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

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

DAVID NEIL WEDLOCK

1988

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ABSTRACT

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

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

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

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

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

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

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

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

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

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To my Mother
and in memory of my Father

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HISTORICAL REVIEW

1. *PACHYSOLEN TANNOPHILUS*

1.1 General Description

The yeast was originally described by Boidin and Adzet (1957). Most strains, including the type strain NRRL Y-2460, were isolated from tree extracts used in the tanning of leather. *Pachysolen tannophilus* is a small budding yeast with cells about half the diameter of *Saccharomyces cerevisiae*. Cells, spheroidal to ellipsoidal (1.5-5.0) x (2.0-7.0) μm , can occur in both haploid (Plate 1) and diploid (Plate 2) phases, although the haplophase is predominant (James and Zahab, 1982). Although diploid cells are larger than haploid cells, budding is bipolar in both phases (Boidin & Adzet, 1957; James & Zahab, 1982). Pseudohyphae are usually present and sometimes highly branched.

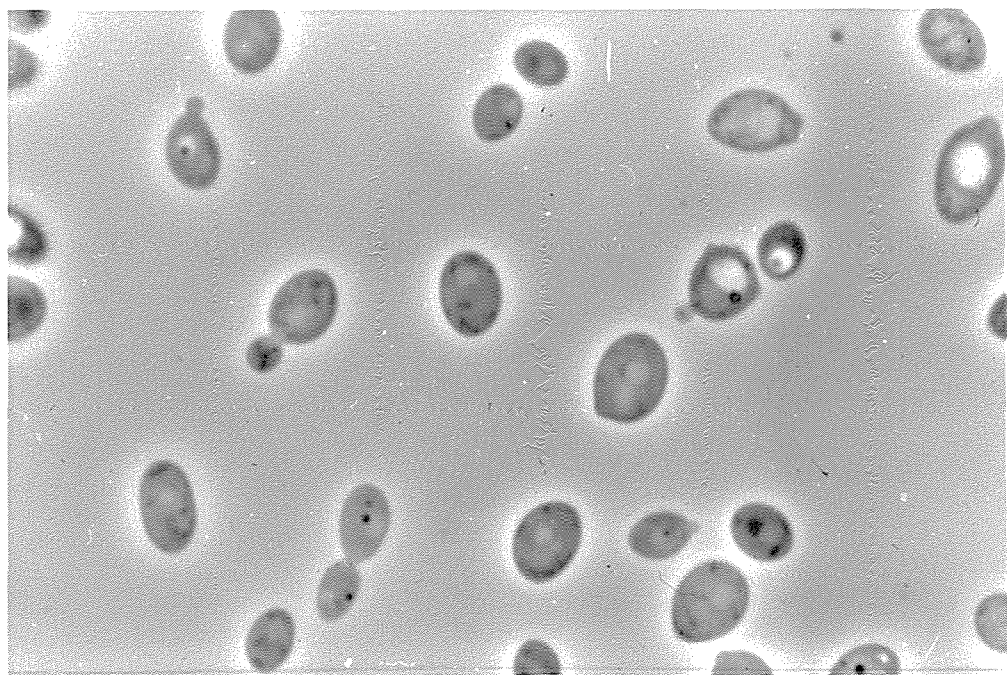
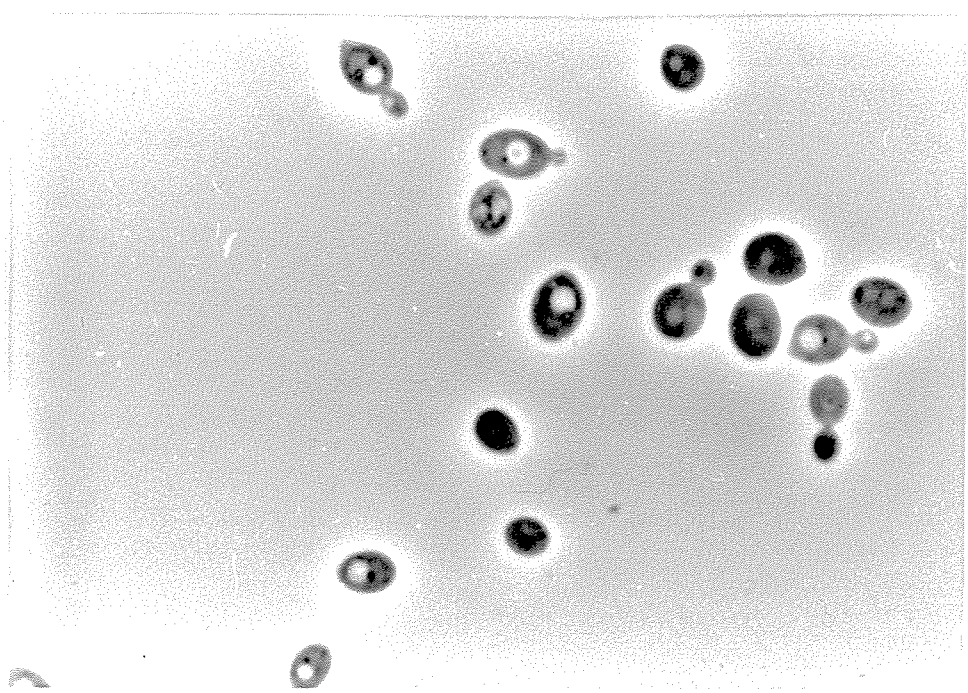
In sporulating cultures, the two phases are more easily distinguished, since the ascophores of a diploid culture are usually free of 'attached' cells and are unconjugated, while those of haploid are conjugated (James & Zahab, 1982; Kurtzman, 1983). The sporulation of diploid cells first becomes apparent with the appearance of enlarged conjugant cells from which long (up to 45 μm) tubes or sometimes branched filaments are extended (James & Zahab, 1982). A conjugant cell may produce a thick walled ascophore at the end of which the ascus carrying up to four hemispheroidal ascospores are formed (Kurtzman,

Plate 1: Haploid Cells of *Pachysolen tannophilus*.

Magnification x 400

Plate 2: Diploid Cells of *Pachysolen tannophilus*.

Magnification x 400



1983). After one or two days, the ascus wall dehisces and the spores are released from the ascus and the cycle is then repeated. The thick walled highly refractile ascospore is a readily identifiable feature of *P. tannophilus* (James & Zahab, 1982; Kurtzman, 1983).

In the normal events of the sexual cycle the diploid phase is usually short-lived. Diploidy and the subsequent formation of an ascophore occurs when sister nuclei fuse, usually within the filament of the conjugant cell (James & Zahab, 1982). Single ascospore isolates from NRRL Y-2460 gave rise to sporogenous colonies, indicating the species to be homothallic with no mating types (Wickerham, 1970).

A system for the genetic manipulation of *P. tannophilus* has been developed by James and Zahab (1982). The fusion of non-sister cells, though relatively infrequent, does occur at a frequency of 1×10^{-6} when the cells are inoculated onto a medium such as YM-YE (malt extract, D-glucose) which is conducive to the formation of conjugant cells. Diploid cells which form as a result of these rare fusions are prevented from undergoing meiosis by transferral onto a medium which encourages mitotic growth. Haploid strains carrying auxotrophic markers can be readily mated and diploids isolated by prototrophic selection. Diploid cells can be induced to sporulate by inoculating onto sporulation medium (YM, yeast extract, malt extract, D-glucose) and incubating for 4-5 days at 30°C. The spores are readily amenable to tetrad analysis.

From a practical viewpoint, *P. tannophilus* can be considered relatively unadapted to commercial conditions. Thus this yeast could be subjected to improvement by genetic modification, either using classical techniques of breeding and selection of mutants or using genetic engineering. A thorough knowledge of the physiology and biochemistry of this yeast is paramount to success.

1.2 Utilisation and Fermentation of Carbon Compounds

Growth of *P. tannophilus* is supported by a number of hexose and pentose sugars, organic acids and sugar alcohols (Kurtzman, 1983). The sugars, D-glucose, D-xylose and D-galactose are assimilated. Other sugars utilised include cellobiose and L-arabinose. Organic acids assimilated include succinic acid and pyruvic acid. Sugar alcohols assimilated include glycerol, ribitol, D-mannitol and D-glucitol. The generation time for vegetative cells is 135 min on solid YEPD (yeast extract, peptone, D-glucose) medium at 30°C (James & Zahab, 1982). Both biotin and thiamine are essential vitamins required for growth (Dellweg *et al.* 1984).

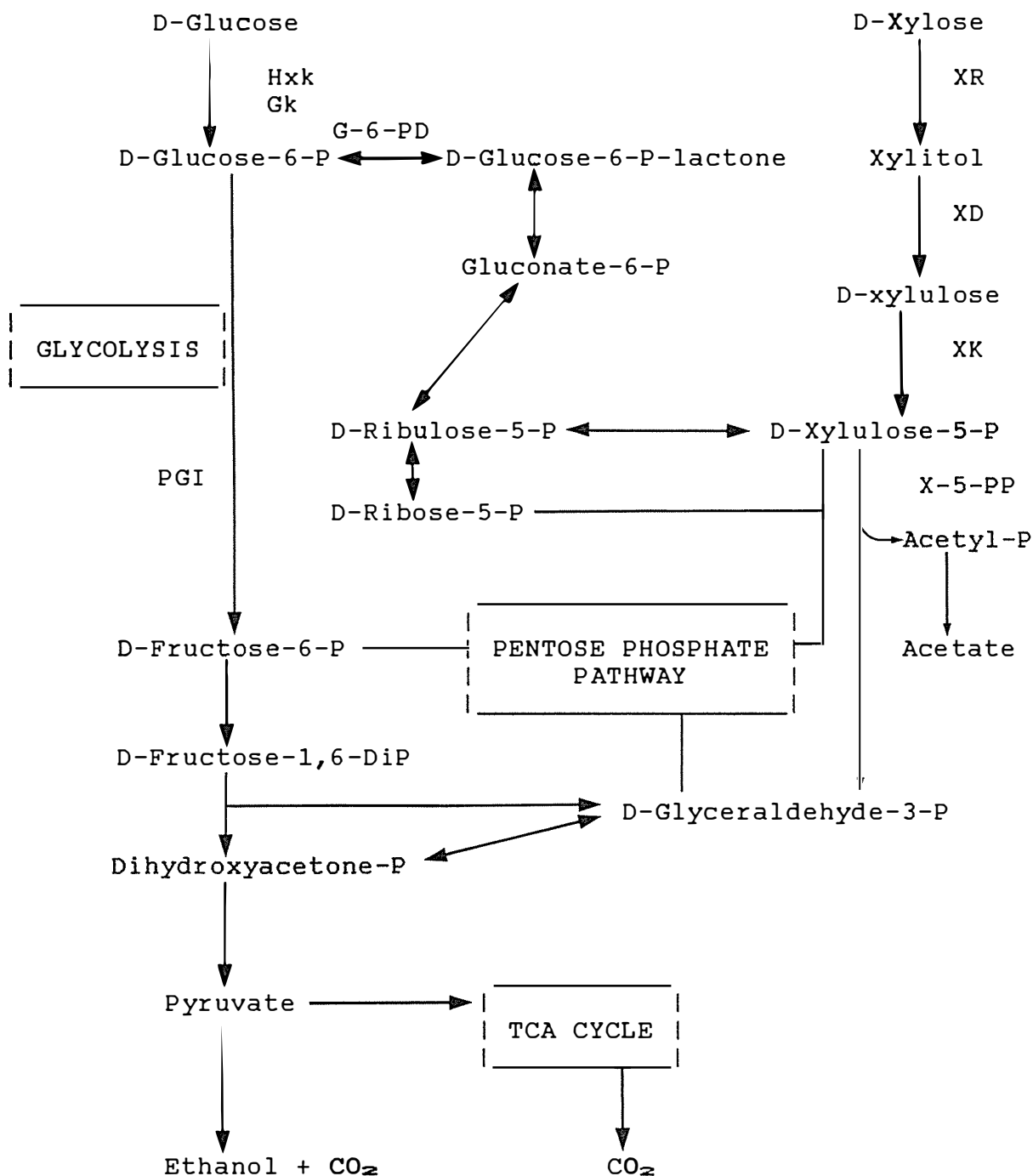
Utilisation of D-xylose involves the intermediary metabolism of the pentose phosphate pathway, the Embden-Meyerhof-Parnas (glycolytic) pathway and the tricarboxylic acid cycle (Debus *et al.*, 1983; Lachke & Jeffries, 1986; Slininger *et al.*, 1987). Entry of D-xylose into these pathways has now been determined to occur by reduction of D-xylose to xylitol and subsequent oxidation to D-xylulose (Maleszka *et al.*, 1983b). D-xylulose is then

phosphorylated to form a key intermediate, D-xylulose-5-phosphate. Various reactions, catalysed by ribulosephosphate-3-epimerase, ribosephosphate isomerase, transaldolase and transketolase convert D-xylulose-5-phosphate to D-glyceraldehyde-3-phosphate and D-fructose-6-phosphate, which are subsequently fermented to ethanol by the glycolytic pathway or used in respiration (Lachke & Jeffries, 1986, Slininger *et al.*, 1987). These major metabolic pathways are shown in Figure 1.

There is evidence for the existence of a D-xylulose-5-phosphate phosphoketolase in *P. tannophilus* (Evans & Ratledge, 1984). This enzyme catalyses the conversion of D-xylulose-5-phosphate to D-glyceraldehyde-3-phosphate and acetyl phosphate. However, most of the xylulose-5-phosphate is thought to enter the pentose phosphate pathway (Slininger, 1987).

Both D-glucose and D-xylose are readily fermented to ethanol. Glycerol and D-galactose have also been reported to be fermentable to ethanol (Maleszka *et al.*, 1982). More ethanol was produced from glycerol under aerobic conditions compared to anaerobic conditions of growth. The maximum ethanol concentration found in YNB-glycerol (0.67% yeast nitrogen base, 20 g l⁻¹ glycerol) medium was 4.0 g l⁻¹ and 0.75 g l⁻¹ under these conditions, respectively. A similar situation was found with D-galactose. Maximum ethanol concentrations were 0.3 g l⁻¹ and 3.5 g l⁻¹ from 20 g l⁻¹ D-galactose under aerobic and anoxic conditions, respectively. However, considerably more ethanol was produced aerobically from this carbon

Fig. 1 Pathways of Hexose and Pentose Metabolism in *Pachysolen tannophilus*



Abbreviations used for enzymes: Hxk, hexokinase; Gk, glucokinase; XR, xylose reductase (NADPH/NADH-dependent); XD, xylitol dehydrogenase (NAD-dependent); PGI, phosphoglucose isomerase; G-6-PD, glucose-6-phosphate dehydrogenase; Xk, xylulokinase; X-5-PP, xylulose-5-phosphate phosphoketolase.

source by employing cell recycling and an ethanol concentration of 7.6 g l^{-1} was achieved. Neither glycerol nor D-galactose supported anaerobic growth. Lee *et al.* (1986) reported maximum ethanol concentrations of 1.6 g l^{-1} and 6.0 g l^{-1} by a wild type strain in YNB medium supplemented with 40 g l^{-1} carbon source, for the aerobic fermentation of D-galactose and glycerol, respectively.

The yeast *Pachysolen tannophilus* has received much attention in recent years, following the discovery of its ability to ferment pentose sugars such as D-xylose to ethanol (Schneider *et al.*, 1981; Slininger *et al.*, 1982b). *Pachysolen tannophilus* is one of comparatively few yeasts able to produce appreciable amounts of ethanol from D-xylose (greater than 1 g l^{-1} ethanol from 20 g l^{-1} (w/v) D-xylose) (Toivola *et al.*, 1984).

In *Pachysolen tannophilus*, there is a lag between the commencement of growth and the initiation of ethanol production (du Preez *et al.*, 1984). Schneider *et al.* (1985) found that the formation of ethanol and other end-products such as xylitol, arabitol and acetic acid from the fermentation of D-xylose correlated with the limitation of growth. Hence these products were considered to be formed as the result of secondary metabolism. Ethanol accumulation during batch growth on D-xylose was found to correlate with a transition of the culture to a state of decreased oxygen consumption (Mahmourides *et al.*, 1985). Ethanol accumulation thus occurs during later stages of cell growth.

An early discovery was the requirement for limited oxygen during the fermentation of D-xylose for optimal ethanol production. Schneider *et al.* (1981) reported ethanol yields obtained during the fermentation of YNB-xylose (20 g l^{-1}) containing 0.4% casamino acids by NRRL Y-2460. Aeration of the culture enhanced alcohol production, a maximum ethanol concentration of 5.3 g l^{-1} was obtained with 'semi-aerobic' conditions. Low ethanol concentrations (less than 0.3 g l^{-1}) occurred under anoxic conditions. Similarly, Debus *et al.* (1983) found that 'semi-aerobic' conditions resulted in optimal or near optimal production of ethanol. Although fermentation could occur anaerobically, lower amounts of ethanol were produced (1.8 g l^{-1}) from 27 g l^{-1} D-xylose. Under these conditions xylitol was a major product, whereas under 'semi-aerobic' conditions less xylitol and correspondingly more ethanol (3.0 g l^{-1} from 25 g l^{-1} D-xylose) was produced.

In another study, aeration was found to increase the rate of ethanol production from 20 g l^{-1} D-xylose and the yield ($0.32 \text{ g ethanol produced (g D-xylose consumed)}^{-1}$) was slightly higher than that obtained under anaerobic conditions (0.28 g g^{-1}) (Delgenes *et al.*, 1986). The greater volumetric productivity achieved with aeration described as 'microaerophilic' compared to anaerobic conditions, was attributed to the greater biomass, since the specific productivity under these two conditions was similar. The volumetric productivity was $1.54 \text{ g ethanol l}^{-1} (\text{day})^{-1}$ under 'microaerophilic' conditions and 0.40 g

ethanol l^{-1} (day) $^{-1}$ under anaerobic conditions. The specific productivity was 0.81 and 0.91 g ethanol (g dry weight of cells) $^{-1}$ day $^{-1}$ under these two conditions, respectively.

During fermenter studies, Watson *et al.* (1984a) found that 'semi-anaerobic' conditions were optimal for ethanol production. Although the greatest amount of ethanol (2.9 g l^{-1} ethanol from 10 g l^{-1} D-xylose) was formed at the lowest oxygen transfer rate (0.05 mmol l^{-1} (h) $^{-1}$), the maximum specific ethanol productivity (0.07 h $^{-1}$) occurred at an oxygen transfer rate between 0.09 and 1.18 mmol l^{-1} (h) $^{-1}$. Polyol production decreased with increased aeration, in agreement with findings by Debus *et al.* (1983), while the cell yield increased.

A similar dependence of ethanol production on aeration conditions was reported by Mütze & Wandrey (1983) during the fermentation of D-xylose in continuous culture. Under conditions of limited aeration a lower cell yield and a greater ethanol yield was obtained. Ligthelm *et al.* (1988) found that low amounts of oxygen had less effect on the efficiency of D-xylose fermentation but stimulated the rate of production. Ethanol yields from 40 g l^{-1} D-xylose were 0.26, 0.28 and 0.10 g ethanol (g D-xylose consumed) $^{-1}$ under anaerobic, 'oxygen-limited' and aerobic conditions, respectively. The highest specific ethanol productivity (0.10 h $^{-1}$) occurred under 'oxygen-limited' conditions. Maximum volumetric and specific ethanol production rates of 0.22 g l^{-1} (h) $^{-1}$ and 0.07 h $^{-1}$, respectively were reported by du Preez *et al.* (1984) from

the fermentation of 50 g l⁻¹ D-xylose with aeration. An ethanol yield of 0.28 g g⁻¹ was similar to that obtained by Ligthelm *et al.* (1988).

Completely aerobic cultural conditions have been found to be conducive to cell growth but little or no ethanol production (Debus *et al.*, 1983; Watson *et al.*, 1984a; Delgenes *et al.*, 1986).

This phenomenon in which limited amounts of oxygen enhances fermentation of D-xylose is common among yeasts. Of fifteen yeast species tested in seven genera that could assimilate D-xylose to ethanol, introduction of air into the media enhanced production in yeasts within six genera including *P. tannophilus* (Maleszka & Schneider, 1982b).

Growth of *P. tannophilus* is stimulated by oxygen. Slininger *et al.* (1982b) found that, in the absence of oxygen, no cell growth occurred, although ethanol was produced. Similarly, Neirinck *et al.* (1984) and du Preez *et al.* (1984) found no growth anaerobically. Some growth under anaerobic conditions was reported by Debus *et al.* (1983), while poor growth was found by Ligthelm *et al.* (1988).

Different explanations have been suggested to explain the lack of growth anaerobically and the improved ethanol production from D-xylose when air has access to the medium. Debus *et al.* (1983) proposed that under anaerobic conditions xylitol acts as a sink for electrons transferred from NADPH and is necessary for the regeneration of NADP. This explained why more xylitol

was accumulated under anaerobic compared to aerobic conditions. The possession of a NADH-linked xylose reductase has been seen as the key to anaerobic fermentation (Bruinenberg, 1984). Metabolism of D-xylose via the NADH-dependent xylose reductase alleviates the imbalance of the NAD/NADH redox system and anaerobic fermentation is thus permitted. Yeasts such as *Pachysolen tannophilus* and *Pichia stipitis*, which possess NADH-linked activity in addition to NADPH-linked xylose reductase activity, can recycle anaerobically the NADH generated during the oxidation of xylitol to D-xylulose. The yeast *Candida utilis* which possesses only a NADPH-dependent xylose reductase can only produce ethanol from D-xylose under aerobic conditions.

Supplementation of D-xylose containing media with lipids such as ergosterol and oleic acid was ineffective in enhancing growth under anaerobic conditions (Maleszka *et al.*, 1982). This suggests that the requirement of oxygen for growth in *P. tannophilus* is different from *Saccharomyces sp.* which grow anaerobically when the medium is supplemented with these unsaturated lipids (Hunter & Rose, 1970).

Functional mitochondria are essential for the growth of *P. tannophilus* and this could explain the dependence of growth on oxygen. Neirinck *et al.* (1984) found that normally functioning mitochondria were necessary for incorporation of D-xylose and D-glucose into trichloroacetic acid-insoluble material. Oxygen was required for the channelling of catabolite intermediates

into biosynthetic routes. Three enzymes associated with the catabolism of D-xylose, xylose reductase, xylitol dehydrogenase and xylulose kinase required oxygen for induction by D-xylose.

Both pH and temperature influence the fermentation of D-xylose. Optimal growth was found to occur at pH 4.8 (Debus *et al.*, 1983), whereas a lower pH of 2.5 favored optimal ethanol production (Slininger *et al.*, 1982b). Dekker (1982) reported ethanol production at pH 2.50-2.75 but no growth occurred when the initial pH of the medium was less than 3.0. Therefore higher pH values than the optimum for ethanol production are required to initiate growth. Optimal growth was found to occur at 31°C (Debus *et al.*, 1983) or 32°C (Slininger *et al.*, 1982b), while 32°C was optimal for ethanol production (Slininger *et al.*, 1982b; Debus, 1983).

The ethanol tolerance of *Pachysolen tannophilus* is considerably less in comparison to a yeast such as *Saccharomyces cerevisiae*; relatively low concentrations of ethanol has been found to inhibit D-xylose fermentation. Slininger *et al.* (1982b) observed severe inhibition of D-xylose uptake at ethanol concentrations as low as 19 g l⁻¹. The rate of production of ethanol from D-xylose was reduced by addition of ethanol at concentrations equal or greater than 24 g l⁻¹ (Watson *et al.* 1984b).

1.3 Fermentation of Hydrolysates

Hydrolysates such as plant hydrolysates and waste liquors from pulp and paper processing, derived from renewable biomass, show considerable potential for production of ethanol. The ability of a yeast such as *P. tannophilus* to ferment D-xylose is of particular importance, since this sugar is abundantly present in many hydrolysates and comprises for example, 29.5% of the sugars in sugar cane bagasse (Tsao *et al.*, 1982) and approximately 30% of a hardwood hydrolysate (Deverell, 1983).

Successful fermentation of a hemicellulose substrate prepared by alkali delignification of wheat straw, followed by acid hydrolysis to yield D-xylose, was reported by Detroy *et al.*, 1982. Production of ethanol was 8.2 g l^{-1} from 46 g l^{-1} D-xylose in 4 days.

Wood hydrolysates prepared by dilute hydrolysis were batch fermented to ethanol (Deverell, 1983). A softwood *Pinus radiata* hydrolysate was fermented with *S. cerevisiae* and the 'beer' consisting of unfermented sugars such as D-xylose and D-galactose subsequently fermented with *P. tannophilus*. Ethanol yield was $0.33 \text{ g ethanol (g sugar consumed)}^{-1}$ and represented a 9% increase in ethanol production above that achieved by using *S. cerevisiae* alone. The yield from a hardwood hydrolysate containing predominately D-xylose and D-glucose was $0.43 \text{ g ethanol (g sugar)}^{-1}$. The fermentation was complete within 35 hr.

Unfortunately, hydrolysates may contain compounds that are potentially inhibitory to fermentation. Toxic compounds present in acid hydrolysates of wood include furfural, 5-hydroxymethyl furfural, organic acids such as levulinic and formic acid and phenolic compounds (Clark & Mackie, 1984; Watson *et al.*, 1984b). Furfural and lignin in wheat straw hydrolysates inhibited fermentation (Detroy *et al.*, 1982). The toxic level of furfural was 2.5-3.0 g l⁻¹ but cell growth and D-xylose utilisation was adversely affected at sub-toxic levels.

Fermentation of hemicellulose hydrolysates prepared from sugar cane was inhibited by factors present (Watson *et al.*, 1984b). Acetic acid and furfural at concentrations of 0.3 and 7.0 g l⁻¹ respectively, inhibited fermentation of D-xylose when added to synthetic medium (Watson *et al.*, 1984b). These levels were similar to those present in an acid hydrolysed bagasse. Metals such as iron, chromium, nickel and copper were also inhibitory but both these components and the acetic acid and furfural could be removed by treating the hydrolysates with ion-exchange resin prior to fermentation. Fermentation of the hydrolysate was then possible, 4.1 g l⁻¹ ethanol being produced in 14 days with a yield of 0.31 g ethanol (g sugar consumed)⁻¹. Another hydrolysate contained less toxic components and was more fermentable, but ethanol production was enhanced by treatment with the ion-exchange resin. Although D-glucose was utilised rapidly in these hydrolysates, D-xylose utilisation was slower and appreciable amounts remained after 20 days.

The method employed for the preparation of hydrolysates can determine its fermentability. A D-xylose rich wheat straw hydrolysate was prepared by treatment with 1 N trifluoroacetic acid (Fanta *et al.*, 1984) and was readily fermented by *Pachysolen tannophilus*. No pre-treatment of the hydrolysate was required other than removal of the trifluoroacetic acid and inhibitors such as furfural and lignin were present only at low concentrations.

2. IMPROVEMENT OF D-XYLOSE FERMENTATION

2.1 Process Control

Ethanol production rates from D-xylose have been enhanced by performing the fermentation with recycled cells in suspension cultures or immobilising the cells in calcium alginate gels (Maleszka *et al.*, 1981). A maximum ethanol concentration of 7.2 g l⁻¹ at 30°C and 7.9 g l⁻¹ at 37°C from 20 g l⁻¹ D-xylose was obtained under 'semi aerobic' conditions and were improvements over previously obtained values of 5.3 g l⁻¹ (Schneider *et al.*, 1981). Continuous aeration of the cultures was not required in order to achieve these values during the fermentation of D-xylose by either recycled or immobilised cells (Maleszka *et al.*, 1981).

Cells entrapped in calcium alginate beads have been successfully used for continuous fermentation of D-xylose at high cell density (Slininger *et al.*, 1982a). Substrate levels influenced the specific production rate and was optimal at between 28 and 35 g D-xylose l⁻¹.

However the optimal yield occurred at a lower substrate concentration than this. They concluded that a compromise in operating conditions was required to achieve a balance between optimal rate of ethanol production and yield.

Jeffries *et al.* (1985) reported an improved aerobic yield of ethanol from D-xylose by the periodic addition of small amounts of D-glucose (5.0 g l^{-1}) to the D-xylose (30 g l^{-1}) fermentation. Yields increased from 28 g g^{-1} to $0.41 \text{ g (g D-xylose consumed)}^{-1}$. This may have been due to a Crabtree effect in which D-glucose inhibits respiration and the oxidation of ethanol.

Periodic addition of a cellulose hydrolysate to a hardwood hemicellulose hydrolysate (Beck, 1986) produced greater yields of ethanol compared to the batch fermentation of either hydrolysate alone or a mixture of the two. Yields of $0.40 \text{ g (g sugar)}^{-1}$ were achieved.

The importance of control of oxygen during the fermentation for maximum ethanol productivity and optimal ethanol yield has already been discussed.

The inhibitory effect of ethanol on D-xylose fermentation has been overcome by *in situ* removal of ethanol (Chung & Lee, 1985). During fermentation, ethanol was absorbed by circulation of the broth through a column of hydrophobic silicalite. Fermentation rates were improved, primarily due to the higher growth rates and cell concentrations obtained. The inhibitory effect of compounds in hydrolysates have been overcome by prior treatment with ion-exchange resins (Watson *et al.*, 1984b) or by selection

of suitable preparatory procedures, as already discussed.

A novel approach to the improvement of D-xylose fermentation involved the addition of acetone, a hydrogen-accepting compound, to the fermentation of D-xylose (Alexander, 1986). Increases in ethanol yields of 50-70%, together with a decrease in xylitol yield were obtained. The conversion of acetone to 2-propanol was thought to provide NAD for xylitol dehydrogenase and hence overcome the imbalance of cellular NAD/NADH.

2.2 Genetic Manipulation

Although *P. tannophilus* is strongly homothallic with a predominately haploid phase, polyploid and aneuploids have been developed (James & Zahab, 1982, 1983). The successful mating of haploid to diploid strains and diploids to diploids was possible due to the absence of stable mating types. Triploids, aneuploids and a tentative tetraploid were produced and confirmed by tetrad analysis. These strains were subjected to fermentation trials (Maleszka *et al.*, 1983a). Polyploids gave both increased rates and yields of ethanol production from D-xylose. The largest increase was found between diploids and haploids. The highest ethanol yield was 83.3% of the theoretical maximum by a probable tetraploid on 20 g l⁻¹ D-xylose in YNB media employing cell-recycling (Maleszka *et al.*, 1981). This represented an improvement of 14.2% over the wild type strain. The rate of ethanol production from D-xylose increased with chromosome number, an aneuploid and the probable tetraploid producing ethanol

1.87 and 1.83 times faster than the wild type, respectively. A similar increase in ethanol production rate from D-glucose with increased ploidy was found. In general, less xylitol was produced by diploids and polyploids compared to haploids.

Neirinck *et al.* (1982) selected for mutants with elevated growth rates on D-galactose. Improved ethanol yields were obtained during the fermentation of simulated softwood and hardwood spent sulphite liquors and a hardboard mill effluent containing a variety of sugars including D-mannose, D-xylose, D-galactose and D-glucose. Ethanol yields for the wild type ranged from 77-84% of the theoretical maximum, values for the mutant were 83-90%.

Part of the inefficiency of ethanol production from D-xylose is due to the concurrent utilisation of ethanol in the presence of D-xylose (Maleszka & Schneider, 1982a). The rate of consumption of ethanol increased with increased aeration of cultures. In YNB-xylose (20 g l⁻¹), the amount of ethanol consumed under 'semi-aerobic' conditions was calculated to be at least 9.8% of that produced. As a result of metabolism of ethanol to cell mass and acetate, ethanol concentrations in the aerobic D-xylose fermentations typically peak and then decline (Slininger *et al.*, 1987).

Mutants defective for growth on ethanol were obtained by UV mutagenesis (Lee *et al.*, 1986). Eleven independent mutant loci conferring the ethanol defective phenotype were identified; three of these produced greater amounts

and yields of ethanol, and at a faster rate, than the wild type. The maximum ethanol concentration produced by the mutants during the fermentation of 40 g l⁻¹ D-xylose in YNB was 1.5 times the wild type value. Mutants *eth2-1* and *eth11-1 sup* produced 0.25 g ethanol (g xylose)⁻¹ compared to 0.16 g g⁻¹ for the wild type strain. These mutants accumulated acetic acid during the fermentation of D-xylose. The biochemical lesion in the *eth2-1* mutant conferred a defect in the malate dehydrogenase enzyme.

Mutant strains which accumulated ethanol at a faster rate and in greater yield than the wild type Y-2460 strain, have been isolated by UV mutagenesis, enrichment in nitrate broth and selection for rapid growth in nitrate medium (Jeffries, 1984). The rationale behind using enrichment in nitrate broth was based on the observation that growth on nitrate stimulated production of enzymes of the pentose phosphate pathway, probably due to a requirement of nitrate reduction for NADPH. Mutant strains capable of faster growth on nitrate should possess higher levels of pentose phosphate pathway enzymes and show improved fermentation of D-xylose. Previously nitrate was shown to stimulate aerobic production of ethanol from D-xylose (Jeffries., 1983). Under anaerobic conditions, nitrate inhibited the production of ethanol from both D-xylose and D-glucose.

Under aerobic conditions, the parental strains produced 0.19 g ethanol (g xylose)⁻¹ consumed from 45 g l⁻¹ D-xylose, while the six best mutants produced 0.25 g g⁻¹. Under anaerobic conditions, ethanol yields were similar to

the parental strain, but specific fermentation rates were 50% higher.

Enzyme analyses conducted on these mutants showed that the best ethanol producers had the highest ratios of NADH to NADPH-linked xylose reductase. In general, the mutants had higher levels of pentose phosphate pathway and other enzymes including xylose reductase, xylitol dehydrogenase, glucose-6-phosphate dehydrogenase, D-xylulokinase, D-xylulose-5-phosphoketolase and alcohol dehydrogenase (Lachke & Jeffries, 1986).

The properties inherent in the *eth2-1* and $\text{NO}_3\text{-NO}_3\text{-4}$ mutants were combined by cross-mating and selecting for hybrids (Clark *et al.*, 1986). The progeny showed improved D-xylose fermentation capabilities, the most significant being a reduced tendency to oxidise the ethanol formed during the fermentation. The hybridisation of these mutants was part of the Ph.D. studies and is discussed in this thesis.

The ability to flocculate was induced in *P. tannophilus* NRRL Y-2461 with a continuous tower fermenter (Deverell & Clark, 1985). At low aeration rates, flocculation occurred and a cell concentration of 16 X was achieved in the fermenter. This induced flocculence in the fermenter may be due to selection of a flocculent mutant or be a physiological response to the environmental conditions imposed. Work done at Massey University (Neil Wedlock, unpublished results) suggested that flocculation in this mutant was under the control of at least one gene.

Pachysoles tannophilus has been manipulated to enhance production of products other than ethanol. Two mutants which produced greater amounts of acetic acid from D-xylose were obtained by UV mutagenesis and screening for inability to grow on pyruvate (Mahmourides *et al.* 1983).

The improvement of D-xylose fermentation for ethanol and other desirable products by genetic manipulation holds considerable promise. For example, selection of ethanol tolerant mutants could alleviate the problem of inhibition by this product. The use of catabolite repression resistant mutants could overcome the problem of hexose repression of D-xylose utilisation.

3 BIOCHEMISTRY OF *P. TANNOPHILUS*

The two enzymes in the xylose catabolic pathway, xylose reductase and xylitol dehydrogenase have received attention, since NADPH-linked D-xylose reductase and NAD-dependent xylitol dehydrogenase were first demonstrated in cell-free extracts of *P. tannophilus* (Smiley and Bolen (1982). Both xylose reductase and xylitol dehydrogenase have been purified and characterised.

A NADPH specific xylose reductase with a molecular weight of 35,000 - 40,000 daltons, was purified by Ditzelmüller *et al.* (1984a). The reduction of D-xylose was inhibited by NADP and the 'anabolic reduction charge' (NADPH/NADP + NADPH). The km value for D-xylose was determined as 162 mM. At physiological pH, the equilibrium for the

catalysis of D-xylose favoured the accumulation of xylitol.

Ditzelmüller et al (1985) reported the existence of two separate xylose reductase enzymes, one NADPH-specific and the other NADH-specific. These were conveniently separated from each other by ion-exchange chromatography or chromatofocusing.

