plots for cell yields and specific cell growth rates of the three strains indicated that their maximal growth responses occurred at temperatures between 28.3° and 28.8° and pH 6.5 (Figures 7.4 and 7.5). Although growth appeared to be favoured by a pH near 6.5, ethanol production was highest between pH 5.6 and 5.8 in each case.

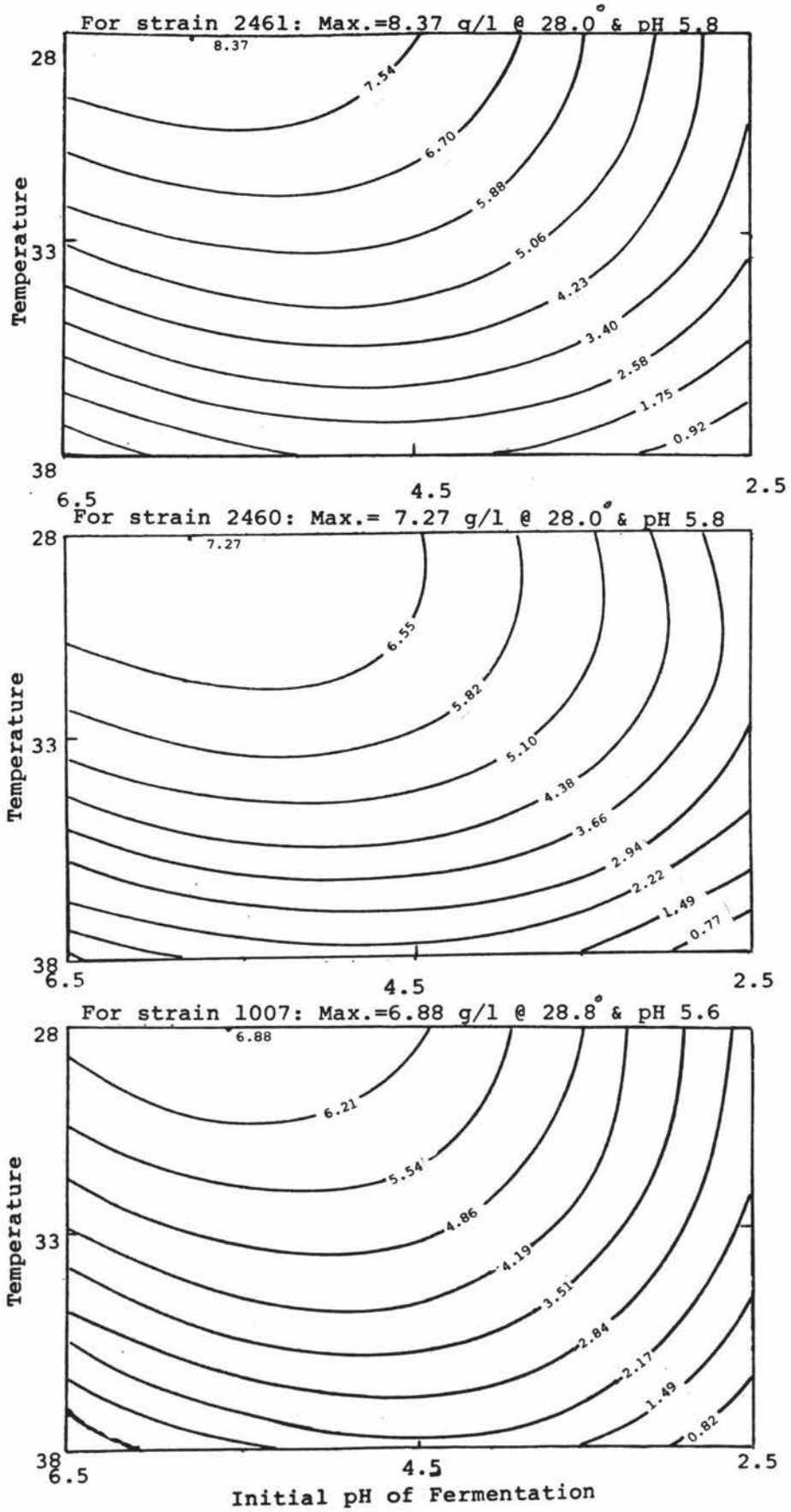



Figure 7.3 A Comparison of the Contour Plots for Final Ethanol Concentrations (g/l) Reached in Test Flasks as Functions of pH & Temperature for Three Strains of *Pachysolen tannophilus* Grown in Pre-hydrolysate.

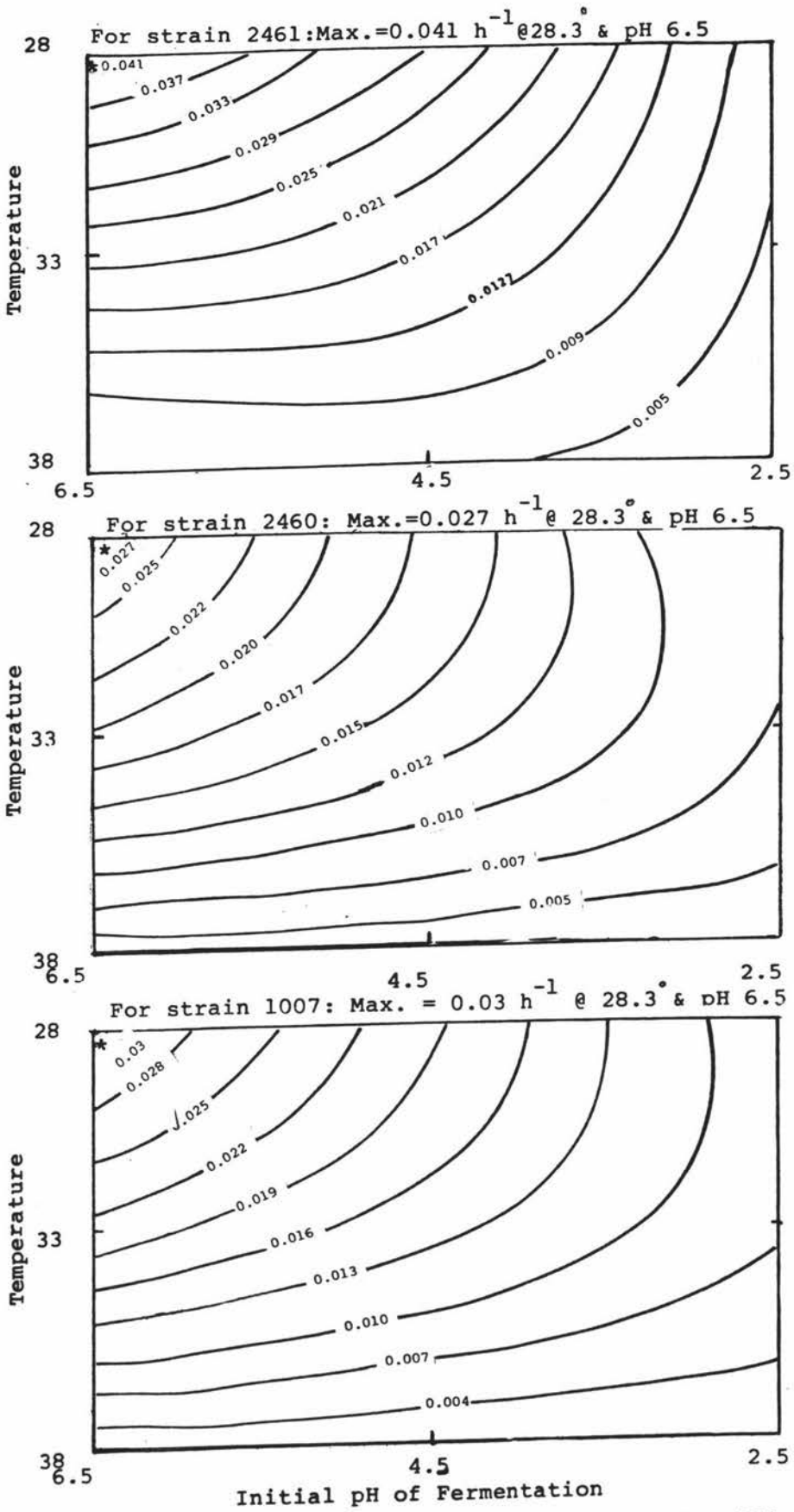



Figure 7.4 A Comparison of Contour Plots for Specific Growth Rates as Functions of pH & Temperature for Three Strains of *Pachysolen tannophilus* Grown in pre-hydrolysate.

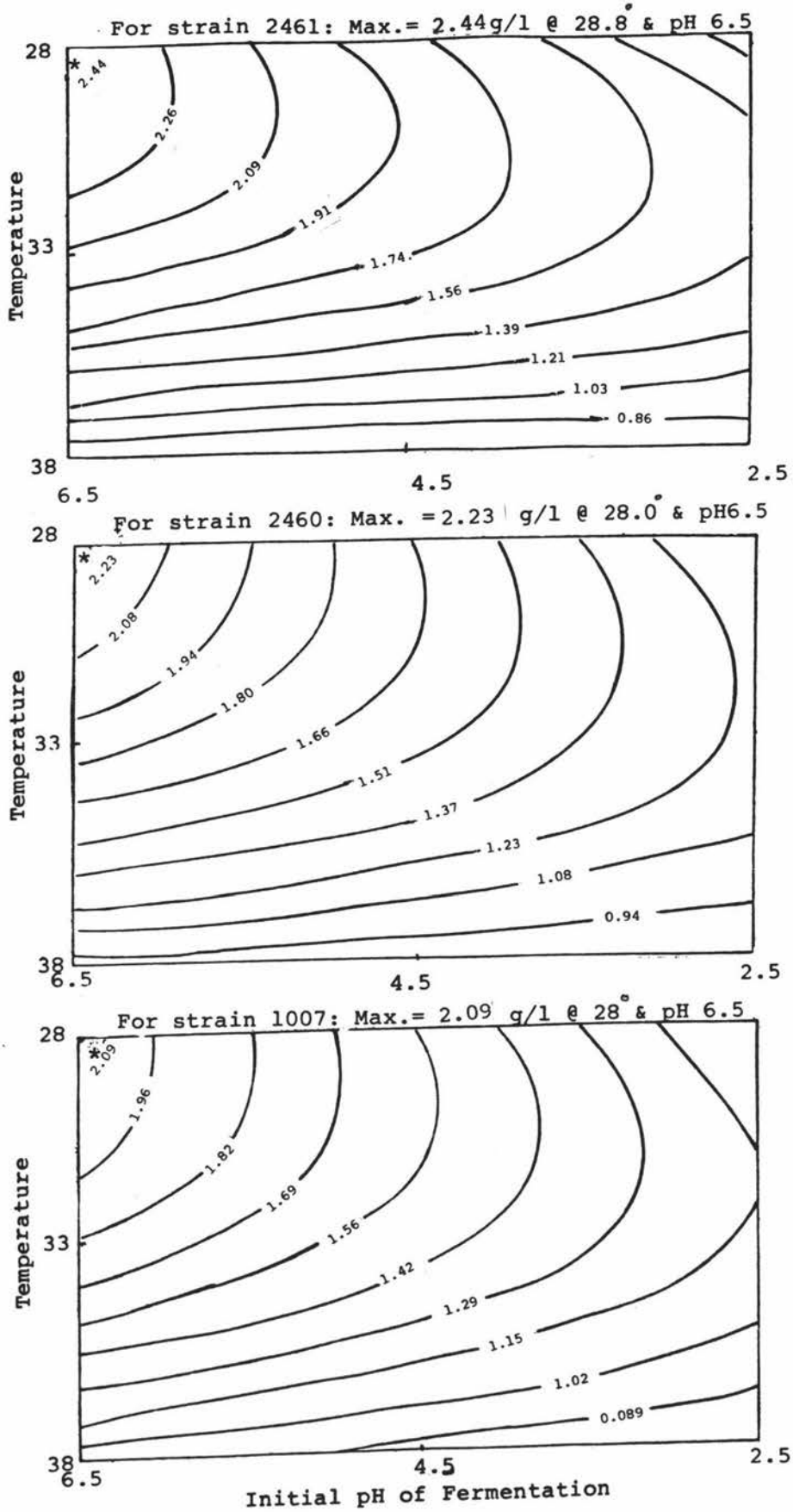



Figure 7.5 A Comparison of the Contour Plots for Yields of Cells (g/l dry-weight) as Functions of Temperature & pH for Three Strains of *Pachysolen tannophilus* Grown in Pre-hydrolysate.

It is interesting to note that no term for nitrogen (N) appears in the parsimonious regression models at a significant level for cells yields and ethanol yields or cell growth rates. However, nitrogen supplementation produced significant interactions with pH and temperature for rates of cell growth and ethanol production (Tables 7.3 to 7.5).

## 7.5 DISCUSSION

As amply demonstrated by the contour plots, the response in terms of growth and ethanol production of all three strains to the varying conditions were similar. This both suggests great similarity in their physiology and lends credence to the results of this investigation. However, Strain 2461 showed slightly greater growth and fermentation rates overall. All three strains grew more vigorously as the temperature fell below 33° and as the pH rose above 4.5. Maximal growth was observed near the lowest temperature and highest pH tested.

The poor performance of all three strains at 38° indicated that Pachysolen tannophilus was a mesophilic yeast with a maximum temperature for growth in prehydrolyzate of about 37°. A number of mesophilic strains of Saccharomyces exhibit optimum cell yields and growth rates in the range of 28 to 37° while 40° has been reported as the maximum temperature at which growth occurs (Jones et al., 1981). It seemed that Pachysolen tannophilus, may not be a useful yeast for any industrial pentose fermentation conducted near or above 38°.

The yield of ethanol for the three strains increased as pH values were increased above 4.5 and temperatures decreased to 28°. Maximum ethanol concentrations using prehydrolyzate were observed at 28°, the limit of the temperature range. It is possible that the optimum temperature for ethanol production was in fact lower than 28°.

Slininger et al. (1982a and b) studied the conversion of D-xylose to ethanol by the yeast Pachysolen tannophilus NRRL Y-2460 and they reported that ethanol formation was optimal at pH 2.5, at 32° with a

substrate concentration not exceeding 50 g/l. In the current study, no growth (relative to the initial yeast concentration) was detected at pH 2.5. In some flasks, no ethanol was detected at all (Table 7.5). Dekker (1982) also found Pachysolen tannophilus unable to grow at an initial pH lower than 3.0. Debus et al. (1983) reported that Pachysolen tannophilus grew at an optimum pH value at 4.8.

Pineault et al. (1977) used a $2^3$ factorial design to study the effect of pH, temperature and stirrer speed on the ethanol yield in glucose fermentation by Saccharomyces cerevisiae, demonstrated that the maximum ethanol yield was at pH 5.0, temperature at 32° and stirrer speed at 700 r.p.m.

The fermentation of whey by Kluyveromyces fragilis was studied by Moresi and Sebastiani (1979) using a central composite designed shaken-flask experiment. These workers determined that fermentation conditions were optimal at 36.4°, pH 5.1 with a salt supplementation of 4.7 g/l; a yeast extract content of 1.14 g/l and an initial lactose concentration of 24.75 g/l. They also observed that temperature and initial lactose concentration exhibited a major influence on the biomass yield. With a 15-litres jar fermenter, Moresi et al. (1980) reported that a lower initial lactose concentration (15.3 g/l) and temperature (31.3°) were required for optimal whey fermentation.

Eroshin et al. (1977) also used a central composite design to investigate the effect of pH and temperature on Saccharomyces cerevisiae metabolism. They found that there was no interaction between pH and temperature and that the maximum growth yield coefficient (Yx/s) was observed at pH 4.1 and at 28.5°.

## 7.6 CONCLUSIONS

Response surface methodology revealed that cell growth of Pachysolen tannophilus was maximal near a neutral pH value of 6.5, whereas final ethanol concentration was highest at lower pH values of 5.6 to 5.8.

The data from studies reported in both Chapters 6 and 7 point to the optimal fermentation conditions for the batch ethanolic fermentation of prehydrolyzate. Table 7.7 lists these conditions.

Table 7.7 Recommended Strain Used and Conditions for the Ethanolic Fermentation of Prehydrolyzate Sugars by Pachysolen tannophilus

| Process Parameter | Fermentation Conditions* |
|---|---|
| Temperature | 28° |
| Initial pH | 5.6 -5.8 |
| Substrate Concentration | to 80 g/l |
| Inoculum Density | 5.5 g cells/l |
| Strain | NRRL Y-2461 |

* Fermentation Conditions are collected from data presented in Chapters 6 and 7.

# CHAPTER EIGHT : FERMENTATION STUDIES OF D-XYLOSE USING *Pachysolen tannophilus*

## 8.1 INTRODUCTION

This Chapter reports on a series of batch and chemostat studies using the yeast Pachysolen tannophilus strain NRRL Y-2461 in a pure D-xylose plus Difco Yeast Nitrogen Base medium. The purpose of these fermentation experiments was to establish the growth constants and fermentation characteristics of this pentose-fermenting yeast under differing conditions of aerobiosis.

A view expressed in Chapter 2 concerning the Pachysolen fermentation was that excess oxygen inhibited fermentation while the absence of oxygen inhibited growth (see also Chapter 5). Therefore, an attempt was made to examine the influence of oxygen on ethanol formation using, batch, chemostat and chemostat with controlled redox potential cultures.

## 8.2 FERMENTATION CONDITIONS

### Batch Fermentation

A Multigen F2000 fermenter vessel was assembled as described in Section 3.6.2. A 1.5 l working volume using the D-xylose (1 8 g/l) and Yeast Nitrogen Base (6.7 g/l) medium was used and the temperature kept at 28° with constant agitation at 100 r.p.m. To review the responses of the yeast to both aerobiosis and anaerobiosis, the culture was sparged in an alternating manner, firstly with nitrogen then with air and this cycle of changing (pulsed) gas phases repeated until the culture had produced maximum ethanol concentration.

Gas flow rate was set so as to deliver 0.37 l of either sterile air or sterile nitrogen per 1 of growth medium per minute.

## 8.3 RESULTS

### 8.3.1 Batch Growth and Fermentation Studies

The batch fermentation results are presented in Figure 8.1 which depicts changes in pH, formation of ethanol, population development and xylose utilization. This fermentation can be regarded as a base line study as no attempt was made here to manipulate the environment of the culture.

#### pH Changes

A marked fall of pH characterized the batch fermentation. Starting at pH 5.1 the medium stabilized at pH 1.8 after approximately 96 hours. The rapid initial fall in pH did not extend over the entire growth period of the culture.

#### Ethanol Formation

Active generation of ethanol by the culture began near the end of exponential growth (about the 100th hour) and reached a maximum concentration of 3.4 g/l at about the 200th hour. The graph suggests that it was after active growth had occurred that ethanol production started.

#### Population Development

In the medium used , the fully adapted culture began growing with virtually no lag period being evident. A maximum specific growth rate of 0.015 $h^{-1}$ was observed. The growth of cells correlated with a fall in both pH (noted above) and substrate concentration.

#### D-Xylose Utilization

Reflecting the increasing demand of an increasing population, D-xylose concentration in the fermenter decreased steadily (Figure

8.1). The maximum rate of uptake was 0.097 g xylose/l/h.

In summary, a number of points can be made. Firstly, while the pH fall was rapid, it did not continue to fall during the entire period of population development. Secondly, the population reached a maximum before all substrate was consumed. Thirdly, ethanol concentration reached a maximum only after cell density reached its maximum.

### 8.3.2. Batch Fermentation with "Pulse" Aeration

Preliminary shake flask studies have indicated that aeration was an important factor affecting the rate of D-xylose fermentation (see Chapter 5). In this section the effects of aerobiosis and anaerobiosis on the ethanolic fermentation were investigated in greater detail.

Alternating periods of sparging, to be termed Phases of Gassing, with oxygen-free nitrogen gas followed by sterile air were arranged. The temperature of the vessel was kept at 28° and agitation was 100 r.p.m. The fermentation results are shown in Figure 8.2 where the arrows indicate commencement of the particular phases of gas sparging. Phase I of gassing (0-46h) was sparging with nitrogen, Phase II of gassing (64-90h) with air, this cycle, the first, was repeated in Phases of gassing III (90-130h) and IV (130-147h), the second cycle. Phase V of gassing was where all gases were switched off. Thus, Cycle 1 covers Phases of gassing I and II while Cycle 2 covers Phases of gassing III and IV.

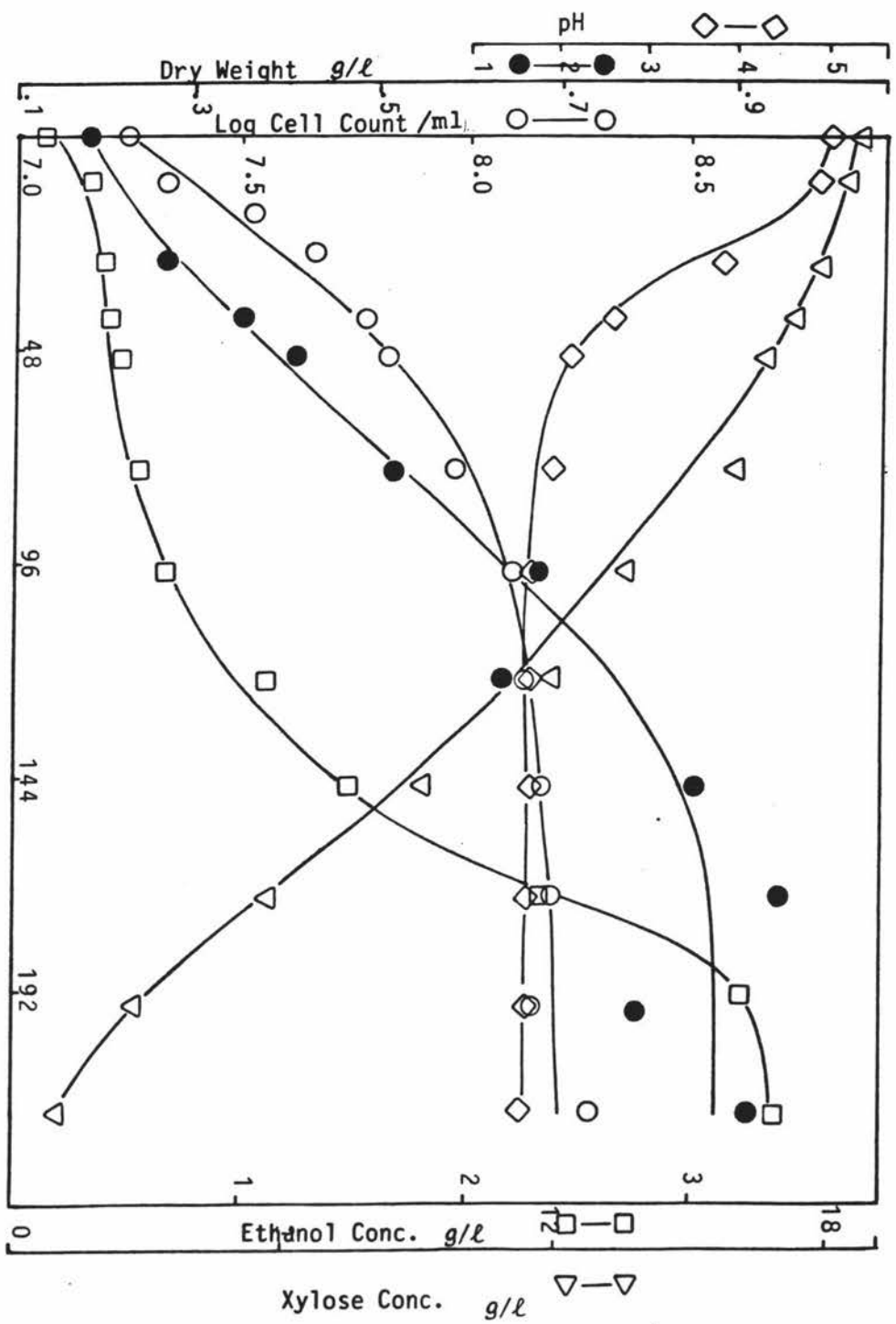



Figure 8.1 The Fermentation of D-xylose (18 g/l) by *Pachysolen tannophilus* strain NRRL y-2461 in Yeast Extract Medium and Under Batch Conditions at 28° and an Initial pH of 5.1 with 100r.p.m. mixing.