Verduyn *et al.* (1985) isolated and partially purified two xylose reductases, enzyme A exhibiting activity with both NADPH and NADH cofactor and enzyme B specific for NADPH. Enzyme A had greater affinity for D-xylose with K_m values of 12.5 and 14.0 when NADH and NADPH were used as the cofactor, respectively. Enzyme B was similar to that reported by Ditzelmüller *et al.* (1984a), with lower affinity for D-xylose (K_m value of 180 mM). Molecular weights were 41,000 and 37,000 daltons for enzyme A and B, respectively. The ratio of NADH- to NADPH-linked activity in cell-free extracts increased with decreasing aeration of the culture, suggesting that oxygen influences the levels of these enzymes.

A xylose reductase exhibiting specificity with both NADPH and NADH was described by Morimoto *et al.* (1987). The k_m value for D-xylose was determined as 12 mM and in this respect and in regard to cofactor specificity, the enzyme was similar to enzyme A reported by Verduyn *et al.* (1985).

Ditzelmüller *et al.* (1984b) partially purified the NAD-dependent xylitol dehydrogenase by a combination of affinity chromatography and fast liquid protein

chromatography. At pH 8.5, the formation of D-xylulose was favoured, whereas at physiological pH values the formation of xylitol was favoured. The activity of the enzyme was found to be under the influence of the 'catabolic reduction' charge (NADH/NAD + NADH) and ATP. At pH 7.0, an increase in the 'catabolic reduction' charge favoured the accumulation of xylitol. ATP inhibited the reaction. The enzyme exhibited a low affinity for xylitol with a K_m value of 70 mM. The kinetic properties of this enzyme and the NADPH specific xylose reductase (Ditzelmüller *et al.*, 1984a) helps explain the tendency for xylitol to be accumulated during the fermentation of D-xylose, especially under anaerobic conditions.

Morimoto *et al.* (1986) reported the purification of an NAD-specific D-xylulose reductase (xylitol dehydrogenase). The molecular weight was determined to be 120,000 daltons with a sub-unit molecular weight of 40,000. The K_m for xylitol was 11 mM.

A xylose reductase and xylitol dehydrogenase were separated on affinity chromatography columns (Bolen *et al.*, 1986). The xylose reductase consisted of a single polypeptide with a molecular weight of 36,500 daltons. The xylose reductase exhibited activity with both NADPH and NADH. The NAD-dependent xylitol dehydrogenase had a molecular weight of 172,000 as determined by gel permeation chromatography with four subunits of about 40,000.

From these studies, it would appear that *Pachysolen tannophilus* possesses at least two xylose reductase enzymes, one possessing activity with both NADPH and NADH and the other NADPH specific. However the results of Ditzelmüller (1985) suggest the presence of a third enzyme specific for only NADH.

There are no references in the literature about the isolation, purification and kinetic properties of other important enzymes in *P. tannophilus*.

INTRODUCTION

1. Hexose-Phosphorylating Enzymes in Yeast

In yeast, entry of D-glucose into the glycolytic pathway involves the phosphorylation at the number 6 carbon to form D-glucose-6-phosphate (Figure 1). Similarly, D-fructose, D-mannose and D-glucosamine are phosphorylated as the initial step in their catabolism. This reaction, requiring adenosine-5'-triphosphate (ATP) and magnesium chloride ($MgCl_2$) is catalysed by two types of enzyme, hexokinase (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1) and glucokinase (ATP : D-glucose 6-phosphotransferase, EC 2.7.1.2) which differ in their sugar specificity and their affinity for D-glucose.

The hexose-phosphorylating enzymes in several species of yeast have been studied.

Saccharomyces cerevisiae possesses two hexokinases, PI and PII (Colowick, 1973). These were formerly referred to as hexokinase A and B (Ramel *et al.*, 1971; Barnard, 1975). A glucokinase is also present (Maitra, 1970; Maitra, 1975; Gancedo *et al.*, 1977). Three unlinked genes *hxx1*, *hxx2* and *glu1* code for the structural genes of hexokinase PI, PII and the glucokinase, respectively (Lobo & Maitra, 1977b; Maitra & Lobo, 1981).

The two hexokinases differ in the rate of D-fructose-phosphorylation to D-glucose-phosphorylation (F/G ratio). Hexokinase PI shows a F/G ratio of 2.8-3.0 (Colowick,

1973), while PII has a ratio of 1.0-1.2 (Colowick, 1973). This marked difference in the rates of phosphorylation of these two hexoses are important distinguishing features.

The D-glucose-phosphorylating enzymes of the *Candida* yeasts were investigated by Hirai *et al.* (1977). Three D-glucose-phosphorylating enzymes were separated from a cell-free extract of *Candida tropicalis* by use of ammonium sulphate fractionation and DEAE-cellulose chromatography and further purified by chromatography on Sephadex G-100 and DEAE-cellulose. Two hexokinases, designated hexokinase I and II, phosphorylated D-fructose 1.5 times faster than D-glucose, while the glucokinase was specific for D-glucose. The yeast *Candida lipolytica* contained at least two D-glucose-phosphorylating enzymes, a hexokinase with an F/G ratio of 1.5 and a glucokinase.

The hexokinase of wild type *Schwanniomyces occidentalis* (CBS 819) was found to have similar properties to the PII hexokinase of *Saccharomyces cerevisiae* (McCann *et al.*, 1987). This enzyme appeared to possess two catalytic sites, one hexokinase-like and associated with catabolite repression and the other glucokinase-like.

There is no report in the literature about the hexose-phosphorylating enzymes in *Pachysolen tannophilus*.

2. The Use of 2-Deoxyglucose to Obtain Hexose-ATP-Kinase Defective Mutants

The toxic analogue of D-glucose, 2-deoxy-D-glucose, has been applied to the yeast *Saccharomyces cerevisiae* to

generate mutants defective in hexose-phosphorylating enzymes (Maitra , 1970; Lobo & Maitra, 1977a, 1977b). In the majority of cases, 2-deoxyglucose resistant mutants unable to grow on D-glucose, had lost the activity of the hexose-phosphorylating enzymes (Lobo & Maitra, 1977a).

The exact mechanism for the toxicity of 2-deoxyglucose has not been fully elucidated but may be due to the depletion of the cell's supply of ATP (Maitra & Lobo, 1981). Evidence for inhibition of the synthesis of cell-wall polysaccharides has been found in *Schizosaccharomyces pombe* (Megnet, 1965).

The yeast *Pachysolen tannophilus* was found to be sensitive to levels of 2-deoxyglucose greater than 2 mM (Allen James, unpublished results). Thus it was assumed that this analogue could be exploited to obtain hexokinase defective mutants in the same manner as in *Saccharomyces cerevisiae*. Direct selection for resistance to the analogue was used. The methionine requiring strain P17-1A (Table I) was plated out (10^7 - 10^8 cells/plate) onto complete YNB-xylose plates supplemented with 2-deoxyglucose at levels of 6 and 8 mM. Resistant colonies were isolated and tested for growth on YNB-glucose plates (Allen James, unpublished results). These were termed the first series. Mutants showing resistance to 6 mM 2-deoxyglucose and reduction of growth on D-glucose and D-mannose were subjected to higher levels of 2-deoxyglucose by plating onto complete YNB-xylose supplemented with 30, 40 and 80 mM 2-deoxyglucose. In this manner a second series of mutants resistant up to 40

mM of the analogue was obtained. These were further checked for growth on YNB media containing either D-glucose, D-fructose, D-mannose or D-xylose as the carbon source.

A series of genetic crosses between the 2-deoxyglucose resistant mutants and wild type auxotrophs were performed (Allen James, unpublished work). Two mutants of the first series were crossed with P471-5D *ade2*. From these crosses two diploids were obtained, P500 and P501. When sporulated, segregation of both resistance to 10 mM 2-deoxyglucose and the ability to grow on D-mannose was 2:2 indicating a lesion within a single gene. The mutation was initially designated *man* and in this study is referred to as *hxx2*. Several of the second series mutants were crossed with P471-5D *ade2* giving rise to diploids P509, P510 and P511. Sporulation and subsequent genetic analysis gave a 2:2 segregation for growth on D-mannose. The segregations for growth on D-glucose and for resistance to 2-deoxyglucose did not give clear results. However, at least one further mutation had been introduced into the second series mutants. This mutation was designated *glu1*. The alleles governing these mutations were recessive, since the diploids grew normally on D-glucose and were sensitive to 2-deoxyglucose. From the above crosses, three segregants, P501-5A, P509-3C and P509-1B (Table I), each with a distinct genotype and phenotype were recovered and subjected to further study in this work.

3. Aims of this Investigation

The objectives of this study were:

1. To characterise the 2-deoxyglucose resistant mutants of *P. tannophilus* by examining their growth on hexose sugars, resistance to 2-deoxyglucose and hexose-phosphorylating activities. The behaviour of these strains on mixtures of D-glucose and D-xylose would also be investigated.

2. Further characterisation of the hexose-ATP-kinase enzymes in *P. tannophilus* by the application of DEAE-cellulose chromatography. In particular, the precise number of enzymes would be determined, since results from the genetic analyses on the 2-deoxyglucose resistant mutants were inconclusive. The role of these enzymes in catabolite repression of D-xylose utilisation would be investigated.

3. Isolate mutant strains which were totally defective for growth on D-glucose and D-fructose. A strain which can utilise and ferment D-xylose in the presence of D-glucose would be sought. Such a strain may have potential for fermentation of phytomass — derived hydrolysates, particularly pentose—rich hemicellulose hydrolysates, to ethanol. Pentose fermentation by the mutant could be carried out leaving the hexoses to be fermented by a more ethanol tolerant yeast such as *Saccharomyces cerevisiae*.

4. Transformation of *P. tannophilus* by the YRp7 plasmid carrying the cloned PII hexokinase gene from *S. cerevisiae* (Fröhlich *et al.*, 1984) would be attempted. As well as developing a method for the transformation of *P. tannophilus*, this could provide further information on the role of the hexokinases in catabolite repression.

5. Genetic hybridisation of two superior ethanol producing strains, the *eth2-1* strain (Lee *et al.*, 1986) and NO₃-NO₃-4 (Jeffries, 1984), for the genetic improvement of fermentation of D-xylose in *P. tannophilus*.

MATERIALS AND METHODS

1. MICROBIOLOGICAL METHODS

1.1 Microbial Strains and Maintenance

The yeast and bacteria strains used in this study are listed in Table 1. Yeast strains were maintained on YEP-glucose or YEP-xylose slopes at 4°C and sub-cultured at intervals of 6-12 months. Cultures were freeze-dried for long-term storage. Bacterial strains were maintained on LB slopes at 4°C and sub-cultured every 3-6 months. Long term storage was by freeze-drying.

1.2 Media and Cultivation

1.21 Yeast Extract Peptone Medium (YEP)

Yeast extract peptone medium (YEP) contained the following (g l⁻¹): yeast extract (Difco), 10.0; peptone, 20.0. D-Glucose, D-mannose, D-fructose, D-xylose or a mixture of these sugars was added to a final concentration of 20-40 g l⁻¹. The solid medium in addition had 20 g l⁻¹ agar.

1.22 Malt Extract Yeast Extract Peptone Glucose (MYGP)

Malt extract yeast extract peptone glucose (MYGP) contained (g l⁻¹): malt extract (Difco), 10.0; yeast extract (Difco), 10.0; peptone (Difco), 20.0; D-glucose, 20.0.

1.23 Yeast Nitrogen Base (YNB)

Yeast nitrogen base medium (YNB) contained (g l⁻¹): yeast nitrogen base (Difco) without added amino acids, 6.7. Sugar

Table I: Strains of Yeast and Bacteria Used in This Study

Strain	Genotype	Source
<i>Pachysolen tannophilus</i>		
P444-3D	Wild Type segregant	NRCC
2530	Wild Type	NRCC
P17-1A	<i>met2-1</i> auxotroph	NRCC
P510-5A	<i>glu1</i>	This study
P509-3C	<i>hxx2</i>	This study
P509-1B	<i>hxx2 glu1</i>	This study
P509-1B # 6	<i>hxx2 glu1 fru</i>	This study
P509-1B D/X A	<i>hxx1 hxx2 glu1</i>	This study
P509-1B F/G 2	<i>hxx1 hxx2 glu1 y</i>	This study
P509-1B F/G 2 #24	<i>hxx1 hxx2 glu1 y eth</i>	This study
NO ₃ -NO ₃ -4		USDA
NO ₃ -NO ₃ -4	<i>lys</i>	A.P. James
P141-10B	<i>eth2-1</i>	NRCC
P446-27A	<i>eth2-1 adel</i>	NRCC
P52-1C	<i>xy12-1 met2-1</i>	NRCC
<i>Saccharomyces cerevisiae</i>		
S288C	Wild Type	MUCC
C631-8A	Wild Type	MUCC

Cont'D...

Table I: Continued

<i>Escherichia coli</i>		
K12 RR1 YRp/HXK2-8	<i>pro leu thi lacY str^R</i> <i>r_k m_k endoI</i> YRp7/HXK2-8	K-D. Entian
HB101	<i>pro leu thi thr lacY str^R</i> <i>r_k m_k recA</i>	MUCC

NRCC National Research Council of Canada, Ottawa,
Ontario, Canada

USDA US Department of Agriculture, Forest Service,
Forest Products Laboratory, Madison, USA

MUCC Massey University culture collection

was added to a final concentration of 20-60 g l⁻¹. For the solid medium, 20 g l⁻¹ agar (Difco or Davis) was added. The medium was either minimal or supplemented with amino acids (Table II). Stock solutions containing all amino acids or lacking in one amino acid were prepared and autoclaved at 110°C for 10 min. The stock solution was added to the YNB medium (10 ml stock solution per 200 ml medium) prior to autoclaving.

1.24 Malt Extract Yeast Extract (YM)

Malt extract yeast extract (YM) had the following composition (g l⁻¹): malt extract (Difco), 10.0; yeast extract, 10.0; D-glucose, 4.0; agar (Difco), 30.0. The pH was adjusted to 5.0 prior to autoclaving. YM-YE contained (g l⁻¹): malt extract (Difco), 10.0; D-glucose, 4.0; and agar (Difco), 20.0.

1.25 Luria Broth (LB)

Luria broth (LB) contained (g l⁻¹): tryptone, 10.0; yeast extract (Difco), 4.0; sodium chloride (NaCl), 10.0. The pH was adjusted to 7.5 with 5 M NaOH before autoclaving. Plates supplemented with ampicillin were prepared by the addition of filter-sterilised ampicillin to a final concentration of 40 µg ml⁻¹ after the medium had been autoclaved.

1.26 Preparation of Media

Sugars were prepared as stock 20% solutions, filter sterilised with 0.22 µm filters and added to the rest of the media which was autoclaved at 121°C for 15 min.

Table II: Amino Acid Supplements for Complete YNB Media

Amino Acid	Concentration* (g per 300 ml stock)	Final Concentration in medium (mg l ⁻¹)
L-arginine	0.12	20
L-histidine	0.12	20
L-lysine	0.18	30
adenine	0.12	20
L-methionine	0.12	20
uracil	0.12	20
L-leucine	0.18	30
L-tryptophan	0.12	20
L-threonine	0.60	100
tyrosine	0.12	20
phenylalanine	0.30	50
isoleucine	0.12	20
valine	0.90	150

* Concentration in stock solution

1.27 Culture Growth and Purity

All stock cultures were inoculated onto either YNB or YEP plates and single colonies used for the inoculation of liquid media.

2. GROWTH EXPERIMENTS

2.1 Materials

2.11 Replica-Plating Velveteens

Velvets were brushed with a clothes brush, placed between parchment paper, wrapped in paper and sterilised by autoclaving at 121°C for 15 min.

2.2 Growth on Solid Media

Each strain was inoculated onto a small area (aprox 1 cm²) of a minimal YNB-xylose plate and incubated 24 hr at 30°C. Each plate was replica-plated onto minimal YNB plates supplemented with various sole carbon sources. Plates were incubated at 30°C for 96 hr and checked at 12-24 hr intervals. The amount of growth was assessed visually.

2.3 Growth in Liquid Media

2.31 Screening Trials and General Vegetative Growth

YEP media was used for screening mutants and for cell cultivation for enzyme assays. YEP-xylose (50 ml) was inoculated from a plate and incubated for 48 hr at 30°C and 180 rpm on a gyratory shaker. This culture was inoculated (0.5 ml) into YEP media containing different carbon sources. For screening trials, 100 ml media in 250 ml Erlenmeyer flasks was used and for enzyme assays, 250 ml in

1 litre flasks. Cultures were incubated at 30°C and 180 rpm on a gyratory shaker. If required, samples (4 ml) were removed at time 0 and after 72 or 96 hr and subjected to HPLC analysis. Growth was assessed by measurement of optical density at 600 nm.

2.32 Growth and Sugar Utilisation

Experiments in which growth and sugar utilisation were measured were done as follows. Minimal YNB-xylose (100 ml) was inoculated from a plate and incubated for 48 hr at 30°C at 180 rpm. The culture was centrifuged (6,000 x g for 10 min at 4°C), the cells washed once with 50 ml water, centrifuged and re-suspended in approximately 10 ml water. The optical density at 600 nm was adjusted to between 34.5 and 36.5 optical density (OD) units.

Minimal YNB or YEP media (100 ml in 250 ml Erlenmeyer flasks) were inoculated, in duplicate, with 0.5 ml (standard density inoculation) or 10.0 ml (high cell density) of this suspension. Final cell concentration was 2.5×10^7 cells ml⁻¹ and 5.0×10^8 cells ml⁻¹, respectively. Samples (4 ml) were removed at 0 time and at intervals of between 3 and 24 hr, centrifuged in a Heraeus Christ Labofuge at 2,500 g for 10 min and frozen at -20°C until required for HPLC analysis. Growth was followed for 72-120 hr or longer in some cases by measurement of optical density.

2.4 Measurement of Growth

The amount of growth was determined by measurement of the absorbance at wavelength 600 nm using a Cecil CE 272

spectrophotometer. A small portion of each culture (0.1 or 0.5 ml) was diluted volumetrically in water to give an absorbance of less than 0.4 and measured against a water blank. An estimation of cell number was obtained by reference to a standard curve which related absorbance to viable cell numbers. During the preparation of cells for UV mutagenesis or transformation, total cell concentration in log phase cultures was estimated by counting cells with a haemocytometer.

2.5 Preparation of Standard Curve

The wild type strain, P444-3D, was inoculated into minimal YNB-xylose (50 ml), incubated for 48 hr and 100 ml YNB-xylose inoculated with 1 ml of this culture. Following incubation for 24 hr, the culture was washed once with water, resuspended and diluted in water to give a range of absorbances (0.1 - 0.5) at 600 nm. Aliquots from each dilution were further diluted in water blanks and plated onto YNB-xylose plates. Viable cell numbers at each absorbance were estimated from the colony count and used to construct a standard curve.

3. SUGAR UTILISATION AND FERMENTATION TRIALS

3.1 Culture Conditions and Sampling

Duplicate cultures using either minimal YNB or YEP media were prepared and inoculated as described in Materials & Methods 2.32. The final cultures were incubated at 30°C on an orbital shaker at 180 rpm. At appropriate time

intervals, samples (4 - 5 ml) were removed and clarified as described before and stored at -20°C until required for analysis. Growth was followed as described previously.

3.2 High Performance Liquid Chromatography (HPLC)

3.21 Preparation of Samples

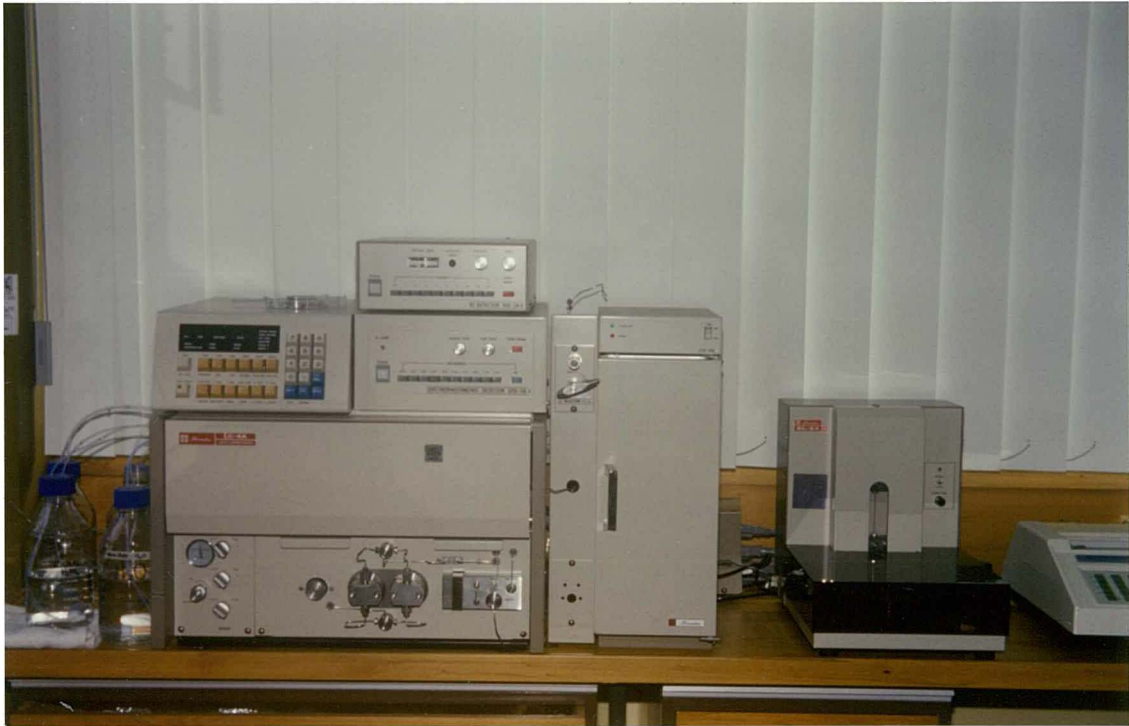
Samples were prepared for HPLC analysis by filtration with $0.45\ \mu\text{m}$ nitrocellulose membrane filters using the Swinnex filter kit (Millipore Corporation, Bedford, Massachusetts, USA).

3.22 Analysis of Sugars and End-products

Sugars and end-products were analysed by high performance liquid chromatography, performed with a Shimadzu LC-4A high performance liquid chromatograph (Plate 3) with a Shimadzu SIL-2A autosampler, a RID-2AS refractive index detector and a Shimadzu C-R3A Chromatopac data processor. All samples were analysed with the Bio-Rad Aminex HPX-87H column with the exception of samples containing both D-fructose and D-xylose which were separated on a Bio-Rad Aminex HXP-87C column. Filtered and degassed sulphuric acid ($0.010\ \text{N}$) and deionised water was used as the mobile phase for the HPX-87H and HPX-87C column, respectively. The oven temperature was 65°C for the H column and 85°C for the C column. Flow rates were $0.8\ \text{ml}\ \text{min}^{-1}$ for both columns. A guard column consisting of the same material as the analytical column was used in-line for protection.

A two point calibration was accomplished by injecting both a high and low standard. A typical high standard was

Plate 3: Shimadzu LC-4A High Performance Chromatograph



(g l⁻¹): D-glucose, D-xylose, or D-mannose or D-fructose, 25.0; xylitol, 10.0; ethanol, 10.0. The low standard contained (g l⁻¹): D-glucose, D-xylose, or D-mannose or D-fructose, 2.5; xylitol, 1.0; ethanol, 2.0. All substances used in standards were AnalaR grade.

3.23 Regeneration of Columns

The HPX-87H column was cleaned by flushing with 30% acetonitrile in 0.01 N H₂SO₄ for 4 hr at a flow rate of 0.1 ml min⁻¹ at ambient temperature. Regeneration was accomplished by flushing with 0.05 N H₂SO₄ for 16 hr at 0.1 ml min⁻¹ and ambient temperature. The HPX-87C column was cleaned by flushing with 30% acetonitrile in water for 4 hr and regenerated by flushing with 0.1 M calcium nitrate (Ca(NO₃)₂, pH 6.3 for 16 hr at 85°C.

4. MEASUREMENT OF ENZYME ACTIVITIES

4.1 Materials

4.11 Buffers

4.111 Tris-HCl

A stock (0.2 M) Tris-HCl solution was prepared by dissolving 12.1 g Trizma base (Sigma) in 400 ml deionised water, adjusting the pH to 7.5, 8.0 or 9.0 with 10 N HCl at room temperature and making the volume up to 0.5 litres in a volumetric flask.

4.112 Potassium phosphate buffer (KPB)

Stock (0.2 M) solutions of potassium dihydrogen phosphate (KH₂PO₄) (BDH, AnalaR) and dipotassium hydrogen orthophosphate (K₂HPO₄) (BDH, AnalaR) were prepared. A

stock potassium phosphate buffer (0.2 M, pH 7.0) was prepared by addition of potassium dihydrogen orthophosphate to dipotassium hydrogen orthophosphate until the desired pH was reached.

4.113 Triethanolamine buffer

Triethanolamine stock buffer (0.3 M) was prepared by dissolving 27.8 g of triethanolamine (Sigma) in 400 ml of deionised water, adjusting the pH to 7.5 with 10 N HCl and making up to 0.5 litre with deionised water.

4.12 Phenylmethanesulphonyl Fluoride (PMSF)

Phenylmethanesulphonyl fluoride (PMSF) was prepared as a 0.1 M stock solution in 95% ethanol.

4.13 Cofactors

Nicotinamide adenine dinucleotide cofactors (NAD, NADP, NADH, NADPH) (Boehringer Mannheim GmbH, grade II) were prepared fresh at a concentration of 1.3 mM in deionised water and stored on ice.

4.14 Enzymes for coupled reactions

Glucose-6-phosphate dehydrogenase (Boehringer Mannheim) and phosphoglucose isomerase (Boehringer Mannheim) were made up fresh in deionised water at a concentration of 2 units ml⁻¹ and stored on ice. Pyruvate kinase/lactate dehydrogenase (Sigma) was prepared at 100 units ml⁻¹ in deionised water.

4.15 Adenosine-5'-Triphosphate (ATP)

Adenosine-5'-triphosphate (ATP) (0.3 M) was prepared by dissolving 1.65 g ATP (Sigma, grade I) in 5 ml deionised water and adjusting the pH to 7.5 with 5 M NaOH and making

up to 10 ml with deionised water. Aliquots were stored at -70°C .

4.2 Preparation of Cell-Free Enzyme Extracts

Cells were pre-grown in YEP medium (50 ml) for 48 hr and inoculated (1-2 ml) into 250 ml YEP medium. Following incubation for 16 or 42 hr, the cells were harvested by centrifugation at $4,000 \times g$ for 10 min at 4°C , washed twice with cold water (100 ml) and resuspended in 10 ml 0.1 M Tris-HCl, pH 7.5, containing 0.2 ml PMSF. Following two passages through a French press at $10,000 \text{ lb in}^{-2}$, cellular debris was removed by centrifugation at $17,500 \times g$ for 20 min at 4°C . The supernatant was used as a cell-free extract and was stored at 0°C on ice until used. Enzyme and protein analyses were performed on the same day as the extraction.

4.3 Enzyme Assays

4.3.1 Measurement of Specific Activities

Enzyme activities were measured by following changes in absorbance of cofactors at 340 nm at 30°C with a Cecil CE 599 recording spectrophotometer. Enzymes were suitably diluted in either 20 mM Tris-HCl, pH 7.5 or 20 mM KPB, pH 7.0 in order to measure activity in the linear range. Reaction mixtures (3 ml total) in cuvettes were set up and the reaction started by the addition of either enzyme, substrate or cofactor. One unit was the reduction or oxidation of 1 μmole cofactor min^{-1} . Specific activity was expressed as units $(\text{mg protein})^{-1}$.

All enzyme assays and protein concentration determinations were done in duplicate. In most cases, analyses were also performed on two independently prepared cell-free extracts. Sugars and sugar alcohols used as substrates were AnalaR grade.

Quartz cuvettes were used for the assays and were cleaned by boiling in 30% nitric acid for 15 min, followed by several rinses in deionised water and a final rinse in 95% ethanol.

4.32 Reaction Mixtures

4.321 Hexose-ATP-kinase (ATP : D-hexose 6-phospho-
transferase, EC 2.7.1.1; ATP : D-glucose 6-phospho-
transferase, EC 2.7.1.2)

The D-glucose-phosphorylating activity of cell-free extracts was assayed by following the increase in NADP absorbance according to the method of Joshi and Jagannathan (1966). The reaction mixture consisted of 15 mM D-glucose, 10 mM ATP, 0.13 mM NADP, 0.6 units glucose-6-phosphate dehydrogenase, 20 mM MgCl₂ in 20 mM Tris-HCl buffer, pH 7.5. D-Fructose-phosphorylating activity was assayed with the same reaction mixture, with the addition of 0.6 units of phosphoglucose isomerase. D-Fructose (15 mM) replaced the D-glucose. The reactions were started by the addition of 0.1 ml suitably diluted enzyme and 0.1 ml stock ATP (0.3 M) and plotted for 15 min. A rate of increase in optical density of between 0.005 and 0.030 min⁻¹ was considered satisfactory. The activity in controls which lacked ATP was also measured and

subtracted from the activity value obtained for the enzyme.

4.322 Phosphoglucose isomerase (EC 5.3.1.9)

The activity of phosphoglucose isomerase in cell-free extracts was followed spectrophotometrically by the method of Noltmann, (1966). The reaction mixture was 0.1 M Tris-HCl, pH 8.0; 1.7 mM D-fructose 6-phosphate, 0.5 mM NADP and 0.6 units glucose-6-phosphate dehydrogenase. After several minutes to allow oxidation of any D-glucose-6-phosphate in the reaction mixture, the phosphoglucose isomerase reaction was initiated by the addition of 15 μ l of diluted cell-free extract. An increase in absorbance between 0.005 and 0.030 min^{-1} was satisfactory. A control reaction without the D-fructose-6-phosphate was also assayed.

4.323 Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

The reaction mixture consisted of 20 mM Tris-HCl, pH 7.5, 20 mM MgCl_2 and 0.13 mM NADP. The diluted enzyme was added (0.1 ml) and the reaction started by the addition of D-glucose-6-phosphate to a final concentration of 15 mM. Mixtures lacking D-glucose-6-phosphate were used as controls. A rate of change in absorbance similar to the hexokinase assay was acceptable.

4.324 Xylose Reductase (Aldose Reductase, EC 1.1.1.21)

Xylose reductase activity was measured by the method of Suzuki & Onishi (1973). The reaction mixture contained 70 mM KPB, pH 7.0, 0.13 mM NADPH and 100 mM D-xylose. The enzyme was diluted and added (0.1 ml) to the reaction mixture and the change in absorbance plotted for several

minutes. A decrease in absorbance of between 0.05 and 0.10 min^{-1} was satisfactory. Controls without substrate were run. In some experiments, activity was also measured using D-glucose as the substrate and also replacing the NADPH cofactor with NADH.

4.325 Xylitol Dehydrogenase (EC 1.1.1.9)

Alditol reductase (xylitol dehydrogenase) activity was measured by the method of Touster & Montesi (1962). The reaction mixture consisted of 4 mM Tris-HCl, pH 9.0, 1 mM L-cysteine (freshly prepared), 5 mM MgCl_2 , 2 mM NAD and 100 mM xylitol. The control lacked the substrate. The enzyme preparation was appropriately diluted in 4 mM Tris-HCl, pH 9.0, the reaction started by the addition of 0.1 ml enzyme and measured at an increase of between 0.005 and 0.010 min^{-1} . Enzyme activity was also measured with D-sorbitol as the substrate.

4.326 Fructokinase (EC 2.7.1.3)

The method of Adelman *et al.* (1967) was used to measure the phosphorylation of D-fructose to D-fructose-1-phosphate. The reaction mixture consisted of 30 mM triethanolamine buffer, pH 7.5, 100 mM potassium chloride (KCl), 0.3 mM NADH, 15 mM ATP, 20 mM MgCl_2 , 3.3 mM phospho-enol-pyruvate, 10 units of pyruvate kinase and lactate dehydrogenase and 5 mM D-fructose. Several controls were necessary. Activity in a control lacking ATP was measured and subtracted. The hexose-ATP-kinase activity with D-fructose as the substrate was also measured and subtracted from the value obtained for the fructokinase activity.

4.33 Determination of Protein Concentration

Protein was determined by the method of Ehresmann *et al.* (1973). The absorbance of the suitably diluted cell-free extract was measured against a water blank at 228.5 and 234.5 nm using a Pye-Unicam SP1800 spectrophotometer. A standard curve was constructed using bovine serum albumin. The absorbance of each protein solution was measured at 228.5 nm and 234.5 nm. The difference between the 228.5 nm value and the 234.5 nm value, multiplied by a factor of 100 was plotted versus protein concentration. The values calculated were linear up to a protein concentration of at least 125.0 $\mu\text{g ml}^{-1}$ (Figure A2).

5. CHROMATOGRAPHY OF CELL-FREE EXTRACTS

5.1 Materials

5.11 Potassium Phosphate Buffer (KPB)

Stock (0.2 M) solutions of potassium dihydrogen phosphate (KH_2PO_4) (BDH, AnalaR) and dipotassium hydrogen orthophosphate (K_2HPO_4) (BDH, AnalaR) were prepared. Stock solutions of buffer were prepared as follows. A stock solution of buffer (stock A, pH 7.4 at 25°C), was prepared by combining 19.0 ml K_2HPO_4 with 81.0 ml of KH_2PO_4 . A stock solution of buffer (stock B, pH 7.9 at 25°C), was prepared by combining 14.0 ml KH_2PO_4 with 93.0 ml K_2HPO_4 . (Munro, 1970). Stock buffers were diluted and combined with EDTA and 2-mercaptoethanol for the preparation of working strength buffers. Stock A was used to prepare 10

mM KPB, pH 7.5 (4°C) and stock B was used to prepare 10 mM KPB, 0.2 M NaCl, pH 7.5 (4°C).

Working strength buffers used for anion-exchange chromatography and enzyme purification were 10 mM KPB containing 2 mM 2-mercaptoethanol and 1 mM EDTA. Elution buffer, in addition, contained 0.2 M NaCl. The final pH of each buffer was 7.5 at 4°C.

5.12 Diethylaminoethyl Cellulose (DEAE-Cellulose)

The anion-exchanger (Whatman DE-52) was prepared, according to the manufacturers instructions, by suspending 60 g dry weight material in 0.2 M KPB, pH 7.5 (15 ml g⁻¹), adjusting the pH to 7.5 with 0.2 M KH₂PO₄ and allowing to settle. The supernatant containing the fines was decanted and the DEAE-cellulose resuspended in 10 mM KPB, pH 7.5 (30 ml g⁻¹) and placed in a 2 litre measuring cylinder. The resin was allowed to settle for 2 hr and 80% of the supernatant was discarded. This slurry was used to pour a column for chromatography. The DEAE-cellulose exchanger was stored at 4°C in buffer containing 0.02% sodium azide.

5.2 Hexose-ATP-Kinase Spot Test

The spot test developed by Schulze *et al.* (1966) was used for the detection of hexose-ATP-kinase activity in eluant fractions. The reagent was prepared by adding 3.0 ml of 0.1 M ATP to 14.0 ml 1.6% MgCl₂ solution containing 0.006% cresol red indicator. NaOH (0.1 M) was added until the colour became reddish-purple (pH approximately 9.0). Following this, 3 ml of 0.1 M glycylglycine-NaOH (pH 9.0) was added and the mixture diluted with deionised water to

30 ml. Prior to use, 5.0 ml of 0.2 M glucose was added. A sample (10 - 20 μ l) of each fraction to be tested was added to 0.24 ml of the reaction mixture in a microtitre plate and the colour change recorded. Fractions with activities of 0.5 unit ml^{-1} or more changed the colour from reddish-purple to yellow within 15 - 30 min, while fractions with lower activities gave colour changes within one to several hours.

5.3 Anion-Exchange Chromatography

Cell-free extracts were subjected to DEAE-cellulose chromatography. The cells were grown in YEP medium for 42 hr and a cell-free extract prepared as described in Materials & Methods 4.2, except the cells were resuspended in 50 mM KPB, pH 7.5 containing either 0.2 mM or 2.0 mM phenylmethanesulphonyl fluoride (PMSF).