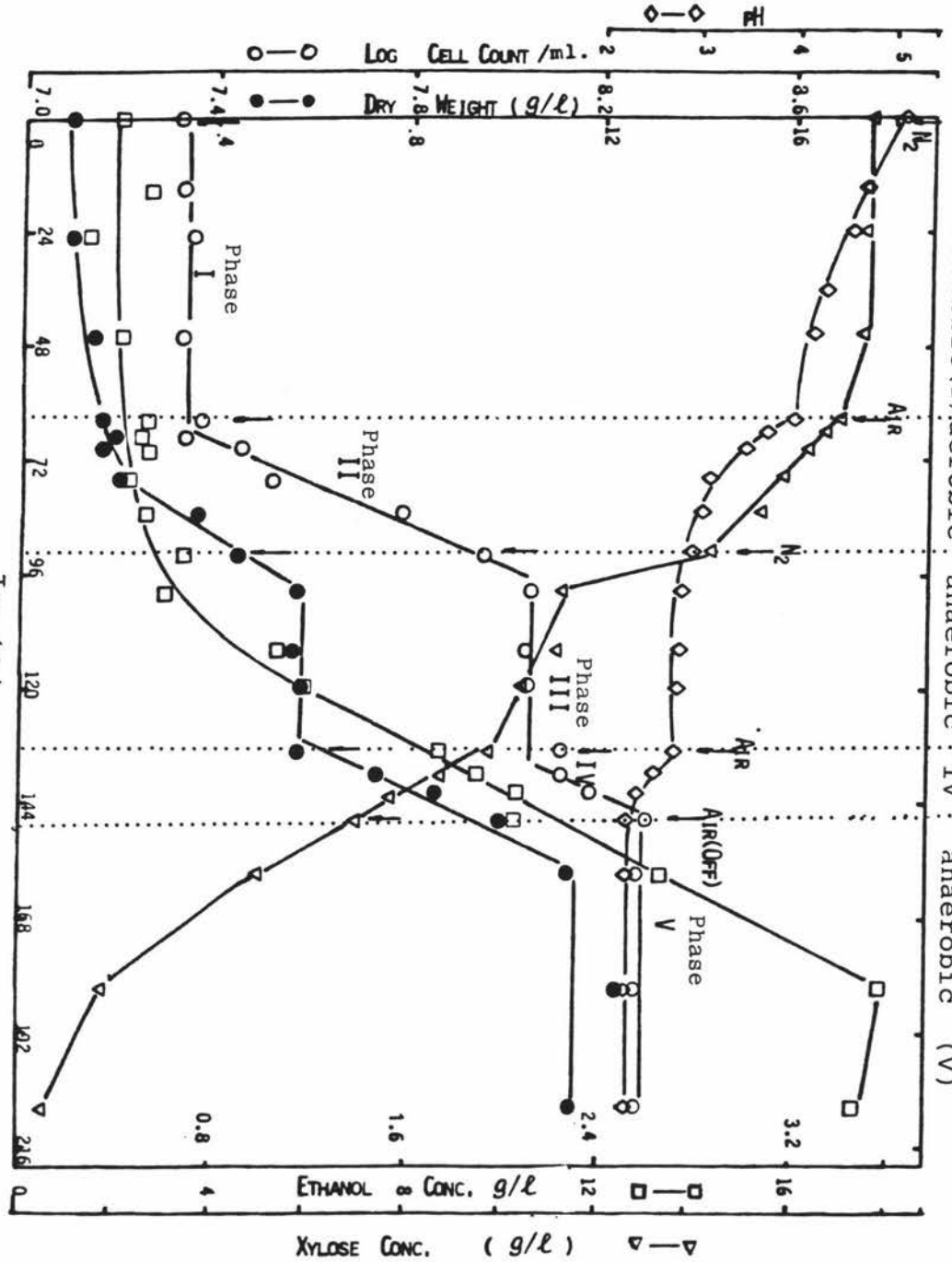



Figure 8.2 A 1.5 ℓ Batch Fermentation of D-xylose (18 g/ℓ) to Ethanol Using *Pachysolen tannophilus* NRRL Y-2461 at 28° and an Initial pH of 5.1. Gaseous Conditions were Alternated Between Anaerobic using Sterile Nitrogen (Phases I, III &V) and Aerobic, using Sterile Oxygen (Phases II & V).

pH Changes

The initial culture pH was 5.1 and gradually declined throughout phase I suggesting some ongoing metabolic activity. Aeration caused a significant drop in pH values from 4 to 3 in the first cycle (Phases I and II) and from pH 3 to pH 2.5 in the second cycle (Phases III and IV). During the anaerobic phases III and V, pH remained steady. The drop in the pH values corresponded with a marked increase in substrate uptake and cell growth during the first and second periods of aeration (phases II and IV, Figure 8.2).

Growth Response

No growth occurred after inoculating Pachysolen tannophilus into an anaerobic system. At 64 hours, aeration was commenced (phase II). Growth started soon afterwards at a rate of 0.039 $h^{-1}$ (as compared to 0.024 $h^{-1}$ in phase IV). . Aeration was terminated at 90 hours and by 96 hours active yeast growth had ceased. No growth, in terms of cell count or cell dry-weight, was observed throughout the second nitrogen sparging period ( that is phase IV from 90-130 h), but it commenced within 3 hours of re-aeration at the 130th hour at the rate of 0.024 $h^{-1}$. A return to anoxic conditions at the 147th hour again terminated yeast growth.

Substrate Consumption and Ethanol Production

D-xylose consumption rates (ds/dt) for the first and second aeration periods (phases II and IV) were 0.088 and 0.176 g xylose/l/h respectively. The maximum specific sugar consumption rates (Qs) were 0.56 and 0.31 g xylose/g dry cell/h respectively (Table 8.1).

Active growth was needed for ethanol production in phases I and II. Once established, ethanol continued to be produced irrespective of the gassing cycles (Figure 8.2).

From figure 8.2, it is apparent that at 92 h and again at 147 h xylose uptake was interrupted.

The quantitative data for the fermentation are summarized in Table 8.1. The highest ethanol yield (Yp/s), was achieved in phase III under anaerobic conditions where a theoretical yield of 82 % or 0.42 g ethanol/g xylose was measured.

Specific ethanol production rates were highest during the anaerobic phase III but dwindled to sightly less than half this value in the anoxic phase V. The cell population at this time was high, ethanol production was continuing and xylose was still present in the medium (Figure 8.2). Under aerobic conditions, maximum specific sugar consumption rates were achieved, namely 0.56 and 0.31 g xylose consumed/g cells/h. High specific growth rates matched these high specific utilization rates.

The overall yields of ethanol in the different phases of batch fermentation are shown in Table 8.2.

Table 8.1 Ethanol Fermentation by Pachysolen tannophilus growing on D-Xylose at 28° and pH 5.1 under Varying Aeration Conditions

| Aeration Condition | Phase | Maximum Specific Growth Rate $\mu_{max}$ $h^{-1}$ | Maximum Specific Sugar Consumption Qs g/g.h | Maximum Specific Ethanol Production Qp g/g.h | Ethanol Yield Yp/s g/g | Ethanol Yield Yp/s % |
|---|---|---|---|---|---|---|
| Aerobic | II | 0.04 | 0.56 | 0.02 | 0.03 | 5.3 |
| Aerobic | IV | 0.02 | 0.31 | 0.05 | 0.16 | 31.0 |
| Anaerobic | III | * | 0.17 | 0.07 | 0.42 | 82.4 |
| Anaerobic | V | * | 0.11 | 0.03 | 0.30 | 57.5 |

* No Cell Growth (by using Cell Count and Dry Weight).

\+ % of Theoretical Yield.

Table 8.2 Ethanol Production by Pachysolen tannophilus in Batch Culture with Anaerobic and Aerobic Sparging Regimes.

| Phase of Gassing | Sparging Condition | Population Response | Ethanol Yield Yp/s + | Proposed Xylose Metabolism |
|---|---|---|---|---|
| II | Aerobic | Growth | 0.03 | Respiration predominant |
| IV | Aerobic | Growth | 0.16 | Respiration and fermentation with possible simultaneous fermentation and consumption of ethanol |
| V | Anoxic | No Growth | 0.30 | As for IV, balance towards fermentation |
| III | Anaerobic | No Growth | 0.42 | Fermentation with presumed minimal consumption of ethanol |
| I | Anaerobic | No Growth | 0 | Low population and undetectable fermentation |

+ Ethanol Yield is presented as g ethanol/g xylose consumed.

### 8.3.3. Continuous Culture Studies

In Section 8.3.2 it was demonstrated that traces of oxygen were important in stimulating the fermentation of D-xylose to ethanol. Chemostat studies were now performed so that the amount of oxygen or air required for best ethanol production might be established. The 1.35 l batch culture as described in Section 8.3.1 was converted to continuous operations when the culture of Pachysolen tannophilus NRRL Y-2461 had reached maximum density. Conditions as described in Sections 8.2 and 3.6.2.1. were used and sterile air was sparged at 0.37 l/l.min. The dilution rates were varied so that their influence on ethanol productivity could be evaluated during each steady-state. Steady-states were maintained for at least 3 retention times (that was 18 days). Together with ethanol production, cell yields, substrate consumption and the amounts of xylitol produced were measured.

A near linear relationship between ethanol production and substrate consumption was observed over the tested range (see Figure 8.3). The product yield was 0.17 g ethanol/g substrate consumed as calculated from the regression line. The increase in ethanol yield with increasing D-xylose consumption was not unexpected. On extrapolating the regression line, it was calculated that 1.9 g/l of substrate was consumed without any production of ethanol. This quantity (1.9 g/l) of substrate presumably contributed to the formation of biomass prior to the commencement of ethanol production. The previous batch fermentations (Sections 8.3.1 and 8.3.2) indicated that about 2 g/l of D-xylose were consumed initially to form biomass before ethanol production commenced (Figure 8.1 at 48th hour and Figure 8.2 at 84th hour) which is in accord with this chemostat derived value.

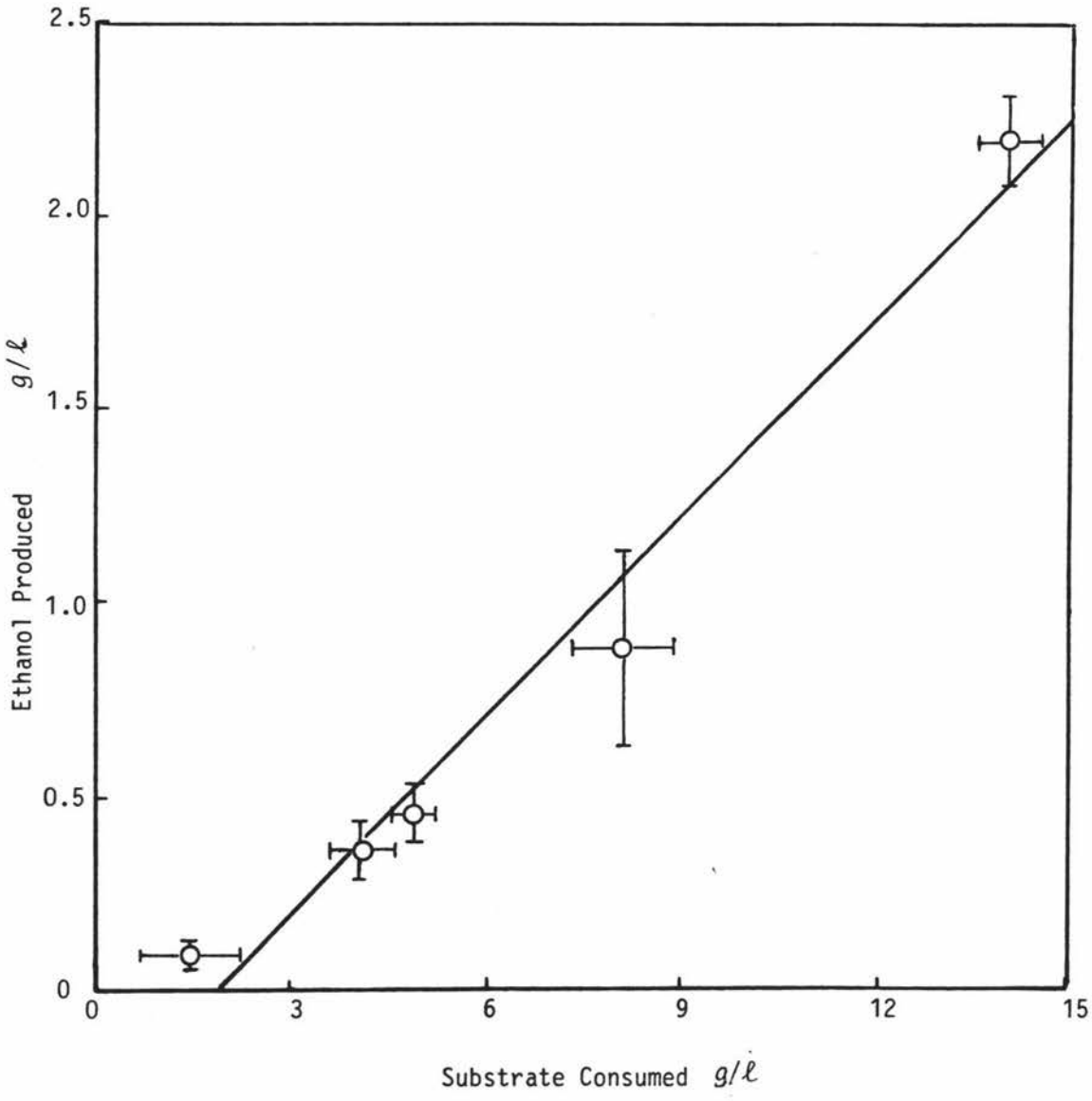



Figure 8.3 Ethanol Concentration as a Function of the Substrate Consumed. *Pachysolen tannophilus* strain NRRL Y-2461 was used in the Chemostat. Error flags Associated with the Analyses for Ethanol and D-xylose are Shown.

The biomass and xylitol yields per g of substrate consumed were 0.04 g dry weight cells and 0.135 g of xylitol respectively (Figures 8.4 and 8.5). Biomass increased linearly with increasing consumption of substrate. However, on extrapolating the curve, it could be seen that 0.34 g of cells were produced without any consumption of the substrate. Consequently, maintenance energy and the true growth yield calculations were made in an attempt to account for this discrepancy in the data (Figure 8.11).

Yields of the product ethanol (Yp/x) and the by-product xylitol (Yxo/x) in terms of biomass were calculated from Figure 8.6 as 4.2 and 3.4 g product or by-product/g dry cells respectively. Furthermore, there was a linear correlation between product and by-product which is depicted in Figure 8.7. From the curve, 1.25 g of ethanol was produced/g xylitol excreted. That is, the product to by-product ratio was 5:4. Such information was useful when considering the stoichiometry of the fermentation.

The extrapolated regression lines in Figure 8.6 suggested that 0.42 and 0.28 g of cells were produced in the absence of ethanol and xylitol production respectively. Both batch fermentations (see previous section) revealed that about 0.41 and 0.38 g/l of cells were produced prior to the commencement of ethanol production.

The Lineweaver-Burk plot of dilution rate against residual sugar concentration is depicted in Figure 8.8. The maximum specific growth rate ($\mu$) and the value for Ks were calculated as 0.046 $h^{-1}$ and 13 g/l respectively.

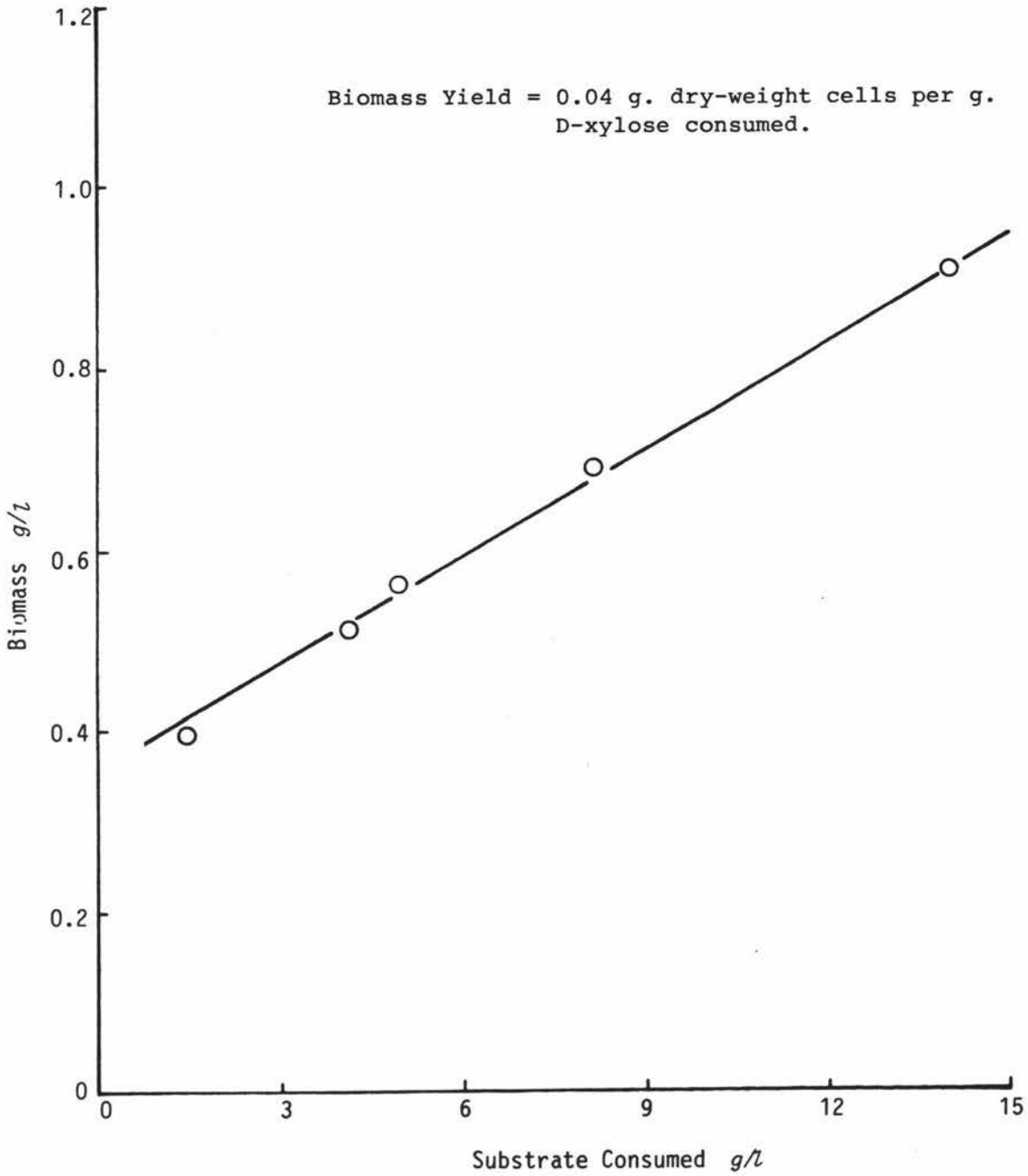



Figure 8.4 Biomass as a Function of the Substrate Consumed. For Experiment, *Pachysolen tannophilus* NRRL Y-2461 was Grown in a 2 ℓ Chemostat at Limiting D-xylose Concentrations. D=0.007 $h^{-1}$; T = 28°; Agitation 100r.p.m. and 0.37 ℓ/ℓ/min. Sterile Air.

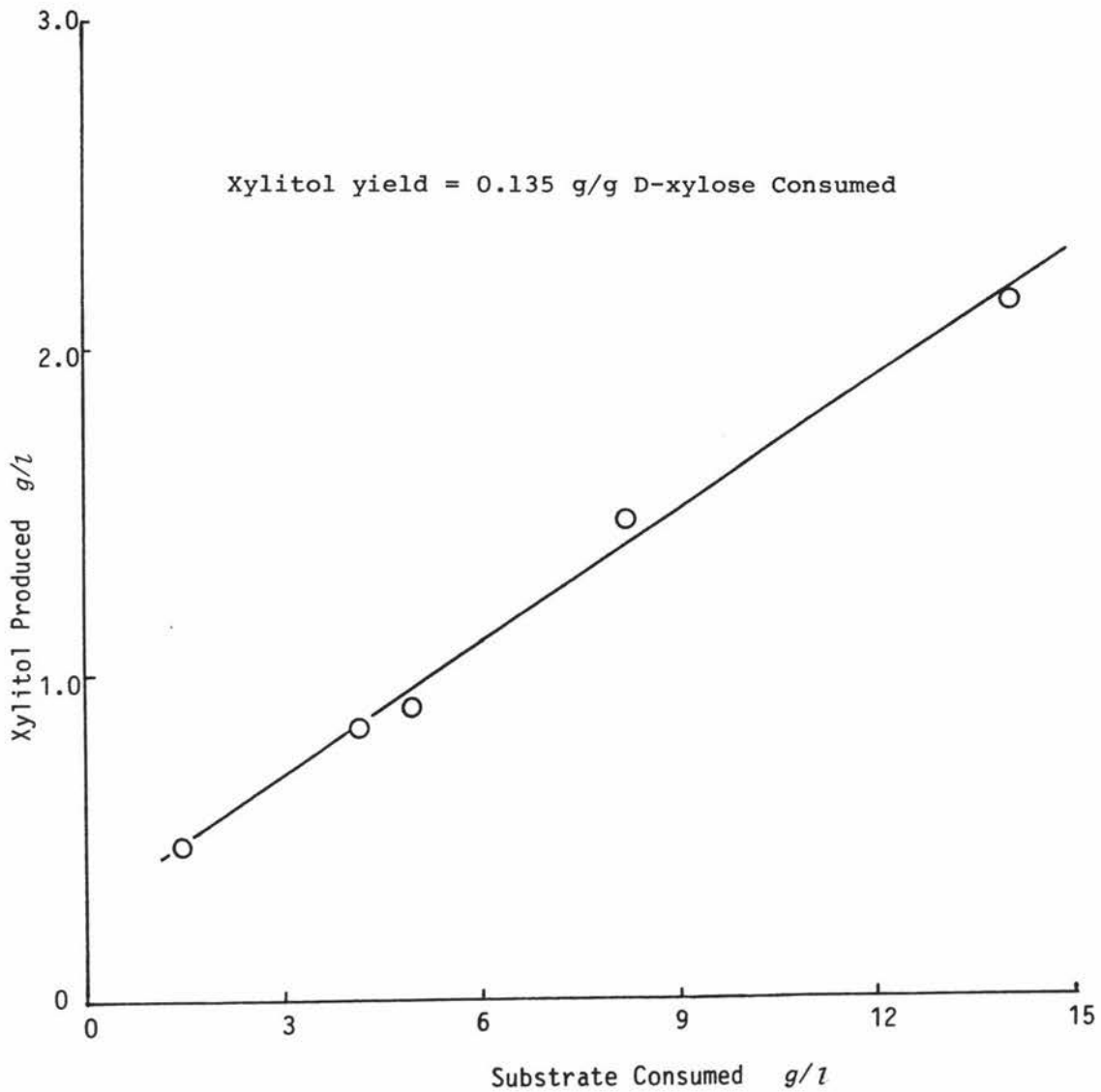



Figure 8.5 By-product (Xylitol) as a Function of the Substrate Consumed. *Pachysolen tannophilus* strain NRRL Y-2461 was the Yeast Used in a 2.5 ℓ Chemostat at Limiting D-xylose Concentrations. D = 0.007 $h^{-1}$ T = 28°; Agitation 100r.p.m. & air 0.37ℓ/ℓ/m.

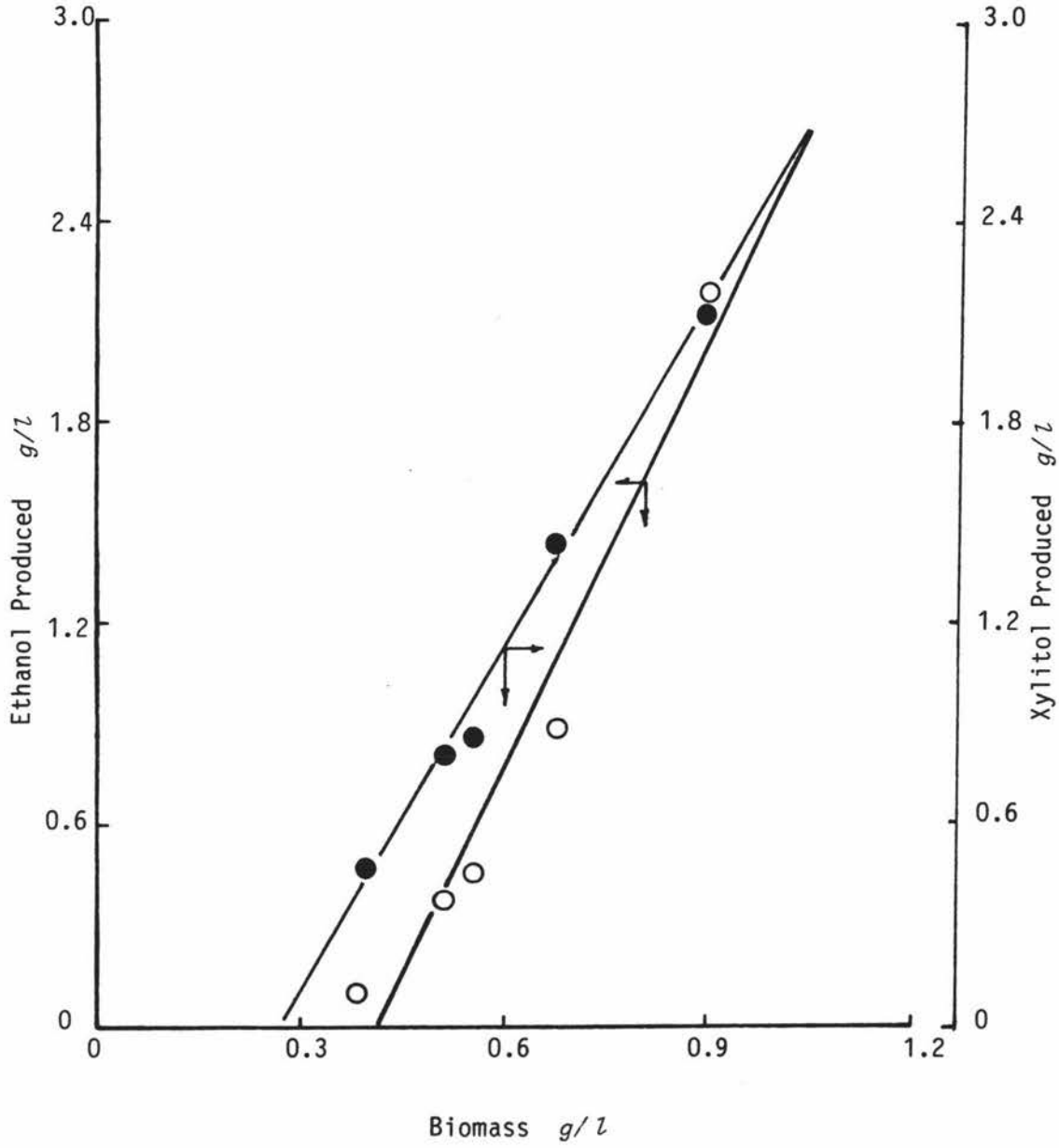



Figure 8.6 Ethanol and Xylitol Produced as a Function of Biomass Growth. *Pachysolen tannophilus* NRRL Y-2461 was the Yeast Used in a 2.5 ℓ Chemostat at Limiting D-xylose Concentration. $D = 0.007\ h^{-1}$; T = 28°; Agitation at 100 rpm. and Air admitted at 0.37 ℓ/ℓ/min. Xylitol Formation "Paralleled" that of Ethanol.

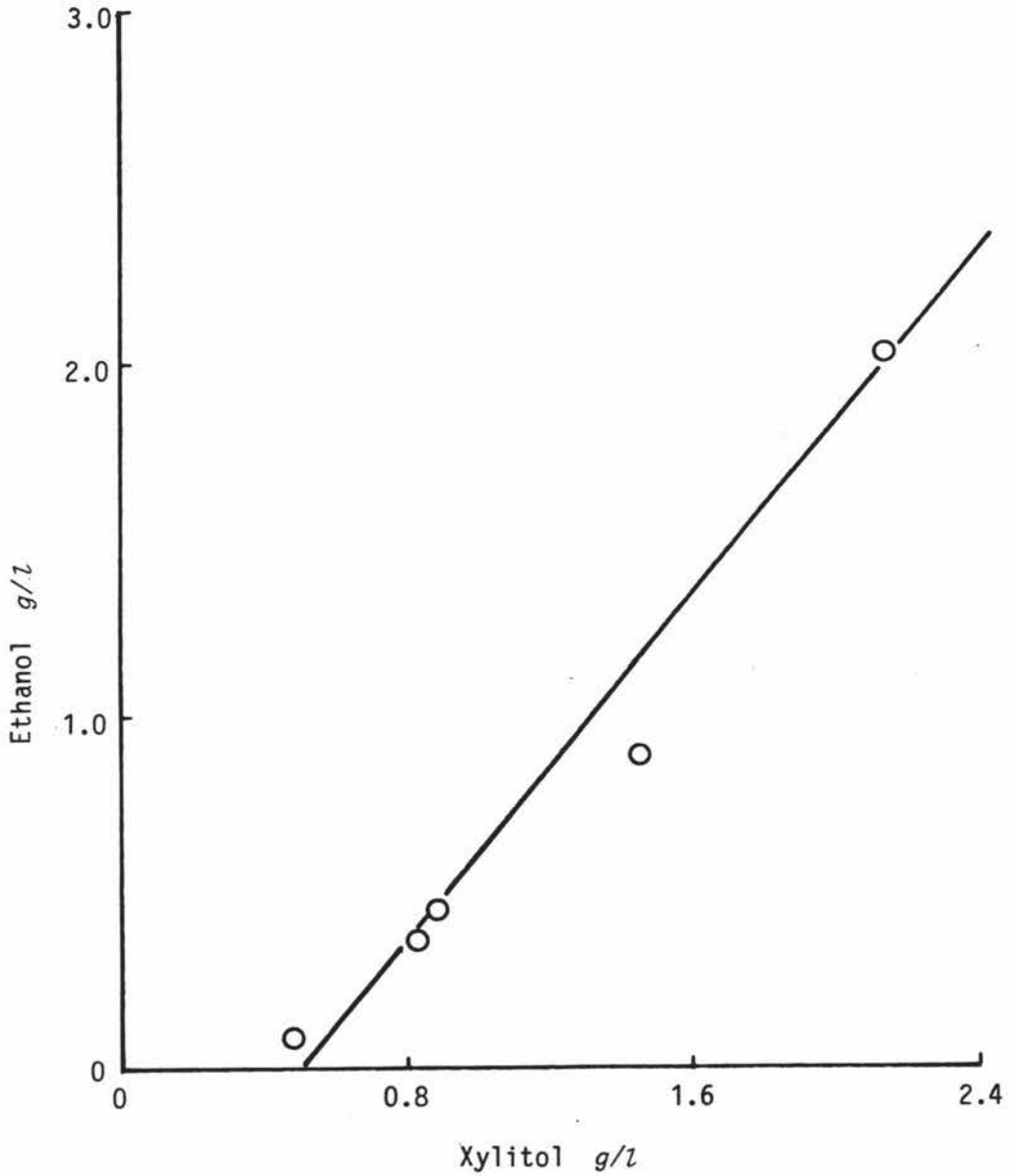



Figure 8.7 Ethanol Produced as a Function of By-Product (Xylitol) Produced

The chemostat data suggested that for *Pachysolen tannophilus* NRRL Y-2461 there existed an interdependence of ethanol and xylitol production under the growth conditions used. EtOH:Xlitol = 1.25.

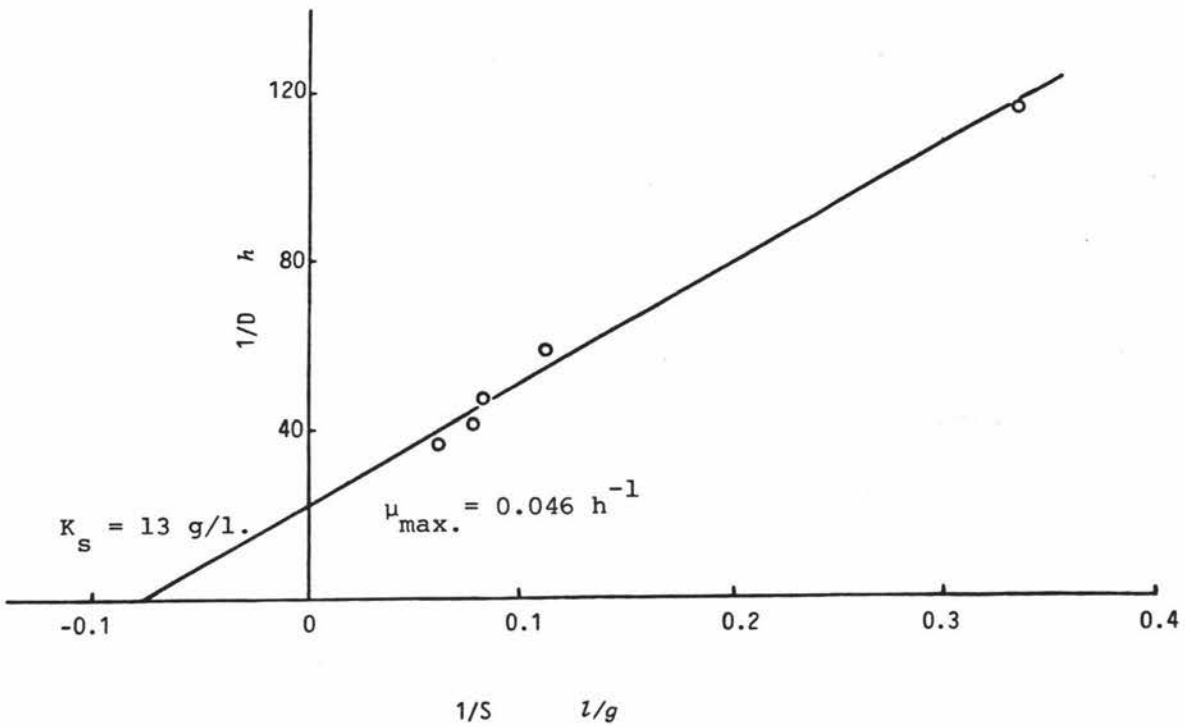



Figure 8.8 Lineweaver-Burk Plot (1/D versus 1/S) to Demonstrate the Effect of Limiting Substrate Concentrations on the Specific Growth Rate of the Yeast *Pachysolen tannophilus* NRRL Y-2461 When Grown in a Chemostat Under Conditions of T = 28° Agitation was 100 r.p.m. and Aeration was set at 0.37 ℓ air/ ℓ medium / minute.

Expressing biomass yields and/or product yields as a function of specific growth rate, in this case equated with dilution rate, seemed an appropriate way of graphically demonstrating the possibility that ethanol may inhibit the fermentation (Figure 8.9). End-product inhibition was observed as the non-competitive, growth linked pattern. The Ki, that is the product inhibition constant for growth, was evaluated as 0.5 g/l from Figure 8.10.

The calculation of maintenance energy (m) and true growth yield (Yg) coefficients using continuous culture data involves application of the equation suggested by either Pirt (1975) or Fieschko and Humphrey (1984). These are :

$$1/Y = 1/Yg + m/D \quad \text{.....(1)}$$

or

$$Qs = D/Y + m \quad \text{.....(2)}$$

where, Y = observed growth yield (g cell/g substrate)

Yg = true growth yield (g cell/g substrate)

m = maintenance energy coefficient
(g substrate/g dry cell.h)

Qs = specific substrate consumption rate
(g substrate/g dry cell.h)

D = dilution rate (specific growth rate, $h^{-1}$)

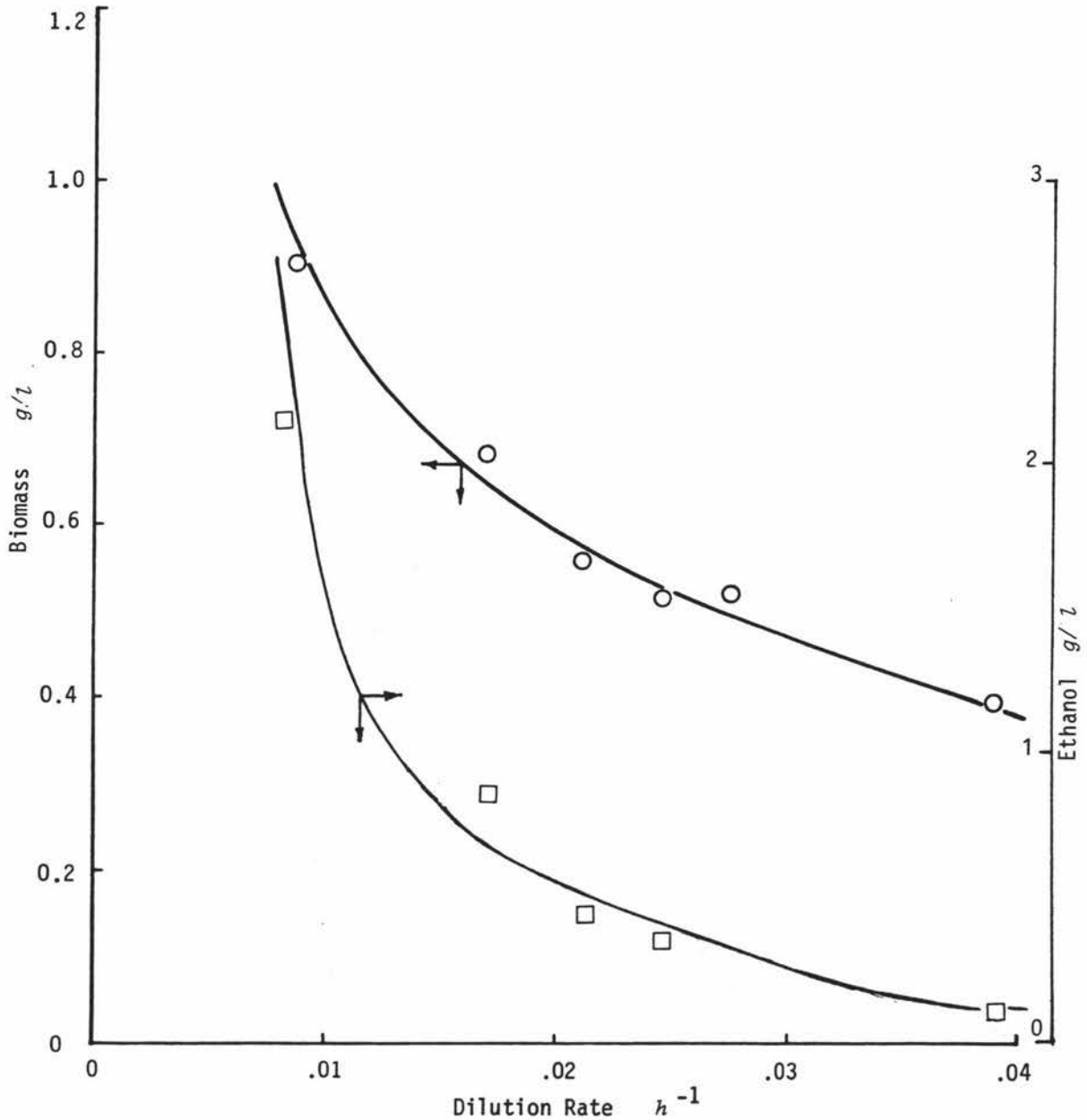



Figure 8.9 Biomass Yield and Ethanol Concentration as a Function of Dilution Rate

*Pachysolen tannophilus* NRRL Y-2461 was grown in a D-xylose limiting chemostat, T = 28°, air was sparged at a rate of 0.35 $\ell$ / $\ell$ / minute. For the growth system $\mu_{max}$ = 0.046 $h^{-1}$ & $K_S$ = 13 g/$\ell$. High dilution rates (above 0.01 ) were shown to be counter-productive in terms of ethanol formation.

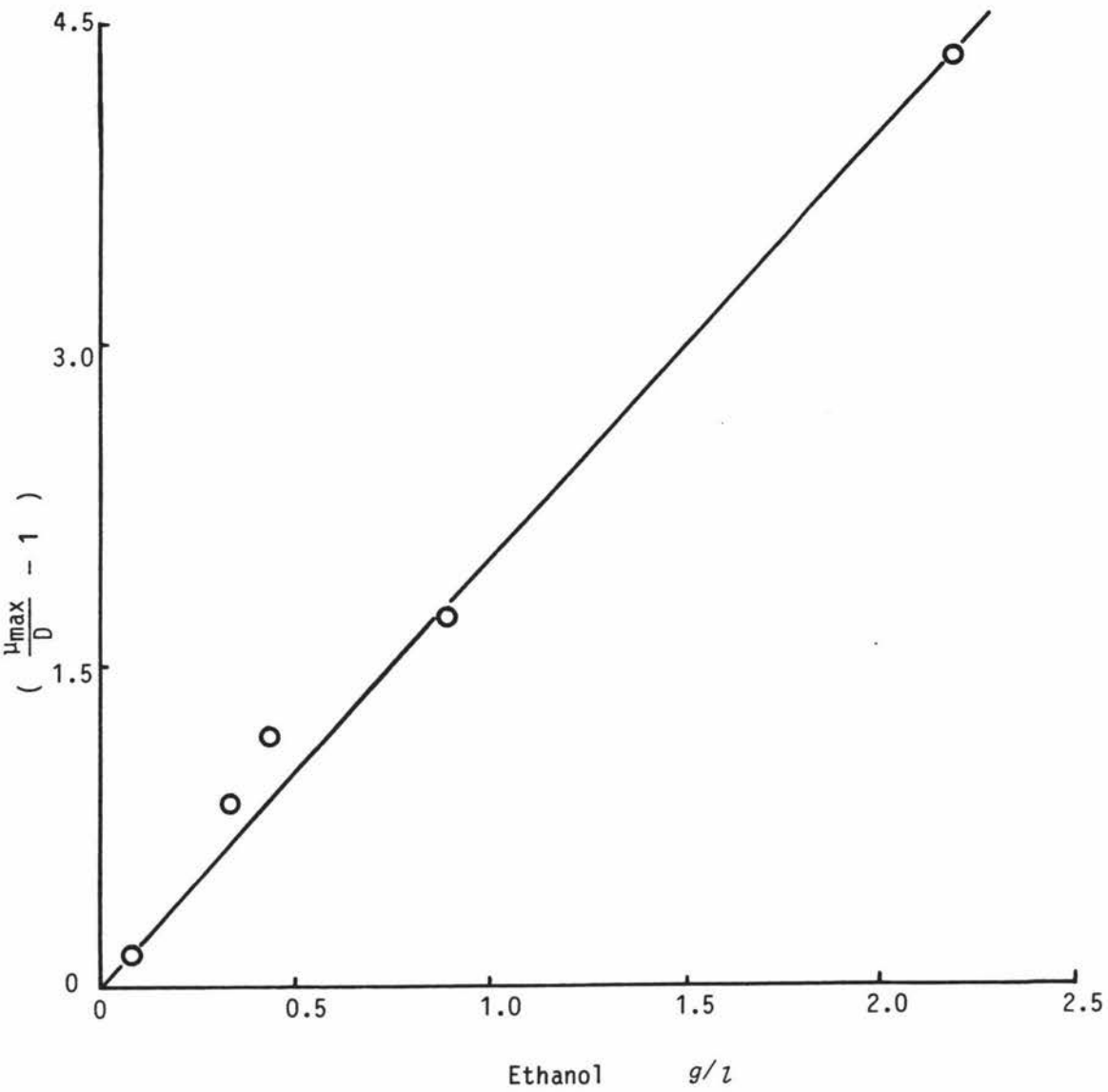



Figure 8.10 Effect of Ethanol Production on Cell Growth

*Pachysolen tannophilus* NRRL Y-2461 was grown under D-xylose limiting conditions in a 1.35 ℓ chemostat at 28°. Agitation was set at 100 r.p.m. and air sparging at 0.37 ℓ/ℓ/minute.
D = 0.007 reciprocal hours; $K_{i(ethanol)}$ = 0.5 g/ℓ was calculated.

Therefore, using equation 1, a double reciprocal plot of the observed growth yield against dilution rate should give a straight line having a slope m and an intercept 1/Yg. Figure 8.11(A) however, indicated that such a linear relationship did not exist. Likewise, using equation 2, a plot of the specific rate of D-xylose consumption versus dilution rate was not linear (Figure 8.11,B). Both plots consisted of two linear portions from which maintenance energy and true growth yield coefficients were calculated for D < or D > 0.017 $h^{-1}$. Using on Fieschko and Humphrey's (1984) suggestion, equation 1 was used for calculating maintenance energy coefficient, and equation 2 for the true growth yield coefficient. The calculated maintenance energy coefficients for D < and D > 0.017 $h^{-1}$ were 0.06 and 0.20 respectively ( Table 8.3 ). The true growth yield coefficients for D < and D > 0.017 $h^{-1}$ were evaluated as 0.11 and 1.15 respectively.

Table 8.3 Maintenance Energy and Growth Yield Coefficients Calculated from Figures 8.11(A) and (B)

| Plot | Dilution Rate $h^{-1}$ | Regression Equation | Maintenance Coefficient[1] g/g. | Yield Coefficient[2] g/g |
|---|---|---|---|---|
| 1/Y vs 1/D | <0.017 | 1/Y = 9.2 + 0.06 1/D | 0.06 | 0.11 |
| | >0.017 | 1/Y = 1.3 + 0.20 1/D | 0.20 | 0.79 |
| Qs vs D | <0.017 | Qs = 0.06 + 9.16 D | 0.06 | 0.11 |
| | >0.017 | Qs = 0.21 - 0.87 D | 0.21 | 1.15 |

Pachysolen tannophilus NRRL Y-2461 was grown in a chemostat under conditions indicated in text.

1 Maintenance energy coefficient (m) is expressed as g substrate/g dry cells/h

2 Yield coefficient (Yg) is expressed as g cells produced/g substrate consumed

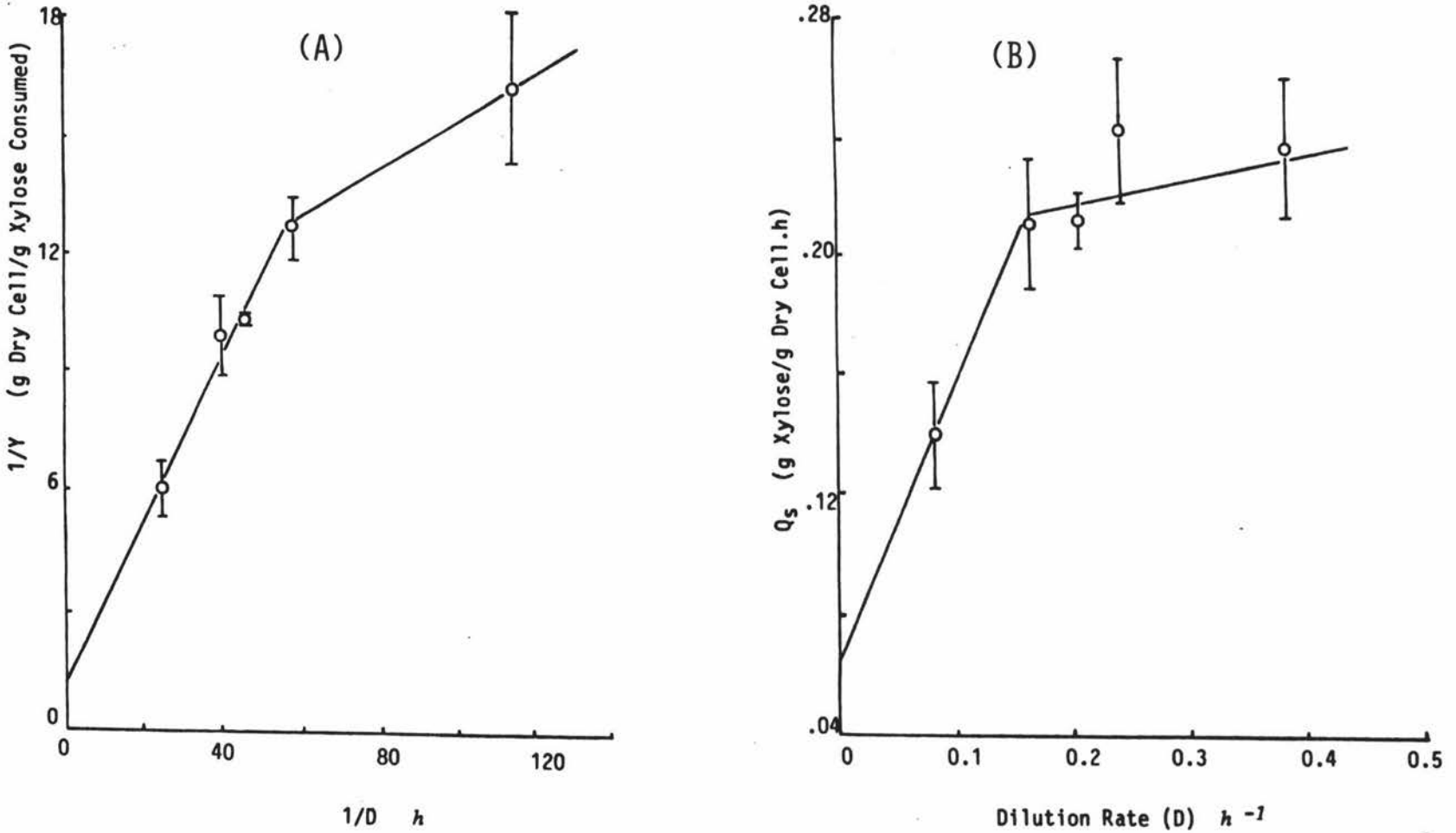



Figure 8.11 Graphical Estimations of Growth Yields for *Pachysolen tannophilus* NRRL Y-2461. Using A) The Double Reciprocal Plot of Cell Growth Yield (Y) versus Dilution Rate (D)
and B) The Specific Rate of Substrate Consumption ($Q_s$) versus Dilution Rate (D). The Chemostat was run at 28°. A volume of 1.35 ℓ was employed with D-xylose the limiting substrate.

Under oxygen-limited continuous culture conditions, xylose was fermented to xylitol, ethanol and carbon dioxide. It is suggested that the mass balance may be represented by the following equation :

$$7\ \text{Xylose} + 15\ O_2 \text{ -----> } \text{Xylitol} + 4\ \text{Ethanol} + 2\ \text{Cell} + 20\ CO_2 + 15\ H_2O$$

| | | | | |
|---|---|---|---|---|
| Theoretical : (g mole/g mole xylose) | 0.145 | 0.175 | 0.047 | 0.838 |
| Experiment : | 0.135 | 0.170 | 0.040 | 0.655 |

can be seen that calculated values based on the above stoichiometry are matched closely by the empirical values, thus emphasizing the validity of the proposed conversion reaction.