The enzyme was desalted by one of two methods. The extract was dialysed against 10 mM KPB, pH 7.5. Dialysis tubing was prepared by boiling in deionised water for 10 minutes and the extract was placed in the tubing. Dialysis was conducted for 2 hr against 10 mM KPB (1 litre) at 4°C and then overnight against a further 1 litre.

Alternatively, the cell-free extract was desalted by passing through a column (2.5 x 10.0 cm) of Sephadex G-25 (Pharmacia) equilibrated with 10 mM KPB, pH 7.5 containing 2 mM PMSF.

Chromatography was conducted at 4°C. Typically, fifteen units of D-glucose-phosphorylating activity were

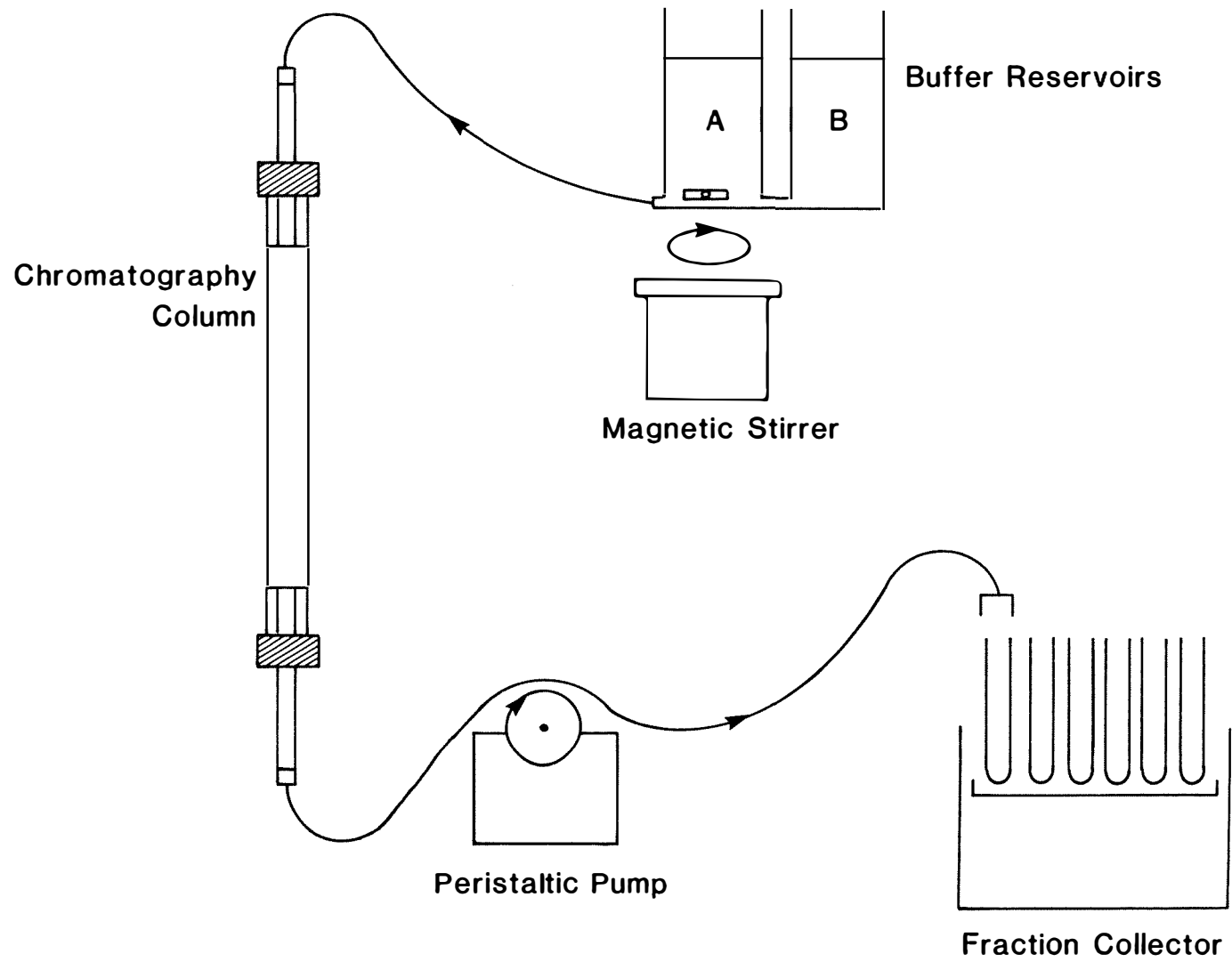
chromatographed on a DEAE-cellulose column (Pharmacia, 1.6 x 20.0 cm) equilibrated with 10 mM KPB, pH 7.5. The extract was loaded onto the column, washed with 2 bed column volumes (80 ml) of buffer and eluted with a linear gradient of 0-0.2 M NaCl in 250 ml buffer. The flow rate was maintained at 30 ml hr⁻¹ with a peristaltic pump. Fractions (5 ml) were collected with a LKB Ultrorac 7000 fraction collector and assayed for D-glucose and D-fructose-phosphorylating activity. The chromatography procedure is schematically represented in Plate 4. The shape of the gradient was checked with a Philips PW92525 conductivity meter. The protein concentration of the fractions was determined by measurement of absorbance at 280 nm against a blank containing 10 mM KPB.

6. KINETIC STUDIES ON HEXOKINASE A

6.1 Preparation of Cibacron Blue F3G-A (Blue Sepharose)

The method described by Atkinson *et al.* (1981) was used to covalently link the triazine dye, Cibacron Blue F3G-A, to Sepharose 6B-CL. An additional wash with Tris-HCl (pH 9.0) was introduced into the method to remove any dye unstable at the higher pH. Sepharose (6B-CL) (150 ml packed volume) was washed once with 500 ml deionised water and water was added to give a volume of 420 ml. Triazine dye (Cibacron Blue F3G-A) was added (1.64 g dissolved in 120 ml water) followed by the addition of sodium chloride (NaCl) (60 ml of 4 M solution). Sodium hydroxide (NaOH) was added (6 ml of 10 M solution) and the Sepharose was incubated at 30°C for 48 hr with gentle swirling from an overhead

Plate 4: Schematic Diagram of Anion-Exchange Chromatography.



stirrer. Following this, the Sepharose was placed in a sintered glass funnel and washed with 1 litre of each of the following in order: deionised water, 1 M NaCl/25% ethanol, deionised water, 1 M NaCl/ 0.2 M KPB (pH 7.0), deionised water, 0.2 M Tris-HCl (pH 9.0) and deionised water. The Blue Sepharose was equilibrated with 0.2 M KPB (pH 7.5) and stored in this buffer with 0.02% sodium azide at 4°C.

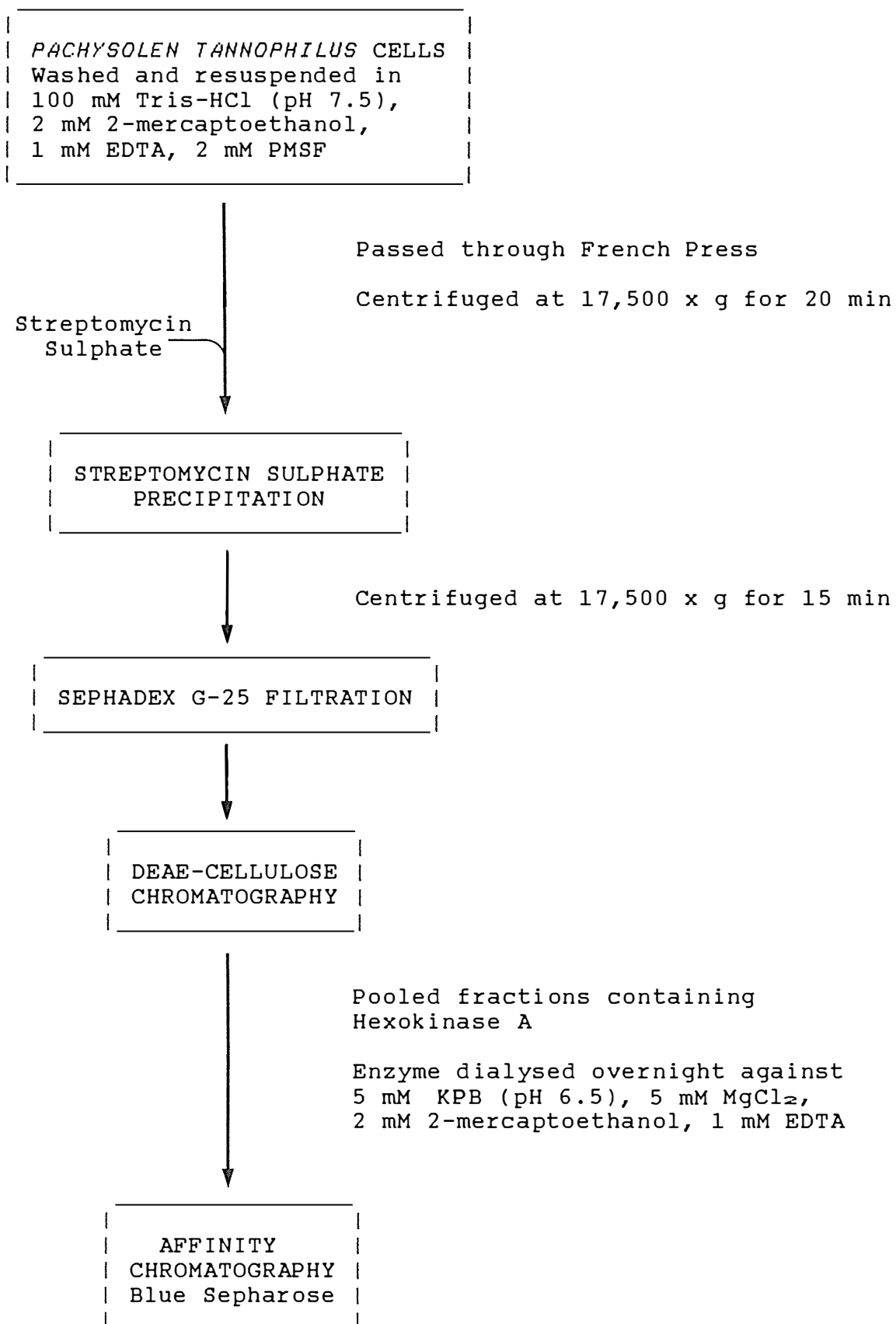
6.2 Enzyme Purification

For purification of the hexokinase the cell-free extract was prepared as described in Materials & Methods 4.2. Cells were cultivated in YEP-glucose for 42 hr. PMSF was added to the extraction buffer at a final concentration of 2 mM. The purification was conducted at 4°C and the procedure is summarised in Figure 2.

The extract was subjected to precipitation with streptomycin sulphate. The cell-free extract was placed in a small beaker which was then placed inside a larger beaker filled with ice. Streptomycin sulphate powder (Glaxo) was added slowly to a final concentration of 0.7% while stirring with a magnetic stirrer. The extract was left for 20 min, centrifuged at 17,500 x g for 15 min and the supernatant purified further.

The cell-free extract was desalted rapidly by passing through a Sephadex G-25 column (2.5 cm x 10.0 cm) equilibrated with 10 mM KPB (pH 7.5) containing 2 mM PMSF. The extract was loaded onto a DEAE-Cellulose column (1.6 x 20.0 cm), washed with 150 ml of buffer and eluted with a

Figure 2: Purification of Hexokinase A



0-0.2 M NaCl gradient. Hexokinase activity was detected in the fractions with the spot test. The peak fractions (typically 6), corresponding to hexokinase A, were pooled and dialysed overnight against 5 mM KPB (pH 6.5) containing 5 mM MgCl₂.

The enzyme was further purified by affinity chromatography on Blue Sepharose as described by Easterday & Easterday (1974). The enzyme was loaded onto a Blue Sepharose column (1.6 cm x 18.0 cm), equilibrated with 5 mM KPB (pH 6.5) containing 5 mM MgCl₂. The enzyme was loaded onto the column at a flow rate of 10 ml min⁻¹. The column was washed with 200 ml 5 mM KPB (pH 6.5), 5 mM MgCl₂ and the enzyme eluted with 10 mM Tris-HCl buffer (pH 8.5) containing 15 mM ATP at a flow rate of 20 ml min⁻¹. The enzyme eluted in several 2 ml fractions and was stored on ice.

6.3 Polyacrylamide Disc Gel Electrophoresis

6.31 Hexose-ATP-Kinase Activity Stain

Hexose-ATP-kinase activity stain was similar to that used by Pilkis (1975) and consisted of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 6 mM ATP, 1.3 mM NADP, 1.75 units ml⁻¹ glucose-6-phosphate dehydrogenase, 0.6 mM nitro blue tetrazolium (Sigma), 10 mM phenazine methosulphate (Sigma), 15 mM D-glucose.

6.32 Coomassie Brilliant Blue Stain for Protein

Protein was stained with Coomassie Brilliant Blue R-250 (Sigma), prepared by dissolving Coomassie Blue at a

concentration of 20 mg ml^{-1} in methanol : acetic acid : water (2:1:7).

6.33 Disc Gel Electrophoresis

Disc gel electrophoresis was based on the method of Gabriel (1971).

6.331 Preparation of stock solutions

The acrylamide/methylene-bis-acrylamide was prepared as a stock solution by dissolving 34.1 g acrylamide in about 70 ml of water, stirring until the solution had returned to room temperature, followed by addition of 0.9 g methylene bis-acrylamide. The solution was made up to 100 ml and filtered through Whatman No. 1 filter paper and stored at 4°C in the dark.

Running gel buffer (x 5 stock solution) (1.0 M Tris-HCl, pH 8.8) was prepared by dissolving 60.5 g (Trizma base, Sigma) in about 300 ml deionised water and adjusting the pH to 8.8 with 10 N HCl and making the volume up to 500 ml. Stacking gel buffer (x 5 stock) (0.5 M Tris-HCl, pH 6.8) was prepared by dissolving 30.25 g (Trizma base, Sigma) in 300 ml deionised water, adjusting the pH to 6.8 with 10 N HCl and making the volume up to 500 ml. Reservoir buffer was prepared as a stock solution (x 10) by dissolving 6.0 g (Trizma base, Sigma) and 28.8 g glycine in 1 litre of deionised water. The final pH was 8.3. Prior to use, bromophenol blue tracking dye was added to the reservoir buffer at a final concentration of 0.0004 %. All buffers were stored at 4°C for up to one month.

Tetraethylmethylethyldiamine (TEMED) was prepared by adding 100 μ l to 20 ml water. Ammonium persulphate was prepared fresh by dissolving 20 mg in 5 ml water. Sucrose (50%) was prepared by dissolving 5 g in 10 ml deionised water.

The gels were prepared by addition of stock solutions as follows:

(a) Running gel

Acrylamide/bis	5.0	ml
Tris-HCl, pH 8.8	5.0	ml
TEMED	2.5	ml
Ammonium persulphate	5.0	ml
deionised water	7.5	ml

(b) Stacking gel

Acrylamide/bis	2.0	ml
Tris-HCl, pH 6.8	5.0	ml
TEMED	5.0	ml
Ammonium persulphate	5.0	ml
Sucrose	5.0	ml
deionised water	3.0	ml

Final concentration of the running gel was 7% acrylamide, 0.18% bis-acrylamide, 0.08% ammonium persulphate, 0.2 M Tris-HCl, pH 8.8 and 2.8% acrylamide, 0.072% bis-acrylamide, 0.08% ammonium persulphate, 0.1M Tris-HCl, pH 6.8, 10% sucrose for the stacking gel. For both gels, the ammonium persulphate and TEMED were added last. After the addition of each stock solution, the ingredients were thoroughly mixed with gentle swirling to avoid the formation of bubbles. Gels were then poured immediately.

6.332 Preparation of gels

Small glass tubes (1.0 x 10.0 cm) were blocked at the lower end with Parafilm™ and placed vertically. Running

gel was prepared and carefully poured into the tubes to within 30 mm of the top, making sure no air bubbles were trapped. Air was excluded by gently placing water on the surface of the gel with a pasteur pipette. The acrylamide was allowed to polymerise for 1 hr and the water was gently removed by blotting with tissue paper. Stacking polyacrylamide was prepared and poured on top of the polymerised running gel to within 20 mm of the top of the tube. Following the addition of water to the surface, polymerisation was allowed to take place for 30 min. The tubes were placed in the electrophoresis tank (Bio-Rad Model 175 Tube Cell) and the parafilm™ removed from the tubes. Prior to loading on protein, the water was gently removed by blotting with a piece of tissue.

6.333 Electrophoresis

The protein (100 μ l or 200 μ l) was loaded onto the top of the gels and electrophoresis was performed at a current of 16 mA for approximately 30 min at 4°C. After the dye front had reached the interface between the stacking gel and the running gel, the current was increased to 32 mA. Gels were run for 1 hr until the tracking dye was within 0.5 cm from the bottom of the gels. Following removal from the glass tubes, gels were stained for protein by immersing in Coomassie Blue reagent for 1 hr followed by immersion in 5% trichloroacetic acid for 10 min.

Gels were destained by immersion in butanol:acetic acid:water (2:1:7) with several changes over several days. Hexokinase was stained by immersion in the hexose-ATP-kinase activity stain for 15 min at room temperature,

followed by immersion in the butanol: acetic acid: water mixture.

For photography, gels were placed in 1.2 x 10.0 cm test-tubes, placed on an illumination box and photographed using Tech Pan 10.5 x 12.7 cm film.

6.4 Determination of Km Values

The activity of hexokinase A with both D-glucose and D-fructose as substrates, was measured at a number of different substrate concentrations. The reaction mixture was similar to that used in Materials & Methods 4.321, except ATP was added to a final concentration of 2.5 mM, instead of 10 mM. The reciprocal value of each initial activity ($1/V_0$) was plotted against the reciprocal value of the substrate concentration $1/[S]$ (Lineweaver-Burke plot).

The best fit line for each plot was calculated by applying linear regression using a basic program run on an Atari 520ST computer. The x- and y-intercepts were calculated from the equation for the line and used to estimate the K_m and V_{max} values. The y intercept was the $1/V_{max}$ value and the x intercept was the $-1/K_m$ value.

6.5 Inactivation by D-Xylose

Reaction mixtures were set up in glass cuvettes and placed in the heated holder of the Cecil CE 599 recording spectrophotometer at 30°C. Purified enzyme (0.5 units D-glucose-phosphorylating activity) was incubated with 10.0 mM $MgCl_2$, 4.0 mM ATP and 20 mM Tris-HCl (pH 7.5) and either 100 mM or 250 mM D-xylose in a total volume of 1 ml.

Samples were removed at 0 time and at 15 min intervals and the hexokinase activity measured as described in Materials & Methods 4.321. Control reaction mixtures lacking D-xylose were also set up and activities measured at 15 min intervals.

7. PREPARATION OF DNA

7.1 Materials

7.11 Ethylene diamine tetraacetic acid (EDTA)

EDTA (Ethylene diamine tetraacetic acid ($\text{CH}_2\text{N}(\text{CH}_2\text{COOH})(\text{CH}_2\text{COONa})_2 \cdot 2\text{H}_2\text{O}$) was prepared at 0.25 M by dissolving 46.53g Na_2 EDTA in 400 ml deionised water, adjusting the pH to 8.0 with 5 M NaOH and making up to 0.5 litre.

7.12 Ampicillin

Ampicillin (Sigma) was prepared as a stock solution at 25 mg ml^{-1} in deionised water, filter sterilised and stored at -20°C .

7.13 Lysozyme

Lysozyme (Sigma) was prepared at 10 mg ml^{-1} in Tris-HCl, pH 7.6, (10 mM) and stored at -20°C .

7.14 Protease K

Protease K (Sigma) was prepared as a 10 mg ml^{-1} stock in deionised water, incubated at 37°C for 1 hr to digest impurities and stored at -20°C .

7.15 Ribonuclease A

Ribonuclease A (Sigma) was prepared at a concentration of

1 mg ml⁻¹ in deionised water, heated at 90°C for 10 min to destroy any DNase activity and stored at -20°C.

7.16 Sodium Dodecyl Sulphate (SDS)

Sodium dodecyl sulphate (BDH) was prepared as a stock solution (10%) in deionised water.

7.17 STET Buffer

STET buffer contained: sucrose, 8 g l⁻¹; 50 mM Tris-HCl, pH 8.0; Triton X-100 (5 ml l⁻¹); 50 mM EDTA, pH 8.0.

7.18 Tris-saturated Phenol

Tris-saturated phenol (BDH, AnalaR) was prepared by dissolving 500 g of phenol in a minimal amount of deionised water (62.5 ml) by warming. A further 500 ml of deionised water and 62.5 ml of 1.0 M Tris-HCl (pH 8.0) was added to the cooled phenol solution.

The mixture was shaken to give an even suspension and poured into a separating funnel. The bottom layer containing the Tris-saturated phenol was collected and stored under a layer of Tris-HCl at -20°C.

7.19 Sodium Acetate

A stock solution (3.0 M) was prepared by dissolving 40.81 g sodium acetate (CH₃COONa.3H₂O) in 80 ml deionised water and adjusting the pH to 5.2 with glacial acetic acid and making up to 100 ml.

Reagents for DNA extraction and transformation of *P. tannophilus* and *E. coli* were sterilised by autoclaving at 121°C for 15 min or by filter sterilisation, according to advice in Maniatis *et al.* (1982).

7.2 Large Scale Preparation of Plasmid DNA

Plasmid DNA was extracted from *E. coli* by a modification of the rapid boiling method (Holmes and Quigley, 1981) as developed by Bret Gold, The Biological Laboratories, Harvard University. An overnight culture of *E. coli* (200 ml) in LB supplemented with ampicillin ($40 \mu\text{g ml}^{-1}$) was centrifuged at $4,000 \times g$ for 10 min at 4°C . The cells were resuspended in 45 ml of STET buffer in polypropylene tubes, 1 ml of stock lysozyme added and the mixture incubated at room temperature for 10 min. The tubes were placed in boiling water for 90 sec and the mixture centrifuged for 15 min at $17,500 \times g$. The supernatant was collected and the DNA precipitated by the addition of an equal volume of isopropanol and allowing to stand for 1 hr at -20°C . After centrifugation at $12,000 \times g$ for 10 min, the pellet was washed with 66% ethanol containing 0.9% NaCl. The ethanol was added, the tubes vortexed and allowed to stand at -20°C for 15 min. After centrifugation the pellet was desiccated to dryness with a vacuum desiccator.

Following resuspension in 9.6 ml TE buffer (0.1 M Tris-HCl, pH 8.0, 1 mM EDTA) containing 0.01% SDS, the DNA was cleaned up as follows: RNase A was added to a final concentration of $20 \mu\text{g ml}^{-1}$ and the DNA incubated for 15 min at 37°C . Protease K was added to a final concentration of $200 \mu\text{g ml}^{-1}$ and the mixture incubated at 65°C for 10 min. The DNA was phenol/chloroform extracted by adding 10 ml of phenol/chloroform (50:50 v/v) and vortexing, followed by centrifugation at $12,000 \times g$ for 5 min at 4°C . The aqueous phase was re-extracted with chloroform and the

organic phase with TE buffer by the addition of an equal volume of chloroform and TE buffer, respectively and then vortexing and centrifuging to separate the two phases. The aqueous phases were pooled and extracted two more times with chloroform. The combined aqueous phase was adjusted to 0.3 M with respect to sodium acetate and the DNA precipitated by adding two volumes of ethanol and allowing to stand 1 hr at -20°C . The DNA was centrifuged at 12,000 x g for 10 min at 4°C and the pellet desiccated to dryness and the DNA resuspended in 0.4 ml of TE buffer.

RNase was added to a final concentration of $100\ \mu\text{g ml}^{-1}$ and the DNA incubated 10 min at 37°C . Protease K was added to a final concentration of $1\ \text{mg ml}^{-1}$ and the DNA incubated 10 min at 65°C . The DNA was further phenol/chloroform extracted, ethanol precipitated, centrifuged and the pellet desiccated to dryness and resuspended in TE buffer. The DNA was stored at 4°C for short periods and at -70°C for longer periods. The concentration of the DNA was estimated by running 1 μl or 5 μl on an agarose gel and comparing the intensity of the ethidium bromide fluorescence with standard quantities of *E. coli* lambda DNA.

7.3 Rapid Preparation of Plasmid DNA

The rapid boiling method of Holmes and Quigley (1981) was used to obtain small amounts of plasmid DNA. *E. coli* was grown overnight in 5 ml of LB supplemented with $40\ \mu\text{g}$ ampicillin ml^{-1} at 37°C . Following centrifugation at 4,000 x g the cells were resuspended in 350 μl of STET buffer in a microfuge tube. Lysozyme was added (25 μl of stock

solution) and the microfuge tube placed in boiling water for 40 sec, followed by centrifugation at 12,000 x g for 10 min in an Eppendorf microfuge at 4°C. The DNA in the supernatant was precipitated with an equal volume of isopropanol at -20°C for 60 min. The DNA was sedimented by centrifugation at 12,000 x g for 10 min and the pellet washed with 95% ethanol. The pellet was dessicated to dryness and the DNA resuspended in either water or TE buffer.

If required, the DNA was further incubated with RNase and protease K to digest the RNA and protein and then phenol/chloroform extracted by the addition of an equal volume of phenol/chloroform (50:50 v/v). The suspension was thoroughly mixed and centrifuged at 12,000 x g for 4 min to separate the two phases. The organic phase was re-extracted with water and the aqueous phase extracted with chloroform to remove phenol. The DNA in the combined aqueous phases was finally precipitated with ethanol, dessicated to dryness and resuspended in water or TE buffer.

7.4 Restriction of Plasmid DNA

Plasmid DNA (15 µl) or *E. coli* lambda DNA (New England BioLabs) (1 µg in 15 µl deionised water) was added to 25 µl deionised water, 5 µl Amersham E4 buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 7 mM MgCl₂, 7 mM 2-mercaptoethanol) in a microfuge tube and 1 µl *EcoRI* added. The mixture was incubated at 37°C for 1 hr and then at 65°C for 10 min.

7.5 Electrophoresis of DNA

7.51 Tris-Acetate Buffer

A stock solution (x 50) consisted of 0.4 M Trizma base (Sigma), 0.1 M glacial acetic acid, 0.5 M EDTA, pH 8.0.

7.52 Sucrose-Bromophenol Blue Loading Buffer

Sucrose-bromophenol blue loading buffer contained 50% (w/v) sucrose in deionised water and 0.5% bromophenol blue.

7.53 Gel Electrophoresis

DNA molecules were separated and visualised by agarose gel electrophoresis in a Bio-Rad mini sub-cell flat bed gel apparatus. Tris-acetate buffer (50 ml) containing 0.7 % agarose (Sigma, low gelling temperature) was heated in a reflux apparatus to melt the agarose, cooled and poured into the horizontal gel apparatus and the wells formed with a teflon comb. After the gel had set, the DNA samples (10- 15 μ l) were mixed with 5 μ l loading buffer and loaded into the wells with an autopipette. Tris-acetate buffer was added to the gel apparatus, the gel submerged and 60 V applied. After the gel had run (2 - 2 $\frac{1}{2}$ hr), the DNA was stained by placing the gel in ethidium bromide (0.5 μ g (ml water)⁻¹ for 20 min and the gel viewed on an ultraviolet transilluminator at 265 nm. The gels were photographed on Kodak Tri-X 12.2 x 10.7 cm film. The film was developed in Kodak D-19 developer for 5 min at ambient temperature, fixed in Ilford Hypon fixer for 5 min at ambient temperature, washed under running water for 10 min and dried at room temperature.

8. TRANSFORMATION WITH PLASMID DNA

8.1 Transformation of *Escherichia coli*

Cells of *E. coli* were made competent and transformed by a modification of the method of Cohen *et al.* (1972). All buffers, reagents and centrifuges were chilled to 4°C prior to the transformation and maintained at this temperature. The *E. coli* strain HB101 was inoculated into 25 ml LB and incubated at 37°C and 180 rpm overnight on a gyratory shaker. The culture was inoculated (1 ml) into 50 ml LB and shaken at 37°C and 180 rpm for approximately 2 hr. The cells (optical density 0.4-0.45 at 600 nm) were centrifuged at 6,000 x g for 10 min, resuspended in 10 ml of 60 mM CaCl₂ and incubated for 20 min on ice.

The cells were centrifuged at 6,000 x g for 10 min and resuspended in 0.25 ml 60 mM CaCl₂ in a microfuge tube. The cells (100 µl) were mixed with 10 µl DNA and 90 µl TEC buffer (10 mM Tris-HCl, pH 8.0, 0.25 mM EDTA, 30 mM CaCl₂) and incubated on ice for 1 hr. The cells were heat shocked by incubation at 42°C for 2 min, incubated on ice for 15 min and 0.9 ml LB added. Following incubation at 37°C for 1 1/2 hr, aliquots (0.2 ml) were plated onto LB plates supplemented with ampicillin (40 µg ml⁻¹). Plates were incubated overnight at 37°C. Cells treated similarly, but without DNA, were plated out as controls.

8.2 Transformation of *Pachysolen tannophilus*

A modification of the lithium method of Ito *et al.* (1983) was used to transform cells of *Pachysolen tannophilus*.

Strain D/X A was grown into early exponential phase (approximately $5-7 \times 10^6$ cells ml^{-1}) by inoculating YEP-xylose (100 ml) with 100 μl of a 48 hr YEP-xylose culture. The cells were incubated for 16 hr at 30°C and 180 rpm on a gyratory shaker. The culture was washed once with deionised water, centrifuged at $6,000 \times g$ for 10 min and resuspended in 1 ml of 0.1 M LiCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA in a microfuge tube. Following incubation for 1-4 hr, 0.1-0.5 μg YRp/HXK2-8 DNA in 10 μl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 10 μl herring sperm DNA (4 mg ml^{-1}) was added and the mixture incubated for 30 min at 30°C . A total of 0.7 ml PEG-LiCl (40% polyethylene glycol 4,000, 0.1 M LiCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added and the cells incubated for a further 30 min. The cells were heat shocked by incubation at 42°C for 5 min and washed twice with TE buffer. Following resuspension in 1 ml TE, 0.2 ml aliquots were plated onto minimal YNB-glucose plates. Cells treated with DNA and without DNA were plated onto YNB-xylose and YNB-glucose plates respectively as controls. Plates were incubated at 30°C and transformant colonies appeared within 5-7 days.

9. DEMONSTRATION OF GLUCOSE CONVERSION IN CELL-FREE EXTRACTS

9.1 Reaction Mixtures

The conversion of D-glucose and D-sorbitol in cell-free extracts was followed by high performance liquid chromatography. Cell-free extracts from strain D/X A were prepared as described in Materials & Methods 4.2 and

dialysed in 1 litre of 10 mM Tris-HCl, pH 7.0 for 2 hr at 4°C and then overnight in a further 1 litre of buffer. The extract (3 mg protein) was added to reaction mixtures consisting of 50 mM sugar; 50 mM cofactor, pH 7.0; 10 mM MgCl₂; 100 mM Tris-HCl, pH 7.0, in a total volume of 1 ml. At 0 time, 24 or 48 hr, an aliquot (0.45 ml) was removed and placed in a microfuge tube. Trichloroacetic acid (50% v/v) was added (10 µl) to precipitate the protein, and the samples frozen at -20°C until required for HPLC analysis.

9.2 HPLC analysis of Samples

Each sample was centrifuged 10 min at 12,000 x g in an Eppendorf microfuge and the supernatant (300 µl in limited volume inserts) analysed. A standard sample containing 50 mM D-glucose, 50 mM D-fructose and 50 mM D-sorbitol was used for calibration. HPLC analysis was carried out using the HPX-87H column as described in Materials & Methods Section 3.22.

10. GENETIC TECHNIQUES

10.1 Ultraviolet Light (UV) Mutagenesis

The method used for UV irradiation of *P. tannophilus* cells was that used by Allen James (personal communication). Strains to be mutagenised with UV light were grown for 16-20 hr in YEP-xylose (50 ml) in a 125 ml flask at 180 rpm and 30°C to a cell density of 5×10^7 cells ml⁻¹. The cells were diluted in water blanks and aliquots (50 and 100 µl, 10^9 - 2×10^9 cells) spread over YEP-xylose plates.

The plates, with the lid removed, were placed 35 cm from a Philips Fluorescent UV tube (574 P/40 OA, TUV, 15 W) in a UV box and irradiated for 30 sec. Following irradiation, the plates were incubated for 3-4 days at 30°C in a light proof box at 30°C. Un-irradiated plates were also incubated at 30°C as controls.

Survivor colonies were replica-plated onto either minimal YNB medium supplemented with the appropriate carbon source, or omission YNB medium.

10.2 Hybridisation of Haploid Strains

Haploid strains carrying auxotrophic or other markers were mated according to the method of James and Zaheb (1982). Strains to be mated were grown into log phase by 24 hr incubation on either YEP-glucose or YEP-xylose. The two cultures were mixed and streaked onto a YM-YE plate and incubated at 30°C for 4 days. The growth from the inoculum area was picked up with a toothpick, and spread over the surface of a minimal YNB plate. Following incubation for 2-3 days at 30°C, prototrophic diploid colonies appeared.

10.3 Sporulation of Diploid Strains

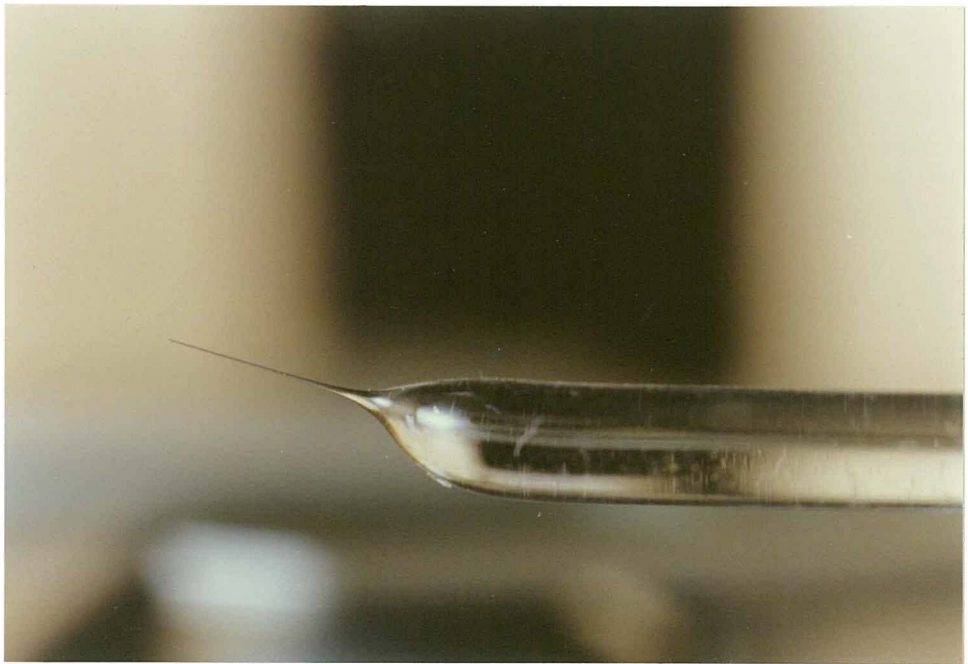
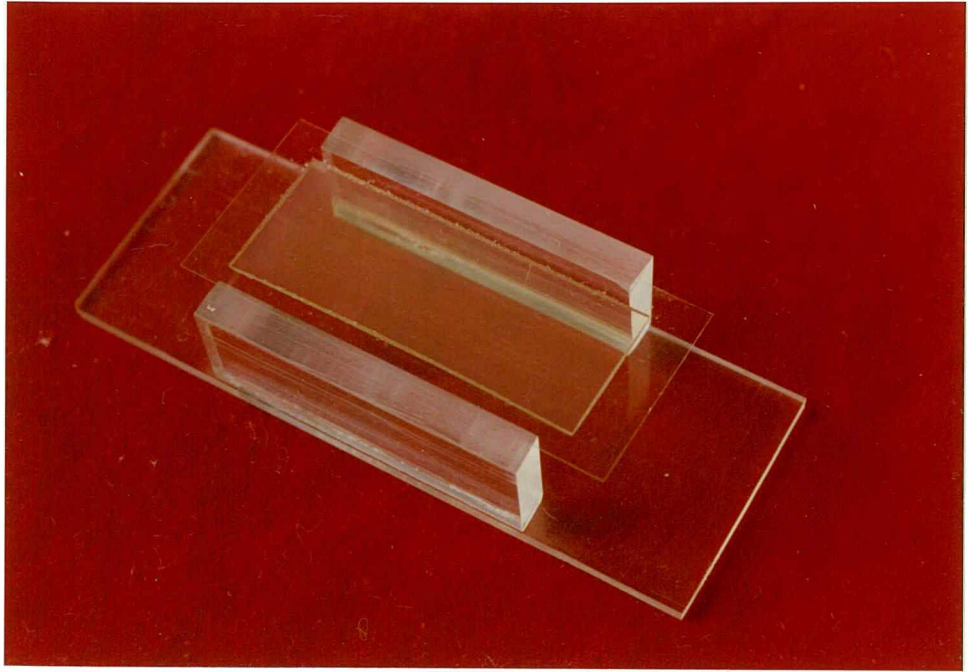
Diploid strains were sporulated by streaking onto YM plates and incubating at 30°C for 4-5 days. Cultures were checked microscopically for the presence of asci.