8.3.4 The Redox Controlled Chemostat

The chemostat experiments as described in the previous section (8.3.3) were conducted under "semi-aerobic" conditions. Air was sparged at 0.37 l/l.min and mixed at 100 r.p.m. Those experiments established base- line kinetic values for the fermentation operating without redox control.

The purpose of the work described in this present section was to evaluate the fermentation kinetic data with the 1.5 l chemostat operating under redox potential conditions (Pirt, 1975). A redox measuring electrode and control system was assembled according to Clark (1982) and steady-states were established at +30, -50, -80 mV. Considerable difficulty prevented the establishment of steady-state values below -80 mV. Throughout, the 18 g/l D-xylose plus 6.7 g/l

Yeast Nitrogen Base medium was used in a 1.5 l vessel operating at D = 0.007 $h^{-1}$. All other conditions were as described in Sections 3.6.2.1. and 8.3.3.

The results (in Table 8.4) suggested that there was some advantage in keeping the redox potential value at -50 mV because high sugar utilization (13.6 g/l) resulted in high ethanol concentrations (3.4 g/l) with low xylitol yields (1.2 g/l).

The product yield per unit biomass (Yp/x) seemed to remain constant with decreasing redox potential values. Higher cell and ethanol yields at negative redox potentials (Table 8.5) were observed. Xylitol production per g xylose consumed was minimal at -50 mV while the rates of volumetric ethanol production (0.024 g/l.h) and substrate consumption (0.095 g/l.h) were maximal at -50 mV (Table 8.6). This suggests some advantage in maintaining the fermentation at -50 mV.

Table 8.4 Ethanolic Fermentation of D-Xylose by Pachysolen tannophilus NRRL 2461 under Continuous Flow and Redox Controlled Conditions*

| Redox Potential mV | Xylitol Produced g/l | Xylose Residual g/l | Xylose Utilized g/l (%) | Biomass g/l | Ethanol in Vessel g/l |
|---|---|---|---|---|---|
| +30 | 1.3 | 11.4 | 5.3 (29.4) | 0.19 | 0.8 |
| -50 | 1.2 | 3.2 | 13.6 (75.5) | 0.85 | 3.4 |
| -80 | 2.1 | 6.5 | 9.4 (52.2) | 0.54 | 2.4 |
| N.R.** | 2.1 | 1.9 | 14.0 (77.8) | 0.90 | 2.2 |

* Fermentation conditions : 28°, 100 r.p.m., D=0.007 $h^{-1}$, So=18 g/l

** N.R. No Reading (D=0.009 $h^{-1}$, So=18 g/l) Uncontrolled.

Table 8.5 Derived Fermentation Characteristics for *Pachysolen tannophilus* NRRL 2461 in Continuous Culture with Controlled Redox Potential*

| Redox Potential mV | Yield Ratios | | | |
|---|---|---|---|---|
| | Product Yp/x g/g | Ethanol Yp/s g/g | Biomass Yx/s g/g | Xylitol Yxo/s g/g |
| +30 | 4.21 | 0.15 | 0.04 | 0.25 |
| -50 | 4.00 | 0.25 | 0.06 | 0.09 |
| -80 | 4.44 | 0.26 | 0.06 | 0.22 |
| N.R.** | 2.44 | 0.16 | 0.07 | 0.15 |

* Fermentation conditions : 28°, 100 r.p.m., D=0.007 $h^{-1}$, So=18 g/l
** N.R. No Reading (D=0.009 $h^{-1}$, So=18 g/l) Uncontrolled.

Table 8.6 Volumetric Ethanol and Xylitol Productivities and Specific Growth Rates of Pachysolen tannophilus NRRL 2461 in Continuous Culture with Controlled Redox Potential*

| Redox Potential mV | Volumetric Rates | | | |
|---|---|---|---|---|
| | Ethanol Production g/l.h | Substrate Consumption g/l.h | Xylitol Production g/l.h | Specific Growth Rates $h^{-1}$ |
| +30 | 0.006 | 0.037 | 0.009 | 0.007 |
| -50 | 0.024 | 0.095 | 0.008 | 0.007 |
| -80 | 0.017 | 0.066 | 0.015 | 0.007 |
| N.R.** | 0.020 | 0.126 | 0.019 | 0.009 |

* Fermentation conditions : 28°, 100 r.p.m., D=0.007 $h^{-1}$, So=18 g/l
** N.R. No Reading (D=0.009 $h^{-1}$, So=18 g/l) Uncontrolled.

Figures 8.12 (A) to (C) present plots of (A) ethanol concentration at steady state versus cell concentration, (B) sugar consumed and in (C) is shown a plot of cells versus sugar consumed. Data, from the redox controlled system were plotted with those obtained from a chemostat without redox control. Thus the enhancement of ethanol yields under redox control can be seen immediately.

The product yields (Yp/x) and other derived yield ratios are presented in Table 8.7. The product yields (Yp/x) were very similar (approximate 4 g/g) irrespective of whether or not redox control was used. Thus redox control, although it increased Yp/s, did not affect Yp/x. Such an observation may be useful for application to larger scale fermentation as the higher ethanol yields were very encouraging. Furthermore, the low xylitol yields were particularly noteworthy.

Table 8.7 Comparison of Yield Ratios of Pachysolen tannophilus in Continuous Culture with and without Controlled Redox Potential

| Yield Ratios g/g | Chemostat System: With Series of Redox Potential Controlled | Chemostat System: Under Oxygen-limited Conditions Without Any Redox Potential Controlled |
|---|---|---|
| Xylitol ( Yxo/s ) | * | 0.14 |
| Product ( Yp/x ) | 4.00 | 4.20 |
| Ethanol ( Yp/s ) | 0.31 | 0.17 |
| Biomass ( Yx/s ) | 0.08 | 0.04 |

* Xylitol yield coefficient varied with different redox values in the culture. Hence, the value was not available.

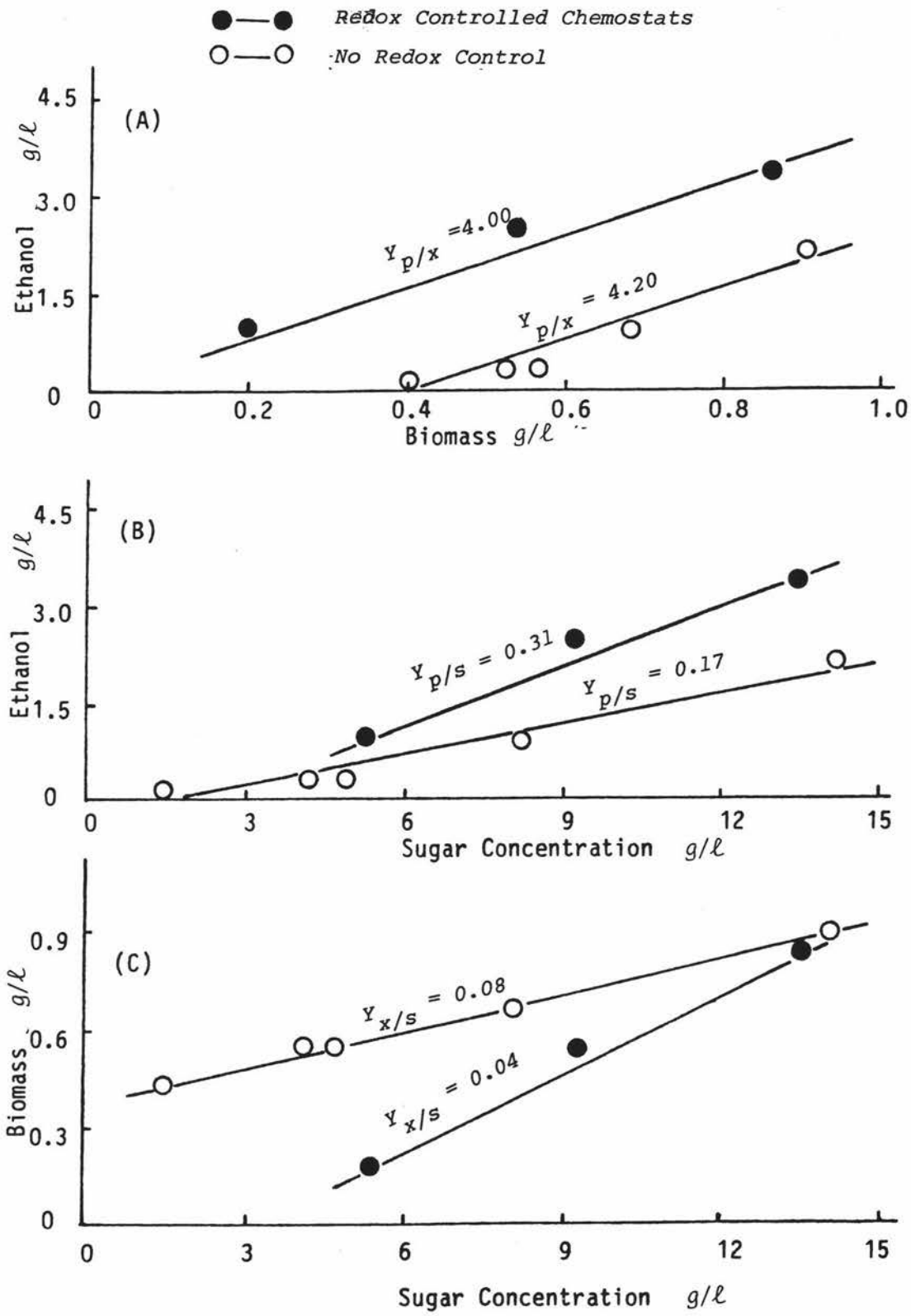



Figure 8.12 The Effect of Redox Control of Culture Growth Medium on a Number of Cell Growth Characteristics. *Pachysolen tannophilus* NRRL Y-2461 was Grown in a 1.35 ℓ Chemostat Under Substrate Limitation. Redox Control was Set at +30, -50 & -80 mV.

## 8.4 DISCUSSION

In the "pulse" aerated batch fermentation, Section 8.3.2, it was observed that the ethanol yield under anoxic conditions was 0.31 g/g as compared with 0.30 and 0.28° g/g reported by Slininger et al. (1982) and du Preez et al. (1984) respectively. These differences are minor. A possible explanation for the varying ethanol yields in the different phases is that in phase I, no cell growth and no ethanol was measured and the bulk of xylose remained unused, due to complete anaerobiosis. It is suggested that the absence of oxygen had prevented the cyclic operation of the TCA cycle which would supply NADPH for the xylose reductase reaction (as proposed by Jeffries, (1981b and 1983) and Smiley and Bolen, (1982)) and also supply of NAD for fermentation. Debus et al. (1983) and Bruinenberg et al. (1983) demonstrated that the major source NADPH in yeast was the hexose monophosphate (HMP) pathway, whereas the isocitrate dehydrogenase reaction was only a minor source of NADPH. Hence, it is also suggested that the presence of oxygen seems to initiate the TCA cycle which generates ATP for phosphorylation of xylulose-5-phosphate and also generates NADPH as required in the first step of xylose metabolism. Subsequently, the NADPH generation proceeds in the HMP pathway by glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase action.

In aerobic phase (II), from 64 to 100 h, air was introduced into the culture and rapid cell growth was observed, which led to high maximum specific growth rate and extremely low specific ethanol production rates and ethanol yields. Respiration predominated over fermentation during this phase.

Following the aerobic phase, a shift to total anaerobiosis resulted in an uninterrupted continuous formation of ethanol and further xylose consumption. No growth was shown however throughout the anaerobic conditions (phase III). A similar result was reported by Bruinenberg et al. (1984) who demonstrated a very slow xylose consumption and ethanol formation when a culture of Pachysolen tannophilus was shifted from full aerobiosis to anaerobiosis. An

aerobic fermentation was induced in phase IV where both cell growth and ethanol production and consumption were observed. Maleszka and Schneider (1982b) have reported a similar observation.

During phase V, air was not supplied to the culture. Oxygen however may not totally be depleted because of entrainment from the head space. No cell growth was shown and lower ethanol production was detected as compared with aerobic conditions (phase III). The possible explanation was the concurrent production and consumption of ethanol under anoxic conditions (Maleszka and Schneider, 1982b; Debus et al., 1983) and the formation of by-product, xylitol, late in the fermentation by Pachysolen tannophilus (Schneider et al., 1982; Debus et al., 1983). These proposals listed above which attempt to explain the observations may be visualised as reaction sequences shown in Figure 8.13.

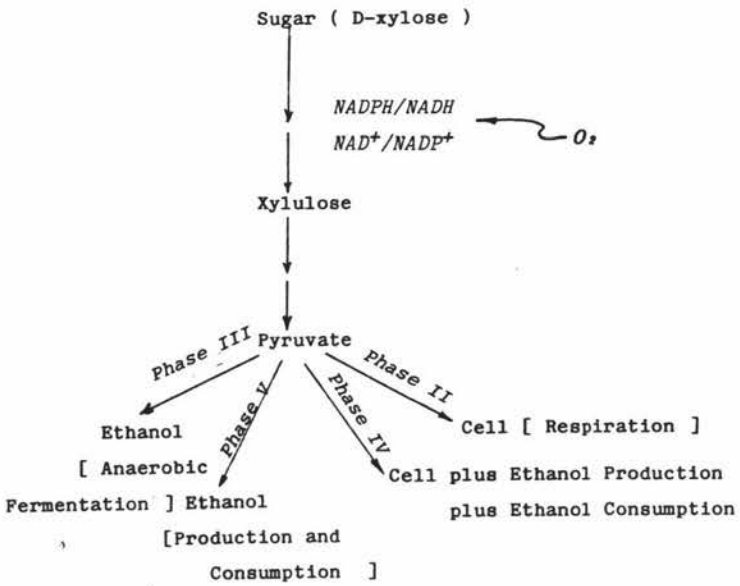



Figure 8.13 Suggested Fate of D-xylose during the Growth of Pachysolen tannophilus under "Pulse" Aerated Conditions of the Culture

Gaseous conditions prevailing in the batch culture, i.e. the gaseous "phases" noted in Fig. 8.2. directed the nature of products formed from D-xylose by the yeast (strain 2461).

These observations under anoxic conditions are in agreement with the data of Slininger et al., (1982b) who reported that Pachysolen tannophilus could convert xylose to ethanol anaerobically, although no growth occurred. Apparently these researchers did not de-aerate the fermentation flask and the oxygen initially present in the culture and entrained from the head-space meant that conditions were not totally anaerobic. Consequently, ethanol production may have been stimulated.

The data presented in Figure 8.2, indicated that Pachysolen tannophilus required oxygen for both growth and ethanol formation. This finding is supported by du Preez et al., (1984) and Jeffries, (1982). An efficient fermentation of xylose to ethanol by Pachysolen tannophilus would be obtained under anaerobic conditions only if cell synthesis, ethanol assimilation and xylitol production could be blocked. It thus appears necessary to use a very high population of cells together with very careful control of the oxygen supply to achieve a successful ethanolic fermentation of xylose.

An alternative approach to a fermentation system with carefully controlled oxygenation rates, would be to use a mutant culture with a metabolic block which prevents ethanol consumption. This may prove to be a better option and recently, Purdue University researchers have successfully isolated an ethanol-tolerant mutant of Pachysolen tannophilus which produces 36 g/l ethanol from D-xylose in less than 48 h with a conversion efficiency of 85 to 90% (cited by Arthur, 1983).

Under a continuous flow system, ethanol, xylitol and cell production were related linearly to substrate consumption. However, on extrapolation, each curve did not pass through the graphs' origin. The discrepancy could be explained in terms of the observation of two values for the maintenance energy (see Graphs 8.11 (A) and (B)). A similar observation was cited by Kirsch and Sykes (1971) when re-plotting the data presented by Tempest et al. (1967). Also, for a plot of specific substrate consumption rate (Qs) versus dilution rate (D), Beyeler et al. (1984) likewise reported that non-linear behaviour was observed for Zymomonas mobilis growing at relatively high dilution rates.

According to Maleszka and Schneider (1982) and Schneider *et al.*,(1983), *Pachysolen tannophilus* can convert more than 15% of the initial xylose to xylitol. Under anaerobic chemostat conditions, immobilized *Pachysolen* cells excreted xylitol in amounts as high as 13% of initial isomerate concentration (Suihko and Poutanen, 1984). The presently described work demonstrated that 13.5% of xylose was converted to xylitol.

Xylitol yield coefficients (Yxo/x) are influenced by aeration (du Preez *et al.*, 1984; Debus *et al.*, 1983). The values noted by Debus *et al.* *viz*; 0, 0.52, and 0.85 g/g for aerobic, "semi-aerobic" and anaerobic conditions respectively were twice as high as the values published by du Preez *et al.* and certainly very much higher than those measured in the present studies using the redox controlled chemostat. The significance of xylitol formation has been explained by Debus *et al.* (1983) in terms of its acting as an electron sink for NADPH, generated in the hexose monophosphate pathway.

The stoichiometry of xylose conversion to ethanol under anaerobic conditions has been postulated by Debus *et al.* (1983) as:

$$5\ \text{Xylose} + 2\ H_2O \longrightarrow 4\ \text{Xylitol} + \text{Ethanol} + 3\ CO_2$$

Bruinenberg *et al.* (1983 and 1984) also have proposed stoichiometric equations based on the discrepancy existing between the production and consumption of cellular pools of NADH and NADPH. Equations were proposed as follows,

either via : NADP-linked xylose reductase/NADH-linked xylitol dehydrogenase

$$6\ \text{Xylose} \longrightarrow 9\ \text{Ethanol} + 9\ CO_2$$

or via : both NAD(P) and NAD(P)H+ -linked xylose reductase and xylitol dehydrogenase

6 Xylose ----------> 10 Ethanol + 10 $CO_2$

It will be noted that these conversions do not take into account any xylitol synthesis whatsoever, nor do they take into account acetate formation.

A possible scheme for D-xylose conversion to xylitol, ethanol and carbon dioxide is presented in Figure 8.14.

In this scheme, xylose is first reduced to xylitol by NADPH-dependent xylose reductase. Low activity of an NADH-linked xylose reductase has also been suggested (Maleszka et al., 1983; Smiley and Bolen, 1982 and Bruinenberg et al., 1984). Xylitol is then oxidized to xylulose by an NAD-dependent xylitol dehydrogenase. Both NADPH and NAD+ required for the xylose reductase and xylitol dehydrogenase reactions are possibly generated in the TCA cycle. The presence of oxygen is required to trigger both the operation of this cycle and the formation of NAD+ and NADPH. This is deduced from the observed oxygen requirement by Pachysolen tannophilus during the fermentation of xylose.

Once xylose metabolism is established, the major source of NADPH formation is from the hexose monophosphate pathway (Bruinenberg et al., 1983) and/or the pentose phosphate cycle (Sols et al., 1971).

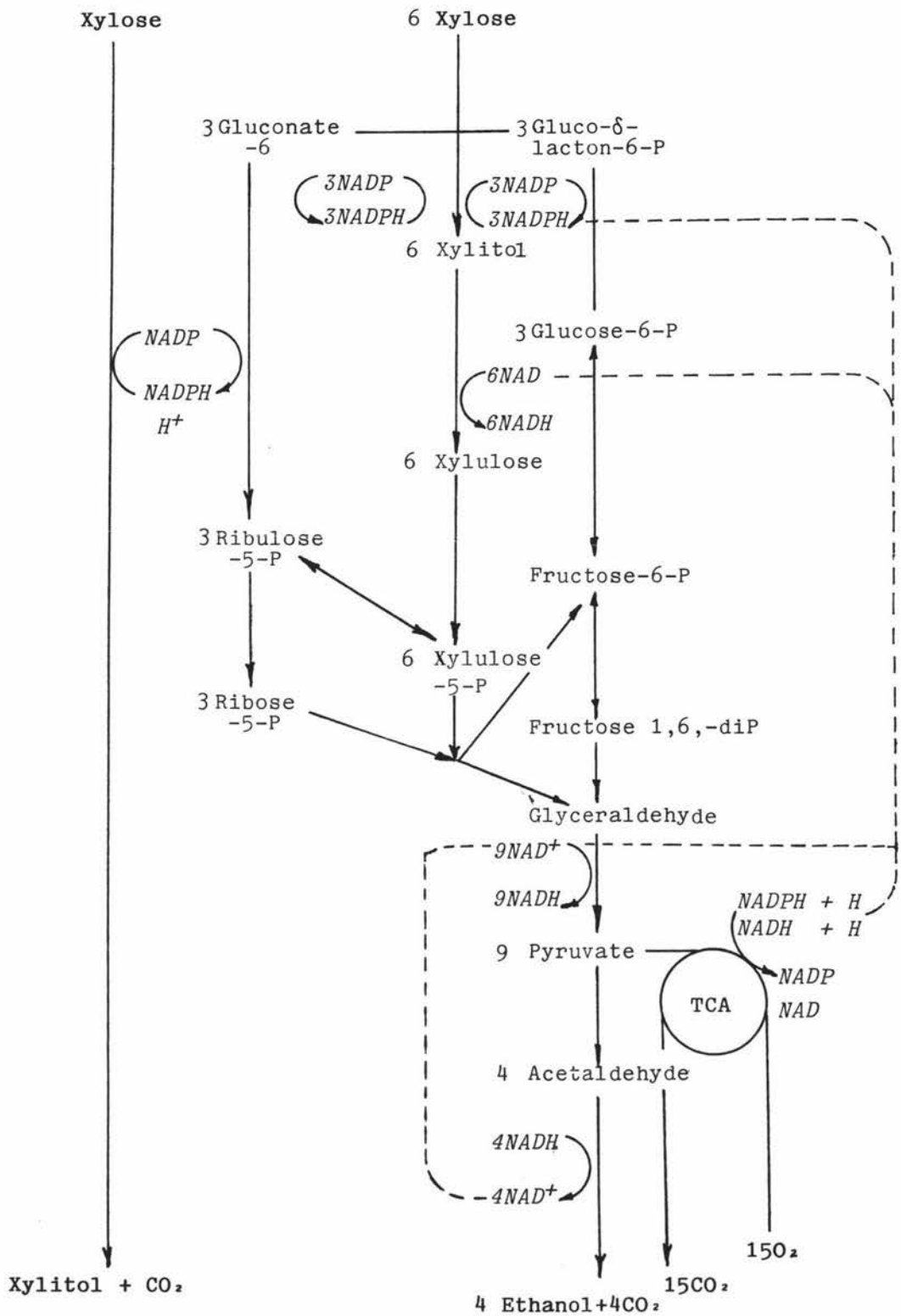



Figure 8.14 Proposed D-xylose Metabolic Scheme of Pachysolen tannophilus under Oxygen-limited Conditions

The production of xylitol is often observed late in the fermentation (Schneider, 1982; du Preez et al., 1984). This may be explained by a depletion of the culture's dissolved oxygen concentration with the overproduction of NADP which causes the phenomenon observed by Debus et al., (1983). There, four moles of xylitol were produced under anaerobic conditions. The lower ethanol yield observed under anaerobic conditions by Debus et al. might thus be explained in terms of overproduction of NADH (Bruinenberg et al., 1984) which cannot be reoxidized in the absence of oxygen. Xylitol is thus seen to accumulate.

## 8.5 CONCLUSIONS CONCERNING D-XYLOSE FERMENTATION BY

### Pachysolen tannophilus

The D-xylose fermentation by Pachysolen tannophilus NRRL Y-2461 growing in batch culture has been studied. A decrease in culture pH coincided with cell growth and substrate uptake. The growth of the yeast responded characteristically to the presence or absence of oxygen in the culture during experiments in which the culture was sparged with either nitrogen or air. However, ethanol formation did not follow the same pattern of production as did cell development. The production of ethanol was concomitant with the late "exponential" phase of cell growth.

In a continuous flow system, ethanol, xylitol and cell production were all linearly related to substrate consumed. Furthermore, two distinct maintenance coefficients values were calculated for a range of dilution rates. At a very low dilution rate, i.e. $< 0.017\ h^{-1}$, the maintenance energy coefficient was 0.06 g energy source/g dry biomass/h. The true growth yield was evaluated as 0.11 g/g. However, at dilution rates higher than $0.017\ h^{-1}$, different values for the maintenance energy coefficient and the true growth yield were obtained, namely 0.20 g energy source/g dry biomass/h and 1.15 g/g respectively.

Pachysolen tannophilus did not appear to tolerate high ethanol concentrations and its ethanol inhibition constant ( Ki ) for growth

was 0.5 g ethanol/l.

When controlling the redox potential of the chemostat culture at -50 mV, a relatively high ethanol yield was achieved as compared with oxygen-limited conditions, that is, when aeration rate was 0.37 l/l.min. The xylitol yield at -50 mV was greatly reduced which was an encouraging aspect of controlling the cultures redox potential.

Nearly two-fold increases in the yields of ethanol and cell mass were also demonstrated under redox potential controlled conditions as compared with yields from non-redox-controlled or oxygen-limited conditions.

The variable aeration batch culture study, the oxygen-limited continuous study and the redox controlled chemostat studies all revealed that Pachysolen tannophilus requires oxygen for both growth and the initiation of ethanol fermentation. The extent of xylitol formation appeared to be determined by the culture's redox potential, that is, the dissolved oxygen status of the culture. The proposed fermentation stoichiometry suggested that under oxygen-limited conditions, a maximal ethanol yield could be obtained by carefully controlling the dissolved oxygen tension status of the culture.

Further, it is suggested that an optimal redox potential, (i.e. an optimal dissolved oxygen concentration), can be established which permits the balanced production and consumption of both NADPH and NADH in Pachysolen tannophilus.

# CHAPTER NINE : GENERAL CONCLUSIONS

Experimentation in the early part of this decade drew attention to the D-xylose fermenting ability of the yeast Pachysolen tannophilus. This focus of attention was a clearly defined starting point for this thesis which has considered not only various aspects of the fermentation characteristics of Pachysolen tannophilus but also sought to find other yeasts with similar abilities. Yeasts closely related, both phylogenetically and physiologically, were considered in the first instance. No Hensenula species were found superior in character to Pachysolen tannophilus. Taking a broader view, Candida species were reviewed and again no tested yeast surpassed Pachysolen tannophilus in D-xylose fermenting ability. Since that time of testing, however, Candida shehatae has been reported as being useful. However, this currently described work could not identify any yeast superior in fermentative abilities to Pachysolen tannophilus and consequently the ongoing research involved its use.

Studies of fermentation characteristics of Pachysolen tannophilus in shake-flask cultures, showed that this yeast could ferment to ethanol all of the sugars of pine-wood acid hydrolyzate. Some variation of cell growth on hexoses and pentoses and ethanol formation was noted. The slower utilization of D-xylose and L-arabinose and D-galactose as compared with the rapid fermentation of D-glucose and D-mannose was observed. Pachysolen tannophilus could easily assimilate 4 g/l of ethanol as substrate but a lag period of 4 days was observed when this substrate's concentration was increased four-fold (i.e. to 16 g/l of ethanol).

Neutralization of inhibitory substances in the hydrolyzate was achieved with 2 g/l of anhydrous sodium sulphite. Large amounts of initial yeast inoculum was also recommended for reducing the fermentation time. This, however, decreased the specific growth rate of the yeast.

At the rapid agitation speed of 200 r.p.m., the shaken culture exhibited a low final ethanol concentration but a high cell population. Static culture, that is no agitation, yielded traces of ethanol and also a low cell population. Mild agitation at 50 and 100 r.p.m. proved to be the most suitable aeration conditions. Similarly, at the high agitation speed of 200 r.p.m. and with high inoculum concentration, the admission of pure oxygen-free nitrogen after 24 hours of incubation markedly diminished the cell formation although the yield of ethanol remained high. Thus it seems appropriate that this fermentation not be conducted under either totally anaerobic or totally aerobic conditions as both extremes are inhibitory to ethanol formation.

Attempts to elucidate the role of oxygen in the fermentation and in particular its influence on ethanol production were made using both batch and continuous cultures of Pachysolen tannophilus NRRL Y-2461. Both D-xylose synthetic media and the prehydrolyzate based medium was used. Batch fermentation studies demonstrated that cell growth, substrate uptake rate and culture pH responded strongly to oxygen supply , but that the production of ethanol was concomitant with the period of late exponential cell growth phase. This suggested that aeration was at least a pre-requisite for achieving ethanol production but may not have been essential towards the end of active growth.

A continuous flow homogeneous culture was set up and aerated at 0.37 l air/l.min. so that there was no residual dissolved oxygen in the culture. Ethanol, xylitol production, and cell yield were linearly correlated to the amount of substrate consumed. The calculated kinetic data were as follows; maximum specific growth rate, 0.046 $h^{-1}$; biomass yield, 0.04 g/g; ethanol yield, 0.17 g/g; Ks value, 13 g/l. When attempting to establish the maintenance energy coefficient, it was found that two values could be obtained. At low dilution rates (less than 0.017 $h^{-1}$) a value of 0.06 was calculated while at higher dilution rates (greater than 0.017 $h^{-1}$) a value of 0.20 g energy source/g dry biomass/h was found.

End-product inhibition was observed as the non-competitive

growth-associated type and the Ki value was calculated as 0.5 g/l.

At extremely low values of dissolved oxygen tension approaching anaerobic conditions, it seemed appropriate to measure and control the overall oxidation-reduction potential of the culture so that pre-selected values of limited oxygenation could be achieved. On controlling the redox potential at -50 mV, there was a 55% increase in the ethanol and a 43% reduction in xylitol production compared with those values observed without redox control. Such a finding may have commercial application.

A quantitative study of the effects of fermentation process variable interactions on the pentose fermentation by the yeast Pachysolen tannophilus growing in a synthetic medium and in soft wood prehydrolyzate was achieved using statistically designed experiments. Empirical models resulting from these experiments showed that the ethanol yields and maximum average ethanol production rates were linear functions of the statistically coded initial substrate concentrations in the culture. Temperature and pH variables had marked effects on yields of cell and ethanol and also on specific growth rates for each strain tested. In these later studies strain NRRL Y-2461 seemed to perform better than the other two strains (NRRL Y-2460 and IFO 1007). Cell growth appeared to be favoured at pH 6.5 but the production of ethanol was enhanced if the initial culture pH value was kept from 5.6 to 5.8.

**REFERENCES**

[ 1 ] Abbott, B.J. 1978
Immobilized Cells.
Annual Reports of Fermentation Processes, 2:91-123.

[ 2 ] Adams, G.A. and Strainer, R.Y. 1945
2,3-Butanediol (III) Carbohydrate Fermentation by
Aerobacillus polymyxa
Canadian Journal of Research, 37F:48-71.

[ 3 ] Aiba, S., Shoda, M. and Nagatani, M. 1968
Kinetics of Product Inhibition in Alcohol Fermentation.
Biotechnology and Bioengineering, 10:845-864.

[ 4 ] Antrim, R.L., Colilla, W. and Schnyder, B. 1979
Glucose Isomerase Production of High-Fructose Syrups.
Applied Biochemistry and Bioengineering, 2:97-155.

[ 5 ] Arthur, D. Little International Incorporation 1983
A Critical Review of Hydrolysis and Fermentation of
Cellulose as a Route to Liquid Fuel.
Liquid Fuels Trust Board, Report No. LF 5002.
pp.132-149. Wellington, New Zealand.

[ 6 ] Auling, G., Bellgardt, K.H., Diekmann, H. and Thoma, M. 1984
Dynamics of Growth in Batch and Continuous of Saccharomyces cerevisiae during Shifts from Aerobiosis to Anaerobiosis and Reverse
Applied Microbiology and Biotechnology, 19:353-357.

[ 7 ] Azhar, A.F., Brey, M.K., Colcord, A.R., Roberts, R.S. and Corbitt, G.V. 1981
Factors Affecting Alcohol Fermentation of Wood Acid Hydrolyzate.
Biotechnology and Bioengineering Symposium, 11:293-300.

[ 8 ] Baillargeon, M.W, Jansen, N.B., Gong, C.S. and Tsao, G.T. 1983
Effect of Oxygen Uptake Rate on Ethanol Production by a Xylose-Fermenting Yeast Mutant, Candida sp XF 217.
Biotechnology Letters, 5(5):339-344.

[ 9 ] Banerjee, N., Bhatnagar, R. and Viswanathan, L. 1981
Inhibition of Glycolysis by Furfural in Saccharomyces cerevisiae.
European Journal of Applied Microbiology and Biotechnology, 11:226-228.

[ 10] Barnett, J.A. 1968
Biochemical Differentiation of Taxa with Special Reference to the Yeasts.
In : The Fungi Volume 3. Edited by Ainsworth, G.C. and Sussman, A.S. pp.557-595. Academic Press, New York, U.S.A.

[ 11] Barnett, J.A. 1976
The Utilization of Sugars by Yeasts.
Advances in Carbohydrate Chemistry and Biochemistry, 32:125-234.

[ 12] Batter, T.R. and Wilke, C.R. 1977
A Study of the Fermentation of Xylose to Ethanol by Fusarium oxysporum.
Lawrence Berkeley Laboratory, Publication No. 6351.
University of Califonia, Berkeley, U.S.A.

[ 13] Bazua, C.D. and Wilke, C.R. 1977
Ethanol Effects on the Kinetics of a Continuous Fermentation with Saccharomyces cerevisiae.
Biotechnology and Bioengineering Symposium, 7:105-118.

[ 14] Berenson, M.L., Levine, D.M. and Goldstein, M. 1983
Intermediate Statistical Methods and Applications - A Computer Package Approach
Prentice-Hall Inc., Englewood Cliffs, U.S.A.

[ 15] Beyeler, W., Rogers, P.L. and Fiechter, A. 1984
A Simple Technique for the Direct Determination of Maintenance Energy Coefficient : An Example with Zymomonas mobilis
Applied Microbiology and Biotechnology, 19:277-280.

[ 16] Boidin, J. and Adzet, J.M. 1957
Deux curieuses levuses isolees d'extraits tannants d'origine vegetale : Pachysolen (nov. gen.) tannophilus nov. sp. et Pachysolen pelliculatus nov. sp.
Bulletin de la Societe Mycologique de France, 73:331-342.

[ 17] Box, G.E.P., Hunter, W.G. and Hunter, J.S. 1978
Statistics for Experimenters : An Introduction to Design, Data Analysis and Model Building.
John Wiley and Sons, New York, U.S.A.

[ 18] Brown, D.E. and Zainudeen, M.A. 1978
Effect of Inoculum Size on the Aeration Pattern of Batch Cultures of a Fungal Micro-organism.
Biotechnology and Bioengineering, 20:1045-1061.

[ 19] Brown, S.W., Oliver, S.G., Harrison, D.E.F. and Righelato, R.C. 1981
Ethanol Inhibition of Yeast Growth and Fermentation : Differences in the Magnitude and Complexity of the Effect.
European Journal of Applied Microbiology and Biotechnology, 11:151-155.

[ 20] Bruinenberg, P.M., de Bot, P.H.M., van Dijken, J.P. and Scheffers, W.A. 1984
NADH-linked aldose reductase:
The Key to Anaerobic Alcoholic Fermentation of Xylose by Yeasts.
Applied Microbiology and Biotechnology, 19:256-260.

[ 21] Bruinenberg, P.M., de Bot, P.H.M., van Dijken, J.P. and Scheffers, W.A. 1983
The Role of Redox Balances in the Anaerobic Fermentation of Xylose by Yeasts.
European Journal of Applied Microbiology and Biotechnology, 18:287-293.

[ 22] Bucke, C. 1977
Industrial Glucose Isomerase.
In : Topics in Enzyme and Fermentation Biotechnology, Volume 1. Edited by Wiseman, A. pp.141-171.
Ellis Horwood Limited, Chichester, U.K.

[ 23] Bucke, C. 1983
There is more to Sweeteners than Sweetness.
Trend in Biotechnology, 1(3):67-69.

[ 24] Campbell, I. 1973
Numerical Analysis of Hensenula, Pichia and Related Yeasts Genera.
Journal of General Microbiology, 77:427-441.

[ 25] Charkravorty, M., Veiga, L.A., Bacila, M. and Horecker, B.L. 1962
Pentose Metabolism in Candida.
II. The Diphosphopyridine Nucleotide-Specific Polyol Dehydrogenase of Candida utilis.
Journal of Biological Chemistry, 237:1014-1020.

[ 26] Chen, S.L. 1981
Optimization of Batch Alcoholic Fermentation of Glucose Syrup Substrate
Biotechnology and Bioengineering, 23:1827-1836.

[ 27] Chen, T.S. 1983
Personal Communication.

[ 28] Chen, W.P. 1980a
Glucose Isomerase ( A Review ).
Process Biochemistry, 15(5):30-35.

[ 29] Chen, W.P. 1980b
Glucose Isomerase ( A Review ).
Process Biochemistry, 15(6):36-41.

[ 30] Chiang, C. and Knight, S.G. 1960
The Metabolism of D-Xylose by Molds.
Nature(London), 188:79-81.

[ 31] Chiang, L.C., Gong, C.S., Chen, L.F. and Tsao, G.T. 1981a
D-Xylose Fermentation to Ethanol by Saccharomyces cerevisiae.
Applied and Environmental Microbiology, 42(2):284-289.

[ 32] Chiang, L.C., Hsiao, H.Y., Flickinger, M.C., Chen, L.T. and Tsao, G.T. 1982
Ethanol Production from Pentoses by Immobilized Micro-organisms.
Enzyme and Microbial Technology, 4(3):93-95.

[ 33] Chiang, L.C., Hsiao, H.Y., Ueng, P.P. and Tsao G.T. 1981b
Enzymatic and Microbial Preparation of D-Xylulose from D-Xylose.
Applied and Environmental Microbiology, 42(1):66-69.

[ 34] Chiang, L.C., Hsiao, H.Y., Ueng, P.P., Chen, L.F. and Tsao, G.T. 1981c
Ethanol Production from Xylose by Enzymic Isomerization and Yeast Fermentation.
Biotechnology and Bioengineering Symposium, 11:263-274.

[ 35] Clark, T.A. 1982
The 19-Hydroxylation of Cortexolone by the Fungus Pellicularia filamentosa.
Ph. D. Thesis, Massey University, Palmerston North, New Zealand.

[ 36] Clark, T.A. snf Mackie, K.L. 1984
Fermentation Inhibitors in Wood Hydrolyzates Derived from the Softwood Pinus radiata.
Journal of Chemical Technology and Biotechnology, 34B :101-110.

[ 37] Claus, D. and Wilmanns, H. 1974
Enrichment and Selective Isolation of Sarcina maxima Lendner.
Archive of Microbiology, 96:201-204.

[ 38] Cysewski, G.R. 1976
Fermentation Kinetics and Process Economics for the Production of Ethanol.
Ph. D. Thesis, University of California, Berkeley, U.S.A.

[ 39] Cysewski, G.R. and Wilke, C.R. 1976
Utilization of Cellulosic Materials through Enzymatic Hydrolysis. I. Fermentation of Hydrolyzate to Ethanol and Single Cell Protein.
Biotechnology and Bioengineering, 18:1297-1313.

[ 40] Dasari, G., Roddick, F., Connor, M.A. and Pamment, N.B. 1983
Factors Affecting the Estimation of Intracellular Ethanol Concentrations.
Biotechnology Letters, 5(11):715-720.

[ 41] Davis, B.J. 1964
Disk Electrophoresis. II. Method and Application to Human Serum Proteins.
Annals of the New York Academy Sciences, 121:404-427.

[ 42] Debus, D., Methner, H., Schulze, D. and Dellweg, H. 1983
Fermentation of Xylose with the Yeast Pachysolen tannophilus.
European Journal of Applied Microbiology and Biotechnology, 17:287-291.

[ 43] Dekker, R.F.H. 1982
Ethanol Production from D-Xylose and Other Sugars by the Yeast Pachysolen tannophilus.
Biotechnology Letters, 4(7):411-416.

[ 44] Detroy, R.W., Cunningham, R.L. and Herman, A.I. 1982a
Fermentation of Wheat Straw Hemicelluloses to Ethanol by Pachysolen tannophilus.
Biotechnology and Bioengineering Symposium, 12:81-89.

[ 45] Detroy, W.R., Cunningham, R.L., Bothast, R.J., Bagby,.M.O. and Hermam, A. 1982b
Bioconversion of Wheat Straw Cellulose/Hemicellulose to Ethanol by Saccharomyces uvarum and Pachysolen tannophilus.
Biotechnology and Bioengineering, 24:1105-1113.

[ 46] Deverell, K.F. 1983
Ethanol Production from Wood Hydrolyzates using
Pachysolen tannophilus.
Biotechnology Letters, 5(7):475-480.

[ 47] Deverell, K.F. and Whitworth, D.A. 1980
Fermentation of Wood Sugars from a Pilot Plant
Acid Hydrolysis Percolation Reactor.
New Zealand Forest Service, Forest Research Institute,
Forest Products Laboratory Report, No. WC/59, 1980.
(Unpublished)

[ 48] Dische, Z. and Borenfreund, E. 1951
A New Spectrophotometric Method for the Detection and
Determination of Keto Sugars and Trioses.
Journal of Biological Chemistry, 192:583-587.

[ 49] Egamberdiev, N.B. and Ierusalimskii, N.D. 1968
Effect of Ethanol Concentration on Rate of Growth of
Saccharomyces vini (RACE Pr-1).
Microbiology, 37(4):686-690.

[ 50] Ericsson, E.O. 1947
Alcohol from Sulfite Waste Liquor.
Chemical Engineering Progress, 43(4):165-167.

[ 51] Eroshin, V.K., Utkin, I.S., Ladynichev, S.V., Samoylov, V.V., Kuvshinnikov, V.D. and Skryabin, G.K. 1976
Influence of pH and Temperature on the Substrate Yield Coefficient of Yeast Growth in a Chemostat
Biotechnology and Bioengineering, 18:289-295.

[ 52] Esser, K. and Karsch, T. 1984
Bacterial Ethanol Production: Advantages and Disadvantages.
Process Biochemistry, 19(7):116-121.

[ 53] Fieschko, J. and Humphrey, A. 1984
Statistical Analysis in the Estimation of Maintenance and True Growth Yield Coefficients
Biotechnology and Bioengineering, 26:394-396.

[ 54] Finn, R.K., Bringer, S. and Sahm, H. 1984
Fermentation of Arabinose to Ethanol by Sarcina ventriculi.
Applied Microbiology and Biotechnology, 19:161-166.

[ 55] Flickinger, M.C. 1980
Current Biological Research in Conversion of Cellulosic Carbohydrates into Liquid Fuels: How Far Have We Come?
Biotechnology and Bioengineering, 22(Suppl. 1):27-48.

[ 56] Fullbrook, P. and Vabo, B. 1977
Industrial Enzymatic Conversion of Starch to Sugars.
Advances in Enzyme and Membrane Technology,
Institute of Chemical Engineering (London) Symposium,
No. 51 :31-44.

[ 57] Gibbs, M., Cochrane, V.W., Paege, L.M. and Wolin, H. 1954
Fermentation of D-Xylose-1-C by Fusarium lini.
Archives of Biochemistry and Biophysics, 50:237-242.

[ 58] Gleason, F.H. 1971
Alcohol Dehydrogenase in Mucorales.
Mycologia, 63:906-910.

[ 59] Gong, C.S. 1983
Recent Advances in D-Xylose Conversion by Yeasts
Annual Reports on Fermentation Processes, 6:253-297.

[ 60] Gong, C.S., Chen, L.F., Flickinger, M.C. and Tsao, G.T. 1981a
Conversion of Hemicellulose Carbohydrates.
Advances in Biochemical Engineering/Biotechnology,
20:93-118.

[ 61] Gong, C.S., Chen, L.F., Flickinger, M.C., Chiang, L.C. and Tsao, G.T. 1981b
Production of Ethanol from D-Xylose by using D-Xylose Isomerase and Yeasts.
Applied and Environmental Microbiology, 41(2):430-436.

[ 62] Gong, C.S., Claypool, T.A., McCracken, L.D., Maun, C.M., Ueng, P.P. and Tsao, G.T. 1983
Conversion of Pentoses by Yeasts.
Biotechnology and Bioengineering, 25:85-102.

[ 63] Gong, C.S., Ladisch, M.R. and Tsao, G.T. 1981c
Production of Ethanol from Wood Hemicellulose Hydrolyzates by a Xylose-Fermenting Yeast Mutant, Candida sp. XF217.
Biotechnology Letters, 3(11):657-662.

[ 64] Gong, C.S., Maun, C.M and Tsao, G.T. 1981d
Direct Fermentation of Cellulose to Ethanol by a Cellulolytic Filamentous Fungus, Monilia sp.
Biotechnology Letters, 3(2):77-82.

[ 65] Gong, C.S., McCracken, L.D. and Tsao, G.T. 1981e
Direct Fermentation on D-Xylose to Ethanol by a Xylose-Fermenting Yeast Mutant, Candida sp XF 217.
Biotechnology Letters, 3(5):245-250.

[ 66] Haraldson, A. and Rosen, C.G. 1982
Studies on Continuous Ethanol Fermentation of Sugar Cane Molasses
II. Continuous Alcohol Fermentation and Product Removal in a Laboratory Scale Plant.
European Journal of Applied Microbiology and Biotechnology, 14:220-224.

[ 67] Harris, E.E., Hannan, M.L., Marquardt, R.R. and Bubl, J.L. 1948
Fermentation of Wood Hydrolyzates by Torula utilis.
Industrial and Engineering Chemistry, 40:1216-1220.

[ 68] Harris, G.S., Leamy, M.L., Fraser, T., Dent, J.B., Brown, W.A.N., Earl, W.B., Fookes, T.W. and Gilbert, J. 1979
The Potential Energy Farming for Transport Fuels in New Zealand.
New Zealand Energy Research and Development Committee, Report No. 46. pp.29-41. Auckland University, Auckland, New Zealand.

[ 69] Hespell, R.B. and Canale-Parola, J. 1973
Glucose and Pyruvate Metabolism of Spirocheata litoralis, an Anaerobic Marine Spirochate
Journal of Bacteriology, 116(2):931-937.

[ 70] Himmelblau, D.M. 1970
Process Analysis by Statistical Methods
John Wiley and Sons, Inc., New York, U.S.A.

[ 71] Ho, N.W.Y., Rosenfeld, S., Stevis, P. and Tsao, G.T. 1983
Purification and Characterization of the D-Xylose Isomerase Gene from Escherichia coli.
Enzyme and Microbial Technology, 5(6):417-420.

[ 72] Hofer, M., Betz, A. and Kotyk, A. 1971
Metabolism of the Obligatory Aerobic Yeast Rhodotorula gracilis IV. Introduction of an Enzyme Necessary for D-Xylose Catabolism.
Biochimica et Biophysica Acta, 252:1-12.

[ 73] Hoppe, G.K. and Hansford, G.S. 1982
Ethanol Inhibition of Continuous Aerobic Yeast Growth.
Biotechnology Letters, 4(1):39-44.

[ 74] Horecker, B.L. 1962
Pentose Metabolism in Bacteria. pp.19-31.
John Weily and Sons, New York, U.S.A.

[ 75] Hsiao, H.Y., Chiang, L.C., Chen, L.F. and Tsao, G.T. 1982
Effects of Borate on Isomerization and Yeast Fermentation of High Xylulose Solution and Acid Hydrolyzate of Hemicellulose.
Enzyme and Microbial Technology, 4(1):25-31.

[ 76] Ingram, L.O. 1976
Adaptation of Membrane Lipids to Alcohols.
Journal of Bacteriology, 125:670-678.

[ 77] James, A.P. and Zahab, D.M. 1982
A Genetics System for Pachysolen tannophilus,
a Pentose-Fermenting Yeast.
Journal of General Microgiology, 128:2297-2301.

[ 78] James, A.P. and Zahab, D.M. 1983
The Construction and Genetic Analysis of Polyploids and
Aneuploids of Pentose-Fermenting Yeast,
Pachysolen tannophilus.
Journal of General Microbiology, 129:2489-2494.

[ 79] Jansen, N.B. and Tsao, G.T. 1983
Bioconversion of Pentose to 2,3-Butandiol by Klebsiella
pneumoniae.
Advances in Biochemical Engineering/Biotechnology,
27:85-100.

[ 80] Jeffries, T.W. 1981a
Fermentation of Xylulose to Ethanol Using Xylose Isomerase
and Yeasts.
Biotechnology and Bioengineering Symposium, 11:315-324.

[ 81] Jeffries, T.W. 1981b
Conversion of Xylose to Ethanol under Aerobic Conditions by Candida tropicalis.
Biotechnology Letters, 3(5):213-218.

[ 82] Jeffries, T.W. 1982
A Comparison of Candida tropicalis and Pachysolen tannophilus for Conversion of Xylose to Ethanol.
Biotechnology and Bioengineering Symposium, 12:103-110.

[ 83] Jeffries, T.W. 1983a
Utilization of Xylose by Bacteria, Yeasts and Fungi.
Advances in Biochemical Engineering/Biotechnology, 27:1-32.

[ 84] Jeffries, T.W. 1983b
Effect of Nitrate on Fermentation of Xylose and Glucose by Pachysolen tannophilus.
Biotechnology, 1(6):503-525.

[ 85] Jeffries, T.W. 1984
Mutants of Pachysolen tannophilus Showing Enhanced Rates of Growth and Ethanol Formation from D-xylose
Enzyme and Microbial Technology, 6(6):254-258.

[ 86] Jeffries, T.W. 1984
Mutants of Pachysolen tannophilus showing enhanced rates of growth and ethanol formation from D-xylose.
Enzyme and Microbial Technology, 6:254-258.

[ 87] Jones, R.P., Pamment, N. and Greenfield, P.F. 1981
Alcohol Fermentation by Yeasts - the Effect of Environmental and Other Variables
Process Biochemistry, 16:42-49.