10.4 Micromanipulation

Tetrad s were dissected by micromanipulation on a thin agar slab (Plate 5). The agar medium (MYGP) was conveniently

Plate 5: Dissection Chamber and Agar Slab for
Micromanipulation.

Plate 6: Needle for Dissection of Asci.



dispensed in 5 ml aliquots and melted prior to use. The medium (1 ml) was pipetted onto the surface of a flamed glass coverslip (22 x 50 mm), allowed to solidify and the edges trimmed with another flamed coverslip. A drop of sterile water was placed on the surface of the sporulated culture with minimal disruption of the surface. A loopful of water, containing suspended asci, was quickly picked up and inoculated along the edge of the agar slab. The agar slab was inverted over the dissecting chamber (Plate 5) and examined microscopically at 200 x magnification.

A dissecting needle (Plate 6) was prepared by joining two 3 mm soda glass rods together over a bunsen flame and quickly drawing apart at an angle. A micromanipulator, equipped with a dissecting needle (Plate 7) was used to separate individual ascospores from the intact tetrads. Each ascospore was picked up by the needle and moved to the position 2-3 mm apart along a grid pattern on the surface of the agar. The slab was then removed from the coverslip with a sterile spatula and placed on the surface of a YEP-glucose plate and incubated at 30°C for 2-3 days.

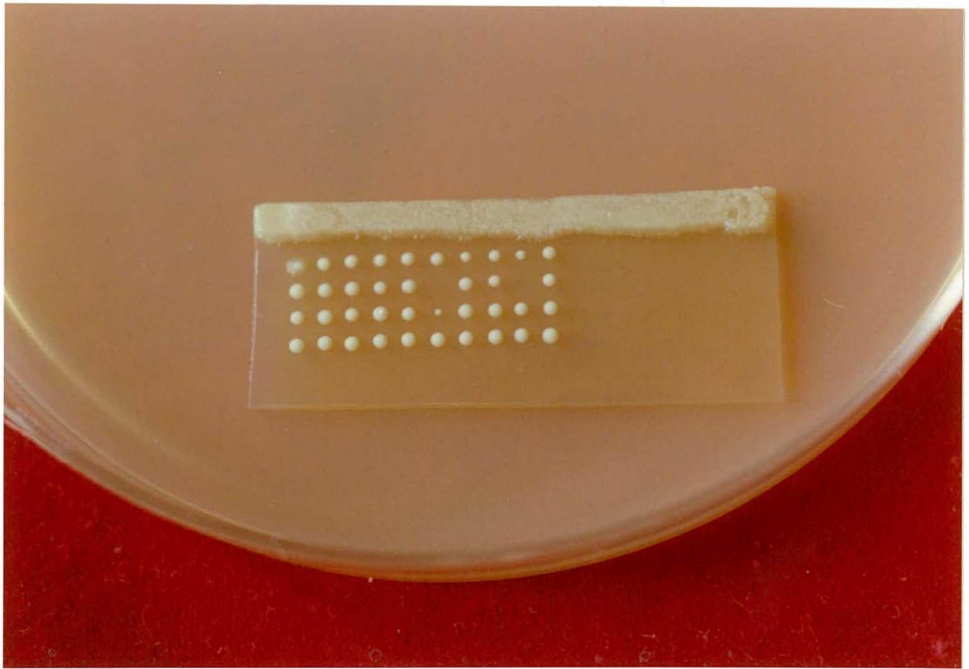
10.5 Tetrad analysis

The haploid segregants (Plate 8) were picked and inoculated onto the appropriate minimal or complete YNB medium. Following growth, segregants were replica-plated onto omission medium or media supplemented with the appropriate carbon source to identify the mutations.

Plate 7: Micromanipulator and Microscope.

Note dissection chamber and agar slab
in position on the stage

Plate 8: Dissected Asci on Agar Slab.



11. SELECTION FOR HEXOSE-NEGATIVE MUTANTS

11.1 Selection for Fructose-Negative Mutants

Strain P509-1B *hxx2 glul* was subjected to UV mutagenesis in order to eliminate the ability to grow on D-fructose. Following UV mutagenesis (Materials & Methods 10.1), the survivor colonies were replica-plated onto minimal YNB-fructose plates and incubated for 2-3 days at 30°C. All colonies that grew more slowly on minimal YNB-fructose than the parent P509-1B strain were picked and re-inoculated onto YNB-fructose and the growth rate compared.

11.2 Selection for Glucose-Negative Mutants

Strain P509-1B *hxx2 glul* was inoculated into minimal YNB-xylose (100 ml) in a 250 ml Erlenmeyer flask, supplemented with 40 mM 2-deoxyglucose. Standard density inoculation conditions were used as described in Materials & Methods 2.32. The culture was incubated at 180 rpm and 30°C. Growth, as monitored by measurement of absorbance, ceased after 48 hr but resumed after 4-5 days. The culture was left incubating for 10 days and the resultant strain was isolated. Two independent single colony isolates were obtained from two separate cultures.

12. STRAIN IMPROVEMENT IN *PACHYSOLEN TANNOPHILUS*

12.1 Selection for Xylose Utilisation in the Presence of Glucose

Strain D/X A *hxx1 hxx2 glul* was mutagenised with UV light. The strain was pre-cultured for 48 hr on a minimal

YNB-glycerol plate and inoculated into 50 ml minimal YNB-glycerol and cultured as already described. The cells were plated out onto minimal YNB-glucose/xylose plates and exposed to UV light. Survivor colonies that appeared to grow more rapidly on this medium were grown on minimal YNB-glycerol, and then replica-plated onto minimal YNB-glucose/xylose and incubated for 2-4 days. Colonies that grew rapidly were re-inoculated onto minimal YNB-glucose/xylose. The growth rate of each isolate was compared with the D/X A parent strain. Strains that appeared to grow more rapidly were inoculated into YEP-glucose/xylose (50 ml) as described in Materials & Methods 2.31. The utilisation of the sugars was analysed by HPLC after 72 and 96 hr.

12.2 Elimination of Ethanol Utilisation

Exposure to UV light was used to mutagenesis the F/G 2 strain and introduce an ethanol defective type mutation. The strain was cultured in YNB-xylose, plated onto minimal YNB-xylose and mutagenised as already described. Survivor colonies were replica-plated onto minimal YNB-xylose and minimal YNB-ethanol plates. Colonies that grew on the former medium but not on the latter were picked and plated onto YNB-ethanol media and checked for loss of growth on this carbon source. Growth was also checked on D-xylose and D-glucose.

12.3 Hybridisation of the *eth2-1* Strain to NO₃-NO₃-4

NO₃-NO₃-4 *lys* was hybridised with P446-27A *eth2-1 adel* as described in Materials & Methods 10.2. Following

sporulation of the diploid culture and dissection of tetrads, strains carrying the *adel* auxotrophic marker in addition to the *eth2-1* mutation were back-crossed with the $\text{NO}_3\text{-NO}_3\text{-4}$ *lys* strain. The presence of the *eth2-1* gene in a segregant was confirmed by replica-plating onto complete YNB-ethanol media.

RESULTS

1. RESISTANCE TO 2-DEOXYGLUCOSE

The resistance of the wild type and glucose-negative mutants to 2-deoxyglucose is summarised in Table III. The wild type strain P444-3D, was able to grow on YNB-xylose plates supplemented with 2 mM 2-deoxyglucose but at 4 mM concentration, inhibition of growth was evident. No growth occurred at analogue concentrations greater than 4 mM. All of the mutants were able to grow at higher concentrations of analogue, compared to the wild type. The resistance of P510-5A was only slightly greater than the wild type and growth was moderately inhibited at 4 mM concentration. Both P509-1B and P509-1B #6 could grow in the presence of 20 mM 2-deoxyglucose but growth was inhibited at 40 mM, while D/X A was able to grow relatively well in the presence of 120 mM analogue.

Although the double mutant P509-1B could grow on a YNB-xylose plate supplemented with levels of 2-deoxyglucose of up to 40 mM concentration, poor growth was found when this strain was inoculated into liquid YNB-glucose supplemented with this level of analogue (Figure 3). When inoculated at the standard cell density, a maximum cell yield of 4.25×10^8 cells ml⁻¹ occurred after 48 hr incubation. No significant increase in optical density occurred upon further incubation for 3 days. However, when the culture was left on the shaker for a longer period as described in Materials & Methods 11.2, cell growth resumed after

Table III: Resistance of *Pachysolen tannophilus* Strains to 2-Deoxyglucose on Solid Media

Strain/ Genotype	Growth on D-xylose (2 % w/v) + 2-deoxyglucose (mM)						
	2	4	10	20	40	80	120
P444-3D WT	+	(+)	-	-	-	-	-
P17-1A <i>met2-1</i>	+	(+)	-	-	-	-	-
P510-5A <i>glu1</i>	+	(+)	-	-	-	-	-
P509-3C <i>hxx2</i>	+	+	(+)	-	-	-	-
P509-1B <i>hxx2 glu1</i>	+	+	+	+	(+)	(+)	(+)
P509-1B #6 <i>hxx2 glu1 fru</i>	+	+	+	+	(+)	(+)	(+)
D/X A <i>hxx1 hxx2 glu1</i>	+	+	+	+	+	+	+

+ Good growth observed within 1-3 days

(+) Inhibition of growth

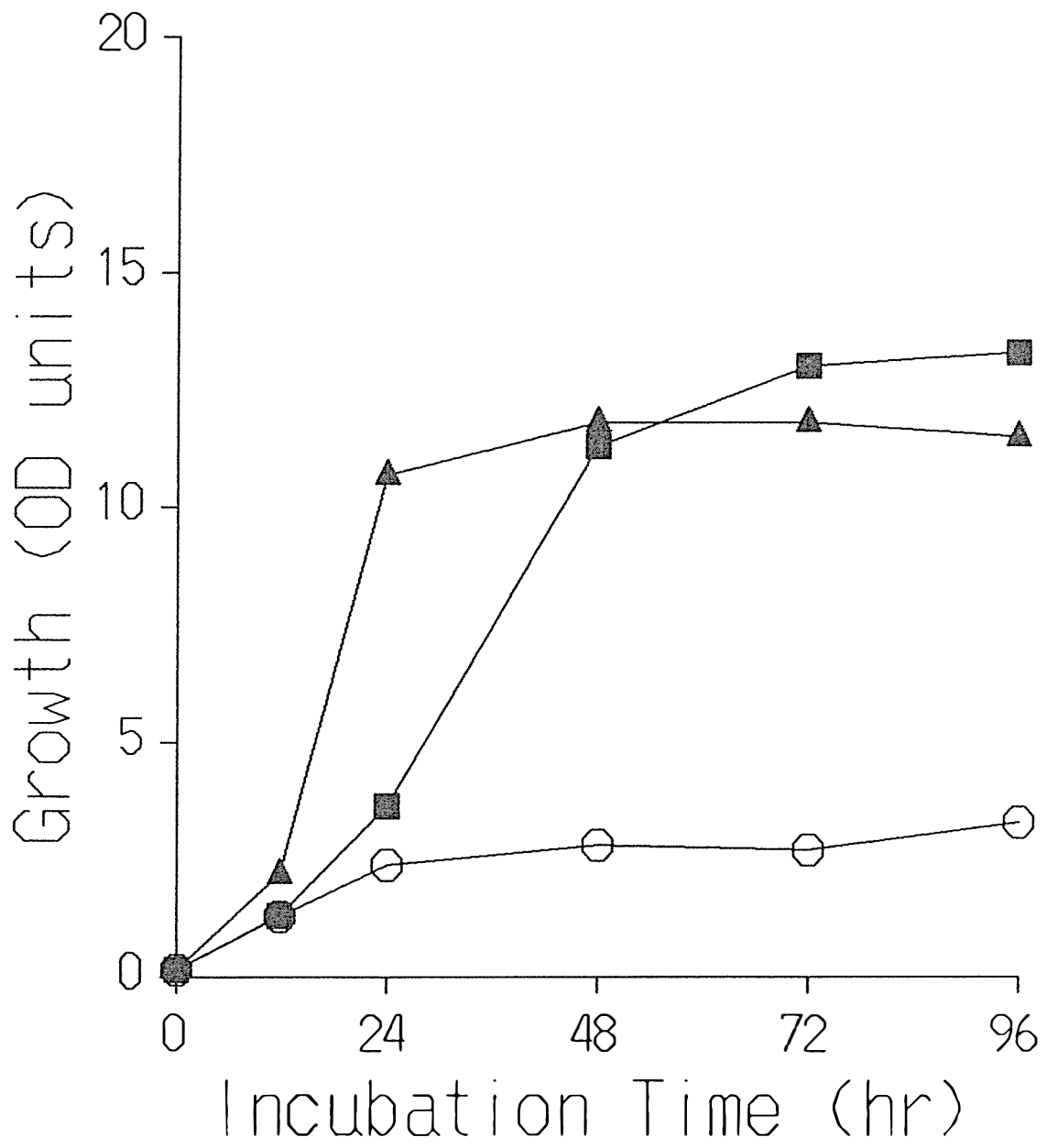
- No growth

Fig. 3: Growth of Strains P509-1B and D/X A in YNB-Xylose (2% w/v) Supplemented with 2-Deoxyglucose.

○ , P509-1B, 40 mM 2-deoxyglucose;

▲ , D/X A, 40 mM 2- deoxyglucose;

■ , D/X A, 120 mM 2-deoxyglucose



about 5 days. The resultant strain was isolated and plated out for single colonies. Two separate isolates from two separate cultures were made; one of these, designated D/X A, was examined further. Strain D/X A was very resistant to 2-deoxyglucose and could grow well at 40 mM and relatively well in liquid media at 120 mM concentration (Figure 3).

2. GROWTH AND SUGAR UTILISATION

2.1 Growth on Solid Media

The rate of growth of several of the mutant strains on different carbon sources was determined and the results are summarized in Table IV. The amount of growth was scored as follows: +++ confluent growth evident after 2 days; ++ less growth compared to the wild type strain, P444-3D; + slow growth which became apparent after 2-3 days incubation; - no growth evident after 6 days. The wild type *P. tannophilus* strain, P444-3D, grew well on all four carbon sources; growth on D-glucose was slightly faster than on D-xylose.

All the mutant strains exhibited a similar rate of growth on D-xylose in comparison to the wild type strain (Table IV). The mutant strain P510-5A grew at a similar rate to the wild type on all three hexose sugars. The other mutants however, exhibited reductions in the rate of growth on the hexose sugars. Growth by P509-3C on D-glucose was only slightly reduced, while the growth rate on D-mannose was markedly reduced. The double mutant,

Table IV: Growth of Wild Type and Mutants on Different Carbon Sources on Solid Media

Strain/ Genotype	Carbon Source			
	D-Xylose	D-Glucose	D-Mannose	D-Fructose
P444-3D WT	+++	+++	+++	+++
P510-5A <i>glu1</i>	+++	+++	+++	+++
P509-3C <i>hxx2</i>	+++	++	+	+++
P509-1B <i>hxx2 glu1</i>	+++	-	-	+
P509-1B #6 <i>hxx2 glu1 fru</i>	+++	-	-	-
D/X A <i>hxx1 hxx2 glu1</i>	+++	-	-	-

+++ Good growth (confluent)

++ Reduced growth

+ Slow growth (not apparent until 2-3 days)

- No growth at 6 days

P509-1B, and the triple mutants, P509-1B #6 and D/X A, were unable to grow on D-glucose and D-mannose as the sole carbon source. However, D-fructose did support growth by strain P509-1B, but growth only became apparent after about 2-3 days incubation. Both strains P509-1B #6 and D/X A were unable to grow on this sugar as the sole carbon source.

The wild type and mutants all grew slowly on D-galactose and glycerol as sole carbon sources, with growth on glycerol more rapid than on D-galactose.

2.2 Growth in Liquid Medium

During the isolation of the glucose-negative strains, tentative mutants were initially screened for growth in YEP liquid media (Materials & Methods 2.31). Growth and sugar utilisation by a number of the mutant strains, was then followed in YNB media employing a standardised procedure for pre-culturing the cells and using a standard inoculum size (Materials & Methods 2.32). This enabled more precise comparisons on the rates of growth and sugar utilisation to be made.

The utilisation of hexose sugars and D-xylose by the wild type strain, P444-3D and mutants P510-5A, P509-3C and P509-1B, when inoculated into YNB media at the standard cell density, is displayed graphically in Figures 4-7. Growth was also routinely monitored in these cultures by measurement of optical density at 600 nm and an estimation of cell numbers was obtained by reference to a standard curve (Figure A1).

The rate and amount of growth shown by each strain in the liquid medium agreed with the results found with the solid medium. Utilisation of each carbon source also closely correlated with the amount of growth on that substrate.

Strain P510-5A (Figure 5) differed little in growth and sugar utilisation rate from the wild type strain (Figure 4) on D-glucose and on D-fructose. Utilisation of D-mannose, after a slight lag period of 12 hr, was complete within 48 hr incubation. The mutant, P509-3C, exhibited a slower rate of growth on D-glucose and a markedly reduced rate of growth on D-mannose with correspondingly slower rates of utilisation of these sugars (Figure 6). Consumption of D-mannose was evident only after a lag phase of 48 hr and only 60% of the sugar had been consumed after 96 hr. Utilisation of D-glucose and D-fructose was complete at 48 hr; in comparison, strains P444-3D and P510-5A consumed most of the D-glucose and D-fructose within 24 hr.

The double mutant, P509-1B, was completely negative for growth on D-glucose and D-mannose and utilisation of these sugars was negligible (Figure 7). The final cell concentration after 96 hr incubation in YNB-glucose, was estimated as 2.3×10^8 cells ml⁻¹. Since the initial cell density at inoculation was 2.5×10^7 cells ml⁻¹, a ten-fold increase in cell numbers occurred during this period. In comparison, 24 hr after inoculation into YNB-glucose the wild type culture had reached a cell density of 1.45×10^9 cells ml⁻¹. Utilisation of D-fructose by P509-1B

Fig. 4: Utilisation of Hexoses and D-Xylose (2% w/v)
in YNB Media by Wild Type Strain, P444-3D.

○ , D-glucose; ▲ , D-fructose;
□ , D-mannose; ■ , D-xylose

Fig. 5: Utilisation of Hexoses and D-Xylose (2% w/v)
in YNB Media by Strain P510-5A.

○ , D-glucose; ▲ , D-fructose;
□ , D-mannose; ■ , D-xylose

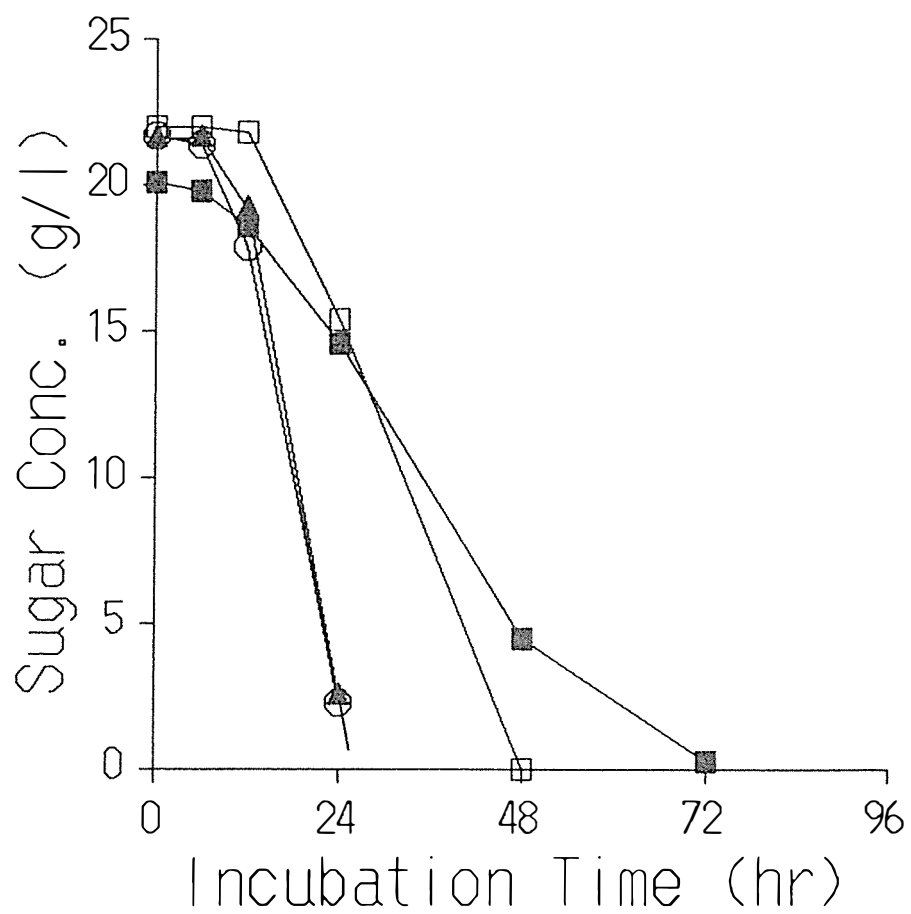
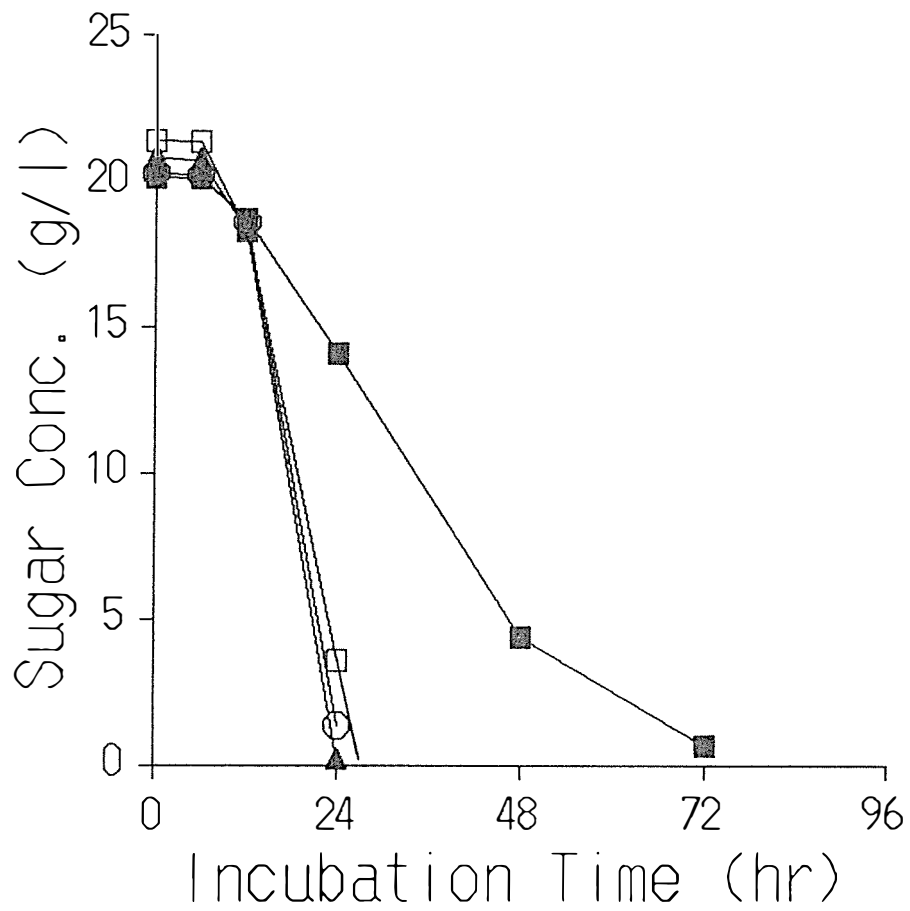
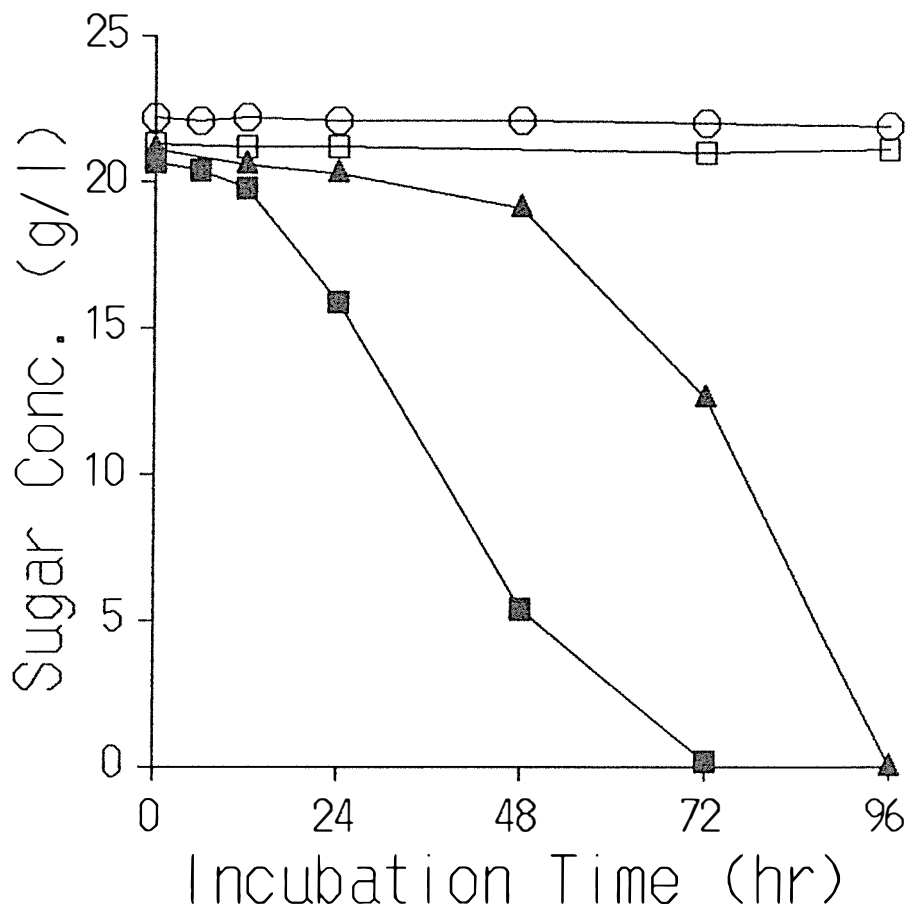
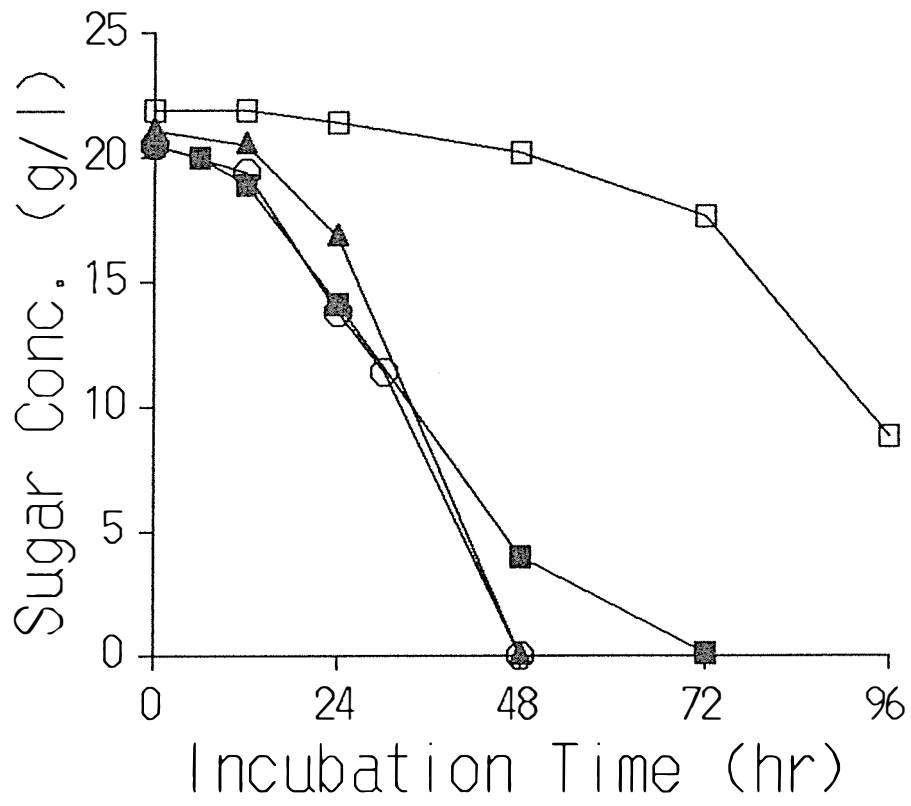


Fig. 6: Utilisation of Hexoses and D-Xylose (2% w/v)
in YNB Media by Strain P509-3C.

○ , D-glucose; ▲ , D-fructose;
□ , D-mannose; ■ , D-xylose

Fig. 7: Utilisation of Hexoses and D-Xylose (2% w/v)
in YNB Media by Strain P509-1B.

○ , D-glucose; ▲ , D-fructose;
□ , D-mannose; ■ , D-xylose.



only became apparent after 48 hr incubation. Growth on this carbon source, although initially slow, did build up to final population density of $1.7 \times 10^9 \text{ ml}^{-1}$. Growth on D-xylose and utilisation of this sugar was similar for all the strains.

The growth of the wild type and glucose-negative strains on 6% D-glucose in YNB medium is shown in Figure 8. The growth and D-glucose utilisation by the double mutant, P509-1B, was minimal; an increase in optical density only apparent at 72 hr. The growth and sugar utilisation exhibited by strain P510-5A was similar to the wild type, all the D-glucose being consumed within 48 hr. Strain P509-3C showed slower growth and sugar utilisation, with the complete consumption of D-glucose taking 96 hr. Strains P444-3D and P510-5A grew most rapidly during the first 24 hr, but growth of these strains and P509-3C continued at a slower rate during the rest of the 96 hr period. This presumably occurred due to metabolism of the ethanol, which was produced during the fermentation of the D-glucose.

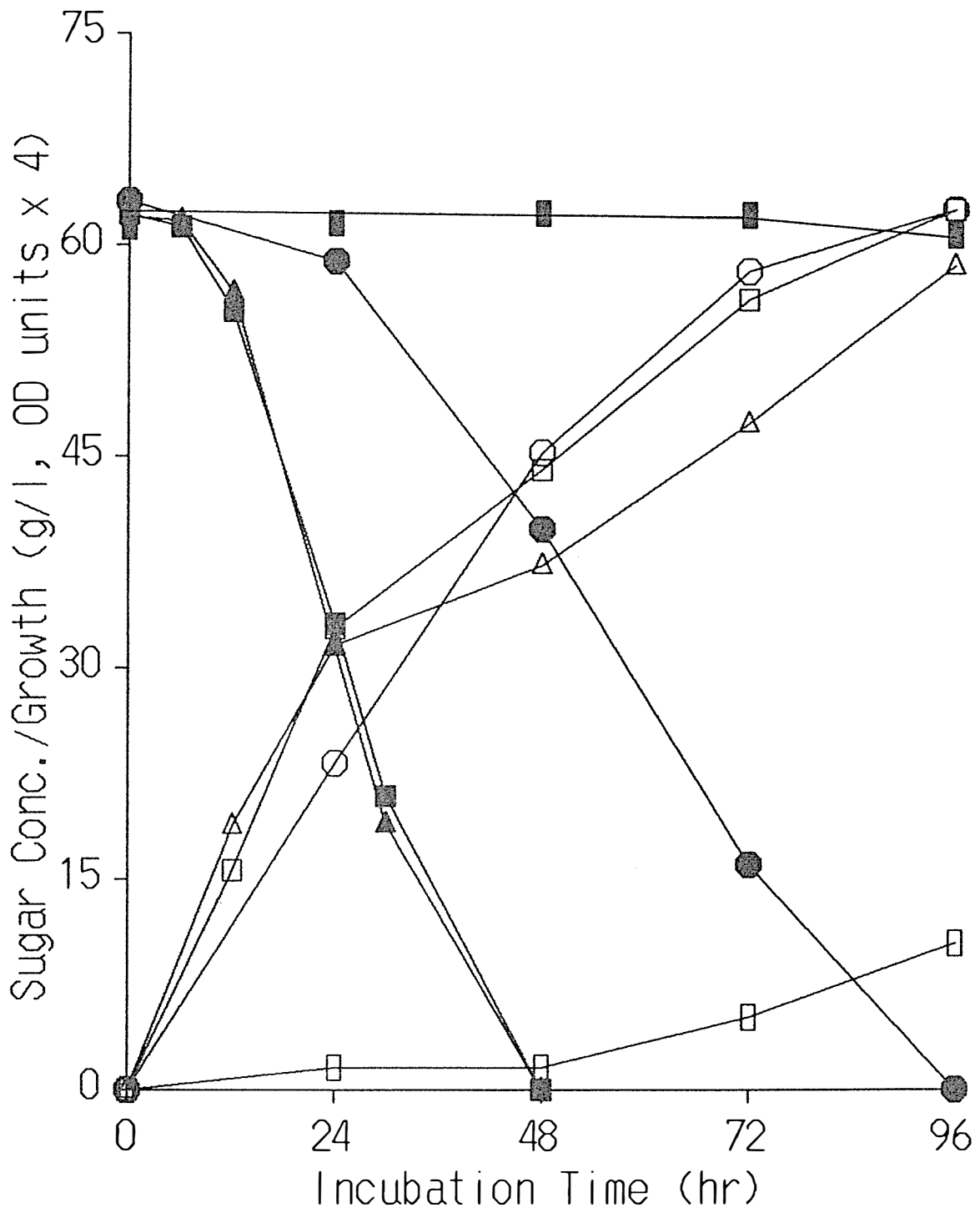
2.3 Sugar Utilisation in Media containing Two Carbon Sources

The sugar utilisation of the wild type strain, P444-3D, the double and triple mutants P509-1B and D/X A, when grown on a mixture of D-glucose and D-xylose are shown graphically in Figures 9-11. The standard density inoculation conditions were employed. The wild type strain characteristically consumed the D-glucose first, D-xylose utilisation commencing after most of the D-glucose

Fig. 8: Growth and D-Glucose Utilisation in YNB-Glucose (6% w/v) by Wild Type strain, P444-3D and Mutant Strains P510-5A, P509-3C and P509-1B.

Growth: □, wild type; △, P510-5A;
 ○, P509-3C; □, P509-1B

Glucose: ■, wild type; ▲, P510-5A;
 ●, P509-3C; ■, P509-1B



had been depleted from the medium (Figure 9). Most of the D-glucose was utilised within 24 hr and all the D-xylose after 96 hr. The rate of D-glucose in the sugar mixture was similar to the rate when present as a sole carbon source, while the complete utilisation of the D-xylose took longer. D-glucose was also preferentially consumed over D-xylose by strain P510-5A; this phenomenon was less obvious with the P509-3C mutant.

Although P509-1B was glucose-negative when D-glucose was the sole carbon source, D-glucose was utilised for both growth and the formation of end-products when D-xylose was additionally present in the medium (Figure 10). The maximum ethanol concentration in the medium (6.62 g l^{-1}) was much greater than the maximum ethanol produced from the fermentation of D-xylose as a sole carbon source (0.87 g l^{-1}). Therefore, metabolism of D-glucose to ethanol as an end-product was presumed to have occurred. This was more clearly seen during the time-course for the fermentation of a 6:2% (w/v) mixture of D-glucose and D-xylose. After 120 hr incubation, 46.2 g l^{-1} D-glucose was consumed but only 8.1 g l^{-1} D-xylose. An ethanol concentration of 15.4 g l^{-1} was reached and is clearly much greater than the theoretical maximum possible from the fermentation of D-xylose alone.

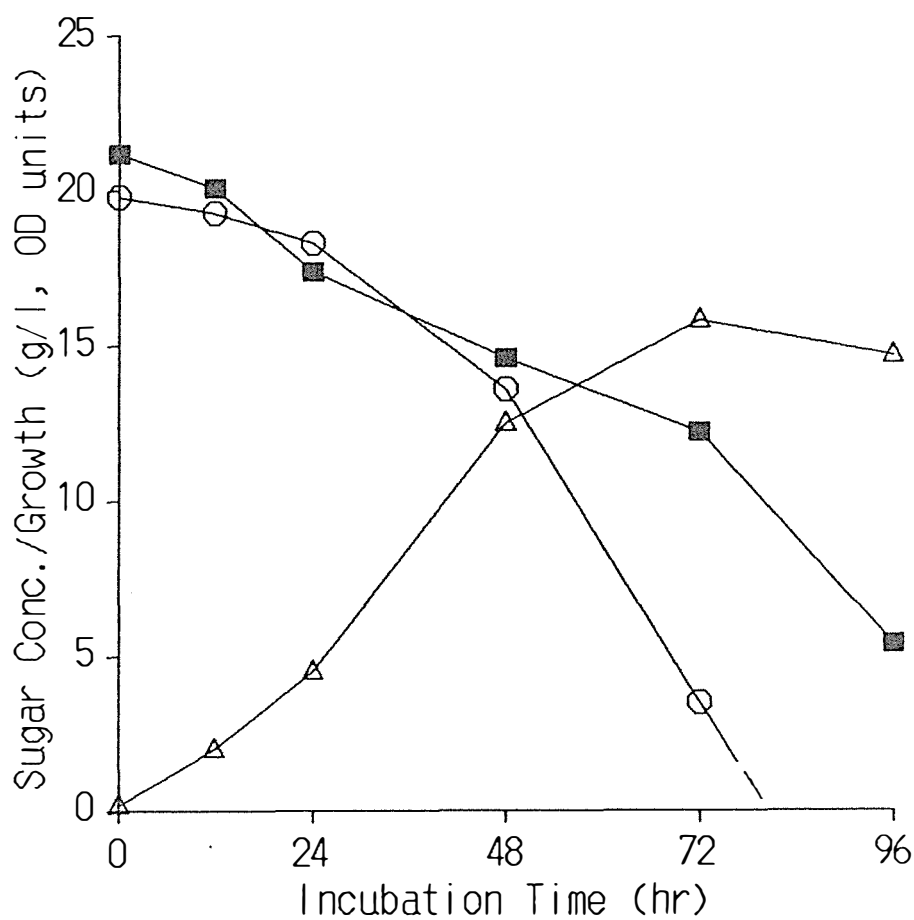
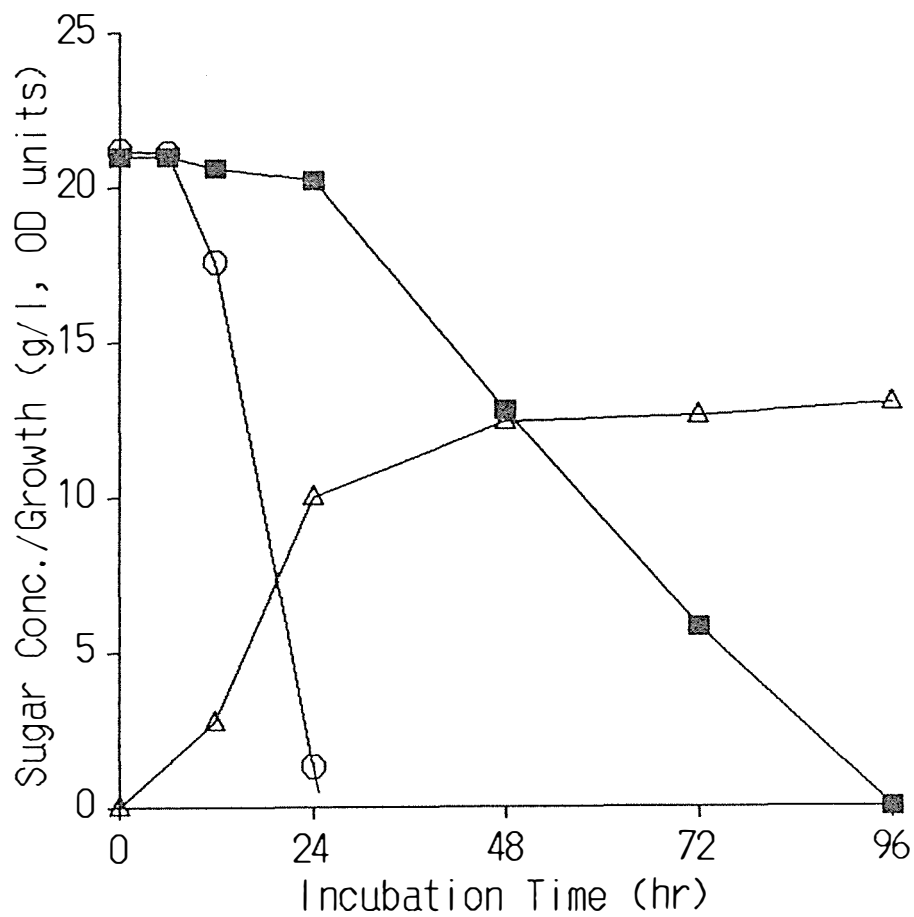
The rate of D-glucose utilisation by P509-1B on the D-glucose/D-xylose mixture was characteristically slow during the first 24 hr, but after 48 hr the rate of consumption had increased. During the period, 24-72 hr, the rate of D-xylose utilisation was inhibited but

Fig. 9: Utilisation of D-Glucose and D-Xylose (2%:2% w/v) in YNB by Wild Type Strain, P444-3D.

△ , growth; ○ , D-glucose; ■ , D-xylose

Fig. 10: Utilisation of D-Glucose and D-Xylose (2%:2% w/v) in YNB by Strain P509-1B.

△ , growth; ○ , D-glucose; ■ , D-xylose



increased after 72 hr, corresponding to the time at which most of the D-glucose had been consumed. At 72 hr, 82% of the D-glucose had been utilised, while only 42% of the xylose had been consumed. In comparison, when D-xylose was the sole carbon source, most of the sugar was consumed within 72 hr (Figure 7). The growth rate was slower in comparison to the wild type strain, but the maximum cell density reached (at 72 hr) was greater. The presence of the D-xylose in the medium appeared to be essential for the utilisation of the D-glucose, since when the cells were recycled from the YNB-glucose/xylose medium into medium containing only D-glucose as the carbon source, no D-glucose utilisation nor cellular growth occurred.

Strain P509-1B # 6 was found to exhibit a similar pattern of sugar utilisation to that shown by P509-1B. Although negative for growth and utilisation of D-glucose when presented as the sole carbon source, utilisation of D-glucose occurred in YNB-glucose/xylose medium.

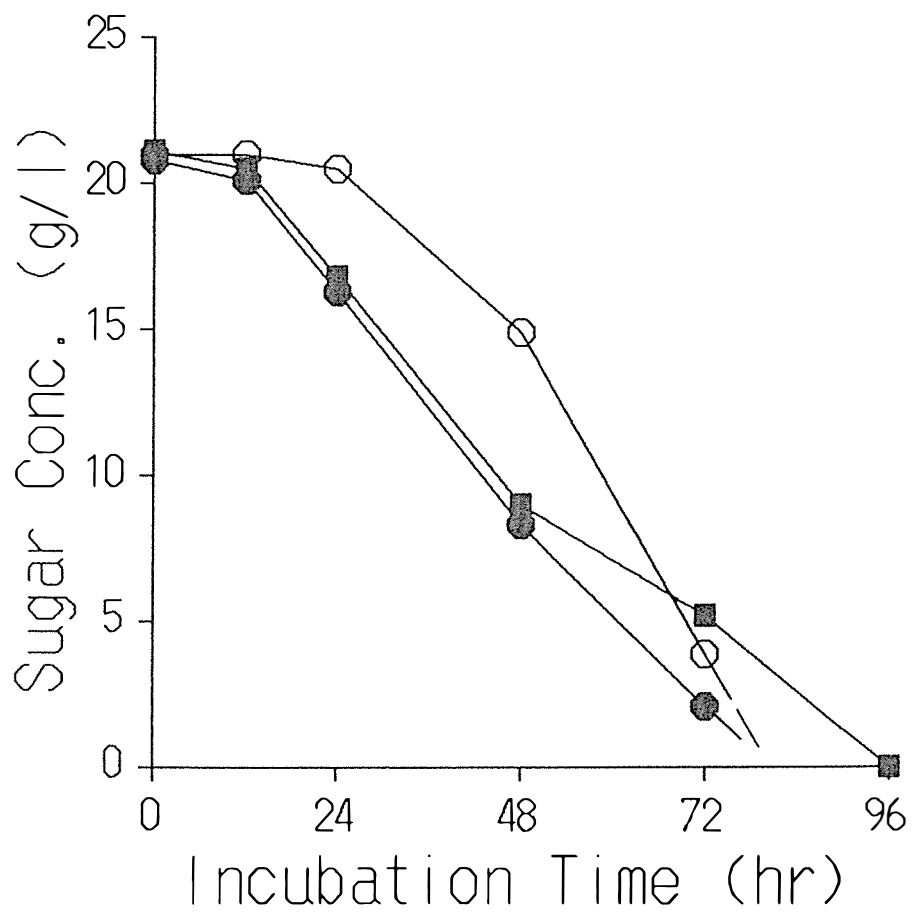
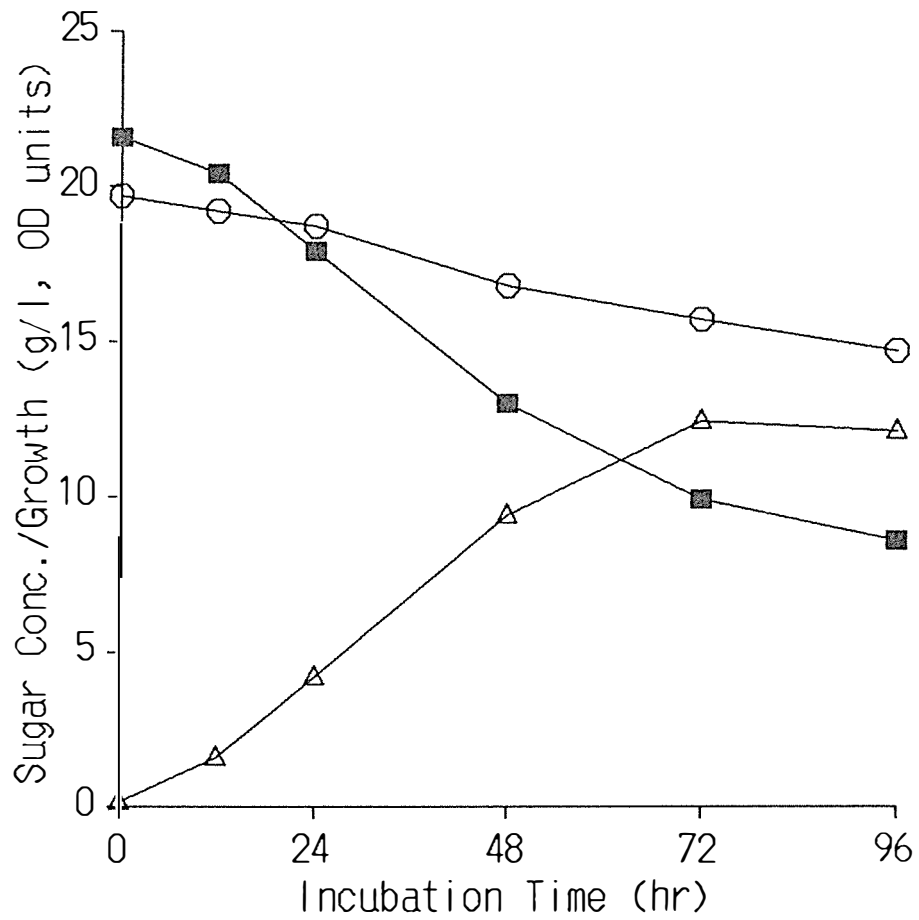
The mutant D/X A, behaved differently to P509-1B or P509-1B #6 in the D-glucose/D-xylose sugar mixture. This strain failed to completely utilise the D-xylose during the incubation period (Figure 11). The presence of D-glucose in the medium adversely affected the utilisation of the D-xylose. Although only 28% of the D-glucose was consumed at 96 hr, 39% of the D-xylose remained in the medium after this period. Corresponding with the slower rate of sugar utilisation was a slower rate of growth.

Fig. 11: Utilisation of D-Glucose and D-Xylose (2%:2% w/v) in YNB by Strain D/X A.

△ , growth; ○ , D-glucose; ■ , D-xylose

Fig. 12: Utilisation of D-Glucose and Glycerol (2%:2% w/v) in YNB by Strain P509-1B.

○ , D-glucose;
⊗ , glycerol (alone);
■ , glycerol (mixture)



The utilisation of D-fructose and D-mannose by P509-1B was also followed in media which contained D-xylose as a carbon source in addition to the hexose sugar (YNB-fructose/xylose and YNB-mannose/xylose, respectively). Both D-fructose and D-mannose were concurrently utilised with D-xylose by strain P509-1B, in a similar manner to that which occurred with D-glucose. The fate of D-glucose was also followed in media, which in addition to the D-glucose contained either glycerol or L-malic acid. D-Glucose was consumed in YNB-glucose/glycerol (Figure 12) and in YNB-glucose/malic acid. The pattern of D-glucose utilisation was again very similar to that found in the YNB-glucose/xylose media. However, the utilisation of the glycerol was not inhibited by the D-glucose to the same extent as the D-xylose. The utilisation of the L-malic acid was inhibited by the D-glucose. After 72 hr, 27% of the L-malic acid and 74% of the D-glucose was consumed in the mixed sugars, compared to 64% of the L-malic acid as a sole carbon source.

2.4 Glucose Utilisation at High Cell Density

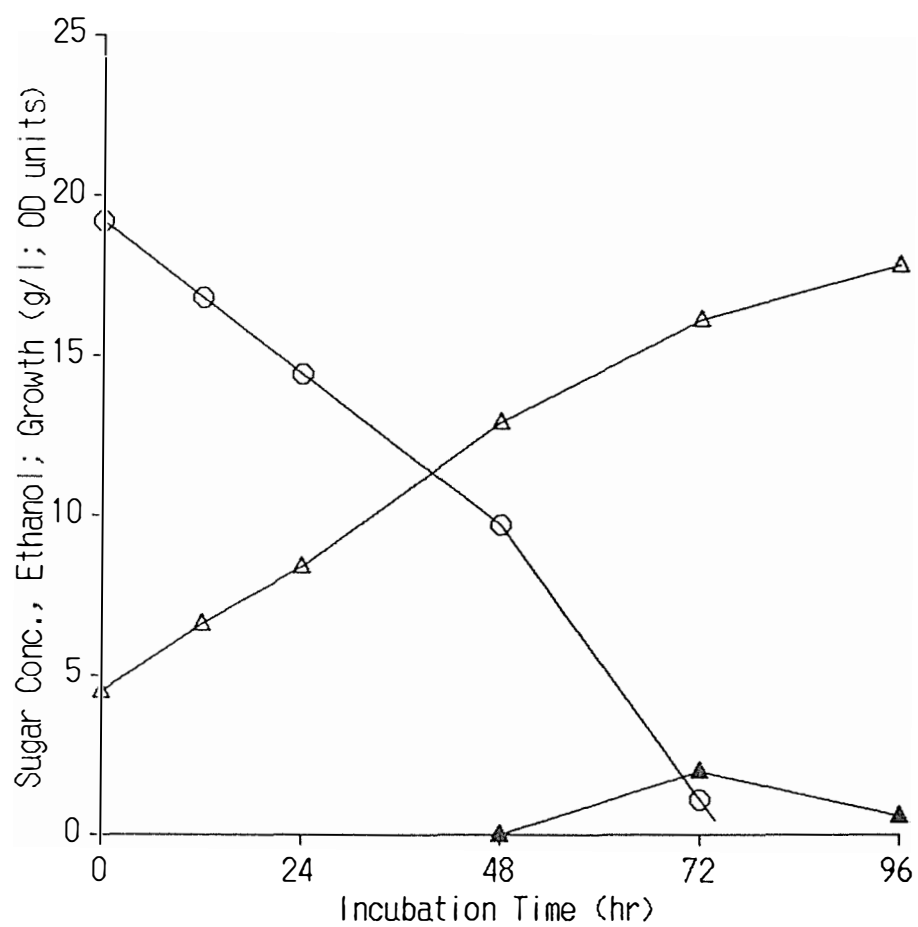
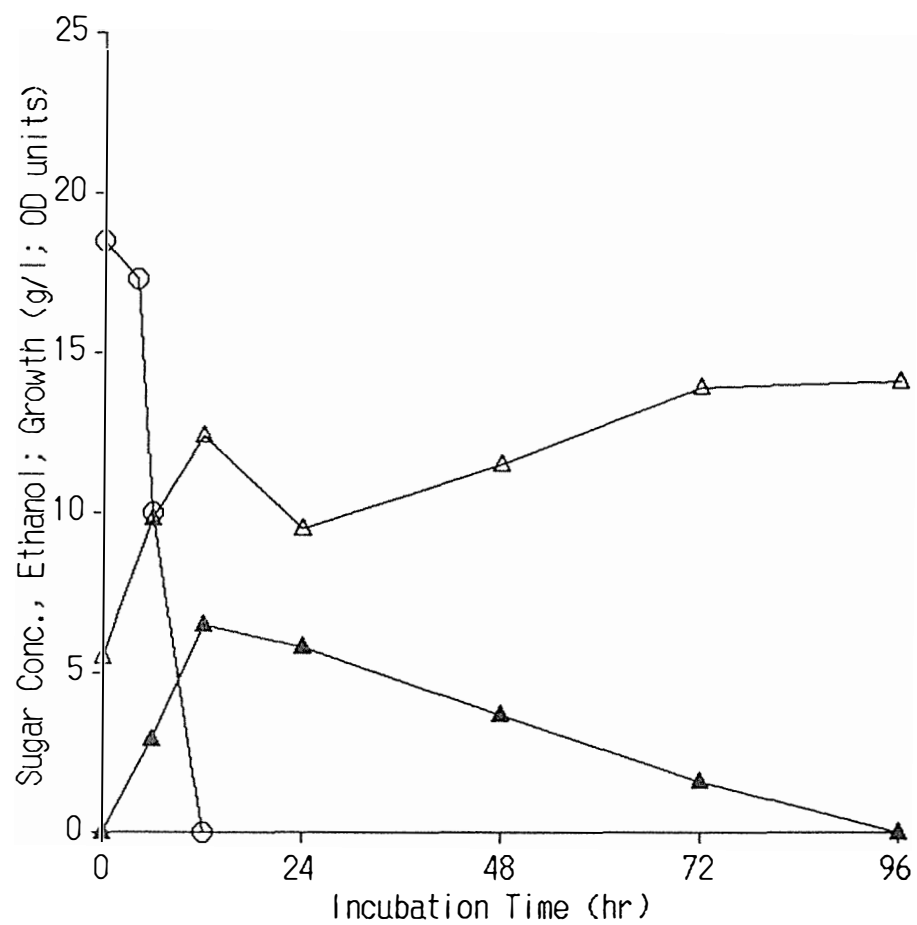
Both P509-1B and D/X A were inoculated into YNB-glucose at high cell density as described in Materials & Methods 2.32. Under these inoculation conditions, P509-1B was found to utilise D-glucose (Figure 14) at a greatly reduced rate compared to the wild type. After 72 hr, 94% of the D-glucose was used, whereas the wild type strain, P444-3D, consumed all the D-glucose within 12 hr (Figure 13). The growth rate of P509-1B was very much slower than the wild type strain but the maximum cell

Fig. 13: Time Course of Wild Type, P444-3D, in YNB-Glucose (2% w/v) at High Cell Density.

△ , growth; ○ , D-glucose; ▲ , ethanol

Fig. 14: Time Course of Strain P509-1B in YNB-Glucose (2% w/v) at High Cell Density.

△ , growth; ○ , D-glucose; ▲ , ethanol

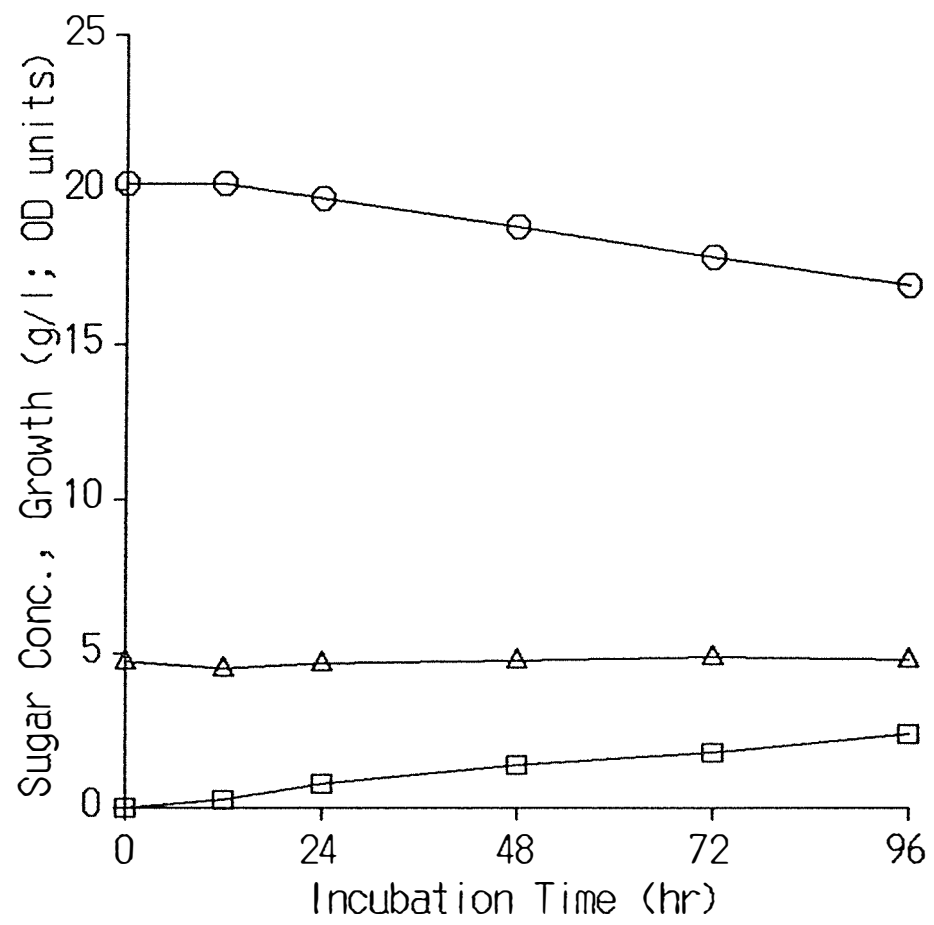


concentration reached was higher (2.7×10^9 cells ml^{-1} at 96 hr and 1.9×10^9 cells ml^{-1} at 12 hr for P509-1B and wild type, respectively). Ethanol was produced by P509-1B but the maximum ethanol concentration in the media was lower than for the wild type strain (2.0 g l^{-1} at 72 hr and 6.5 g l^{-1} at 12 hr for mutant and wild type, respectively).

The triple mutant, D/X A, consumed D-glucose very slowly, only a minimal increase in cell numbers was observed over a 96 hr incubation period (Figure 15). No ethanol was detected in the media, but an unknown compound accumulated during the incubation period. This compound eluted at a similar retention time to that of D-fructose on the Bio-Rad Aminex HPX-87H column used for the analysis of sugar and end-products. The retention time lay within the range of values for D-fructose on this column (Table XI). During the course of this study, the identity of this compound was confirmed as D-fructose by use of C^{13} -Nuclear Magnetic Resonance Spectroscopy and by borohydrate reduction to D-mannitol and D-sorbitol (Paul Bicho, personal communication). The C^{13} -NMR spectrum of the compound was similar to the spectrum of pure D-fructose. The compound was reduced by sodium borohydrate to an approximately equimolar concentration of D-mannitol and D-sorbitol. A theoretical 1:1 ratio of D-mannitol and D-sorbitol would be expected from the reduction of D-fructose. After 96 hr incubation, D/X A had consumed 3.3 g l^{-1} D-glucose and had accumulated 2.4 g l^{-1} D-fructose in the medium.

Fig. 15: Time Course of Strain D/X A in YNB-Glucose
(2% w/v) at High Cell Density.

△ , growth; ○ , D-glucose; □ , D-fructose



3. HEXOSE-ATP-KINASE, PHOSPHOGLUCOSE ISOMERASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITIES

Cell-free extracts of the wild type strain and several glucose-negative mutants were prepared and enzyme activities measured. The hexose-ATP-kinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase activities in the mutant strains were compared to the wild type strain, P444-3D (Table V). The hexose-ATP-kinase activity of the P17-1A strain from which the mutant strains were derived was also measured. All strains were pre-cultured in YEP-glucose/xylose and then grown in YEP-glucose/xylose for 42 hr.

All mutant strains exhibited lower hexose-ATP-kinase activities, with D-glucose as the substrate than the wild type strain, P444-3D, and the parent strain, P17-1A. Strains P510-5A and P509-3C exhibited 43% and 49% of the wild type activity, respectively. The double and triple mutants, P509-1B and D/X A, exhibited 5% and about 1% of the wild type activity, respectively.

The wild type strain and the mutants were also pre-cultured and grown in YEP-glucose and the hexose-ATP-kinase activities measured. With some strains, measurements were also made on cells which had been grown for only 16 hr and were still in the exponential phase of growth. The activities measured in the wild type strain and P509-3C at a later phase of growth (Table VI) were similar to the values obtained when these strains were grown in YEP-glucose/xylose (Table V). The activity in

Table V: Hexose-ATP-Kinase, Phosphoglucose Isomerase and Glucose-6-Phosphate Dehydrogenase Activities of Wild Type and Mutants

Strain/ Genotype	Specific Activity ¹		
	Hexose-ATP- ² -kinase	Phospho- glucose Isomerase	Glucose-6-P Dehydrogenase
P444-3D WT	0.77	3.3	0.17
P17-1A <i>met2-1</i>	0.76	ND	ND
P510-5A <i>glu1</i>	0.33	4.4	0.12
P509-3C <i>hxx2</i>	0.38	3.9	0.24
P509-1B <i>hxx2 glu1</i>	0.042	2.0	0.31
D/X A <i>hxx1 hxx2 glu1</i>	0.0061	1.1	0.34

1 Specific Activity in $\mu\text{moles NADP reduced min}^{-1}$
(mg protein^{-1}).

2 Glucose-phosphorylating activity

ND not determined

Table VI: Glucose and Fructose-Phosphorylating Activity of Wild Type and Mutants

Strain	Carbon Source	Hexose-ATP-Kinase* Activity		
		D-Glucose	D-Fructose	F/G Ratio
P444-3D	D-Glucose ¹	0.80	1.01	1.26/1.00
P444-3D	D-Glucose ²	0.80	0.87	1.10/1.00
P444-3D	D-Xylose ¹	0.56	0.30	0.54/1.00
P510-5A	D-Glucose ²	0.63	0.78	1.24/1.00
P509-3C	D-Glucose ²	0.38	0.11	0.29/1.00
P509-1B	D-Glucose/D-Xylose ¹	0.027	0.085	3.15/1.00
P509-1B	D-Glucose/D-Xylose ²	0.050	0.15	3.00/1.00
D/X A	D-Glucose/D-Xylose ²	0.0061	0.0096	1.57/1.00

Strains P444-3D, P510-5A and P509-3C were grown in YEP-glucose. Strains P509-1B and D/X A were grown in YEP-glucose/xylose.

* Hexose-phosphorylating activity in $\mu\text{moles NADP reduced min}^{-1} (\text{mg protein})^{-1}$

1 16 hr culture

2 42 hr culture

P510-5A was higher than the corresponding activity in YEP-glucose/xylose grown cells.

Since hexose-ATP-kinase activity was measured using D-fructose as well as D-glucose as a substrate, the rate of D-fructose to D-glucose phosphorylation (F/G ratio) could be compared between strains. Only P510-5A had an F/G ratio close to the wild type value; 1.24/1.00 and 1.10/1.00 for P510-5A and wild type grown into stationary phase growth, respectively (Table VI). Under the same conditions of growth, P509-3C exhibited a lower F/G ratio of 0.29/1.00, while P509-1B and D/X A, grown in YEP-glucose/xylose, gave higher values of 3.00/1.00 and 1.57/1.00, respectively.

The wild type strain exhibited little variation in hexose-ATP-kinase activity between cells growing exponentially in YEP-glucose and cells at stationary phase. Hexose-ATP-kinase activity when measured using D-glucose as the substrate, was identical in cells grown into both phases, but the F/G ratio was slightly lower in the latter phase cells. Higher enzyme activities were measured in P509-1B cells at 42 hr compared to 16 hr culture cells, (0.050 and 0.027 $\mu\text{moles NADP reduced min}^{-1}$ (mg protein) $^{-1}$ with D-glucose; 0.15 and 0.085 $\mu\text{moles NADP reduced min}^{-1}$ (mg protein) $^{-1}$ with D-fructose, respectively). The F/G ratio was similar at both phases of growth.

Wild type hexose-ATP-kinase activities were measured in log phase cells grown on D-xylose. Both a lower

specific activity and a lower F/G ratio was found in D-xylose grown cells compared to D-glucose grown cells (0.56 and 0.80 $\mu\text{moles NADP reduced min}^{-1} (\text{mg protein})^{-1}$ with D-glucose; 0.30 and 1.01 $\mu\text{moles NADP reduced min}^{-1} (\text{mg protein})^{-1}$ with D-fructose; F/G ratios 0.54 and 1.26, respectively.

Less variation in specific activities between the wild type and mutant strains was found for phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The double and triple mutants, P509-1B and D/X A gave lower phosphoglucose isomerase activities and higher glucose-6-phosphate dehydrogenase activities compared to the wild type.

Fructokinase activity was not detected in P509-1B nor D/X A.

4. CHROMATOGRAPHY OF HEXOSE-PHOSPHORYLATING ENZYMES ON DEAE-CELLULOSE

4.1 Chromatography of Wild Type and Hexose-ATP-kinase Defective Mutants

Anion-exchange chromatography of cell-free extracts from the wild type strain and the mutants was carried out to confirm the existence of three hexose-phosphorylating enzymes and to investigate some of their characteristics further.

The chromatography of the wild type strain and five mutants is shown in Figures 16-21. Both conductivity and protein concentration in each fraction were routinely

measured for each chromatograph, but the values are only included on the wild type profile. Fifteen units of D-glucose phosphorylating activity was typically chromatographed; only 5 and 1.25 units from strains P509-1B and P509-1B #6 respectively, were chromatographed. A total of 75.0 mg of protein from strain D/X A was chromatographed in comparison to 19.0 mg protein from the wild type strain. The wild type, P510-5A and P509-3C were grown in YEP-glucose for 42 hr, while P509-1B, P509-1B #6 and D/X A were cultured in YEP-glucose/xylose for the same period. All strains were pre-cultured on the same carbon source used for cell cultivation.

Four peaks of hexose-phosphorylating activity eluted when the wild type, P444-3D, extract was chromatographed with the sodium chloride gradient (Figure 16). The first two peaks exhibited F/G ratios of 3.0/1.0, the third peak 1.1/1.0 and the fourth peak 1.3/1.0. The conductivity of the fractions with the maximum activity were 4.4, 6.1, 9.0 and 10.7 mS for the first, second, third and fourth peaks, respectively. A similar chromatographic profile was found with a cell-free extract prepared from an exponential phase culture (16 hr), although the third peak had a lower F/G ratio of 0.9/1.0.

Strain P510-5A exhibited one major peak, with maximum activity eluting at 9.3 mS and a 1.3/1.0 F/G ratio. This was preceded by two smaller peaks, eluting at 4.6 and 6.5 mS, both with F/G ratios of 3.0/1.0. (Figure 17). The extract prepared from strain P509-3C, chromatographed with one major peak exhibiting activity with D-glucose,

Fig. 16: DEAE-Cellulose Chromatography of Cell-Free
Extract from Wild Type Strain, P444-3D.