[ 88] Kent, C.A. and Emery, A.N. 1973
The Preparation of an Immobilized Glucose Isomerase. I. The Production and Selection Propertise of the Partially-purified Enzyme from Lactobacillus brevis.
Journal of Applied Chemistry and Biotechnology, 23:689-703.

[ 89] Kirsch, E.J. and Sykes, R.M. 1970
Anaerobic Digestion in Biological WasteTreatment
Progress in Industrial Microbiology, 9:155-237.

[ 90] Kjaegaard, K. 1976
Influence of Redox Potential on the Glucose Catabolism of Chemostat Grown Bacillus licheniformis.
European Journal of Applied Microbiology, 2:215-220.

[ 91] Kjaegaard, K. and Joergensen, B.B. 1981
The Redox Potential, A Hitherto Seldom Used Parameter in Fermentation Systems.
In : Advances in Biotechnology. Volume 1. Scientific and Engineering Principles. Edited by Moo-Young, M., Robinson, C. W. and Vezina, C. pp. 371-376.
Pergamon Press, Toronto, Canada.

[ 92] Kosaric, N., Wieczorek, A., Cosentino, G.P., Magee, R.J. and Prenosil, J.E. 1983
Ethanol Fermentation.
In : Biotechnology Volume 3. Biomass, Microogranisms for Special Applications, Microbial Products I : Energy from Renewable Resources. Edited by Rehm, H.J. and Reed, G. pp.257-385. Verlag Chemie GmbH, Weinheim, West Germany.

[ 93] Krouwel, P.G. and Braber, L. 1979
Ethanol Production by Yeast at Supraoptimal Temperatures.
Biotechnology Letters, 1(10):403-408.

[ 94] Kurtzman, C.P. 1983
Biology and Physiology of D-Xylose Fermenting Yeast Pachysolen tannophilus.
Advances in Biochemical Engineering/Biotechnology, 27:73-84.

[ 95] Kurtzman, C.P., Bothast, R.J. and Van Cauwenberge, J.E. 1982
Conversion of D-Xylose to Ethanol by the Yeast Pachysolen tannophilus.
U.S. Patent, 4,359,534 16 November 1982.

[ 96] Larsen, V.F. 1981
Personal Communication.

[ 97] Lawlis, V.B., Dennis, M.S., Chen, E.Y., Smith, D.H. and Henner, D.J. 1984
Cloning and Sequencing of the Xylose Isomerase and Xylulose Kinase Genes of Escherichia coli
Applied and Environmental Microbiology, 47(1):15-21.

[ 98] Lee, Y.Y. and McCaskey, T.A. 1983
Hemicellulose Hydrolysis and Fermentation of Resulting Pentoses to Ethanol.
TAPPI, 66:102-107.

[ 99] Leonard, R.H. and Hajny, G. 1945
Fermantation of Wood Sugars to Ethyl Alcohol.
Industrial Engineering and Chemistry, 37:390-395.

[100] Longley, R.P., Dennis, R.R., Heyer, M.S. and Wren, J.J. 1978
Selective Saccharomyces Media Containing Ergosterol and Tween 80.
Journal of Institute of Brewer, 84:341-345.

[101] Mackie, K.L. 1982a
Personal Communication.

[102] Mackie, K.L. 1982b
Wood Hydrolysis - Current Technology.
In : Proceedings of the 14th New Zealand Biotechnology Conference. pp.270-286.
Biotechnology Department, Massey University, Palmerston North, New Zealand.

[103] Maleszka, R. and Schneider, H. 1982a
Fermentation of D-Xylose, Xylitol and D-Xylulose by Yeasts.
Canadian Journal of Microbiology, 28:360-363.

[104] Maleszka, R. and Schneider, H. 1982b
Concurrent Production and Consumption of Ethanol by Cultures of Pachysolen tannophilus Growing on D-Xylose.
Applied and Environmental Microbiology, 44(4):909-914.

[105] Maleszka, R., James, A.P. and Schneider, H. 1983a
Ethanol Production from Various Sugars by Strains of Pachysolen tannophilus Bearing Different Numbers of Chromosomes.
Journal of General Microbiology, 129:2495-2500.

[106] Maleszka, R., Neirinck, L., James, A.P., Rutten, H. and Schneider, H. 1983b
Xylitol Dehydrogenase Mutants of Pachysolen tannophilus and the Role of Xylitol in D-Xylose Catabolism.
FEMS Microbiology Letters, 17(1-3):227-229.

[107] Maleszka, R., Veliky, I.A. and Schneider, H. 1981
Enhanced Rate of Ethanol Production from D-Xylose using Recycled or Immobilized Cells of Pachysolen tannophilus.
Biotechnology Letters, 3(8):415-420.

[108] Maleszka, R., Wang, P.Y. and Schneider H. 1982a
A Col E1 Hybrid Plasmid Containing Escherichia coli Genes Complementing D-Xylose Negative Mutants of Escherichia coli and Salmonella typhimurium.
Canadian Journal of Biochemistry, 60:144-151.

[109] Maleszka, R., Wang, P.Y. and Schneider, H. 1982b
Yeasts that Ferment D-Cellobiose as well as D-Xylose.
Biotechnology Letters, 4(2):133-136.

[110] Maleszka, R., Wang, P.Y. and Schneider, H. 1982c
Ethanol Production from D-Glucose and Glycerol by Pachysolen tannophilus.
Enzyme and Microbial Technology, 4(9):349-352.

[111] Margaritis, A. and Bajpai, P. 1982
Direct Fermentation of D-Xylose to Ethanol by
Kluyveromyces marxianus Strains.
Applied and Environmental Microbiology, 44(5):1039-1041.

[112] Maric, V. and Einsele, A. 1981
Ethanol Production by Candida tropicalis.
In : Advances in Biotechnology. Volume 1. Scientific and
Engineering Principles. Edited by Moo-Young, M., Robinson,
C. W. and Vezina, C. pp. 335-338.
Pergamon Press, Toronto, Canada.

[113] Marshall, R.O. and Kooi, E.R. 1957
Enzymic Conversion of D-glucose to D-Fructose.
Science, 125:648-649.

[114] McCracken, L.D. and Gong, C.S. 1982
Fermentation of Cellulose and Hemicellulose
Carbohydrates by Thermotolerant Yeasts.
Biotechnology and Bioengineering Symposium, 12:91-102.

[115] McCracken, L.D. and Gong, C.S. 1983
D-Xylose Metabolism by Mutant Strains of Candida sp.
Advances in Biochemical Engineering/Biotechnology,
27:38-56.

[116] Mermelstein, N.H. 1975
Immobilized Enzymes Produce High-Fructose Corn Syrup.
Food Technology, 29(6):20-26.

[117] Mitsuhashi, S. and Lampen, J.O. 1953
Conversion of D-Xylose to D-Xylulose in Extracts of
Lactobacillus pentosus.
Journal of Biological Chemistry, 204:1011-1018.

[118] Moresi, M. and Sebastiani, E. 1979
Optimization of Whey Fermentation in a Shaken-Flask
Fermenter
European Journal of Applied Microbiology and Biotechnology,
8:63-71.

[119] Moresi, M., Colicchio, A. and Sanaovini, F. 1980
Optimization of Whey Fermentation in a Jar Fermenter
European Journal of Applied Microbiology and Biotechnology,
9:173-183.

[120] Moser, A. and Steiner, W. 1975
The Influence of the Term Kd for Endogenous Metabolism on
the Evaluation of Monod Kinetics for Biotechnological
Processes.
European Journal of Applied Microbiology, 1:281-289.

[121] Mullen, K. and Ennis, D.M. 1979
Rotatable Desigins in Product Development
Food Technology, 33(7):74-80.

[122] Murphy, T.D. 1977
Design and Analysis of Industrial Experiments
Chemical Engineering, 84(6):168-182.

[123] Murray, A.W. and Szostak, J.W. 1983
Construction of Artificial Chromosomes in Yeasts.
Nature(London), 305:187-193.

[124] Mutze, B. and Wandrey, C. 1983
Continuous Fermentation of Xylose with
Pachysolen tannophilus.
Biotechnology Letters, 5(9):633-638.

[125] Nagodawithana, T.W. and Steinkraus, K.H. 1976
Influence of the Rate of Ethanol Production and
Accumulation of the Viability of
Saccharomyces cerevisiae in "Rapid Fermentation".
Applied and Environmental Microbiology,
31(2):158-162.

[126] Nagodawithana, T.W., Whitt, J.T. and Cutaia, A.J. 1977
Study of the Feedback Effect of Ethanol on Selected Enzymes of the Glycolytic Pathway.
Journal of American Society of Brewery Chemist, 35:179-183.

[127] Nakayama, K. 1981
Sources of Industrial Microogranisms
In : Biotechnology Volume 1. Microbial Fundamentals
Edited by Rehm, H.J. and Reed, G.
pp.335-410. Verlag Chemie GmbH, Weinheim, West Germany.

[128] Navarro, J.M. 1980
Alcohol Fermentation : Influence of Culture Conditions on Inhibition by Ethanol.
Cellular and Molecular Biology, 26(2):241-246.

[129] Navarro, J.M. and Durrand, G. 1978
Alcohol Fermantation : Effect of Temperature on Ethanol Accumulation within Yeast Cells.
Annales of Microbiology (Institute of Pasteur), 129B:215-224.

[130] Navarro, J.M. and Finck, J.D. 1982
Evolution de l'Activite "Hexokinase" de Saccharomyces uvarum Fermentant le Saccharose.
Cellular and Molecular Biology, 28:85-89.

[131] Neirinck, L., Maleszka, R. and Schneider, H. 1982
Alcohol Production from Sugar Mixtures by
Pachysolen tannophilus.
Biotechnology and Bioengineering Symposium, 12:161-169.

[132] Nishio, N., Biebl, H. and Meiners, M. 1983
Effect of pH on the Production of Acetone and Butanol
by Clostridium acetobutylicum in a Minimum Medium.
Journal of Fermentation Technology, 61(1):101-104.

[133] Nishizawa, Y., Dunn, I.J. and Bourne, J.R. 1978
Influence of Oxygen and Glucose on Anaerobic Ethanol
Production.
European Congress on Biotechnology, pp. 207-210.
Verlag Chemie Gmb, Weinheim, West Germany.

[134] Northrop, J.K., Ashe, L.H. and Senior, J.K. 1919
Biochemistry of Bacillus acetoethylicum
with Reference to Formation of Acetone
Journal of Biochemistry, 39:1-21.

[135] Novak, M., Strehaiano, P., Moreno, M. and Goma, G. 1981
Alcohol Fermentation : On the Inhibitory Effect of Ethanol.
Biotechnology and Bioengineering, 23:201-211.

[136] Onishi, H. and Suzuki, T. 1969
Microbial Production of Xylitol from Glucose.
Applied Microbiology, 18:1031-1035.

[137] Perlman, D. 1950
Observations on the Production of Ethanol by Fungi and Yeasts.
American Journal Botany, 37:237-241.

[138] Pharmacia 1976
Gel Filtration - Theory and Practice
Pharmacia Fine Chemicals AB, Uppsala, Sweden.

[139] Pineault, G., Pruden, B.B. and De Kee, D. 1977
Etudes des Parametres d'operation du Procede de Fermentation du d-glucose, d'mannose et d-galactose
Canadian Journal of Chemical Engineering, 55:197-202.

[140] Pironti, F.F. 1971
Kinetics of Alcohol Fermentation.
Ph. D. Thesis, Cornell University, Ithaca, U.S.A.

[141] Pirt, S.J. 1957
The Oxygen Requirements of Growing Cultures of an Aerobacter species Determined by Means of Continuous Culture Techniques.
Journal of General Microbiology, 16:59-75.

[142] Pirt, S.J. 1975
Principles of Microbes and Cell Cultivation.
Blackwell Scientific Publications, Oxford, U.K.

[143] Prescott, S.C. and Dunn, C.G. 1959
Industrial Microbiology. 3rd Edition pp. 117-120.
McGraw Hill Book Company Inc., New York, U.S.A.

[144] Rainbow, C. and Rose, A.H. 1963
Biochemistry of Industrial Micro-organisms
Academics Press, New York, U.S.A.

[145] Righelato, R.C., Rose, D. and Westwood , A.W. 1981
Kinetics of Ethanol Production by Yeast in Continuous Culture.
Biotechnology Letters, 3(1):3-8.

[146] Roman, G.N., Jansen, N.B. and Tsao, G.T. 1984
Ethanol Inhibition of D-Xylulose Fermentation by Schizosaccharomyces pombe.
Biotechnology Letters, 6(1):7-12.

[147] Rosenberg, S.L. 1980
Fermentation of Pentose Sugars to Ethanol and Other Neutral Products by Micro-organisms.
Enzyme and Microbial Technology, 2(7):185-193.

[148] Sakano, I and Mikata, K. 1983
Formation of Ethanol from Pentose by Yeasts.
In : Abstracts of Janpanese Agricultural Chemical Society Annual Conference, 1983. p43.
The Agricultural Chemical Society of Japan, Tokyo, Japan.

[149] Schneider, H., Maleszka, R., Neirinck, L., Veliky, I.A., Wang, P.Y. and Chan, Y.K. 1983
Ethanol Production from D-Xylose and Several Other Carbohydrates by Pachysolen tannophilus and Other Yeasts.
Advances in Biochemical Engineering/Biotechnology, 27:57-71.

[150] Schneider, H., Wang, P.Y., Chan, Y.K. and Maleszka, R. 1981a
Conversion of D-Xylose into Ethanol by the Yeast Pachysolen tannophilus.
Biotechnology Letters, 3(2):89-92.

[151] Schneider, H., Wang, P.Y., Johnson, B.F. and Shopsis, C. 1981b
Pentose Fermentation by Yeasts.
In : Current Developments in Yeast Research.
Edited by Stewart, G.G. and Russell, I. pp.81-85.
Pergamon Press, Toronto, Canada.

[152] Shibai, H., Ishizaki, A., Kobayashi, K. and Hirose, Y. 1974
Simultaneous Measurement of Dissolved Oxygen and Oxidation-Reduction Potentials in the Aerobic Culture.
Agricultural Biological Chemistry, 38:2407-2411.

[153] Slein, M.W. 1955
Xylose Isomerase from Pasteurella pestis,
Strain A-1122.
Journal of American Chemical Society, 77:1663-1667.

[154] Slininger, P.J., Bothast, R.J., Black, L.T. and McGhee, J.E. 1982a
Continuous Conversion of D-Xylose to Ethanol by Immobilized Pachysolen tannophilus.
Biotechnology and Bioengineering, 24:2241-2251.

[155] Slininger, P.J., Bothast, R.J., Van Cauwenberge, J.E. and Kurtzman, C.P. 1982b
Conversion of D-Xylose to Ethanol by the Yeast Pachysolen tannophilus.
Biotechnology and Bioengineering, 24:371-384.

[156] Slodki, M.E., Wickerham, L.J. and Cadmus, M.C. 1961
Phylogeny of Phospho-mannan Producing Yeasts.
II. Phospho-mannan Properties and Taxonomic Relationships.
Journal of Bacteriology, 82:269-274.

[157] Smiley, K.L. and Bolen, P.L. 1982
Demonstration of D-Xylose Reductase and D-Xylitol
Dehydrogenase in Pachysolen tannophilus.
Biotechnology Letters, 4(9): 607-610.

[158] Sols, A., Gancedo, C. and DelaFuente, G. 1971
Energy-Yielding Metabolism in Yeasts.
In : The Yeasts Volume 2 Physiology and Biochemistry of
Yeasts. Edited by Rose, A.H. and Harrison, J.S.
Acedemic Press, London, U.K.

[159] Strainer, R.Y. and Adams, G.A. 1944
The Nature of the Aeromonas Fermentation
Biochemical Journal, 38:168-171.

[160] Strehaiano, P., Mota, M. and Goma, G. 1983
Effects of Inoculum Level on Kinetics of Alcoholic
Fermentation.
Biotechnology Letters, 5(2):135-140.

[161] Stucley, C.R. and Pamment, N.B. 1982
Ethanol Inhibiton in Yeast : The Role of Intracellular Ethanol.
In : Proceedings of V International Symposium on Alcohol Fuel Technology. Volume 1 , pp.291-298.
Auckland, New Zealand.

[162] Suihko, M.L. and Drazic, M. 1983
Pentose Fermentation by Yeasts.
Biotechnology Letters, 5(2):107-111.

[163] Suihko, M.L. and Enari, T.M. 1981
The Production of Ethanol from D-Glucose and D-Xylose by Different Fusarium Strains.
Biotechnology Letters, 3(12):723-728.

[164] Suihko, M.L. and Poutanen, K. 1984
D-Xylose Fermentation by Free and Immobilized Saccharomyces cerevisiae Cells.
Biotechnology Letters, 6(3):189-194.

[165] Suihko, M.L., Suomalainen, I., and Enari, T.M. 1983
D-Xylose Catabolism in Fusarium oxysporum.
Biotechnology Letters, 5(8):525-530.

[166] Suzuki, T. and Onishi, H. 1973
Oxidation and Reduction of D-xylose by Cell-Free Extract of Pichia quercuum.
Applied Microbiology, 25:850-852.

[167] Swings, J. and De Ley, J. 1977
The Biology of Zymomonas
Bacteriological Review, 41(1):1-46.

[168] Tarkow, H., Baker, A., Eickner, H., Eslyn, W., Hajny, G., Hann, R., Koeppen, R., Millet, M., and Moore, W. 1970
Wood.
Encyclopedia of Chemical Technology, 2nd Edition.
22:364-365.

[169] Tempest, D.W., Herbert, D. and Phipps, P.J. 1967
Studies on the Growth of Aerobacter aerogenes
at Low Dilution Rates in a Chemostat
In : Microbial Physiology and Continuous Culture
Edited by Powell, E.O., Evans, C.G.T., Strange, R.E. and Tempest, D.W. pp.240-245 Her Majesty's Stationary Office, London, U.K.

[170] Thomas, D.S. and Rose, A.H. 1979
Inhibitory Effect of Ethanol on Growth and Solute Accumulation by Saccharomyces cerevisiae as Affected by Plasma-Membrane Lipid Composition.
Archives of Microbiology, 122:49-55.

[171] Timell, T.E. 1964
Wood Hemicellulose : Part I.
Advances in Carbohydrate Chemistry, 19:247-302.

[172] Timell, T.E. 1965
Wood Hemicellulose : Part II.
Advances in Carbohydrate Chemistry, 20:410-484.

[173] Toivola, A., Yarrow, D., van den Bosch, E., van Dijken, J.P. and Scheffers, W.A. 1984
Alcoholic Fermentation of D-xylose by Yeasts.
Applied and Environmental Microbioloby, 47(6):1221-1223.

[174] Tomoyeda, M. and Horitsu, H. 1964
Pentose Metabolism by Candida utilis.
Agricultural Biological Chemistry, 28:139-142.

[175] Ueng, P.P. and Gong, C.S. 1982
Ethenol Production from Pentoses and Sugar-cane Bagasse Hemicellulose Hydrolyzate by Mucor and Fusarium spp.
Enzyme and Microbial Technology, 4(5):169-171.

[176] Ueng, P.P., Hunter, C.A., Gong, C.S. and Tsao, G.T. 1981
D-Xylulose Fermantation in Yeasts.
Biotechnology Letters, 3(6):315-320.

[177] Volesky, B. and Szczesny, T. 1983
Bacterial Conversion of Pentose Sugars to Acetone and Butanol.
Advances in Biochemical Engineering/Biotechnology, 27:101-118.

[178] Wang, P.Y. and Schneider, H. 1980
Growth of Yeasts on D-Xylulose.
Canadian Journal of Microbiology, 26:1165-1168.

[179] Wang, P.Y., Johnson, B.F. and Schneider, H. 1980a
Fermentation of D-Xylose by Yeasts using Glucose Isomerase in the Medium to Convert D-Xylose to D-Xylulose.
Biotechnology Letters, 2(6):273-278.

[180] Wang, P.Y., Shopsis, C. and Schneider, H. 1980b
Fermentation of a Pentose by Yeasts.
Biochemical and Biophysical Research Communications, 94(1):248-254.

[181] Watson, K. and Rose, A.H. 1980
Fatty-acyl Composition of Lipids of
Saccharomyces cerevisiae Grown Aerobically or
Anaerobically in Media Containing Different Fatty Acids.
Journal of General Microbiology, 117:225-233.

[182] Wayman, M. 1982
Personal Communication.

[183] Weimer, P.J. 1984
Fermentation of 6- Deoxyhexoses.
Applied and Environmental Microbiology, 47(2):263-267.

[184] Whistler, R.L. and Corbett, W.M. 1957
Polysaccharides : Part I. General Aspects and Phyto and
Microbial Polysaccharides.
In :The Carbohydrates : Chemistry,Biochemistry, Physiology.
Edited by Pigman, W. pp.641-708. Academic Press Inc.,
New York, U.S.A.

[185] White, F.H. 1978
Ethanol Tolerance of Brewing Yeast.
In : Proceedings of the Fifteenth Convention, Institute of
Brewing, Australia and New Zealand Section, 15:133-159.

[186] White, M.G. and Willaman, J.J. 1928a
Biochemistry of Plant Diseases.
X. Fermentation of Pentoses by Fusarium lini.
Biochemical Journal, 22:583-591.

[187] White, M.G. and Willaman, J.J. 1928b
Biochemistry of Plant Diseases.
XI.Fusarium lini and the Pyruvic Acid Theory of Alcoholic Fermentation.
Biochemical Journal, 22:592-595.

[188] Whitworth, D.A. 1976
Production of Liquid Transport Feul from Cellulose (Wood) 1. Economic Considerations of Acid Hydrolysis of Wood for Subsequent Conversion to Ethyl Alcohol.
New Zealand Energy Journal, 49(11):173-177.

[189] Whitworth, D.A. 1977
Production of Liquid Transport Fuel from Cellulose (Wood) 2. Energy Conversion Efficiencies of the Processes.
New Zealand Energy Journal, 50(1):14-17.

[190] Wickerham, L.J. 1970
Genus 14 : Pachysolen Boidin et Adzet
In : The Yeasts: a Taxonomic Study. Edited by Lodder, J. pp.448-454.
North-Holland Publishing Company, Amsterdem, Holland.

[191] Wickerham, L.J. and Burton, K.A. 1961
Phylogeny of Phospho-mannan Producing Yeasts.
I. The Genera.
Journal of Bacteriology, 82:265-268.

[192] Wiegel, J. 1980
Formation of Ethanol by Bacteria. A pledge for the use of Extreme Thermophilic Anaerobic Bacteria in Industrial Ethanol Fermentation Processes.
Experientia, 36:1434-1446.

[193] Wiegel, J., Ljungdahl, L.G. 1979
Ethanol as Fermentation Product of Extreme Thermophilic, Anaerobic Bacteria
In : Technische Mikrobiologie. Edited by Dellweg, H. pp. 117-127. Varlag Versuchs- und Lehranstalt fur Spiritusfabrikation und Fermentationstechnologie im Institut fur Garungsgewerbe und Biotechnologie, Berlin.

[194] Wiegel, J., Ljungdahl, L.G. 1981
Thermoanaerobic ethanolicus gen. nov., spec. nov., a New, Extreme Thermophilic Anaerobic Bacterium
Archives of Microbiology,128:343-348.

[195] Wiegel, J., Ljungdahl, L.G. and Rawson, J.R. 1979
Isolation from Soil and Properties of the Extreme Thermophile Clostridium thermohydrosulfuricum
Journal of Bacteriology, 139(3):800-810.

[196] Wiley, A.J., Johnson, M.J., McCoy, E. and Peterson, W.H. 1941
Acetone-Butyl Alcohol Fermentation of Waste Sulfite Liquor.
Industrial Engineering and Chemistry, 33:606-610.

[197] Wills, C., Kratofil, P. and Martin, T. 1982
Functional Mutants of Yeast Alcohol Dehydroganase
In : Genetic Engineering of Microorganisms for Chemicals
Edited by Hollaender, A., DeMoss, R.D., Kaplan, S.,
Konlsky, J., Dwayne, S. and Wolfe, R.S. pp.305-329.
Plenum Press, New York, U.S.A.

[198] Wood, W.A. 1961
Fermantation of Carbohydrates and Related Compounds.
In : The Bacteria. Volume 2. Edited by Gunsalus, I.C. and
Stanier, R.Y. pp.111-122.
Academic Press, New York, U.S.A.

[199] Wovcha, M.G., Steuerwald, D.L. and Brooks, K.E. 1983
Amplication of D-Xylose and D-Glucose Isomerase Activities
in Escherichia coli by Gene Cloning.
Applied and Environmental Microbiology, 45(4):1402-1404.

[200] Yamada, Y., Okada,T., Ueshima, O. and Kondo, K. 1973
Coenzyme Q System in the Classification of the
Ascosporogenous Yeast Genera Hensenula and Pichia.
Journal of General and Applied Microbiology, 19:198-208.

[201] Yamanaka, K. 1968
Purification, Crystallization and Properties of D-Xylose Isomerase from Lactobacillus brevis.
Biochimica et Biophysica Acta, 151:670-680.

[202] Yamanaka, K. and Takahara, N. 1977
Purification and Properties of D-xylose Isomerase from Lactobacillus xylosus
Agricultural and Biological Chemistry, 41(10):1909-1915.

[203] Yu, E.K.C. and Saddler, J. 1982a
Power Solvent Production by Klebsiella pneumoniae Grown on Sugars Present in Wood Hemicellulose.
Biotechnology Letters, 4(2):121-126.

[204] Yu, E.K.C. and Saddler, J. 1982b
Enhanced Production of 2,3- Butanediol by Klebsiella pneumoniae Grown on Higher Sugar Concentrations in the Presence of the Acetic Acid.
Applied and Environmental Microbiology, 44(4):777-784.

[205] Yu, E.K.C. and Saddler, J. 1983
Feb-batch Approach to Production of 2,3- Butanediol by Klebsiella pneumoniae Grown on High Substrate Concentrations.
Applied and Environmental Microbiology, 46(3):630-635.

[206] Yu, E.K.C., Levitin, N. and Saddler, J. 1982
Production of 2,3- Butanediol by Klebsiella pneumoniae Grown on Acid Hydrolyzate Wood Hemicellulose.
Biotechnology Letters, 4(11):741-746.

[207] Zeikus, J.G., Hegge, P.W. and Anderson, M.A. 1979
Thermoanaerobium brockii gen. nov. spec. nov. A New Caldoactive Anaerobic Bacterium.
Archives of Microbiology,122:41-47.

[208] Zittan, L., Poulsen, P.B. and Hemmingsen, S.H. 1975
Sweetzyme - A New Immobilized Glucose Isomerase
Die Starke, 27(7):236-241.

[209] du Preez, J.C. and van der Walt, J.P. 1983
Fermentation of D-Xylose to Ethanol by a Strain of Candida shehatae.
Biotechnology Letters, 5(5):357-362.

[210] du Preez, J.C., Prior, B.A. and Monteiro, M.T. 1984
The Effect of Areation on Xylose Fermentation by Candia shehatae and Pachysolen tannophilus.
A Comparative Study.
Applied Microbiology and Biotechnology, 19:261-266.

## APPENDIX 1 : COSTING STUDY OF COMMERCIAL D-XYLOSE ISOMERASE FOR ENZYME LINKED PENTULOSE FERMENTATION

The costing study is based on NOVO's Sweetzyme enzymes, by assuming the activities for xylose isomerization equals that for glucose isomerization, and an activity of 5% of the design activity for type Q is used.

The following equation has been suggested by Larsen (1981) :

$$C1 = 0.139\ C2 / ( A.\ Y.\ HL )$$

where, C1 : cost ($) of enzyme/ kg ethanol
C2 : cost ($) of enzyme/ kg enzyme
A : activity m moles (xylose converted)/min g enzyme
kg (xylose converted)/h kg enzyme
Y : yield kg ethanol/kg xylose
HL : number of half-lives the enzyme is used

A cost of approximately $20 to 30/kg has been obatined from NOVO enzymes. An estimated cost of Sweetzyme Q (quoted prices 22.5.1984) of $18.75/kg is used here.

A yield of 0.4 kg/kg is assumed and enzyme useage of 2 half-lives is assumed with 1 HL being 800 hours (Fullbrook and Vabo, 1977).

Thus :

If A : 10 kg (xylose converted)/h kg enzyme and HL : 2

C1 = $0.33/kg ethanol

If A : 20 kg (xylose converted)/h kg enzyme and HL : 3

C1 = $0.11 /kg ethanol

**APPENDIX 2 : REGRESSION ANALYSIS OF CELL COUNT**

**VERSUS DRY WEIGHT**

THE REGRESSION EQUATION IS

DRY-WEIGHT (W) = 0.0056 CELL-COUNT (X) ($\times 10^6$)

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| NOCONSTANT | - | - | - |
| X CELL-COUNT | 0.0055865 | 0.0001175 | 47.54 |

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS

S = 0.04744

WITH ( 21 - 1 ) = 20 DEGREES OF FREEDOM

ANALYSIS OF VARIANCE :

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 1 | 5.08659 | 5.08659 | 2260.7* |
| RESIDUAL | 20 | 0.04501 | 0.00225 | |
| TOTAL | 21 | 5.13160 | | |

* Significant at the 0.1% level.

**APPENDIX 3 : TWO WAY ANALYSIS OF VARIANCE FOR**

**TOTAL REDUCING SUGARS ANALYSIS**

TWO WAY ANALYSIS OF VARIANCE

ANALYSIS OF VARIANCE ON Abs

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| Conc | 4 | 1.511912 | 0.377978 | 2952.95* |
| Day | 7 | 0.027030 | 0.003861 | 30.16* |
| ERROR | 28 | 0.003582 | 0.000128 | |
| TOTAL | 39 | 1.542524 | | |

* Significant at the 1% level.

## APPENDIX 4 : TWO WAY AND ONE WAY ANALYSES OF VARIANCES FOR ETHANOL ANALYSIS

TWO WAY ANALYSIS OF VARIANCE FOR ALL THE DATA :

ANALYSIS OF VARIANCE ON Units

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| Ethanol | 3 | 4432.338 | 1477.446 | 1575.10++ |
| Day | 4 | 33.139 | 8.285 | 8.83+ |
| INTERACTION | 12 | 38.210 | 3.184 | 3.39+ |
| ERROR | 40 | 37.512 | 0.938 | |
| TOTAL | 59 | 4541.199 | | |

ONE WAY ANALYSIS OF VARIANCE FOR EACH ETHANOL CONCENTRATION :

For 6.4 g Ethanol per litre

ANALYSIS OF VARIANCE

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| FACTOR | 4 | 50.32 | 12.58 | 9.11+ |
| ERROR | 10 | 13.81 | 1.38 | |
| TOTAL | 14 | 64.13 | | |

INDIVIDUAL 95 % CI'S FOR MEAN
BASED ON POOLED STDEV

| LEVEL | N | MEAN | STDEV | --------+--------+--------+------- |
|---|---|---|---|---|
| DAY 1 | 3 | 28.44 | 1.00 | (------*-----) |
| DAY 2 | 3 | 23.48 | 1.32 | (-----*-----) |
| DAY 3 | 3 | 27.51 | 1.11 | (------*-----) |
| DAY 4 | 3 | 26.75 | 1.51 | (-----*------) |
| DAY 5 | 3 | 28.42 | 0.82 | (-----*------) |
| | | | | --------+--------+--------+------- |
| POOLED STDEV = | | 1.18 | | 24.0 26.4 28.8 |

For 4.0 g Ethanol per litre

ANALYSIS OF VARIANCE

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| FACTOR | 4 | 18.37 | 4.59 | 3.30* |
| ERROR | 10 | 13.93 | 1.39 | |
| TOTAL | 14 | 32.29 | | |

INDIVIDUAL 95 % CI'S FOR MEAN
BASED ON POOLED STDEV

| LEVEL | N | MEAN | STDEV | -------+--------+--------+-------- |
|---|---|---|---|---|
| DAY 1 | 3 | 18.93 | 1.19 | (--------*--------) |
| DAY 2 | 3 | 16.82 | 0.67 | (--------*--------) |
| DAY 3 | 3 | 17.07 | 1.89 | (--------*--------) |
| DAY 4 | 3 | 16.49 | 0.58 | (--------*--------) |
| DAY 5 | 3 | 19.11 | 1.08 | (--------*--------) |
| | | | | -------+--------+--------+-------- |
| POOLED STDEV = | | 1.18 | | 16.0 17.6 19.2 |

For 2.4 g Ethanol per litre

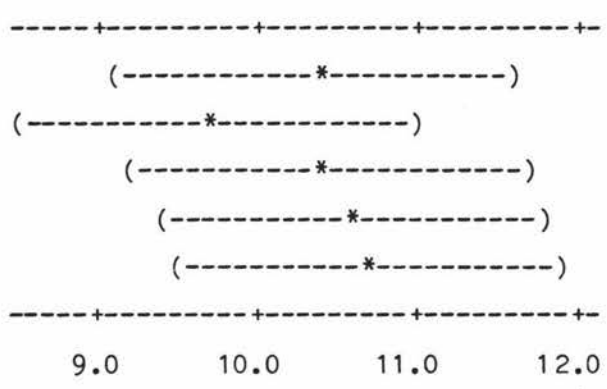
ANALYSIS OF VARIANCE

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| FACTOR | 4 | 1.725 | 0.431 | 0.47* |
| ERROR | 10 | 9.097 | 0.910 | |
| TOTAL | 14 | 10.822 | | |

| LEVEL | N | MEAN | STDEV |
|---|---|---|---|
| DAY 1 | 3 | 10.377 | 1.070 |
| DAY 2 | 3 | 9.740 | 0.106 |
| DAY 3 | 3 | 10.433 | 1.201 |
| DAY 4 | 3 | 10.617 | 1.287 |
| DAY 5 | 3 | 10.707 | 0.542 |

POOLED STDEV = 0.954

INDIVIDUAL 95 % CI'S FOR MEAN
BASED ON POOLED STDEV




For 0.8 g Ethanol per litre

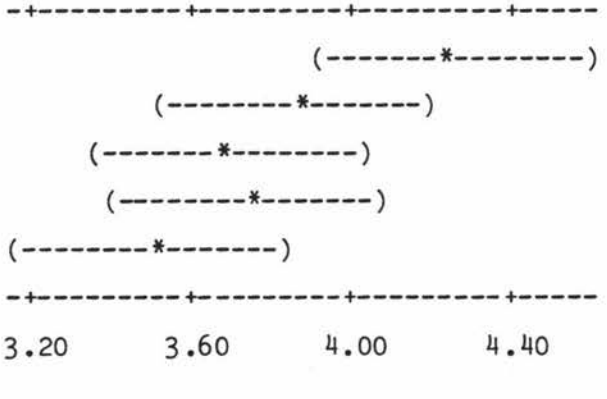
ANALYSIS OF VARIANCE

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| FACTOR | 4 | 0.9369 | 0.2342 | 3.47* |
| ERROR | 10 | 0.6757 | 0.0676 | |
| TOTAL | 14 | 1.6125 | | |

| LEVEL | N | MEAN | STDEV |
|---|---|---|---|
| DAY 1 | 3 | 4.253 | 0.323 |
| DAY 2 | 3 | 3.873 | 0.263 |
| DAY 3 | 3 | 3.697 | 0.064 |
| DAY 4 | 3 | 3.740 | 0.353 |
| DAY 5 | 3 | 3.503 | 0.188 |

POOLED STDEV = 0.260

INDIVIDUAL 95 % CI'S FOR MEAN
BASED ON POOLED STDEV



++ Significant at the 0.1% level.

+ Significant at the 5% level.

* Not significant at the 5% level.

## APPENDIX 5 : STATISTICAL EXPERIMENTAL DESIGNS

(I) Central Composite Rotatable Design :

The central composite rotatable design is composed of three sets of points. (Himmelbau, 1970; Box et al., 1978; Davies, 1978; Mullen end Ennis, 1979).

(i) The factorial points :

$$Nf = 2^k$$

where, Nf = the number of factorial points.
k = the number of factors in the experiment.

here, k = 2
Nf = 4

(ii) The `star' or axial poins, located at the axis with a coded distance $\pm \alpha$ from the origin.

For rotatability, $\alpha = 2^{k/4}$

$$Ns = 2k$$

where, Ns = the number of `star' points.
k = the number of factors in the experiment.

here, Ns = 4
$\alpha$ = 1.414

(iii) The centre points, situated at the origin.

To acheive orthogonality,

$$Nc = \frac{4\alpha^2 ( Nf + \alpha^2 )}{Nf} - Ns$$

where, Nc = the number of centre points.

here, Nc = 8

Hence, 8 replicated observations at the centre of the design plus 4 `star' points at the axes confer on the design rotatability and orthogonality.

This rotatable design can be represented as an octogon diagram and shown in Figure 6.2.1.

(II) Central Composite Design :

A three-factor, $2^3$ factorial, central composite design consists of

(i) The factorial points :

$$Nf = 2^k$$

where, Nf = the number of factorial points.
k = the number of factors used in the designed experiment.
here, Nf = 8

(ii) The `star' or axial points with the value of $\alpha = 1$. (accorded to Murphy's recommendation, 1977).

$$Ns = 2^k$$

here, Ns = 6

(iii) The centre point augmented with the replication runs.

$$Nc = 1 + 12$$
$$= 13$$

The central composite design experiment is depicted as a cube, is shnown in Figure 7.2.1.

**APPENDIX 6 : RAW DATA FOR EXPERIMENT 1**

| FLASK No. | S Coded | I | CELL.YD cells/l | ETOH.YD g/l | M.ETOH.R g/l.h | SpEtOH g/g.h | M.AVETOH g/l.h | 90%ETOHT h |
|---|---|---|---|---|---|---|---|---|
| 1 | -1.414 | 0.000 | 2.12 | 1.97 | 0.019 | 0.012 | 0.014 | 124.8 |
| 2 | -1.000 | -1.000 | 1.97 | 2.37 | 0.026 | 0.016 | 0.013 | 157.2 |
| 3 | 0.000 | -1.414 | 1.35 | 5.01 | 0.112 | 0.086 | 0.027 | 168.0 |
| 4 | 1.000 | -1.000 | 1.82 | 6.79 | 0.158 | 0.113 | 0.034 | 176.4 |
| 5 | 1.414 | 0.000 | 2.07 | 7.22 | 0.176 | 0.114 | 0.036 | 179.4 |
| 6 | 1.000 | 1.000 | 3.70 | 7.42 | 0.077 | 0.354 | 0.039 | 170.4 |
| 7 | 0.000 | 1.414 | 3.74 | 3.83 | 0.044 | 0.016 | 0.029 | 117.6 |
| 8 | -1.000 | 1.000 | 2.80 | 2.01 | 0.022 | 0.011 | 0.017 | 104.4 |
| 9 | 0.000 | 0.000 | 2.05 | 5.29 | 0.050 | 0.024 | 0.025 | 185.4 |
| 10 | 0.000 | 0.000 | 1.63 | 4.42 | 0.029 | 0.018 | 0.023 | 168.6 |
| 11 | 0.000 | 0.000 | 1.95 | 5.17 | 0.047 | 0.030 | 0.028 | 166.8 |
| 12 | 0.000 | 0.000 | 1.81 | 4.54 | 0.038 | 0.023 | 0.024 | 169.8 |
| 13 | 0.000 | 0.000 | 1.72 | 4.77 | 0.044 | 0.032 | 0.024 | 170.4 |
| 14 | 0.000 | 0.000 | 1.66 | 4.74 | 0.044 | 0.030 | 0.024 | 174.0 |
| 15 | 0.000 | 0.000 | 1.56 | 5.13 | 0.058 | 0.037 | 0.028 | 164.4 |
| 16 | 0.000 | 0.000 | 1.87 | 4.66 | 0.048 | 0.033 | 0.025 | 165.6 |

## APPENDIX 7 : PARSIMONIOUS MODELS AND REGRESSION STATISTICS

### FOR EXPERIMENT 1

THE REGRESSION EQUATION IS
CELL.YD = 1.78 + 0.760 I + 0.261 SI + 0.220 SS + 0.445 II

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 1.78311 | 0.07733 | 23.06 |
| I | 0.75979 | 0.07733 | 9.82 |
| SI | 0.2613 | 0.1094 | 2.39 |
| SS | 0.22004 | 0.07735 | 2.84 |
| II | 0.44510 | 0.07735 | 5.75 |

COEFFICIENT OF DETERMINATION :

R-SQUARED = 92.9 PERCENT
R-SQUARED = 90.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE :

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 6.8621 | 1.7155 | 35.89+ |
| I | 1 | 4.6175 | | |
| SI | 1 | 0.2730 | | |
| SS | 1 | 0.3874 | | |
| II | 1 | 1.5842 | | |
| RESIDUAL | 11 | 0.5262 | 0.0478 | |
| LACK OF FIT | 4 | 0.3260 | 0.0815 | 2.85* |
| PURE ERROR | 7 | 0.2002 | 0.0286 | |
| TOTAL | 15 | 7.3883 | | |

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.ETOH.R = 0.0451 + 0.0511 S - 0.0228 I - 0.0192 SI + 0.0223 SS
+ 0.0125 II

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.045143 | 0.004025 | 11.22 |
| S | 0.051121 | 0.004025 | 12.70 |
| I | -0.022802 | 0.004025 | -5.66 |
| SI | -0.019209 | 0.005692 | -3.37 |
| SS | 0.022326 | 0.004026 | 5.55 |
| II | 0.012479 | 0.004026 | 3.10 |

S = 0.01138

COEFFICIENT OF DETERMINATION :

R-SQUARED = 96.1 PERCENT
R-SQUARED = 94.1 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE :

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 5 | 0.0317700 | 0.0063540 | 49.0+ |
| S | 1 | 0.0209036 | | |
| I | 1 | 0.0041588 | | |
| SI | 1 | 0.0014759 | | |
| SS | 1 | 0.0039864 | | |
| II | 1 | 0.0012453 | | |
| RESIDUAL | 10 | 0.0012959 | 0.0001296 | |
| LACK OF FIT | 3 | 0.0007717 | 0.000257 | 3.43* |
| PURE ERROR | 7 | 0.0005240 | 0.000075 | |
| TOTAL | 15 | 0.0330660 | | |

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
90%ETOHT = 171 + 20.3 S - 16.3 I + 11.7 SI - 8.10 SS - 12.8 II

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 170.625 | 2.191 | 77.88 |
| S | 20.304 | 2.191 | 9.27 |
| I | -16.261 | 2.191 | -7.42 |
| SI | 11.700 | 3.098 | 3.78 |
| SS | -8.101 | 2.191 | -3.70 |
| II | -12.752 | 2.191 | -5.82 |

S = 6.196

COEFFICIENT OF DETERMINATION :

R-SQUARED = 95.3 PERCENT
R-SQUARED = 92.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE :

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 5 | 7785.2 | 1557.0 | 40.55+ |
| S | 1 | 3297.4 | | |
| I | 1 | 2115.0 | | |
| SI | 1 | 547.6 | | |
| SS | 1 | 525.0 | | |
| II | 1 | 1300.3 | | |
| RESIDUAL | 10 | 383.9 | 38.4 | |
| LACK OF FIT | 3 | 70.7 | 23.57 | 0.53* |
| PURE ERROR | 7 | 313.2 | 44.74 | |
| TOTAL | 15 | 8169.1 | | |

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
ETOH.YD = 4.71 + 2.16 S

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 4.7128 | 0.1107 | 42.59 |
| S | 2.1583 | 0.1565 | 13.79 |

S = 0.4426

COEFFICIENT OF DETERMINATION :

R-SQUARED = 93.1 PERCENT
R-SQUARED = 92.7 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE :

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 1 | 37.261 | 37.261 | 190.1+ |
| RESIDUAL | 14 | 2.743 | 0.196 | |
| LACK OF FIT | 7 | 2.0318 | 0.2903 | 2.86* |
| PURE ERROR | 7 | 0.7112 | 0.1016 | |
| TOTAL | 15 | 40.004 | | |

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
1/SpEtOH = 38.6 - 30.4 S + 11.5 I

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 38.646 | 2.718 | 14.22 |
| S | -30.449 | 3.844 | -7.92 |
| I | 11.505 | 3.844 | 2.99 |

S = 10.87

COEFFICIENT OF DETERMINATION :

R-SQUARED = 84.7 PERCENT
R-SQUARED = 82.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE :

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 2 | 8474.9 | 4237.5 | 35.85+ |
| S | 1 | 7416.2 | | |
| I | 1 | 1058.8 | | |
| RESIDUAL | 13 | 1536.7 | 118.2 | |
| LACK OF FIT | 6 | 929.32 | 154.886 | 1.78* |
| PURE ERROR | 7 | 607.39 | 86.769 | |
| TOTAL | 15 | 10011.6 | | |

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.AVETOH = 0.0261 + 0.00923 S

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.0260863 | 0.0005864 | 44.49 |
| S | 0.0092314 | 0.0008293 | 11.13 |

S = 0.002346

COEFFICIENT OF DETERMINATION :

R-SQUARED = 89.8 PERCENT
R-SQUARED = 89.1 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE :

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 1 | 0.00068165 | 0.00068165 | 123.94+ |
| RESIDUAL | 14 | 0.00007702 | 0.00000550 | |
| LACK OF FIT | 7 | 0.0000546 | 0.0000078 | 2.44* |
| PURE ERROR | 7 | 0.0000224 | 0.0000032 | |
| TOTAL | 15 | 0.00075867 | | |