- , D-glucose-phosphorylation;
- , D-fructose-phosphorylation;
- , protein; □ , conductivity

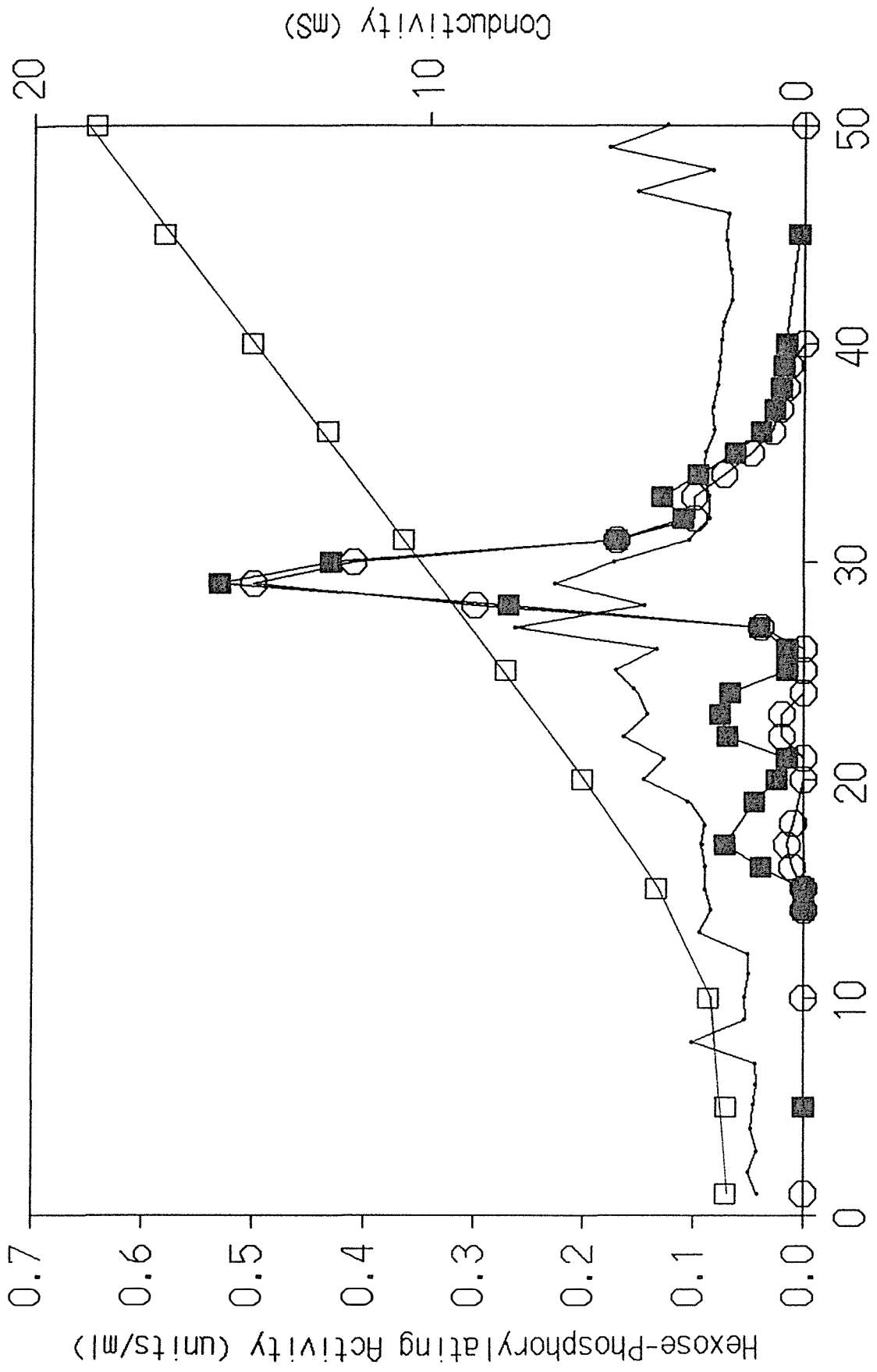
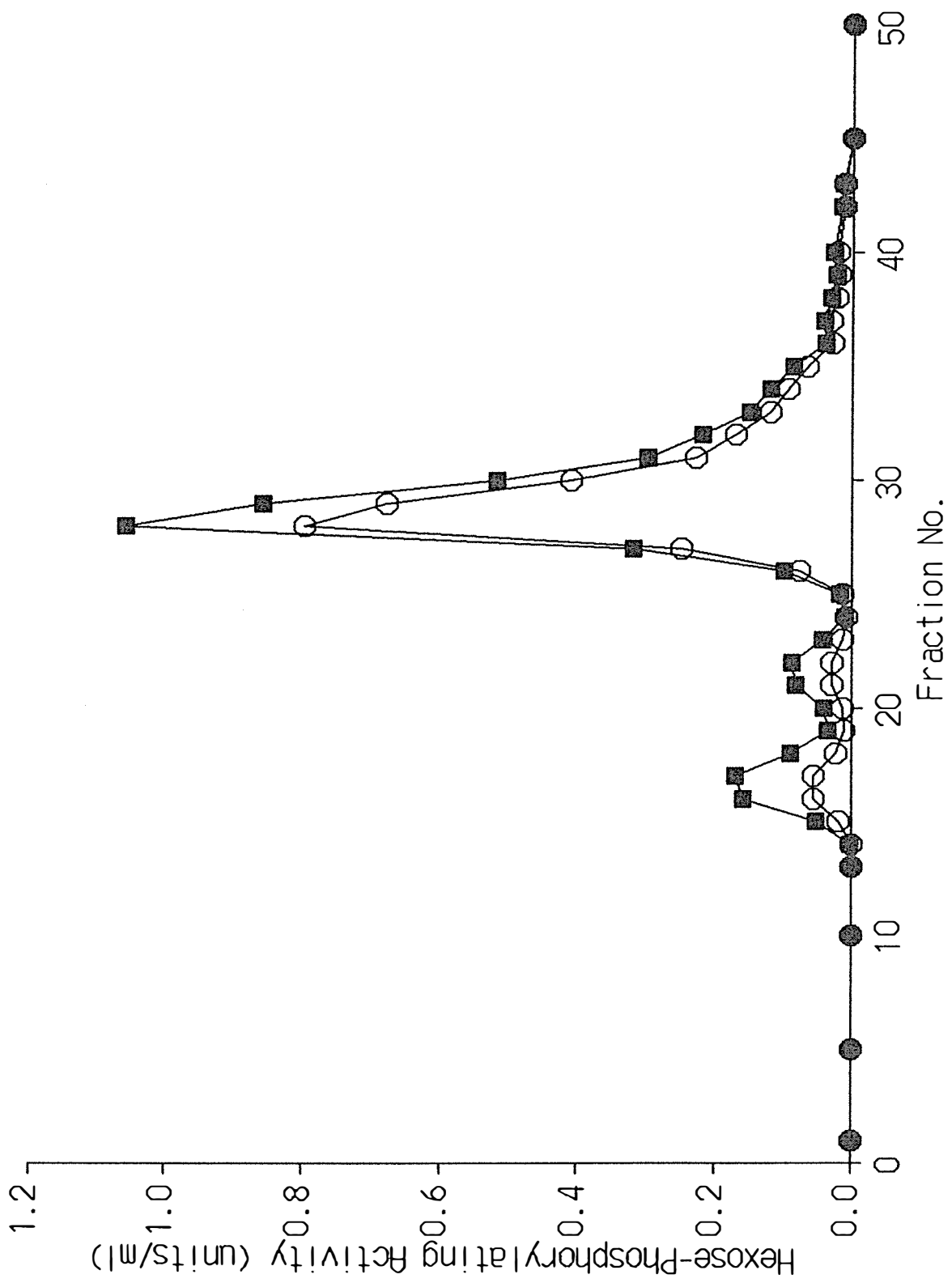


Fig. 17: DEAE-Cellulose Chromatography of Cell-Free
Extract from Strain P510-5A.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation



but only minor activity with D-fructose (Figure 18). Two small peaks of activity, with maximum activities eluting at 4.4 and 5.7 mS and ratios of 3.0/1.0, preceded the major peak which eluted at 8.7 mS.

When the double mutant, P509-1B was chromatographed, two hexose-phosphorylating peaks both with F/G ratios of 3.0/1.0 eluted (Figure 19). Maximum activities eluted at 4.0 and 6.0 mS. The larger of the two peaks eluted first, followed by the minor peak.

Chromatography of P509-1B #6 (Figure 20) showed a similar profile to P509-1B. Preparation of the cell-free extract from this strain differed from the previous strains. The protease inhibitor, PMSF, was included in the extraction buffer at 2 mM instead of 0.2 mM and the cell-free extract was desalted by filtration on Sephadex G-25 instead of overnight dialysis (Material & Methods 5.3). A lower hexose-phosphorylating activity in the P509-1B #6 cell-free extract was measured compared to P509-1B (0.028 and 0.050 $\mu\text{moles NADP reduced min}^{-1} (\text{mg protein})^{-1}$, respectively). The F/G ratio in the crude extract and in the peak fractions of the eluted enzyme was similar for both strains but the order of elution of the peaks was different. With P509-1B #6, the smaller peak eluted first, with maximum activity at 5.0 mS, followed by the larger peak at 6.4 mS.

Only a very small amount of activity eluted at a conductivity of 5.0 when the D/X A extract was chromatographed (Figure 21).

Fig. 18: DEAE-Cellulose Chromatography of Cell-Free
Extract from Strain P509-3C.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation

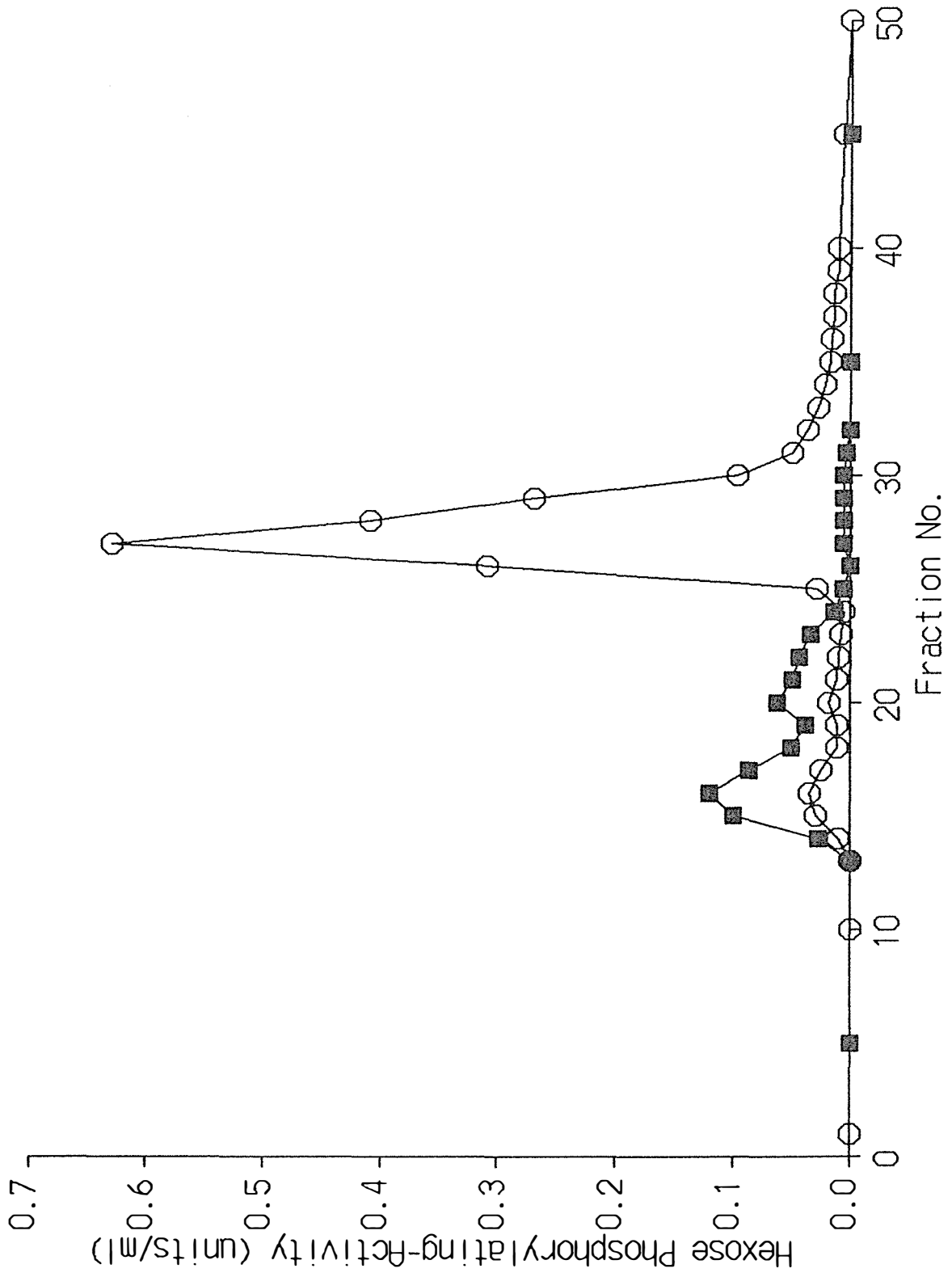


Fig. 19: DEAE-Cellulose Chromatography of Cell-Free
Extract from Strain P509-1B.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation

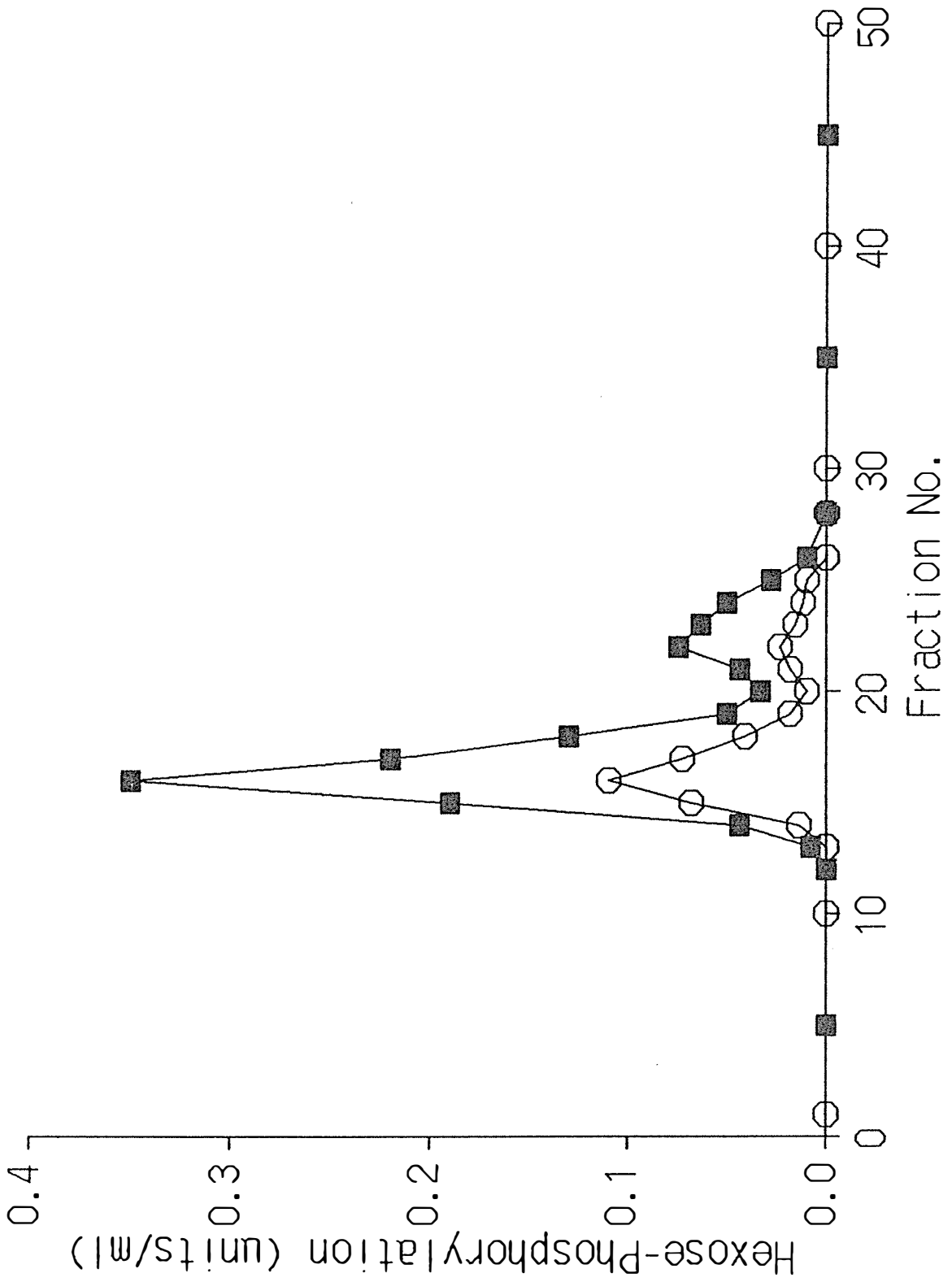


Fig. 20: DEAE-Cellulose Chromatography of Cell-Free
Extract from Strain P509-1B #6.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation

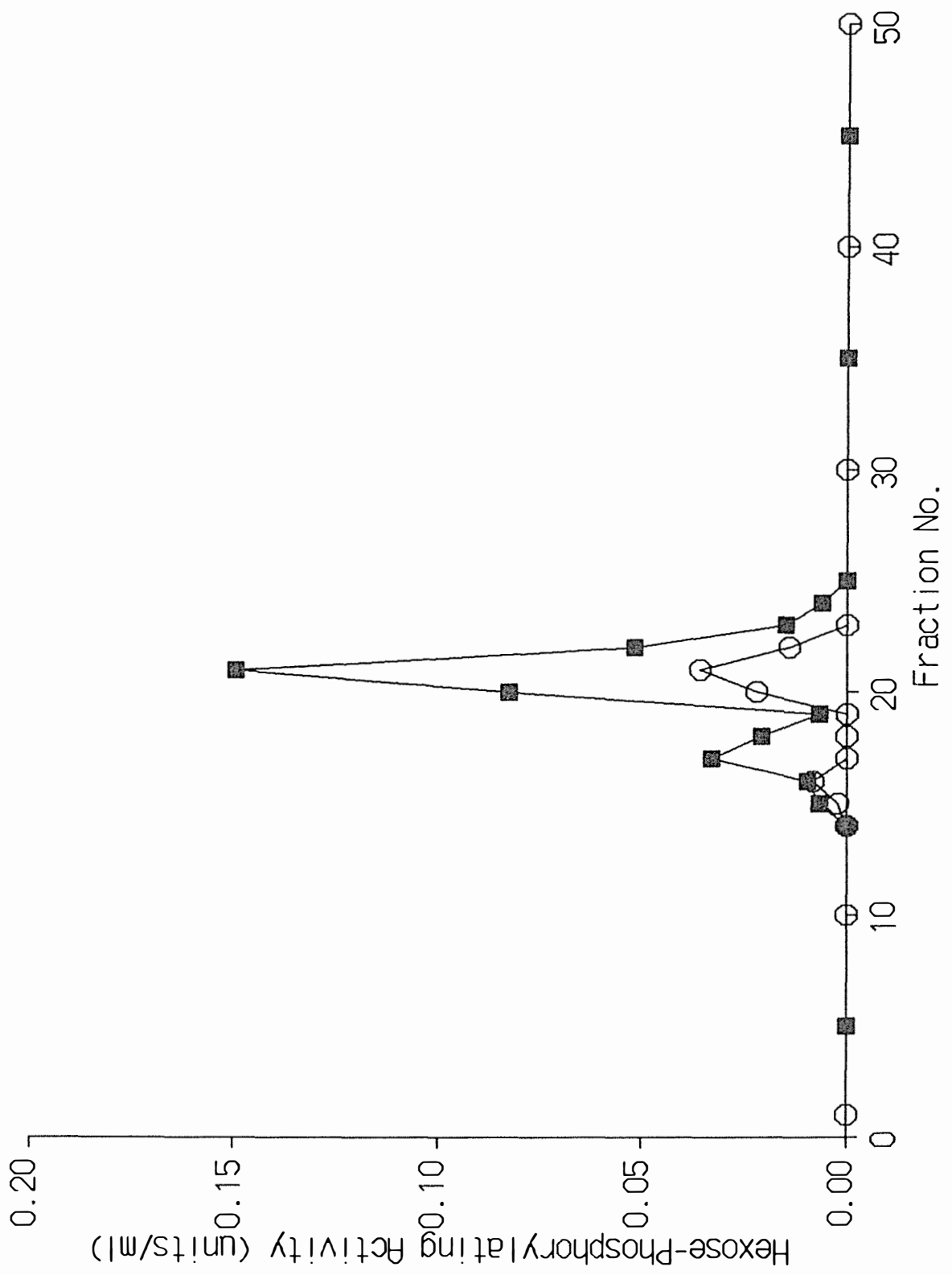
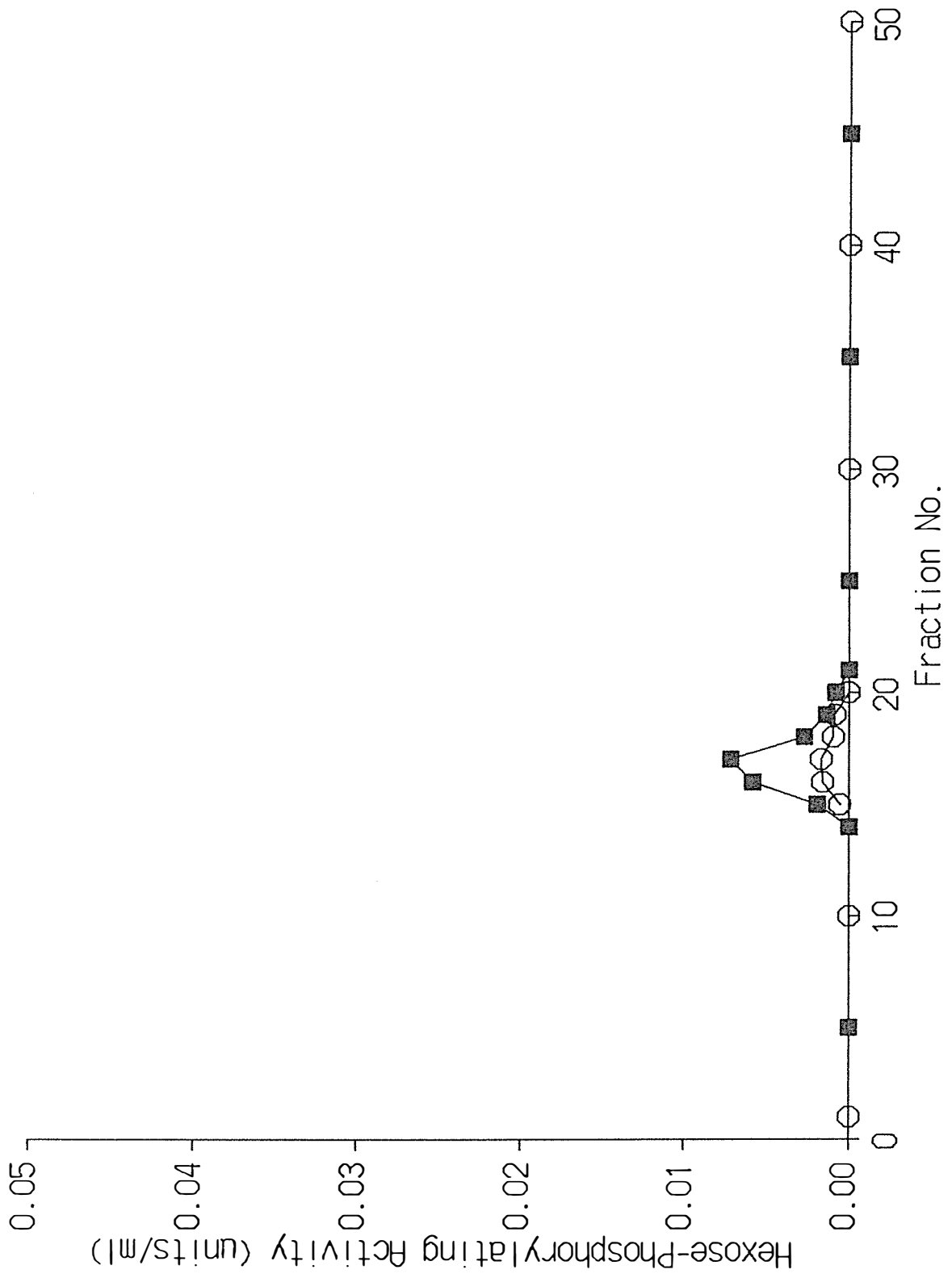


Fig. 21: DEAE-Cellulose Chromatography of Cell-Free
Extract from Strain D/X A.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation



4.2 Chromatography of Wild Type Grown on Different Carbon Sources

Figures 22-24 show the chromatographs of cell-free extracts of wild type, P444-3D, prepared from cells grown on D-glucose, D-xylose and glycerol. For each of these chromatographs, PMSF was used in the extraction buffer at a final concentration of 2 mM and the cell-free extracts were desalted on Sephadex G-25 as explained above.

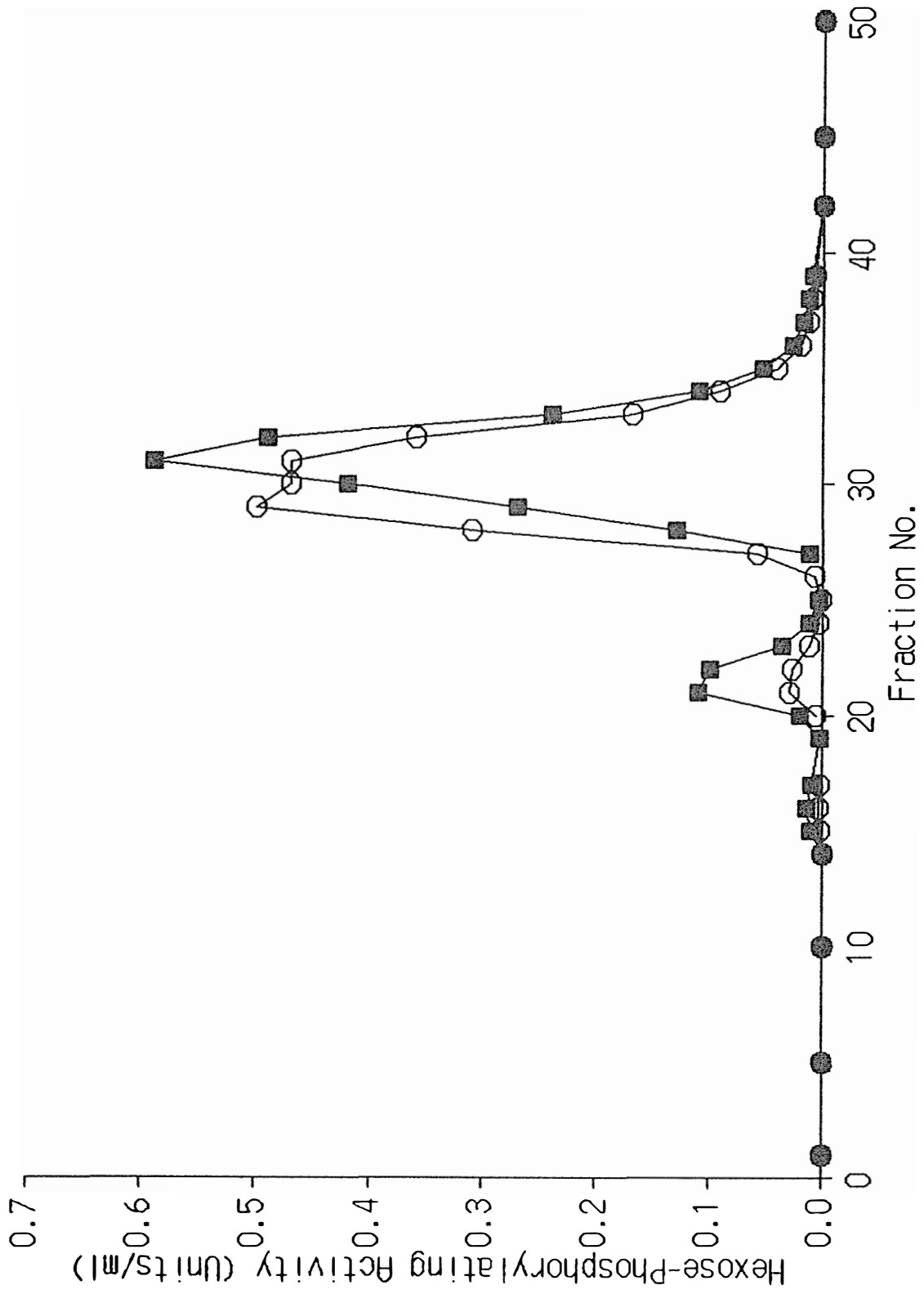
The first eluted enzyme, corresponding to hexokinase B, still eluted as two peaks of activity, irrespective of the concentration of PMSF used in the extraction buffer and the method employed for desalting the extract, but the order of elution of the peaks had changed (Figure 22). When 0.2 mM PMSF was used in the extraction buffer and the extract was dialysed overnight, the larger peak generally eluted first, followed by the smaller or minor peak of activity (Figures 17-19). When the higher concentration of protease inhibitor was used in the extraction buffer and G-25 filtration was employed as the method of salt removal, the minor peak eluted first, followed by the major peak (Figure 22). The F/G 1.3/1.0 peak, corresponding to hexokinase A, chromatographed as a single peak. Hexokinase A was preceded by the the glucokinase; these two enzymes were partially resolved and together they contributed the major activity on the gradient; the contribution of hexokinase B to the overall activity was minor in comparison.

Lower levels of hexokinase A were present, as indicated by the lower level of D-fructose phosphorylation, when the

Fig. 22: DEAE-Cellulose Chromatography of Cell-Free
Extract from Wild Type, P444-3D; Cells Grown
on D-Glucose.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation



cells had been grown on either D-xylose or glycerol (Figures 23 & 24). Both hexokinase A and hexokinase B eluted as two peaks, but the order of elution of hexokinase B was reversed in comparison to chromatographs employing overnight dialysis of the extract (Figures 16-19).

5. XYLOSE REDUCTASE AND XYLITOL DEHYDROGENASE ACTIVITIES

The xylose reductase and xylitol dehydrogenase activities of P510-5A, P509-3C and P509-1B, grown exponentially for 16 hr on a number of carbon sources, were measured and compared to the wild type, P444-3D, activities (Table VII). The wild type strain only showed significant enzyme activities when grown on D-xylose. Much lower activities were found in cells grown on either D-glucose or glycerol and also in cells grown on a mixture of D-xylose and D-glucose.

Similar activities for both xylose reductase and xylitol dehydrogenase, were measured in the mutants when grown on D-xylose. With both P509-3C and P509-1B strains, only small reduction of activities were found in the cells grown on both D-glucose and D-xylose compared to cells grown on D-xylose alone. In contrast, activities for xylose reductase and xylitol dehydrogenase in cells of P510-5A grown on D-glucose and D-xylose were much lower than in the D-xylose grown cells. Xylose reductase and xylitol dehydrogenase activities were 0.039 and 0.054 $\mu\text{moles cofactor reduced or oxidised min}^{-1} (\text{mg protein})^{-1}$ for D-glucose/D-xylose grown cells compared to 0.33 and

Fig. 23: DEAE-Cellulose Chromatography of Cell-Free Extract from Wild Type, P444-3D; Cells Grown on D-Xylose.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation

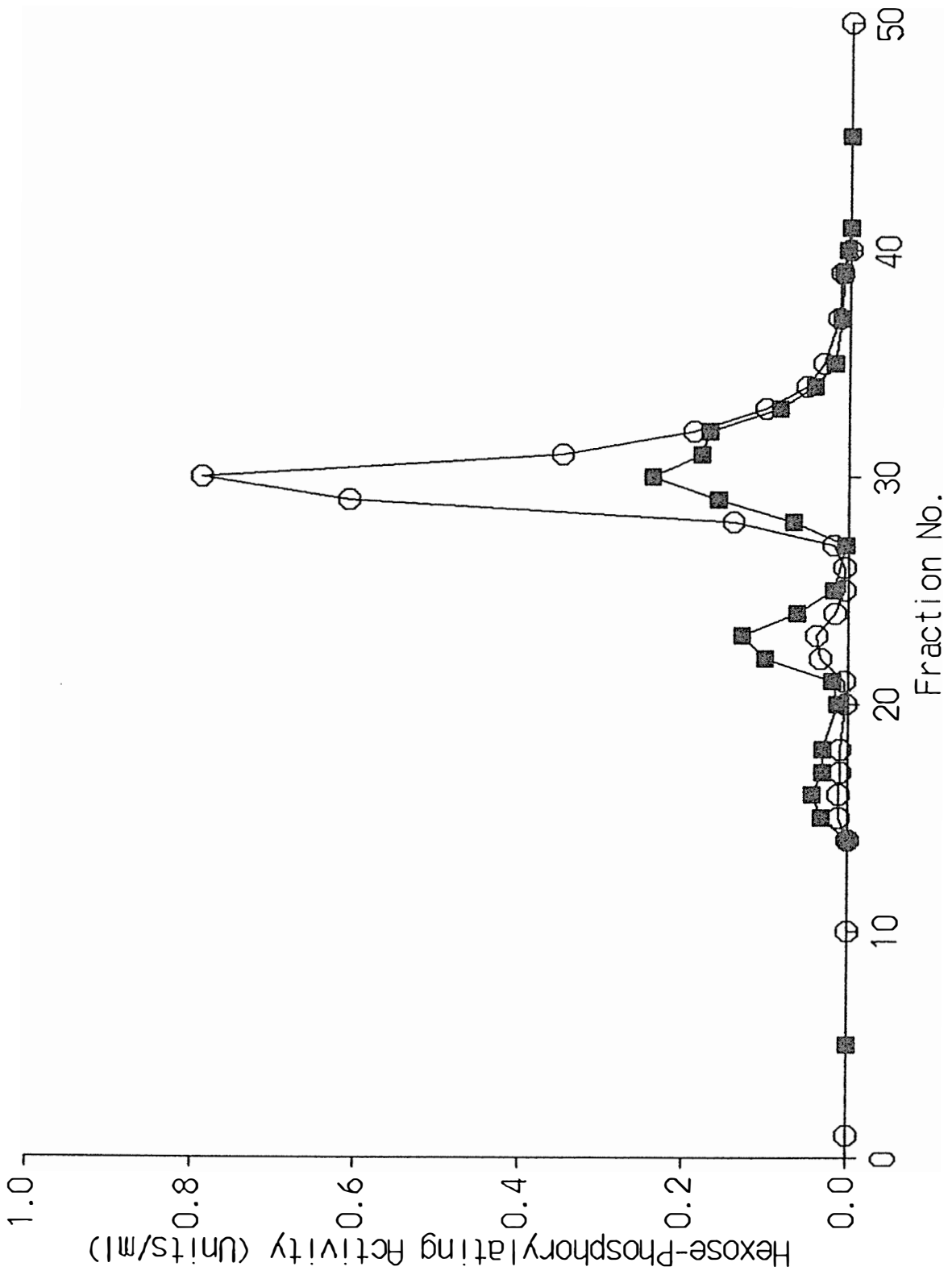


Fig. 24: DEAE-Cellulose Chromatography of Cell-Free Extract from Wild Type, P444-3D; Cells Grown on Glycerol.

○ , D-glucose-phosphorylation;

■ , D-fructose-phosphorylation

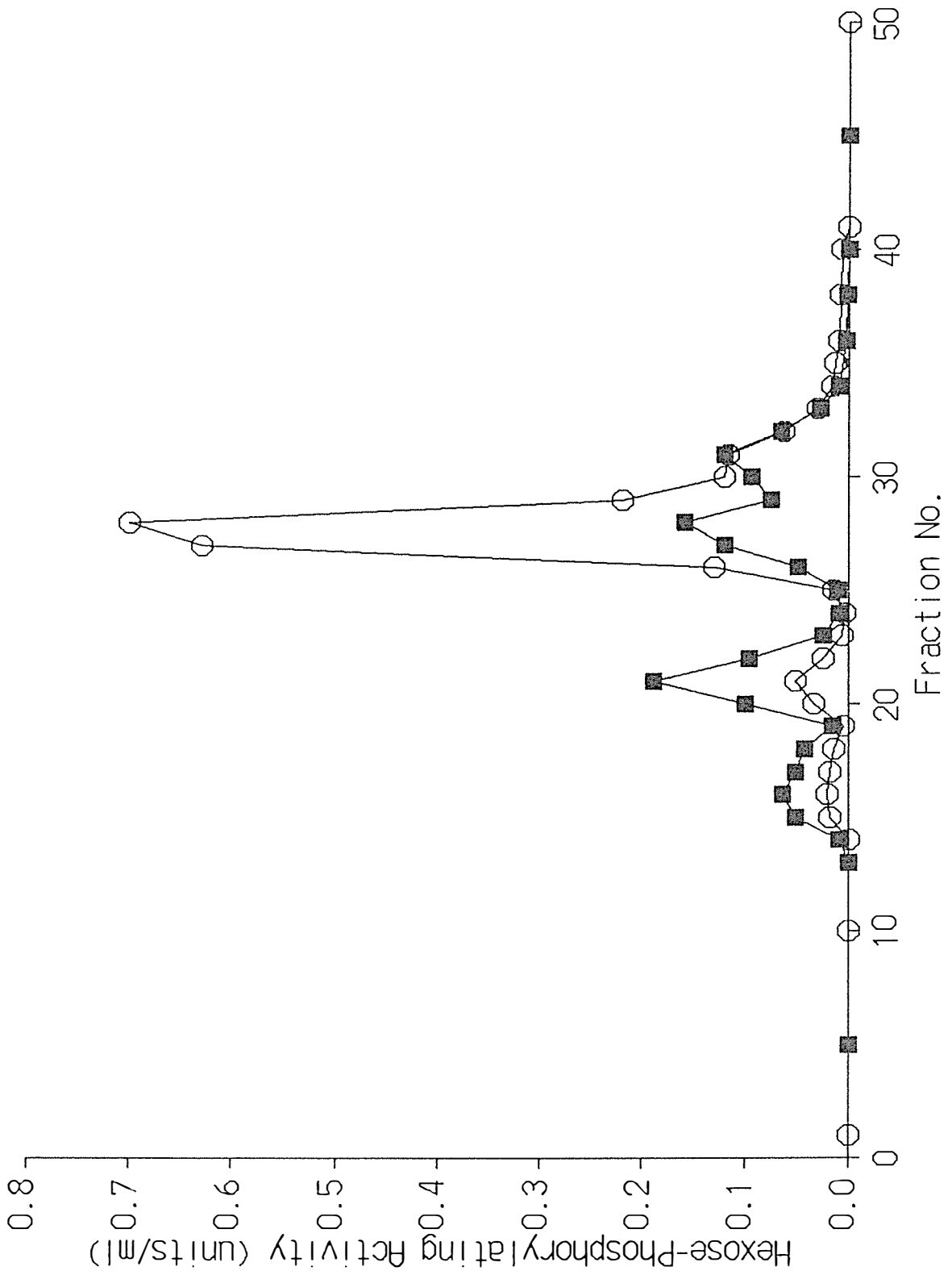


Table VII: Specific Activities of Xylose Reductase and Xylitol Dehydrogenase by Wild Type and Mutants Grown on Different Carbon Sources.