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

**APPENDIX 8 : RAW DATA FOR EXPERIMENT 2**

| Strain | H | N | T | Cell | EtOH | M.Cell.R | M.EtOH | Sp.EtOH | M.AvEtOH | Sp.Cell |
|---|---|---|---|---|---|---|---|---|---|---|
| IFO1007 | -1 | -1 | -1 | 8.15 | 0.75 | 0.059 | 0.0122 | 0.00255 | 0.00652 | 0.0109 |
| | 1 | -1 | -1 | 166.40 | 4.74 | 1.059 | 0.0425 | 0.00063 | 0.03144 | 0.0298 |
| | -1 | 1 | -1 | 8.45 | 0.00 | 0.023 | 0.0000 | 0.00000 | 0.00000 | 0.0033 |
| | 1 | 1 | -1 | 101.00 | 4.43 | 0.926 | 0.0402 | 0.00120 | 0.04566 | 0.0308 |
| | 0 | 0 | -1 | 47.50 | 4.51 | 0.388 | 0.0503 | 0.00176 | 0.04226 | 0.0191 |
| | 0 | 0 | 0 | 24.50 | 3.55 | 0.204 | 0.0293 | 0.00170 | 0.02867 | 0.0146 |
| | 0 | 0 | 0 | 30.00 | 4.26 | 0.125 | 0.0375 | 0.00211 | 0.02670 | 0.0157 |
| | 0 | 0 | 0 | 24.00 | 3.98 | 0.113 | 0.0202 | 0.00116 | 0.01975 | 0.0132 |
| | 0 | 0 | 0 | 20.00 | 4.13 | 0.185 | 0.0352 | 0.00209 | 0.02717 | 0.0289 |
| | 0 | -1 | 0 | 26.80 | 2.32 | 0.175 | 0.0229 | 0.00139 | 0.01872 | 0.0148 |
| | 0 | 1 | 0 | 52.30 | 3.39 | 0.185 | 0.0258 | 0.00125 | 0.02396 | 0.0160 |
| | -1 | 0 | 0 | 8.77 | 0.11 | 0.022 | 0.0000 | 0.00000 | 0.00000 | 0.0044 |
| | 1 | 0 | 0 | 49.50 | 2.37 | 0.188 | 0.0264 | 0.00170 | 0.02405 | 0.0200 |
| | 0 | 0 | 0 | 20.00 | 3.12 | 0.063 | 0.0173 | 0.00121 | 0.01341 | 0.0063 |
| | 0 | 0 | 0 | 38.00 | 3.55 | 0.176 | 0.0391 | 0.00355 | 0.03096 | 0.0104 |
| | 0 | 0 | 0 | 50.50 | 4.30 | 0.545 | 0.0481 | 0.00310 | 0.02978 | 0.0156 |
| | 0 | 0 | 0 | 62.00 | 4.53 | 0.500 | 0.0705 | 0.00624 | 0.02038 | 0.0134 |
| | 0 | 0 | 0 | 62.00 | 3.50 | 0.520 | 0.0310 | 0.00250 | 0.02859 | 0.0158 |
| | -1 | -1 | 1 | 6.50 | 0.77 | 0.000 | 0.0225 | 0.00351 | 0.00434 | 0.0000 |
| | 1 | -1 | 1 | 8.30 | 0.43 | 0.012 | 0.0047 | 0.00067 | 0.00376 | 0.0018 |
| | -1 | 1 | 1 | 7.00 | 0.00 | 0.001 | 0.0000 | 0.00000 | 0.00000 | 0.0015 |
| | 1 | 1 | 1 | 7.50 | 0.31 | 0.000 | 0.0176 | 0.00235 | 0.00164 | 0.0000 |
| | 0 | 0 | 1 | 7.05 | 0.28 | 0.006 | 0.0036 | 0.00054 | 0.00276 | 0.0011 |
| | 0 | 0 | 0 | 26.50 | 3.87 | 0.117 | 0.0329 | 0.00346 | 0.03096 | 0.0070 |
| | 0 | 0 | 0 | 26.50 | 5.21 | 0.096 | 0.0579 | 0.00529 | 0.04005 | 0.0072 |
| | 0 | 0 | 0 | 21.00 | 4.74 | 0.108 | 0.0495 | 0.00387 | 0.03081 | 0.0070 |
| | 0 | 0 | 0 | 23.50 | 5.45 | 0.050 | 0.0639 | 0.00511 | 0.03957 | 0.0113 |
| NRRL2460 | -1 | -1 | -1 | 8.88 | 0.07 | 0.038 | 0.0116 | 0.00131 | 0.00355 | 0.0050 |
| | 1 | -1 | -1 | 146.00 | 4.80 | 1.875 | 0.0560 | 0.00116 | 0.03199 | 0.0306 |
| | -1 | 1 | -1 | 8.00 | 1.09 | 0.050 | 0.0149 | 0.00188 | 0.00529 | 0.0065 |
| | 1 | 1 | -1 | 94.00 | 3.93 | 0.995 | 0.0462 | 0.00177 | 0.04376 | 0.0259 |
| | 0 | 0 | -1 | 47.00 | 4.64 | 0.350 | 0.0542 | 0.00237 | 0.03863 | 0.0135 |
| | 0 | 0 | 0 | 18.00 | 4.00 | 0.202 | 0.0345 | 0.00197 | 0.02267 | 0.0158 |
| | 0 | 0 | 0 | 22.30 | 4.34 | 0.161 | 0.0482 | 0.00246 | 0.02709 | 0.0113 |
| | 0 | 0 | 0 | 20.20 | 3.82 | 0.195 | 0.0296 | 0.00151 | 0.02346 | 0.0154 |
| | 0 | 0 | 0 | 31.30 | 4.02 | 0.385 | 0.0395 | 0.00233 | 0.02441 | 0.0123 |
| | 0 | -1 | 0 | 33.50 | 3.39 | 0.132 | 0.0379 | 0.00308 | 0.03523 | 0.0164 |
| | 0 | 1 | 0 | 44.90 | 4.13 | 0.244 | 0.0270 | 0.00104 | 0.02069 | 0.0115 |
| | -1 | 0 | 0 | 10.56 | 0.30 | 0.145 | 0.0000 | 0.00000 | 0.00460 | 0.0096 |
| | 1 | 0 | 0 | 54.00 | 3.87 | 0.309 | 0.0289 | 0.00109 | 0.02496 | 0.0204 |
| | 0 | 0 | 0 | 18.20 | 4.14 | 0.087 | 0.0402 | 0.00365 | 0.02583 | 0.0082 |
| | 0 | 0 | 0 | 37.00 | 3.67 | 0.229 | 0.0400 | 0.00400 | 0.04297 | 0.0098 |
| | 0 | 0 | 0 | 47.00 | 4.22 | 0.416 | 0.0715 | 0.00624 | 0.02235 | 0.0182 |
| | 0 | 0 | 0 | 64.00 | 4.24 | 0.833 | 0.0437 | 0.00380 | 0.04937 | 0.0201 |
| | 0 | 0 | 0 | 60.00 | 3.75 | 0.556 | 0.0384 | 0.00289 | 0.03002 | 0.0158 |
| | -1 | -1 | 1 | 9.80 | 0.20 | 0.028 | 0.0032 | 0.00036 | 0.00168 | 0.0045 |
| | 1 | -1 | 1 | 10.68 | 0.71 | 0.023 | 0.0082 | 0.00094 | 0.00434 | 0.0029 |
| | -1 | 1 | 1 | 7.60 | 0.52 | 0.000 | 0.0129 | 0.00170 | 0.00321 | 0.0000 |
| | 1 | 1 | 1 | 10.67 | 0.71 | 0.045 | 0.0139 | 0.00141 | 0.00442 | 0.0046 |
| | 0 | 0 | 1 | 8.80 | 0.00 | 0.017 | 0.0000 | 0.00000 | 0.00000 | 0.0022 |
| | 0 | 0 | 0 | 44.50 | 5.49 | 0.184 | 0.0616 | 0.00417 | 0.03878 | 0.0070 |
| | 0 | 0 | 0 | 27.30 | 5.84 | 0.123 | 0.0646 | 0.00442 | 0.03049 | 0.0072 |
| | 0 | 0 | 0 | 43.50 | 5.59 | 0.164 | 0.0658 | 0.00450 | 0.03610 | 0.0098 |
| | 0 | 0 | 0 | 44.00 | 5.37 | 0.198 | 0.0548 | 0.00421 | 0.02954 | 0.0106 |
| NRRL2461 | -1 | -1 | -1 | 7.73 | 0.55 | 0.038 | 0.0050 | 0.00108 | 0.00717 | 0.0064 |
| | 1 | -1 | -1 | 100.00 | 5.18 | 3.906 | 0.0599 | 0.00214 | 0.03531 | 0.0448 |
| | -1 | 1 | -1 | 6.65 | 0.82 | 0.037 | 0.0164 | 0.00247 | 0.00429 | 0.0066 |
| | 1 | 1 | -1 | 106.00 | 5.05 | 1.587 | 0.0606 | 0.00135 | 0.04597 | 0.0339 |
| | 0 | 0 | -1 | 121.00 | 5.64 | 2.474 | 0.0749 | 0.00139 | 0.07102 | 0.0309 |
| | 0 | 0 | 0 | 36.00 | 4.74 | 0.400 | 0.0751 | 0.00245 | 0.02962 | 0.0154 |
| | 0 | 0 | 0 | 32.50 | 4.42 | 0.358 | 0.0658 | 0.00319 | 0.02867 | 0.0171 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 32.50 | 4.06 | 0.357 | 0.0887 | 0.00364 | 0.03057 | 0.0202 |
| 0 | 0 | 0 | 34.80 | 4.36 | 0.357 | 0.0782 | 0.00319 | 0.02930 | 0.0187 |
| 0 | -1 | 0 | 66.00 | 4.50 | 0.537 | 0.0436 | 0.00105 | 0.02820 | 0.0208 |
| 0 | 1 | 0 | 96.00 | 4.10 | 0.625 | 0.0539 | 0.00270 | 0.03989 | 0.0157 |
| -1 | 0 | 0 | 9.28 | 0.37 | 0.014 | 0.0000 | 0.00000 | 0.00000 | 0.0019 |
| 1 | 0 | 0 | 118.00 | 3.69 | 0.486 | 0.0632 | 0.00243 | 0.06312 | 0.0275 |
| 0 | 0 | 0 | 50.00 | 4.81 | 0.172 | 0.0796 | 0.00530 | 0.03918 | 0.0159 |
| 0 | 0 | 0 | 56.00 | 3.08 | 0.655 | 0.0253 | 0.00197 | 0.02283 | 0.0126 |
| 0 | 0 | 0 | 62.00 | 3.79 | 0.774 | 0.0355 | 0.00278 | 0.02740 | 0.0166 |
| 0 | 0 | 0 | 77.00 | 3.96 | 1.125 | 0.0380 | 0.00307 | 0.02867 | 0.0201 |
| -1 | -1 | 1 | 10.10 | 0.00 | 0.036 | 0.0000 | 0.00000 | 0.00000 | 0.0049 |
| 1 | -1 | 1 | 7.45 | 0.90 | 0.000 | 0.0184 | 0.00248 | 0.00628 | 0.0000 |
| -1 | 1 | 1 | 8.45 | 1.10 | 0.014 | 0.0193 | 0.00229 | 0.00512 | 0.0019 |
| 1 | 1 | 1 | 7.35 | 0.00 | 0.000 | 0.0000 | 0.00000 | 0.00000 | 0.0000 |
| 0 | 0 | 1 | 8.40 | 0.00 | 0.020 | 0.0000 | 0.00000 | 0.00000 | 0.0029 |
| 0 | 0 | 0 | 70.50 | 4.89 | 0.365 | 0.0400 | 0.00303 | 0.03152 | 0.0106 |
| 0 | 0 | 0 | 118.00 | 4.89 | 0.833 | 0.0366 | 0.00207 | 0.03365 | 0.0154 |
| 0 | 0 | 0 | 102.00 | 5.53 | 0.667 | 0.0785 | 0.00466 | 0.02409 | 0.0117 |
| 0 | 0 | 0 | 103.00 | 5.11 | 0.930 | 0.0410 | 0.00222 | 0.03152 | 0.0170 |

## APPENDIX 9 : PARSIMONIOUS MODELS AND REGRESSION STATISTICS

### FOR EXPERIMENT 2

For Strain IFO1007

THE REGRESSION EQUATION IS
CELL.YD = 1.47 + 0.328 H - 0.344 T - 0.281 HT - 0.269 TT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 1.47297 | 0.03810 | 38.66 |
| H | 0.32752 | 0.04968 | 6.59 |
| T | -0.34398 | 0.04968 | -6.92 |
| HT | -0.28142 | 0.05554 | -5.07 |
| TT | -0.26891 | 0.06261 | -4.30 |

S = 0.1571

R-SQUARED = 86.0 PERCENT
R-SQUARED = 83.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 3.34482 | 0.83620 | 32.88+ |
| H | 1 | 1.07269 | | |
| T | 1 | 1.18325 | | |
| HT | 1 | 0.63356 | | |
| TT | 1 | 0.45532 | | |
| RESIDUAL | 22 | 0.54299 | 0.02468 | |
| PURE ERROR | 10 | 0.1628 | 0.01628 | 0.51* |
| LACK OF FIT | 12 | 0.3802 | 0.03170 | |
| TOTAL | 26 | 3.88781 | | |

DURBIN-WATSON STATISTIC = 1.32

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
ETOH.YD = 3.90 + 1.06 H - 2.12 T - 1.06 HT - 1.91 HH + 1.07 HHT
- 0.748 TT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 3.8977 | 0.1984 | 19.64 |
| H | 1.0649 | 0.2501 | 4.26 |
| T | -2.1172 | 0.5593 | -3.79 |
| HT | -1.0556 | 0.2796 | -3.78 |
| HH | -1.9056 | 0.4312 | -4.42 |
| HHT | 1.0675 | 0.6253 | 1.71 |
| TT | -0.7482 | 0.4312 | -1.74 |

S = 0.7909

R-SQUARED = 86.0 PERCENT
R-SQUARED = 81.8 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 6 | 76.674 | 12.779 | 20.41+ |
| H | 1 | 11.341 | | |
| T | 1 | 15.957 | | |
| HT | 1 | 8.915 | | |
| HH | 1 | 36.755 | | |
| HHT | 1 | 1.823 | | |
| TT | 1 | 1.884 | | |
| RESIDUAL | 20 | 12.511 | 0.626 | |
| PURE ERROR | 8 | 6.8746 | 0.859 | 1.83* |
| LACK OF FIT | 12 | 5.6364 | 0.469 | |
| TOTAL | 26 | 89.185 | | |

DURBIN-WATSON STATISTIC = 1.06

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
SP.CELLR = 0.0131 + 0.00622 H - 0.00896 T - 0.00575 HT
- 0.00320 TT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.013065 | 0.001142 | 11.44 |
| H | 0.006217 | 0.001489 | 4.18 |
| T | -0.008963 | 0.001489 | -6.02 |
| HT | -0.005749 | 0.001665 | -3.45 |
| TT | -0.003197 | 0.001876 | -1.70 |

S = 0.004709

R-SQUARED = 75.7 PERCENT
R-SQUARED = 71.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 0.00151864 | 0.00037966 | 17.12+ |
| H | 1 | 0.00038647 | | |
| T | 1 | 0.00080341 | | |
| HT | 1 | 0.00026442 | | |
| TT | 1 | 0.00006434 | | |
| RESIDUAL | 22 | 0.00048775 | 0.00002217 | |
| PURE ERROR | 10 | 0.000522 | 0.00005220 | 0.14* |
| LACK OF FIT | 12 | 0.000436 | 0.00003630 | |
| TOTAL | 26 | 0.00200639 | | |

DURBIN-WATSON STATISTIC = 1.48

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.CELL.R = 0.217 - 0.244 T - 0.236 HT + 0.239 NNH

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.21681 | 0.02732 | 7.94 |
| T | -0.24365 | 0.04489 | -5.43 |
| HT | -0.23630 | 0.05019 | -4.71 |
| NNH | 0.23907 | 0.05019 | 4.76 |

S = 0.1420

R-SQUARED = 76.4 PERCENT
R-SQUARED = 73.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 3 | 1.49763 | 0.49921 | 24.77+ |
| T | 1 | 0.59367 | | |
| HT | 1 | 0.44671 | | |
| NNH | 1 | 0.45725 | | |
| RESIDUAL | 23 | 0.46352 | 0.02015 | |
| PURE ERROR | 11 | 0.07388 | 0.00672 | 0.21* |
| LACK OF FIT | 12 | 0.38960 | 0.03247 | |
| TOTAL | 26 | 1.96116 | | |

DURBIN-WATSON STATISTIC = 0.75

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.AVETOH = 0.0267 + 0.00957 H - 0.0197 T - 0.00869 HT
- 0.0150 HH + 0.0105 HHT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.026740 | 0.001618 | 16.52 |
| H | 0.009569 | 0.002110 | 4.54 |
| T | -0.019750 | 0.004718 | -4.19 |
| HT | -0.008690 | 0.002359 | -3.68 |
| HH | -0.014997 | 0.002659 | -5.64 |
| HHT | 0.010515 | 0.005275 | 1.99 |

S = 0.006672

R-SQUARED = 82.5 PERCENT
R-SQUARED = 78.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 5 | 0.00439831 | 0.00087966 | 20.57+ |
| H | 1 | 0.00091571 | | |
| T | 1 | 0.00128552 | | |
| HT | 1 | 0.00060413 | | |
| HH | 1 | 0.00141605 | | |
| HHT | 1 | 0.00017690 | | |
| RESIDUAL | 21 | 0.00093495 | 0.00004452 | |
| PURE ERROR | 9 | 0.0002855 | 0.00003170 | 0.59* |
| LACK OF FIT | 12 | 0.0006494 | 0.00005412 | |
| TOTAL | 26 | 0.00533326 | | |

DURBIN-WATSON STATISTIC = 1.80

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

For Strain NRRL Y-2460

THE REGRESSION EQUATION IS
CELL.YD = 1.52 + 0.318 H - 0.279 T - 0.263 HT - 0.264 TT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 1.51700 | 0.03844 | 39.47 |
| H | 0.31794 | 0.05012 | 6.34 |
| T | -0.27878 | 0.05012 | -5.56 |
| HT | -0.26266 | 0.05603 | -4.69 |
| TT | -0.26357 | 0.06316 | -4.17 |

S = 0.1585

R-SQUARED = 83.4 PERCENT
R-SQUARED = 80.4 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 2.77736 | 0.69434 | 27.64+ |
| H | 1 | 1.01086 | | |
| T | 1 | 0.77720 | | |
| HT | 1 | 0.55192 | | |
| TT | 1 | 0.43738 | | |
| RESIDUAL | 22 | 0.55257 | 0.02512 | |
| PURE ERROR | 10 | 0.1126 | 0.01126 | 0.31* |
| LACK OF FIT | 12 | 0.4399 | 0.03666 | |
| TOTAL | 26 | 3.32993 | | |

DURBIN-WATSON STATISTIC = 1.32

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
ETOH.YD = 4.32 + 1.18 H - 2.32 T - 0.860 HT - 1.60 HH - 1.37 TT
+ 1.35 HHT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 4.3211 | 0.1957 | 22.08 |
| H | 1.1818 | 0.2466 | 4.79 |
| T | -2.3226 | 0.5515 | -4.21 |
| HT | -0.8601 | 0.2758 | -3.12 |
| HH | -1.6018 | 0.4252 | -3.77 |
| TT | -1.3688 | 0.4252 | -3.22 |
| HHT | 1.3539 | 0.6166 | 2.20 |

S = 0.7800

R-SQUARED = 87.5 PERCENT
R-SQUARED = 83.7 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 6 | 84.973 | 14.162 | 23.39+ |
| H | 1 | 13.967 | | |
| T | 1 | 15.364 | | |
| HT | 1 | 5.918 | | |
| HH | 1 | 40.487 | | |
| TT | 1 | 6.304 | | |
| HHT | 1 | 2.933 | | |
| RESIDUAL | 20 | 12.167 | 0.608 | |
| PURE ERROR | 8 | 4.9634 | 0.620 | 1.03* |
| LACK OF FIT | 12 | 7.2036 | 0.600 | |
| TOTAL | 26 | 97.140 | | |

DURBIN-WATSON STATISTIC = 0.97

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
SP.CELLR = 0.0129 + 0.00587 H - 0.00672 T - 0.00524 HT
- 0.00335 TT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.0129387 | 0.0008608 | 15.03 |
| H | 0.005874 | 0.001122 | 5.23 |
| T | -0.006720 | 0.001122 | -5.99 |
| HT | -0.005243 | 0.001255 | -4.18 |
| TT | -0.003353 | 0.001414 | -2.37 |

S = 0.003549

R-SQUARED = 79.7 PERCENT
R-SQUARED = 76.0 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 0.00108741 | 0.00027185 | 21.58+ |
| H | 1 | 0.00034510 | | |
| T | 1 | 0.00045161 | | |
| HT | 1 | 0.00021992 | | |
| TT | 1 | 0.00007078 | | |
| RESIDUAL | 22 | 0.00027711 | 0.00001260 | |
| PURE ERROR | 10 | 0.000611 | 0.0000611 | 0.34* |
| LACK OF FIT | 12 | 0.000216 | 0.0000180 | |
| TOTAL | 26 | 0.00136452 | | |

DURBIN-WATSON STATISTIC = 1.29

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.CELL.R = 0.296 - 0.343 HT - 0.358 HHT + 0.353 NNH

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.29604 | 0.04233 | 6.99 |
| HT | -0.34260 | 0.07777 | -4.41 |
| HHT | -0.35767 | 0.07777 | -4.60 |
| NNH | 0.35275 | 0.07777 | 4.54 |

S = 0.2200

R-SQUARED = 72.7 PERCENT
R-SQUARED = 69.1 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 3 | 2.95791 | 0.98597 | 20.38+ |
| HT | 1 | 0.93900 | | |
| HHT | 1 | 1.02345 | | |
| NNH | 1 | 0.99546 | | |
| RESIDUAL | 23 | 1.11296 | 0.04839 | |
| PURE ERROR | 11 | 0.58104 | 0.05282 | 1.19* |
| LACK OF FIT | 12 | 0.53200 | 0.04433 | |
| TOTAL | 26 | 4.07087 | | |

DURBIN-WATSON STATISTIC = 1.33

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.AVETOH = 0.0287 + 0.00911 H - 0.0110 T - 0.00788 HT
- 0.0151 TT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.028743 | 0.002163 | 13.29 |
| H | 0.009114 | 0.002820 | 3.23 |
| T | -0.010957 | 0.002820 | -3.89 |
| HT | -0.007880 | 0.003153 | -2.50 |
| TT | -0.015053 | 0.003554 | -4.24 |

S = 0.008917

R-SQUARED = 69.3 PERCENT
R-SQUARED = 63.8 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 0.00395475 | 0.00098869 | 12.43+ |
| H | 1 | 0.00083069 | | |
| T | 1 | 0.00120062 | | |
| HT | 1 | 0.00049679 | | |
| TT | 1 | 0.00142665 | | |
| RESIDUAL | 22 | 0.00174920 | 0.00007951 | |
| PURE ERROR | 10 | 0.0008919 | 0.00008919 | 1.25* |
| LACK OF FIT | 12 | 0.0008573 | 0.00007140 | |
| TOTAL | 26 | 0.00570395 | | |

DURBIN-WATSON STATISTIC = 1.89

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

For Strain NRRL Y-2461

THE REGRESSION EQUATION IS
CELL.YD = 1.75 + 0.323 H - 0.323 T - 0.313 HT - 0.511 TT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 1.75190 | 0.05736 | 30.54 |
| H | 0.32259 | 0.07255 | 4.45 |
| T | -0.32252 | 0.07255 | -4.45 |
| HT | -0.31338 | 0.08111 | -3.86 |
| TT | -0.51060 | 0.09248 | -5.52 |

S = 0.2294

R-SQUARED = 80.2 PERCENT
R-SQUARED = 76.4 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 4.4709 | 1.1177 | 21.25+ |
| H | 1 | 1.0406 | | |
| T | 1 | 1.0402 | | |
| HT | 1 | 0.7857 | | |
| TT | 1 | 1.6044 | | |
| RESIDUAL | 21 | 1.1053 | 0.0526 | |
| PURE ERROR | 10 | 0.6422 | 0.0642 | 1.53* |
| LACK OF FIT | 11 | 0.4631 | 0.0421 | |
| TOTAL | 25 | 5.5762 | | |

DURBIN-WATSON STATISTIC = 1.76

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
ETOH.YD = 4.37 + 1.20 H - 2.82 T - 1.13 HT - 1.80 HH - 1.01 TT
+ 1.62 HHT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 4.3739 | 0.1687 | 25.93 |
| H | 1.1976 | 0.2058 | 5.82 |
| T | -2.8242 | 0.4603 | -6.14 |
| HT | -1.1317 | 0.2301 | -4.92 |
| HH | -1.7953 | 0.3556 | -5.05 |
| TT | -1.0093 | 0.3556 | -2.84 |
| HHT | 1.6234 | 0.5146 | 3.15 |

S = 0.6509

R-SQUARED = 92.1 PERCENT
R-SQUARED = 89.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 6 | 93.233 | 15.539 | 36.65+ |
| H | 1 | 14.343 | | |
| T | 1 | 23.271 | | |
| HT | 1 | 10.246 | | |
| HH | 1 | 37.743 | | |
| TT | 1 | 3.412 | | |
| HHT | 1 | 4.217 | | |
| RESIDUAL | 19 | 8.050 | 0.424 | |
| PURE ERROR | 8 | 3.127 | 0.391 | 0.87* |
| LACK OF FIT | 11 | 4.923 | 0.448 | |
| TOTAL | 25 | 101.283 | | |

DURBIN-WATSON STATISTIC = 1.37

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
SP.CELLR = 0.0164 + 0.00844 H - 0.0113 T - 0.00908 HT
- 0.00358 HH

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.0163865 | 0.0008856 | 18.50 |
| H | 0.008443 | 0.001120 | 7.54 |
| T | -0.011294 | 0.001120 | -10.08 |
| HT | -0.009077 | 0.001252 | -7.25 |
| HH | -0.003576 | 0.001428 | -2.50 |

S = 0.003542

R-SQUARED = 91.2 PERCENT
R-SQUARED = 89.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 4 | 0.00272627 | 0.00068157 | 54.16+ |
| H | 1 | 0.00071293 | | |
| T | 1 | 0.00127557 | | |
| HT | 1 | 0.00065908 | | |
| HH | 1 | 0.00007869 | | |
| RESIDUAL | 21 | 0.00026350 | 0.00001255 | |
| PURE ERROR | 10 | 0.0001573 | 0.00001573 | 1.69* |
| LACK OF FIT | 11 | 0.0001027 | 0.00000930 | |
| TOTAL | 25 | 0.00298976 | | |

DURBIN-WATSON STATISTIC = 1.97

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.CELL.R = 0.649 - 0.474 T - 0.395 HT + 0.317 NNH

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.64937 | 0.05136 | 12.64 |
| T | -0.47416 | 0.08281 | -5.73 |
| HT | -0.39492 | 0.09259 | -4.27 |
| NNH | 0.31697 | 0.09259 | 3.42 |

S = 0.2619

R-SQUARED = 74.0 PERCENT
R-SQUARED = 70.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 3 | 4.2997 | 1.4332 | 20.89+ |
| T | 1 | 2.2483 | | |
| HT | 1 | 1.2477 | | |
| NNH | 1 | 0.8038 | | |
| RESIDUAL | 22 | 1.5088 | 0.0686 | |
| PURE ERROR | 11 | 0.5812 | 0.0528 | 0.63* |
| LACK OF FIT | 11 | 0.9273 | 0.0843 | |
| TOTAL | 25 | 5.8085 | | |

DURBIN-WATSON STATISTIC = 2.05

\+ Significant at the 0.1% level.
* Not significant at the 5% level.

THE REGRESSION EQUATION IS
M.AVETOH = 0.174 + 0.126 H - 0.133 T - 0.0306 HT - 0.0468 HH
- 0.0392 TT + 0.0830 HHT - 0.0932 NNH - 0.0246 NHT

| COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|
| | 0.173608 | 0.003879 | 44.75 |
| H | 0.12562 | 0.01058 | 11.87 |
| T | -0.13325 | 0.01058 | -12.59 |
| HT | -0.030556 | 0.005292 | -5.77 |
| HH | -0.046801 | 0.008178 | -5.72 |
| TT | -0.039172 | 0.008178 | -4.79 |
| HHT | 0.08303 | 0.01183 | 7.02 |
| NNH | -0.09315 | 0.01183 | -7.87 |
| NHT | -0.024562 | 0.005292 | -4.64 |

S = 0.01497

R-SQUARED = 97.5 PERCENT
R-SQUARED = 96.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F |
|---|---|---|---|---|
| REGRESSION | 8 | 0.146127 | 0.018266 | 81.54+ |
| H | 1 | 0.026110 | | |
| T | 1 | 0.044654 | | |
| HT | 1 | 0.007469 | | |
| HH | 1 | 0.033012 | | |
| TT | 1 | 0.005140 | | |
| HHT | 1 | 0.011031 | | |
| NNH | 1 | 0.013884 | | |
| NHT | 1 | 0.004826 | | |
| RESIDUAL | 17 | 0.003809 | 0.000224 | |
| PURE ERROR | 6 | 0.00216 | 0.000360 | 2.4* |
| LACK OF FIT | 11 | 0.00165 | 0.000150 | |
| TOTAL | 25 | 0.14993 | | |

DURBIN-WATSON STATISTIC = 2.46

\+ Significant at the 0.1% level.
* Not significant at the 5% level.