Strain	Carbon Source	Specific Activity*	
		Xylose Reductase	Xylitol Dehydrogenase
P444-3D	D-Xylose	0.26	0.24
	D-Xylose/D-Glucose	0.007	0.028
	D-Glucose	0.006	0.006
	Glycerol	0.009	0.049
P510-5A	D-Xylose	0.33	0.29
	D-Xylose/D-Glucose	0.039	0.054
P509-3C	D-Xylose	0.34	0.28
	D-Xylose/D-Glucose	0.21	0.20
P509-1B	D-Xylose	0.34	0.28
	D-Xylose/D-Glucose	0.23	0.25

* $\mu\text{moles cofactor reduced or oxidised min}^{-1} (\text{mg protein})^{-1}$

NADPH and NAD were used as cofactors for the measurement of xylose reductase and xylitol dehydrogenase activity, respectively.

0.29 $\mu\text{moles min}^{-1} (\text{mg protein})^{-1}$ for D-xylose grown cells. This represented an 8.5 and 5.4-fold reduction in activity for xylose reductase and xylitol dehydrogenase, respectively.

The strain P52-1C, presumed to be defective in xylitol dehydrogenase, gave a xylitol dehydrogenase activity of 0.004 $\mu\text{moles cofactor reduced min}^{-1} (\text{mg protein})^{-1}$.

6. KINETIC STUDIES ON HEXOKINASE A

6.1 Purification of Hexokinase A

The hexokinase A isoenzyme from strain P510-5A was partially purified by treatment of a cell-free extract with streptomycin sulphate, fractionation on DEAE-cellulose, followed by affinity chromatography. A summary of the purification is given in Table VIII. By these methods a purification factor of 55 was achieved with about a 10% yield. The application of affinity chromatography resulted in the largest increase of purity in one step.

Magnesium chloride (MgCl_2) was required in the buffer to allow binding of the enzyme to the Blue Sepharose. Low binding occurred if the magnesium chloride was omitted from the buffer. The enzyme was easily released from the Blue Sepharose by increasing the pH of the elution buffer to 8.5. Although ATP was tried as an eluant, it was not as successful in elution of the enzyme but inclusion in the buffer at a concentration of 15 mM aided stability of the enzyme. If ATP was omitted from the elution buffer

Table VIII: Summary of Purification of Hexokinase A from Strain P510-5A

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U mg protein) ⁻¹	Yield (%)	Purification factor
Cell-free extract	245.0	216	0.9	100	1.0
Streptomycin sulphate precipitation	162.0	191	1.2	88	1.4
Anion-exchange chromatography (DEAE-cellulose)	21.0	53	2.6	25	3.0
Affinity chromatography (Blue Sepharose)	0.4	21	48.4	10	55.0

U is one unit, 1 μ mole NADP reduced min⁻¹

almost complete loss of enzyme activity occurred within 24 hr. The enzyme retained activity for at least one week when ATP was present. The addition of glycerol to the purified enzyme at a final concentration of 10% also aided stability.

The purified enzyme was electrophoresed on a polyacrylamide disc gel and stained for both protein and hexokinase. One dominant band and three or four faint bands were visible after staining with Coomassie Blue R-250 (Plate 9). Only one band, corresponding to the dominant band which stained with the Coomassie Blue, stained with the activity stain. In comparison, the wild type demonstrated four bands with the activity stain.

6.2 K_m Values

The K_m values for both D-glucose and D-fructose were determined (Table IX). The K_m values were estimated to be 0.36 mM for D-glucose and 2.28 mM for D-fructose. These values were the average of assessments on two independently purified batches of enzyme. A double-reciprocal (Lineweaver-Burke) plot for the enzyme is shown in Figure 25. The maximum D-fructose/D-glucose phosphorylation ratio (F/G) was 1.50/1.00 (Table IX) as predicted from the V_{max} D-fructose/V_{max} D-glucose ratio. The F/G ratio at the 15 mM concentration of substrate routinely used in the enzyme reaction mixture was 1.32/1.00.

Plate 9: Polyacrylamide Disc Gel Electrophoresis of Partially Purified Hexokinase A.

Plate 10: Agarose Gel Electrophoresis of YRp/HXK2-8 DNA and Plasmid DNA Extracted from *E. coli*.

Lanes 1-6: Lambda:*EcoRI*; YRp/HXK2-8;
Plasmid DNA extracted from *E. coli*;
YRp/HXK2-8 cut with *EcoRI*; DNA from *E. coli*
cut with *EcoRI*; lambda:*EcoRI*

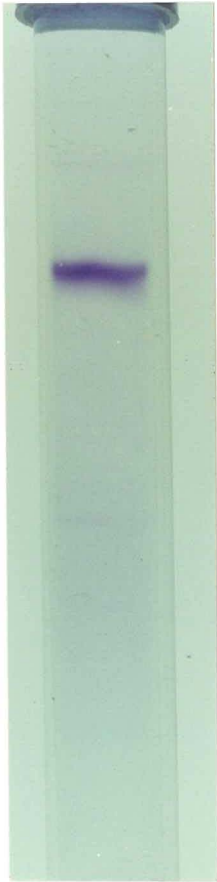


Table IX: Kinetic Properties of Hexokinase A

Substrate Concentration (mM)	Hexokinase Activity*		F/G Ratio
	D-Glucose	D-Fructose	
0.5	0.37	0.12	0.32/1.00
1.0	0.44	0.27	0.61/1.00
2.5	0.48	0.44	0.92/1.00
5.0	0.57	0.62	1.09/1.00
10.0	0.59	0.71	1.20/1.00
15.0	0.60	0.79	1.32/1.00
25.0	0.60	0.89	1.48/1.00
V _{max}	0.60	0.88	1.50/1.00 ¹
K _m ¹	0.36	2.28	

* $\mu\text{moles NADP reduced min}^{-1}$

1 mean of duplicate estimations

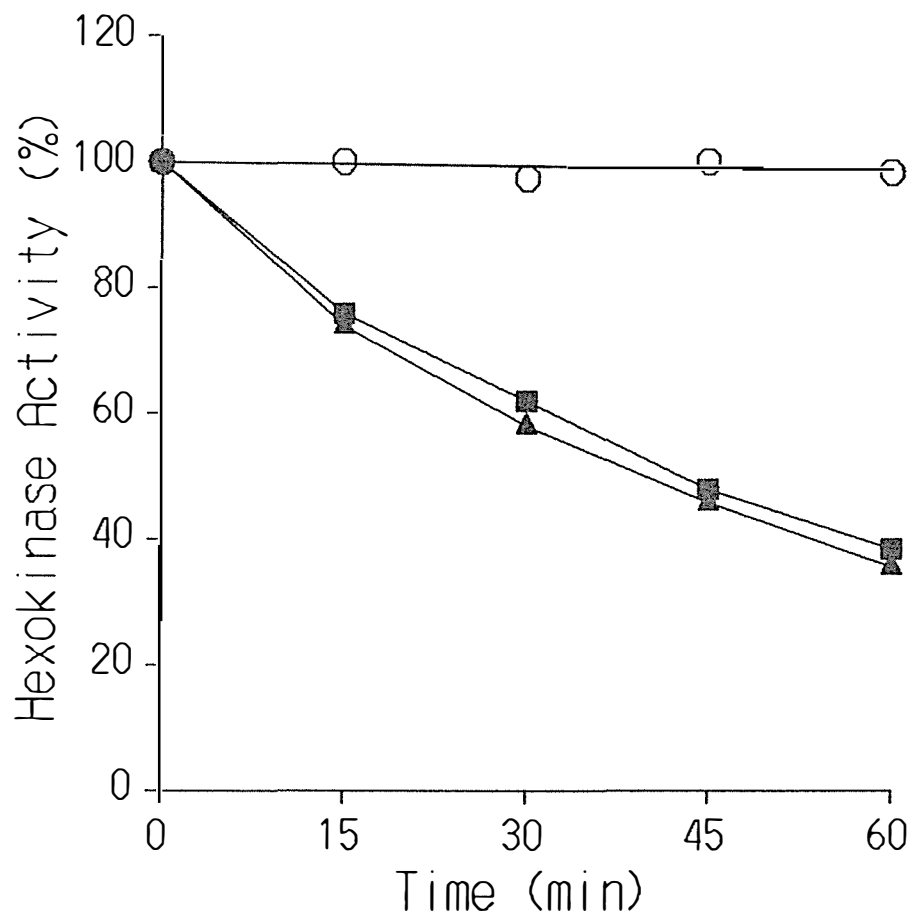
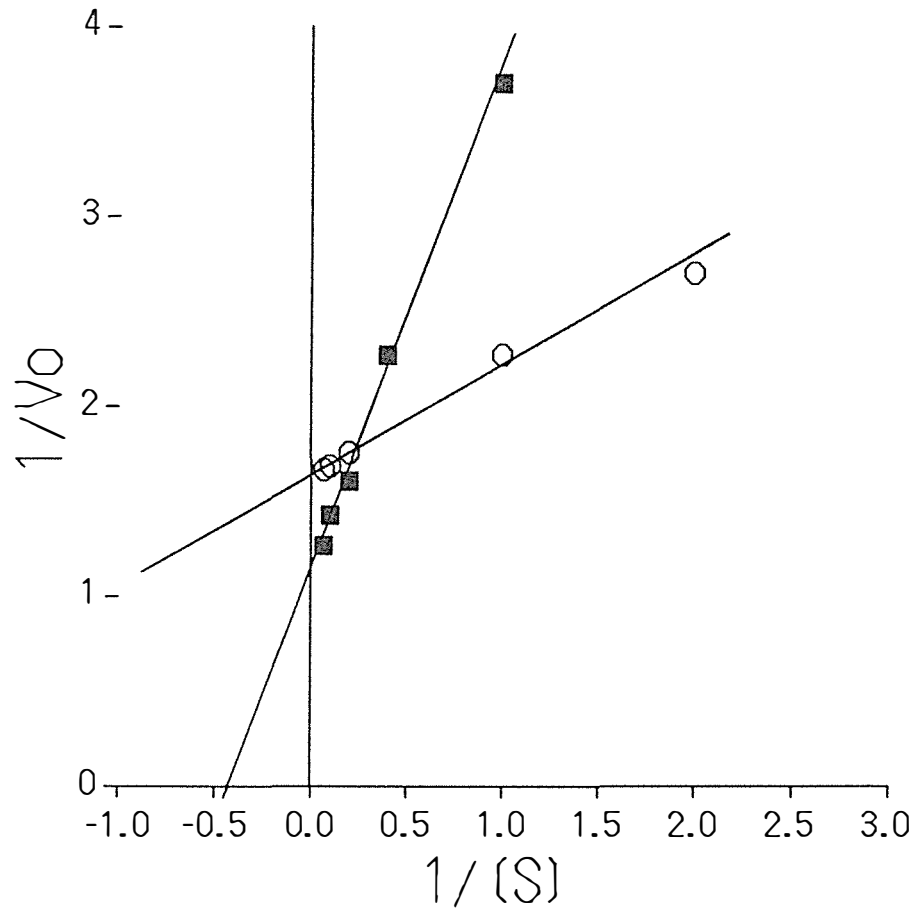
Fig. 25: Double Reciprocal Plot of Hexokinase A.

○, D-glucose; ■, D-fructose

Fig. 26: Inactivation of Hexokinase A by D-Xylose.

○, - D-xylose; ■, 100 mM D-xylose;

▲, 250 mM D-xylose



6.3 Inactivation by D-Xylose

Incubation of the partially purified enzyme with D-xylose in the presence of ATP/MgCl₂ resulted in a loss of activity. The inactivation of hexokinase A is depicted in Figure 26. When D-xylose was incubated with the enzyme, a greater rate of loss of activity was found compared to a control in which the enzyme was incubated in the presence of ATP/MgCl₂ alone. Incubation with 250 mM D-xylose gave a similar inactivation compared to 100 mM D-xylose. Inactivation at both levels of D-xylose was not linear with time; the inactivation rate decreased during the incubation period. After 60 min incubation, 60% and 65% of enzyme activity was lost with 100 mM and 250 mM D-xylose respectively. During this period, less than 10% of activity was lost in the control reaction.

7. TRANSFORMATION OF *PACHYSOLEN TANNOPHILUS* WITH THE YRP/HXK2-8 PLASMID

The mutant D/X A was transformed with the YRp7 plasmid carrying the cloned hexokinase PII gene as described in Materials & Methods 8.2. The frequency of transformation was estimated to be 40-50 transformants ($\mu\text{g DNA}$)⁻¹ after exposure to LiCl for 2 ¹/₂ hr. Most of these transformants were unstable, since when they were grown on YNB-xylose plates for 48 hr and then re-inoculated onto YNB-glucose, the ability to grow on D-glucose had been lost.

All transformants could grow on D-fructose, D-glucose and D-mannose, growth being evident after 3-4 days incubation. Growth on D-fructose was slightly faster

than the growth rate on D-glucose (Figure 27), while growth on D-mannose was slowest. Growth on D-xylose and utilisation of this sole carbon source (Figures 27 & 28) was similar to the parental D/X A strain, but utilisation was slower in the D-glucose/D-xylose mixture (Figure 28).

Two of the unstable transformants, T1 and T2, were assayed for hexokinase activity. Specific activities with D-glucose as the substrate were 0.44 and 0.23 $\mu\text{moles NADP reduced min}^{-1} (\text{mg protein})^{-1}$, respectively. The cell-free extracts from both transformants were chromatographed on DEAE-cellulose. The chromatography of T2 is shown in Figure 29. With both strains, a single peak of hexose-phosphorylating activity eluted at a conductivity of 11.7 mS and exhibited an F/G ratio of 1.2/1.0.

Plasmid DNA was extracted from both the T1 and T2 transformants. The method described in Materials & Methods 7.2 was used except the cells were sphaeroplasted by resuspension in 20 ml sphaeroplasting buffer (100 mM Tris-HCl, pH 7.5, 50 mM EDTA and 1.0 M sorbitol). Cytohelicase (Pharmindustrie, Reactifs IBF, 0.30 ml) and 2-mercaptoethanol (75 mM) were added and the cells incubated at 30°C for 2 hr, prior to resuspension in the STET buffer. When this DNA was electrophoresed on an agarose gel, bands were observed, but these showed different mobility to the original HXK2-8 plasmid isolated from *Escherichia coli*. However, the DNA was successfully used to transform *E. coli* HB101 to ampicillin resistance. A number of transformant colonies

Fig. 27: Growth of Transformant T1 on D-Glucose, D-xylose (2% w/v), D-Glucose and D-Xylose (2%:2% w/v) in YNB.

○ , D-glucose; ■ , D-xylose;
□ , D-glucose/D-xylose (mixture);

Fig. 28: Utilisation of D-Glucose, D-Xylose (2% w/v), D-Glucose and D-Xylose (2%:2% w/v) in YNB by Transformant T1.

○ , D-glucose; ■ , D-xylose;
△ , D-glucose (mixture);
● , D-xylose (mixture)

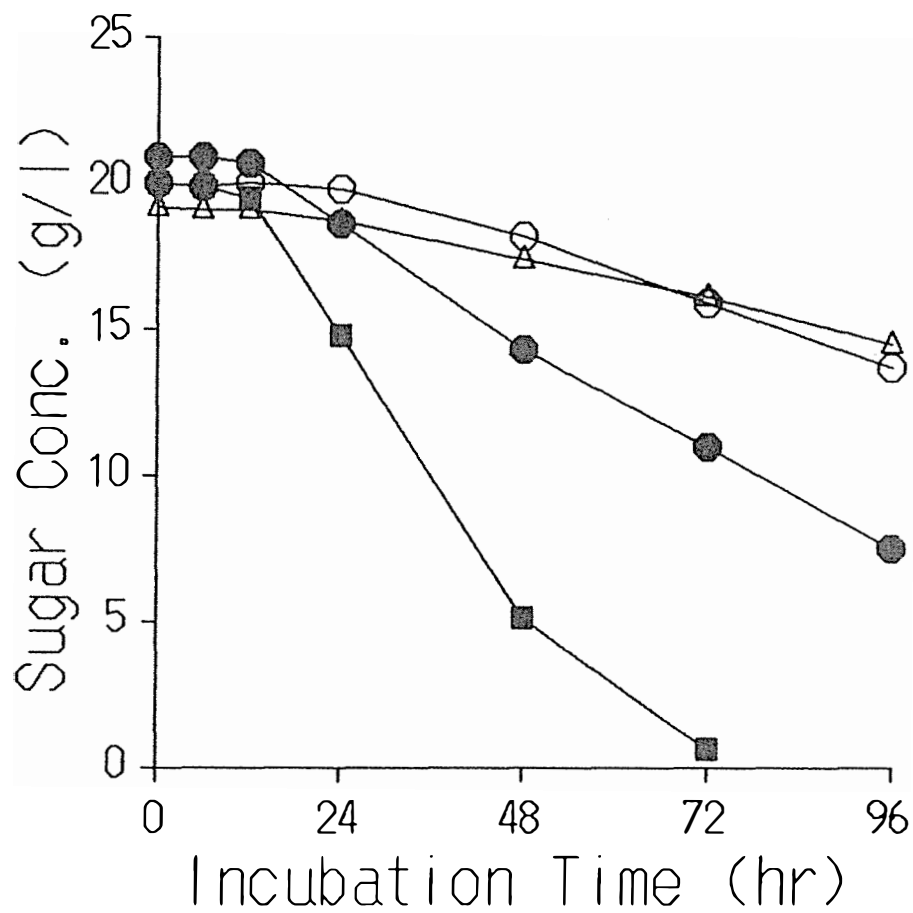
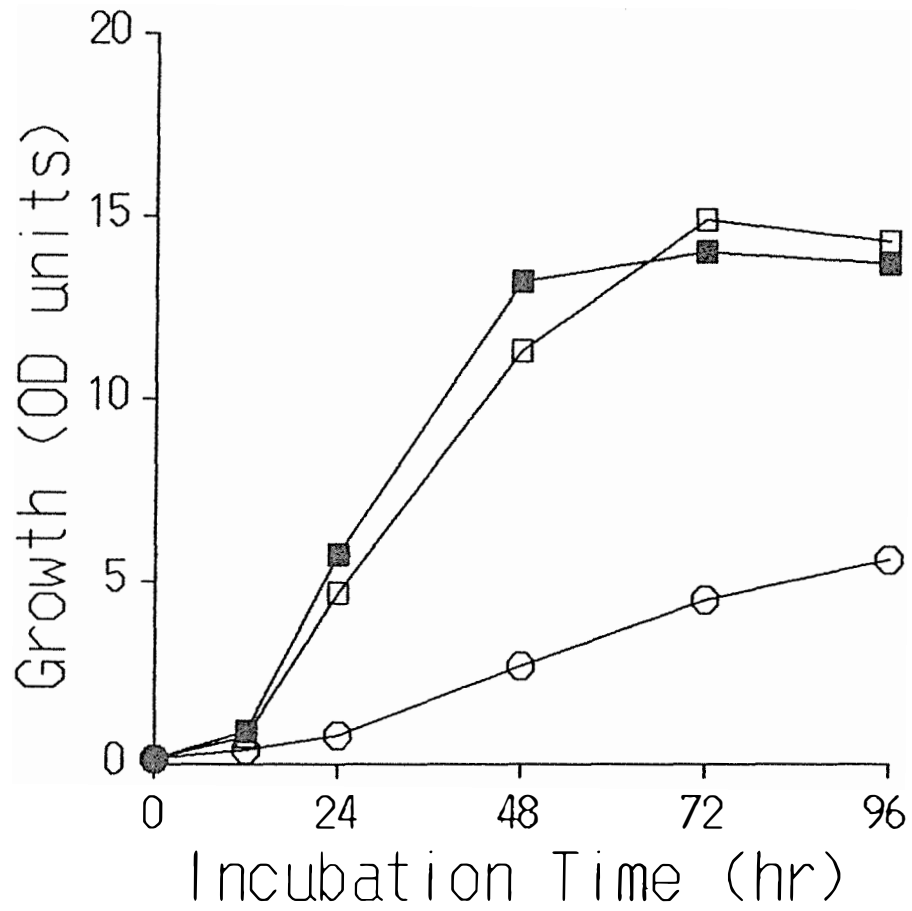
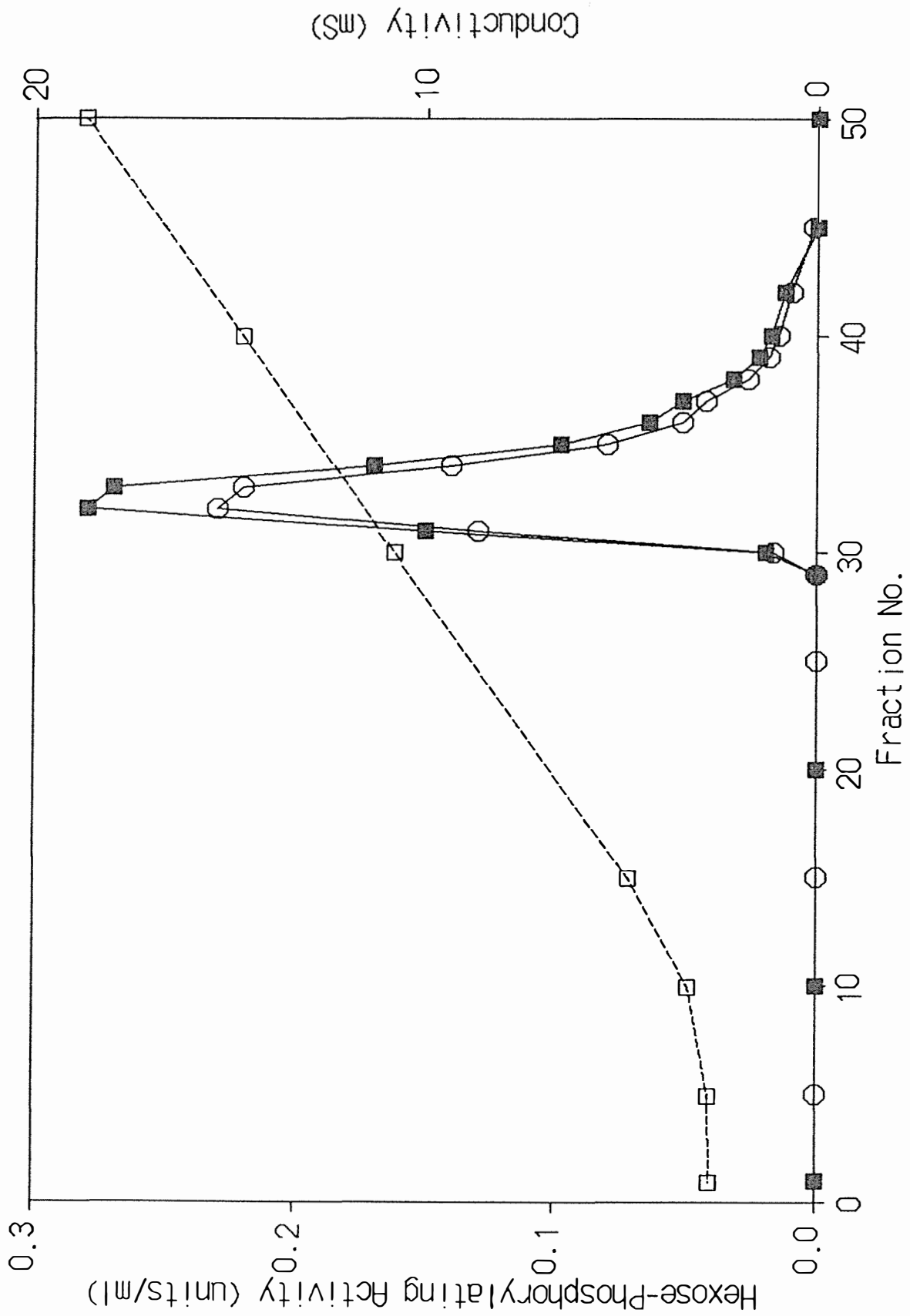


Fig. 29: DEAE-Cellulose Chromatography of Cell-Free
Extract from Transformant T2.

- , D-glucose-phosphorylation;
- , D-fructose-phosphorylation;
- , Conductivity



on LB/ampicillin plates were obtained and plasmid DNA was extracted from one colony picked at random. Plasmid DNA exhibiting a similar electrophoretic mobility to the YRp/HXK2-8 plasmid was demonstrated (Plate 10). This plasmid also gave a similar *Eco*R1 restriction pattern to the original plasmid, with one large and one small DNA fragment visible.

A shorter exposure period to LiCl of 1 hr produced fewer transformants. A modification of the basic method as employed by Bröker (1987) for the transformation of *Schizosaccharomyces pombe*, produced no transformants. This procedure was similar to that successfully used in this study but with several differences. The cells were grown in YNB-xylose and resuspended in the LiCl buffer at 10^9 cells ml⁻¹, treated with 0.1 M LiCl for 1 hr, heat shocked at 42°C for 15 min following the transformation procedure and then directly plated onto YNB-glucose.

8. CONVERSION OF GLUCOSE TO FRUCTOSE

8.1 Growth of strains on Sorbitol.

Wild type and mutant strains of *P. tannophilus* and two *S. cerevisiae* strains were checked for growth on D-sorbitol using the methods described in Materials & Methods 2.2. The results are shown in Table X. Neither of the two *S. cerevisiae* strains were able to grow on D-sorbitol, whereas both wild type *P. tannophilus* strains grew well. Commencement of growth was slow initially and only became apparent after 2-3 days incubation. This 'lag phase' was

Table X: Growth of *S. cerevisiae* and *P. tannophilus* Strains on D-Sorbitol and Other Carbohydrates

Strain/ Genotype	Carbohydrate			
	D-Xylose	D-Glucose	D-Fructose	Sorbitol
<i>Saccharomyces cerevisiae</i>				
C631-8A WT	-	+++	+++	-
S288C WT	-	+++	+++	-
<i>Pachysolen tannophilus</i>				
P444-3D WT	+++	+++	+++	+++
2530 WT	+++	+++	+++	+++
P510-5A <i>glu1</i>	+++	+++	+++	+++
P509-3C <i>hxx2</i>	+++	++	+++	++
P509-1B <i>hxx2 glu1</i>	+++	-	+	++
P509-1B #6 <i>hxx1 hxx2 glu1 fru</i>	+++	-	-	-
D/X A <i>hxx1 hxx2 glu1</i>	+++	-	-	-
F/G 2 <i>hxx1 hxx2 glu1 y</i>	+++	-	-	-
P52-1C <i>xy12-1 met2-1</i>	-	+++	+++	+

Growth recorded on solid media
after 96 hr incubation at 30°C

+++ Good growth

++ Moderate growth

+ Poor growth

- No growth

slightly longer if the cells were pre-cultured on YNB-glycerol instead of YNB-xylose prior to replica-plating onto YNB-sorbitol plates. All of the mutant strains, with the exception of P510-5A grew more slowly on D-sorbitol compared to the wild type strains, P444-3D and 2530. Strains P509-1B and P509-3C showed some reduction of growth, while strains P509-1B #6, and D/X A and F/G 2 were completely negative for growth on D-sorbitol. Strain P52-1C, defective in xylitol dehydrogenase, grew only very slowly on this carbon source. Growth of this strain and the two *S. cerevisiae* strains on D-xylose was negative.

8.2 Conversion of Glucose by cell-free Extracts

The activities of xylose reductase and xylitol dehydrogenase in a cell-free extract prepared from the wild type strain, P444-3D, were measured using D-glucose and D-sorbitol respectively as substrates. When D-glucose was substituted for D-xylose in the xylose reductase assay, the activity with NADPH as cofactor was approximately 6% of the activity which was measured using D-xylose as the substrate (0.26 and 0.016 $\mu\text{moles NADPH oxidised min}^{-1} (\text{mg protein})^{-1}$, respectively). No NADH-dependent activity was measured with D-glucose as a substrate. When NADH was substituted as the cofactor in the reaction mixture, the activity with D-xylose was only 4% of that obtained using NADPH as the cofactor. When D-sorbitol was substituted for xylitol in the xylitol dehydrogenase assay, the activity was 71% of that measured with xylitol as the substrate (0.24 and 0.17 $\mu\text{moles NAD}$

reduced $\text{min}^{-1} (\text{mg protein})^{-1}$ for xylitol and D-sorbitol, respectively).

Plates 11 - 14 show HPLC chromatographs of the reaction mixtures at 0 time and 24 hr, set up as described in Materials & Methods 9.1. When NADPH was present in the reaction mixture, a small amount of D-sorbitol and D-fructose was detected after a 24 hr incubation period (Plate 11). More D-sorbitol was present than D-fructose. When both NADPH and NAD were present, only a small amount of D-sorbitol and no D-fructose was detected (Plate 12). No conversion of D-glucose to either D-fructose or D-sorbitol occurred in control mixtures which lacked either the cofactors or the cell-free extract. No further conversion of D-glucose to either D-sorbitol or D-fructose was demonstrated in extracts incubated for 48 hr. D-Sorbitol was readily converted to D-fructose when NAD was present as the cofactor (Plate 13). More than 50% of the D-sorbitol had been converted to D-fructose after either a 24 hr or 48 hr incubation. No conversion was found in control mixtures which lacked either cofactor or cell-free extract.

As an additional control, the reduction of D-xylose to xylitol was demonstrated. D-Xylose was readily converted to xylitol in a reaction mixture which contained NADPH and the cell-free extract (Plate 14). After 24 hr, more than 50% of the D-xylose had been converted to D-xylitol.

The identity of the end-products of these conversions was confirmed by identification with HPLC. As a further check

Plate 11: HPLC Chromatograph: Conversion of D-Glucose to D-Sorbitol and D-Fructose by Cell-Free Extract From D/X A (NADPH).

A, 0 time

D-Glucose, 7.217

B, 24 hr

D-Glucose, 7.208

D-Fructose, 7.767

D-Sorbitol, 8.233

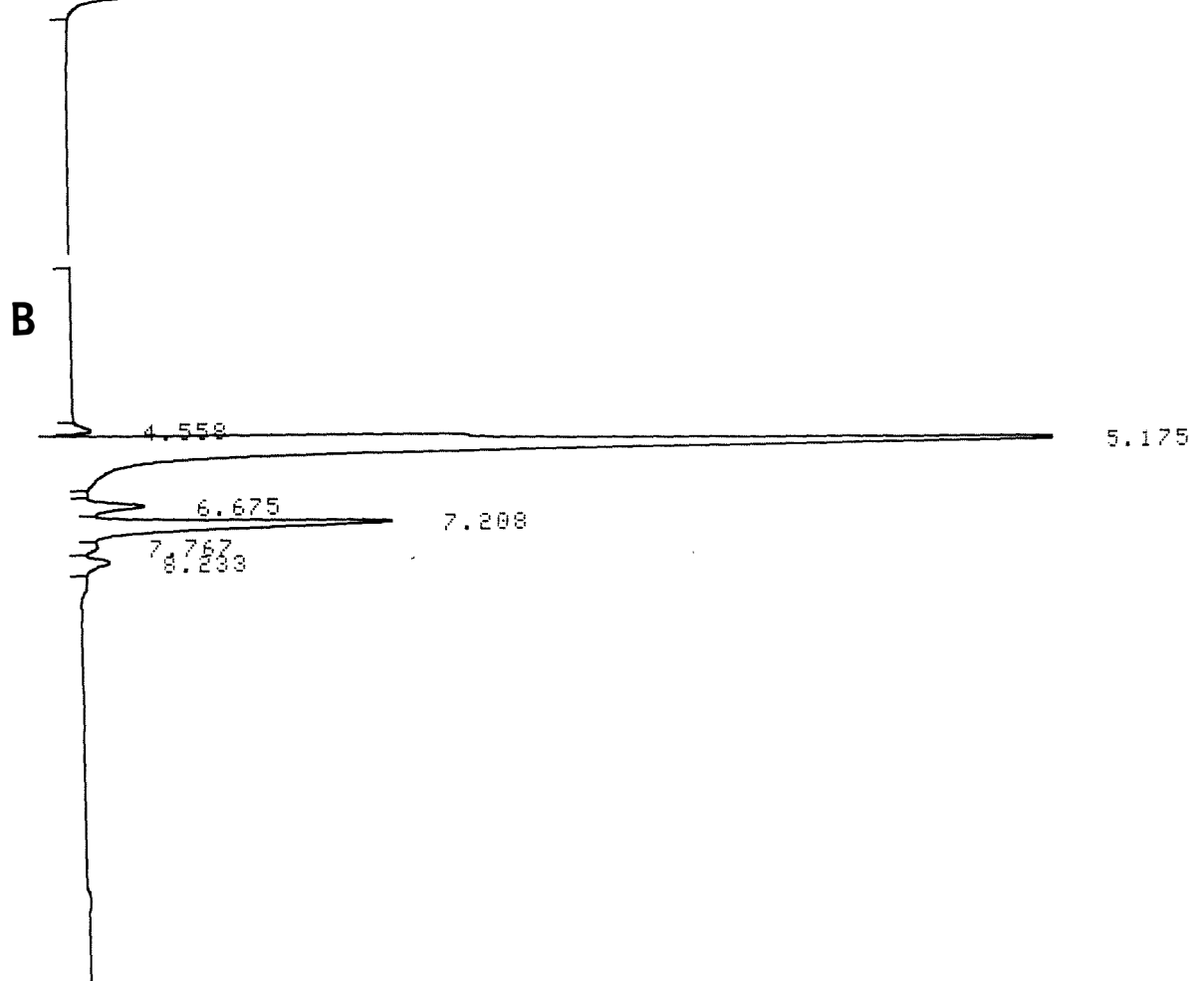
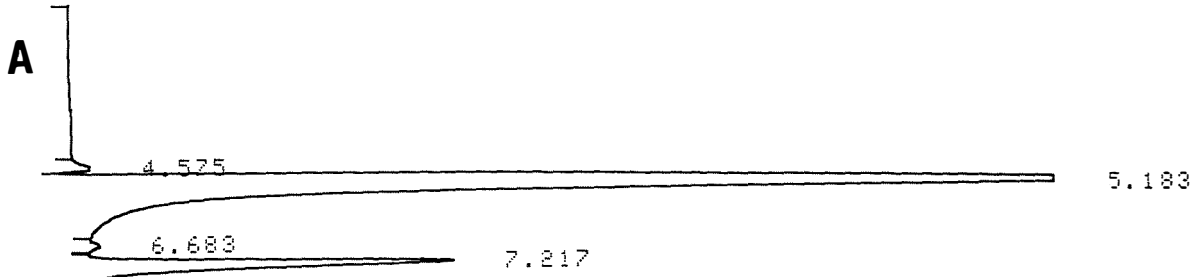


Plate 12: HPLC Chromatograph: Conversion of D-Glucose
to D-Sorbitol by Cell-Free Extract From D/X A
(NADPH and NAD).

A, 0 time

D-Glucose, 7.225

B, 24 hr

D-Glucose, 7.217

D-Sorbitol, 8.242

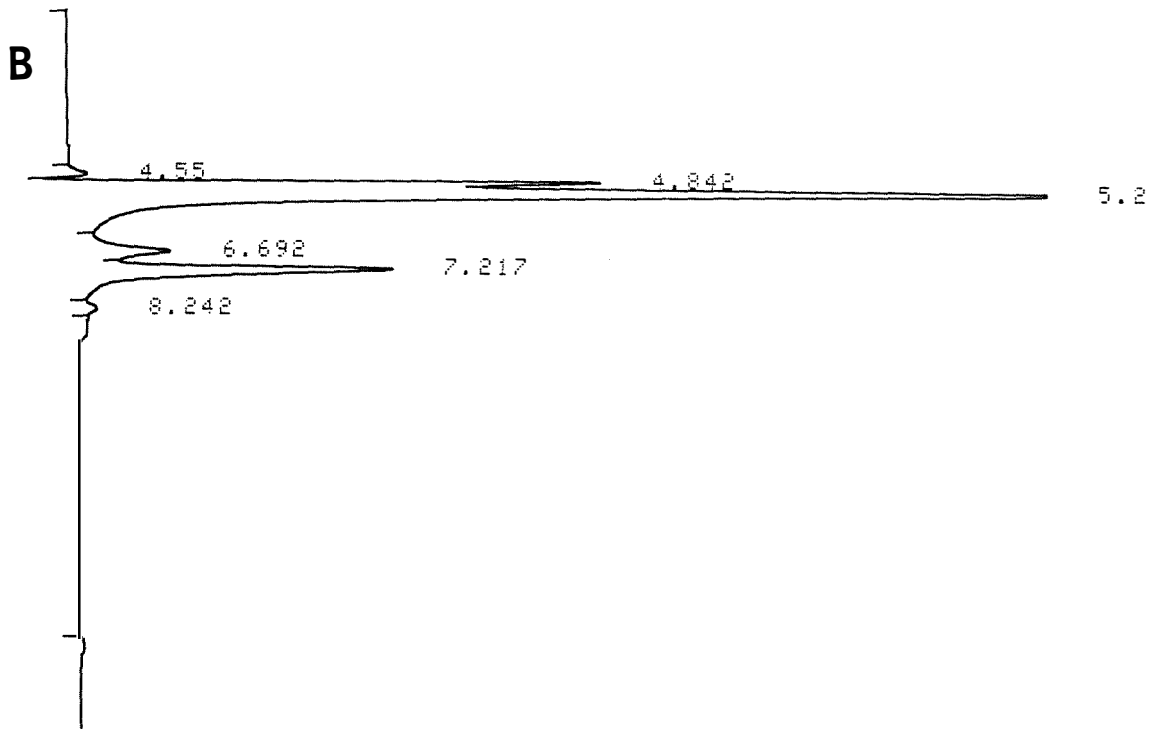
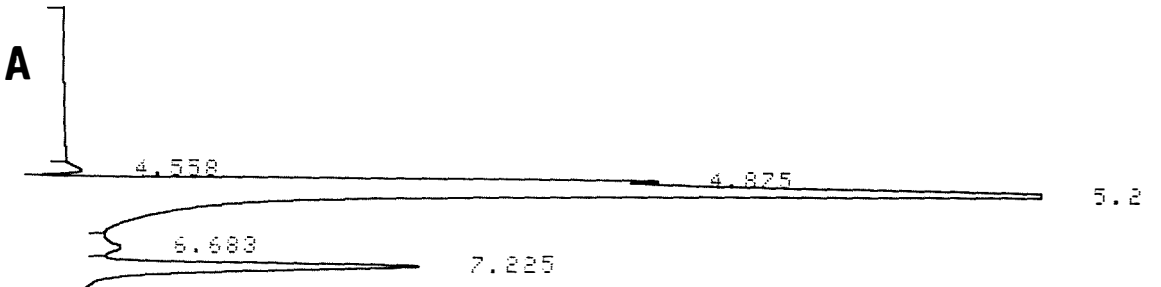


Plate 13: HPLC Chromatograph: Conversion of D-Sorbitol to D-Fructose by Cell-Free Extract from D/X A (NAD).

A, 0 time

D-Sorbitol, 8.242

B, 24 hr

D-Fructose, 7.792

D-Sorbitol, 8.233

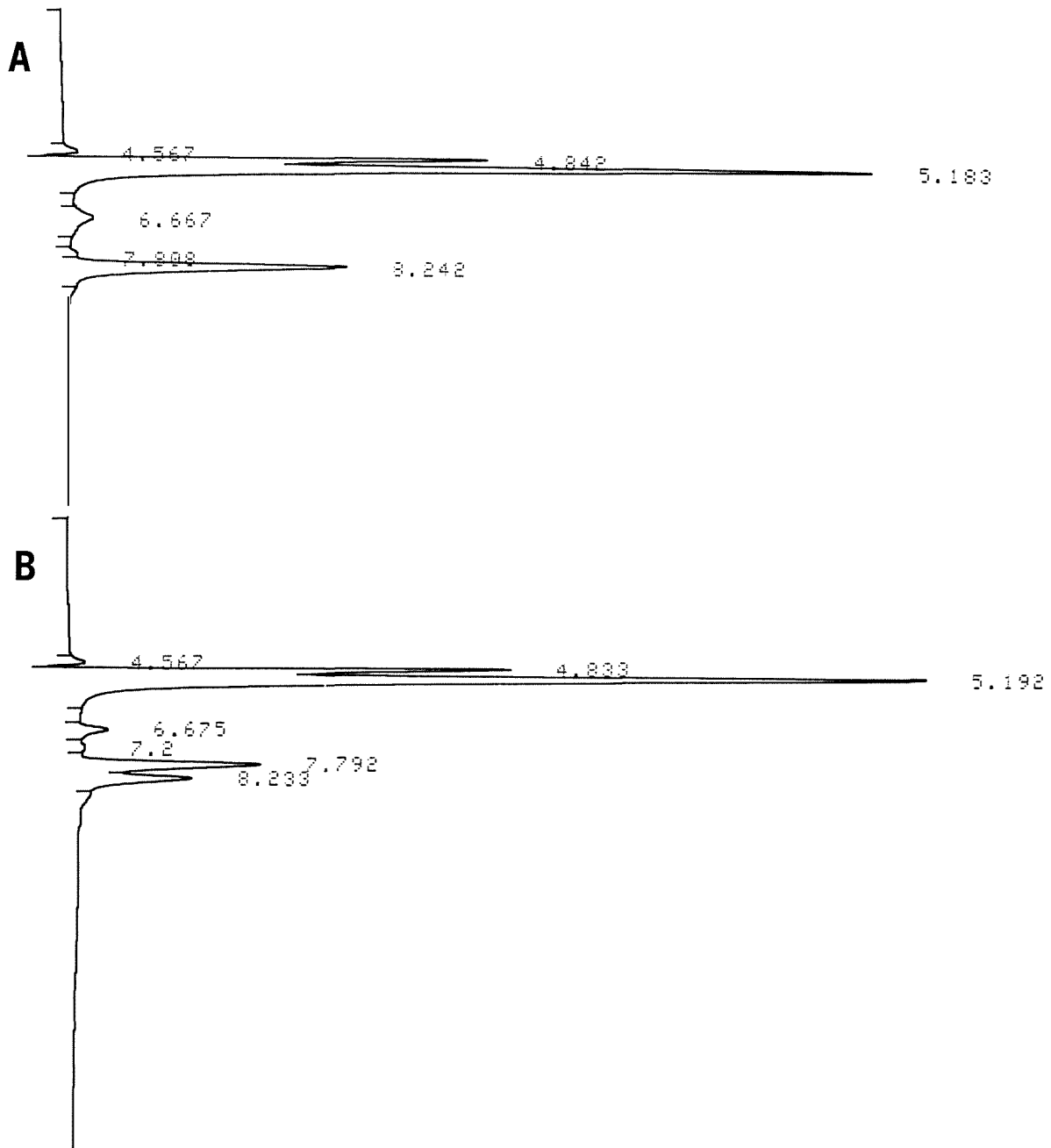


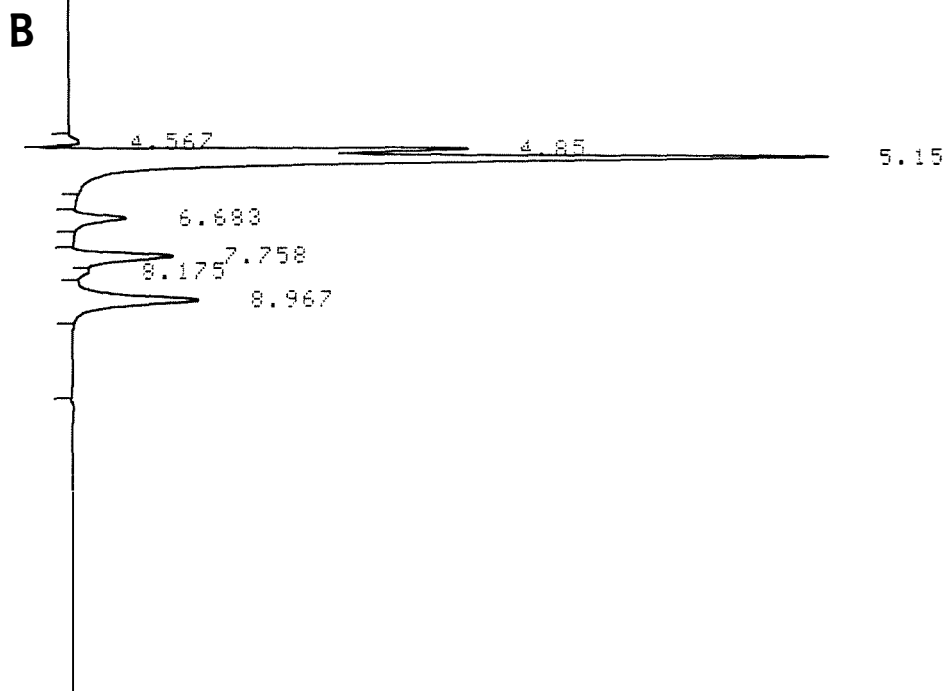
Plate 14: HPLC Chromatograph: Conversion of D-Xylose to Xylitol by Cell-Free Extract From D/X A (NADPH).

A, 0 time

D-Xylose, 7.758

B, 24 hr

D-Xylose, 7.758
Xylitol, 8.967



on the identities, the retention times of these end-products were compared to retention times of known standards (Table XI). The retention times in the chromatographs shown in plates 11-14 of tentative D-fructose was 7.792 and 7.767; D-sorbitol, 8.233 and 8.242 and xylitol, 8.967. These values were similar to the retention times given in Table XI.

9. GENETIC IMPROVEMENT OF *P. TANNOPHILUS*

9.1 Hexose-Negative Mutants

Mutants exhibiting different growth rates on D-fructose were obtained following UV mutagenesis of strain P509-1B, as described in Materials & Methods 11.1. Two of these mutants grew more slowly on D-fructose; P509-1B # 6 was completely negative for growth on D-fructose, while P509-1B # 12 grew very slowly on this carbon source. A third mutant (P509-1B #22) grew more rapidly on D-fructose compared to P509-1B. Only the P509-1B #6 strain was investigated further in this study.

Two independent isolates (designated D/X A and D/X B) were obtained when P509-1B was cultured in YNB-xylose (2% w/v) supplemented with 40 mM 2-deoxyglucose as described in Materials & Methods 11.2. Both strains isolated behaved identically when inoculated into YNB-glucose/xylose. The D/X A strain was used in further studies and has been described in this thesis.

A hexose-negative mutant capable of complete utilisation of D-xylose in the presence of D-glucose was selected by

Table XI: Retention Times of Sugars and Sugar Alcohols
on the Bio-Rad Aminex HPX-87H Column

Compound	Retention Time (mins)
D-Glucose	7.22 +/- 0.02
D-Fructose	7.79 +/- 0.03
D-Xylose	7.76 +/- 0.01
D-Sorbitol	8.24 +/- 0.02
Xylitol	8.95 +/- 0.02

Retention times are stated as the average
from 4-6 independent chromatographs, +/- standard
deviation

use of the selection scheme described in Materials & Methods 12.1. The rationale behind adopting this selection scheme, was based on the observation that when both P509-1B and D/X A were pre-cultured on glycerol, the initial growth rate upon transferral onto YNB-glucose/xylose was slower than the corresponding growth rate on YNB-xylose. By selecting for a more rapid growth on YNB-glucose/xylose, it was hoped that a strain no longer subject to D-xylose inhibition by D-glucose would be isolated. A total of six strains that grew faster on YNB-glucose/xylose plates than the D/X A strain were isolated and subjected to screening trials in YEP-glucose/xylose. Of these six strains, only two showed promising results. These were designated F/G 1 and F/G 2. Only the latter strain was sufficiently better than the D/X A to warrant use in fermentation trials. The behaviour of this strain in YNB-glucose/xylose is shown in Figure 30. D-Glucose utilisation was minimal, but D-xylose utilisation was still inhibited and ceased after 48 hr. However, complete utilisation of D-xylose occurred in YEP-glucose/xylose medium (Figure 32).

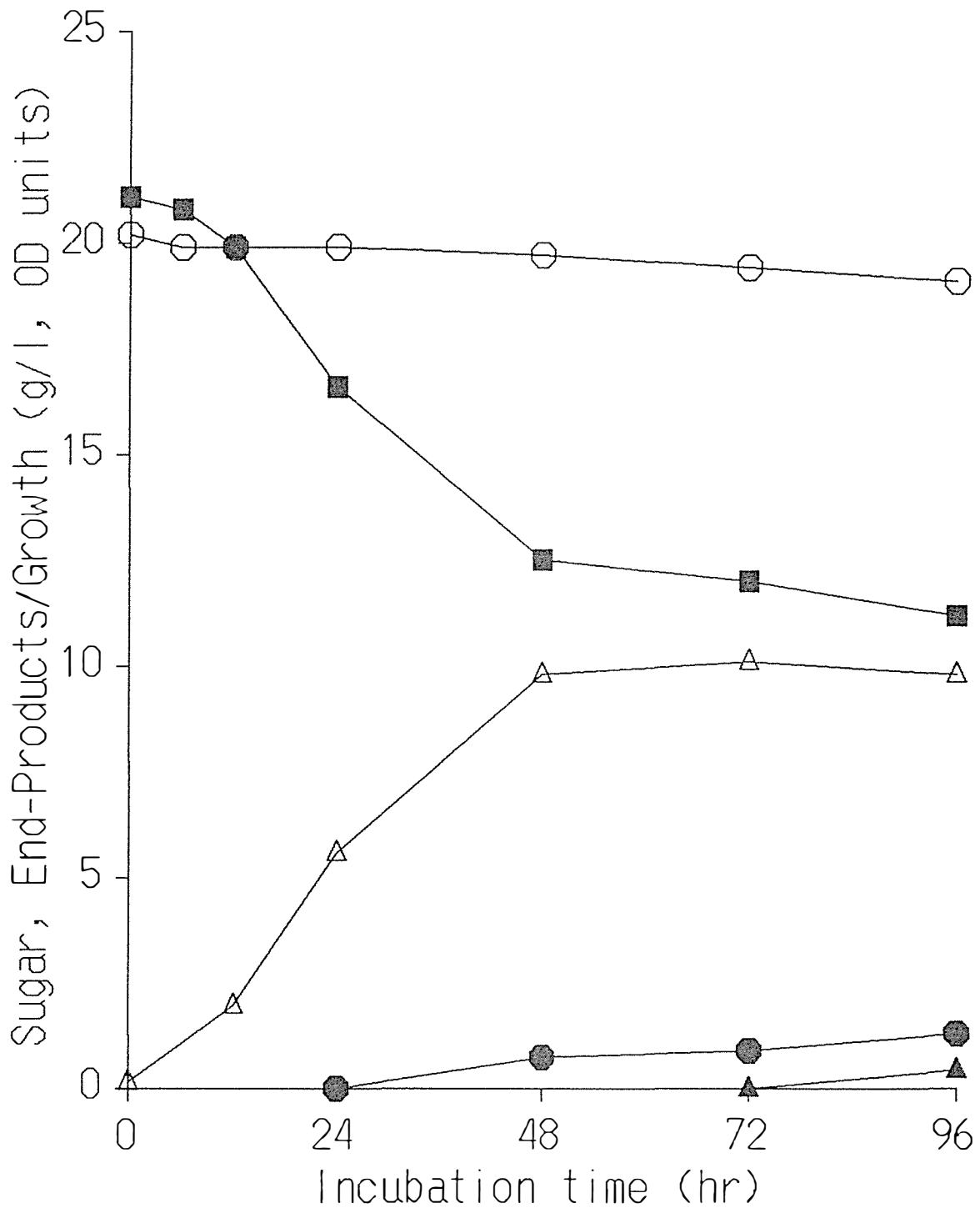
Two ethanol-negative derivatives of F/G 2 were obtained by UV mutagenesis and selection for absence of growth on YNB-ethanol plates (Materials & Methods 12.2). These strains, designated F/G 2 #24 and F/G 2 #30, grew on D-xylose at a similar rate to F/G 2 but growth on ethanol was greatly reduced. The former strain was included in the fermentation experiments described in Results Section 10.

Fig. 30: Time Course of Strain F/G 2 in YNB-glucose/
xylose (2%:2% w/v).

○ , D-glucose; ■ , D-xylose;

● , D-fructose; ▲ , ethanol;

△ , growth



9.2 Hybridisation of the *Eth2-1* Strain with $\text{NO}_3\text{-NO}_3\text{-4}$

Table XII summarises the cross between the *eth2-1* strain and the backcrosses between segregants carrying the *eth2-1* gene with the mutant $\text{NO}_3\text{-NO}_3\text{-4}$. After three backcrosses, a total of 8 *eth2-1* segregants (P727), without auxotrophic markers, were isolated. These strains were subjected to fermentation trials at the Forest Research Institute, in both synthetic D-xylose media and in wood hydrolysates. Some of these results are presented in the paper in the Appendix.

10. FERMENTATION TRIALS

Several hexose-negative mutants were subjected to fermentation trials employing 'semi-aerobic', batch fermentation conditions' (Materials & Methods 3.1). Fermentations were carried out in both YNB and YEP media in duplicate cultures. Maximum ethanol concentrations in the media (g l^{-1}) and yields ($\text{g ethanol produced (g D-xylose or sugar consumed)}^{-1}$) were averaged and reported in Table XIII. Figures 31 & 32 show the time course of the fermentations of D-glucose/D-xylose by wild type, P444-3D and F/G 2, respectively.

Strains D/X A and F/G 2 accumulated more ethanol and gave higher yields than the wild type strain on both YNB-xylose and YEP-xylose. Both the wild type strain and the mutants produced higher concentrations and yields of ethanol in the YEP medium compared to the YNB medium. Higher ethanol concentrations and yields were produced by mutants

Table XII: Summary of Crosses Between the *eth2-1* Strain and $\text{NO}_3\text{-NO}_3\text{-4}$

Cross/ Backcross	Parental Strains	Diploid
Cross ¹	$\text{NO}_3\text{-NO}_3\text{-4 } lys$ x P446-27A <i>eth2-1 adel</i>	P581 ²
1	$\text{NO}_3\text{-NO}_3\text{-4 } lys$ x P581-3A <i>eth2-1 adel</i>	P664 ²
2	$\text{NO}_3\text{-NO}_3\text{-4 } lys$ x P664-6C <i>eth2-1 adel</i>	P723 ²
3	$\text{NO}_3\text{-NO}_3\text{-4 } lys$ x P723-5D <i>eth2-1 adel</i>	P727 ²

1 Initial cross performed by Allen James

2 Segregation of the auxotrophic markers and growth on ethanol was 2:2

Table XIII: Maximum Ethanol Concentrations and Yields from the Fermentation of YNB and YEP-Xylose and YNB and YEP-Glucose/Xylose

Strain	Maximum Ethanol Conc. (g l ⁻¹)/ Yield (g (g D-xylose consumed) ⁻¹)							
	D-Xylose				D-Glucose/D-Xylose			
	YNB		YEP		YNB		YEP	
	g l ⁻¹	g g ⁻¹	g l ⁻¹	g g ⁻¹	g l ⁻¹	g g ⁻¹	g l ⁻¹	g g ⁻¹
P444-3D	1.10	0.054	3.00	0.17	3.60	0.17	10.60	0.26
P509-1B	0.87	0.049	ND	ND	6.62	0.19	ND	ND
D/X A	1.60	0.10	3.40	0.22	0 ¹	0 ¹	0 ¹	0 ¹
F/G 2	2.80	0.14	4.70	0.25	0.04	0.034	4.10	0.19
F/G 2 #24	ND	ND	1.90	0.11	ND	ND	4.20	0.20

ND not determined

1 none detected

D/X A and F/G 2 on YEP-xylose, compared to the wild type strain (3.40 g l^{-1} , 0.22 g g^{-1} ; 4.70 g l^{-1} , 0.25 g g^{-1} compared to 3.00 g l^{-1} and 0.17 g g^{-1}). For strains D/X A and F/G 2, this was 43% and 51% of the theoretical maximum ethanol yield. These values compared favourably to the wild type, which gave 31% of the theoretical maximum. Strain F/G 2 #24 accumulated considerably less ethanol at a lower yield than the wild type or the F/G 2 strain during the fermentation of D-xylose in YEP media.

Strain P509-1B produced greater amounts of ethanol compared to the wild type strain on the YNB-glucose/xylose mixture (6.62 g l^{-1} compared to 3.60 g l^{-1}). The ethanol yield was also slightly greater (0.19 g g^{-1} compared to 0.17 g g^{-1}). The maximum concentration and yield of ethanol by the mutants, D/X A, F/G 2 and F/G 2 #24 were lower than the wild type for the fermentation of the D-glucose/D-xylose mixture. No ethanol was produced by strain D/X A during the fermentation of D-glucose and D-xylose in either YNB or YEP media. This correlated with the incomplete utilisation of the D-xylose, when D-glucose was present. The mutant F/G 2 produced little ethanol from the fermentation of D-glucose and D-xylose in YNB (Figure 30) but greater yields were obtained in the YEP media (Figure 32). A maximum ethanol concentration and yield of 4.10 g l^{-1} and 0.19 g g^{-1} respectively, was obtained (Table XIII). At the point of maximum ethanol concentration, only 3.0 g l^{-1} D-glucose had been consumed, 2.2 g l^{-1} accounted for by the conversion to D-fructose (Figure 32).

Fig. 31: Fermentation of D-Glucose and D-Xylose (2%:2% w/v) in YEP Medium by Wild Type Strain, P444-3D.

○ , D-glucose; ■ , D-xylose; ▣ , xylitol;
▲ , ethanol; △ , growth

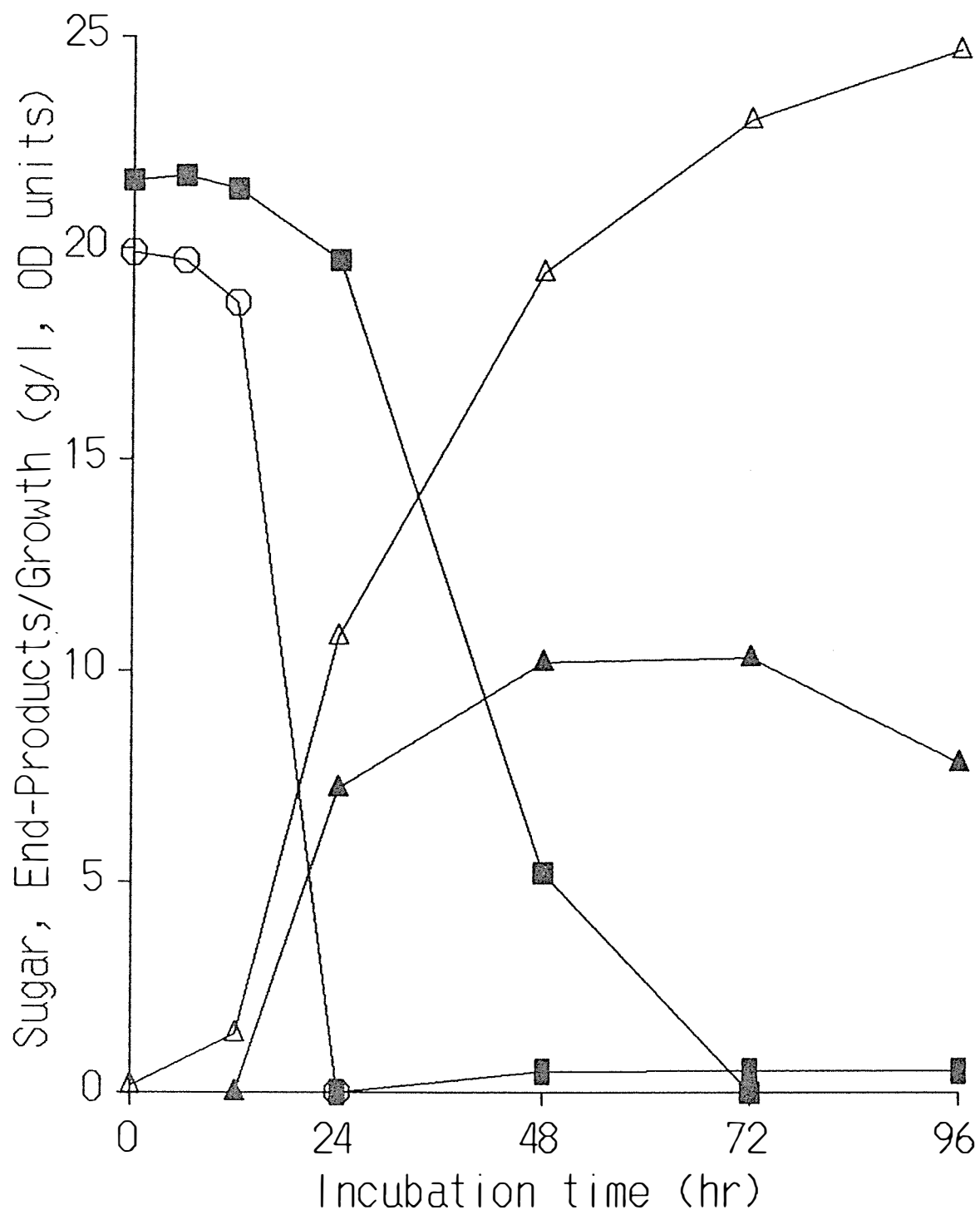
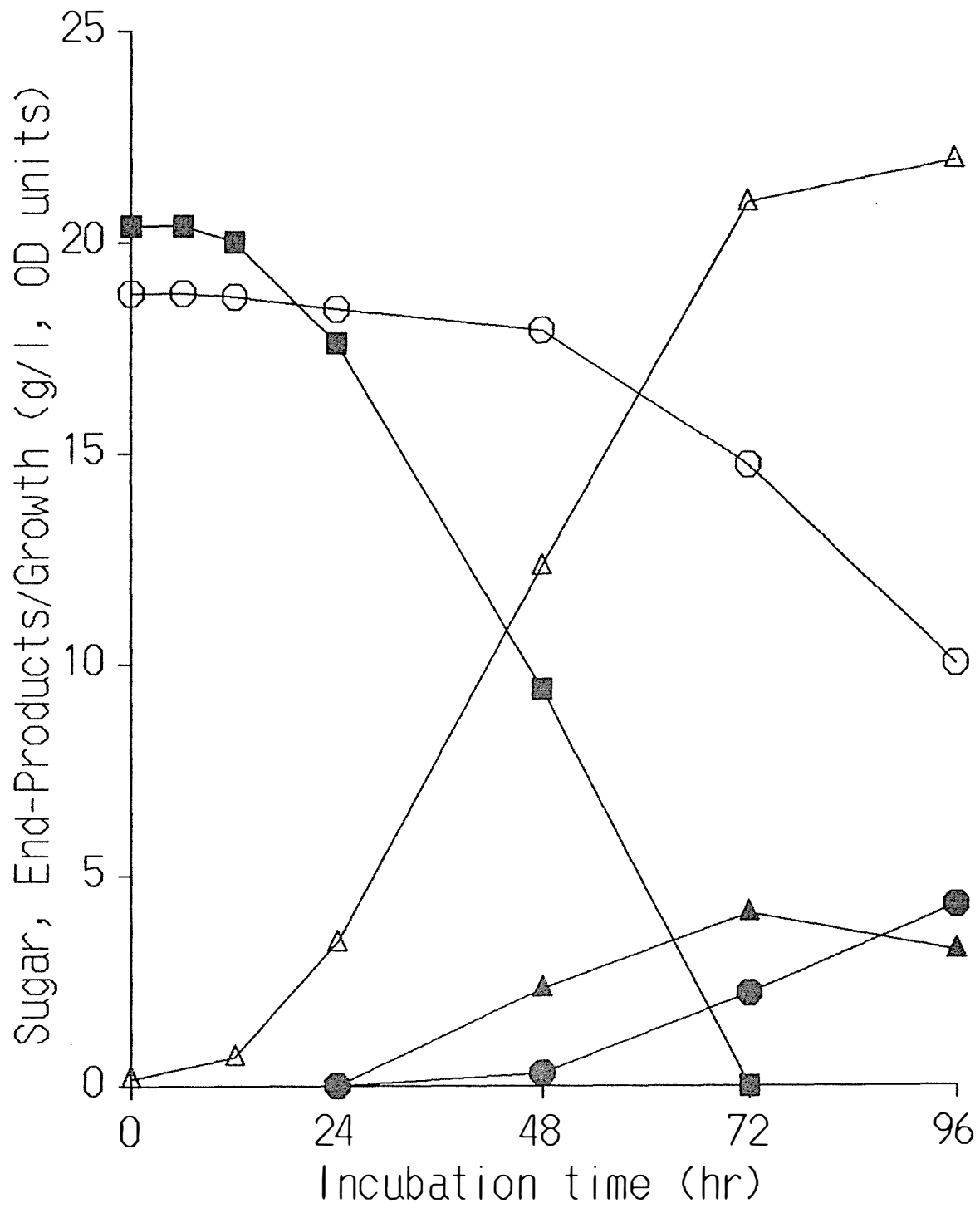


Fig. 32: Fermentation of D-Glucose and D-Xylose (2%:2% w/v) in YEP Medium by Strain F/G 2.

○ , D-glucose; ■ , D-xylose;
● , D-fructose; ▲ , ethanol; △ , growth



Strain F/G 2 #24 was marginally better than F/G 2, with a maximum concentration and yield of 4.20 g l^{-1} and 0.20 g g^{-1} , respectively (Table XIII).

Xylitol accumulation was also measured during the fermentation of D-xylose and D-glucose/D-xylose. The wild type produced more xylitol than the D/X A and F/G 2 mutants in YNB-xylose (maximum concentrations of xylitol were 4.40 , 3.33 and 2.60 g l^{-1} for these strains, respectively). The lower amounts of xylitol accumulated by the mutants correlated with the improved ethanol production and yields. A similar trend was found with the YEP-xylose media. Maximum xylitol accumulation was 1.60 and 0.95 g l^{-1} for the wild type and F/G 2 strains respectively. During the fermentation of YEP-glucose/xylose, the wild type produced 0.55 g l^{-1} , while F/G 2 produced only 0.015 g l^{-1} .

DISCUSSION

1. GROWTH CHARACTERISTICS, ENZYME ACTIVITIES AND RESISTANCE TO 2-DEOXYGLUCOSE OF MUTANTS

A number of mutants of *Pachysolen tannophilus* have been isolated by Allen James and during this study by selecting for resistance to 2-deoxyglucose and mutagenesis with UV light. Several of these mutants were characterised by their growth on different carbon sources, resistance to 2-deoxyglucose and their hexose-ATP-kinase activities. All these mutants, with the exception of strain P510-5A, were phenotypically different in growth compared to the wild type strain, P444-3D.

Genetic analyses conducted by Allen James, suggested that the segregants, P510-5A and P509-3C, were each defective at one independent locus, while strain P509-1B was defective at two independent loci. The latter strain was negative for growth on D-glucose and initially it was thought to be unable to grow on D-fructose as well. In this study, a further mutation designated as *h_xk1* was introduced into P509-1B and the resultant mutant, D/X A, was phenotypically different from the P509-1B strain. The D/X A strain was unable to grow on both D-glucose and D-fructose.

When strains of *Saccharomyces cerevisiae*, carrying one hexose-phosphorylating enzyme, were subjected to the selective pressure of 2-deoxyglucose, the majority of resistant mutants were defective in that enzyme (Lobo & Maitra, 1977a). The resistance to 2-deoxyglucose,

conferred on the glucose-negative mutants isolated by Allen James, was presumed to be the consequence of mutations occurring within the genes coding for or regulating the hexose-phosphorylating enzymes. Results in this study have confirmed this to be correct.

Strains P510-5A and P509-3C, presumed to be defective in one hexose-phosphorylating enzyme, in general exhibited little or no reduction of growth or sugar utilisation on the three hexose sugars tested (Table IV and Results Section 2.1 and 2.2). The exception was P509-3C, which grew extremely slowly on D-mannose and utilised this sugar only after an initial lag period. In comparison, strains presumed defective in two hexose-phosphorylating enzymes, showed greatly diminished growth or complete lack of growth on the hexose sugars.

Strain P509-1B did not grow on D-glucose, nor utilise D-glucose, when inoculated at the standard cell density into medium containing this sugar as the sole carbon source (Figure 7). Slow growth on D-glucose and utilisation of D-glucose was observed by inoculating media at the higher cell density (Figure 14). Although hexose-ATP-kinase activity was low in this strain, there was a measureable level (Tables V & VI). In comparison, hexose-ATP-kinase activity was almost completely lacking in the subsequently isolated D/X A strain, which did not exhibit any growth on D-glucose, even when inoculated into media at the higher cell density (Figure 14). Thus, on the basis of these results, it seemed possible that the mutant P509-1B was able to grow on D-glucose because of the existence of a

third hexose-phosphorylating enzyme, which presumably would be non-functional or absent in strain D/X A. This contrasted with the initial interpretation that P509-1B was negative for growth on D-glucose on solid media, due to defects in the two hexose-phosphorylating enzymes that were present in this yeast. This existence of a third enzyme was unrealised at that stage.

The hexose-ATP-kinase activities in the mutants were measured and the results correlated with the observed growth pattern on the hexose sugars. These results, together with the chromatography profiles (Results Section 4), confirmed that mutations within the hexose-ATP-kinase genes were responsible for the diminished growth on the hexose sugars and the increase in resistance to 2-deoxyglucose. Thus strains P510-5A and P509-3C, defective in one hexose-phosphorylating enzyme (glucokinase and hexokinase A, respectively), were still capable of growth on the hexoses. These strains possessed a functional hexokinase A and glucokinase respectively, in addition to hexokinase B. However, mutations within both hexokinase A and glucokinase greatly diminished the ability of a strain to grow on hexose sugars. Growth was still possible by virtue of the existence of hexokinase B, but was slow, particularly on D-glucose and D-mannose. In contrast to the original observation, P509-1B was shown to be able to grow slowly on D-fructose. The presence of hexokinase B also explains the ability of this strain to grow slowly on this sugar, since the kinetic properties possessed by this enzyme permit faster phosphorylation of D-fructose than D-

glucose (Results Section 3 & 4.1). The mutations which affected the hexose-phosphorylating activity did not affect the ability of strains to grow on D-xylose, D-galactose or glycerol. All the mutants grew equally well on these carbon sources as the wild type strain.

Although the levels of 2-deoxyglucose at which the original resistant mutants isolated by Allen James were known, the resistance of the segregants had not been determined. The resistance of each mutant used in this study was determined on solid YNB-xylose supplemented with the analogue. The wild type strain was inhibited in the presence of 4 mM 2-deoxyglucose (Table III). Strains defective in either hexokinase A or the glucokinase exhibited some increase in resistance to the analogue. Strain P509-3C was able to grow in the presence of 4 mM 2-deoxyglucose and was considered resistant at this level. However, strain P510-5A exhibited only a slight increase in resistance compared to the wild type strain. The double and triple mutants, P509-1B and D/X A, were resistant at the higher levels of 20 mM and 120 mM, respectively. Therefore, in a strain defective in all three hexose-phosphorylating enzymes, the analogue had virtually no toxic effect; resistance to the analogue correlating with an almost complete loss of hexose-ATP-kinase activity.

Some differences in 2-deoxyglucose sensitivity were found between *Pachysolen tannophilus* and *Saccharomyces* species. *S. cerevisiae* and *S. diastaticus* strains did not grow at 1 mM 2-deoxyglucose (Jones *et al.*, 1986), while Maitra (1970)

reported that *S. cerevisiae* failed to grow in a salt-vitamin-galactose medium supplemented with 0.3 mM of the analogue. In comparison, wild type *P. tannophilus* was resistant at 2 mM and was capable of diminished growth at 4 mM 2-deoxyglucose. The biochemical basis of this apparently higher intrinsic resistance to the analogue is unknown.

From the combined data in Results Section 1-4 it was concluded that, in *Pachysoles tannophilus*, there exists three hexose-phosphorylating enzymes, hexokinase A and B and a glucokinase. Loss of both hexokinase A and the glucokinase, greatly reduced the ability of a strain to grow on D-glucose, D-fructose and D-mannose but a third enzyme, hexokinase B, did permit growth on these sugars albeit at a greatly reduced rate.

The behaviour of these glucose-negative mutants when grown in media containing both D-glucose and D-xylose as the carbon sources was investigated. The wild type strain, P444-3D, utilised D-glucose preferentially, repressing D-xylose utilisation until almost all the D-glucose was depleted (Figures 9 & 31). A similar pattern of sugar utilisation in mixed sugars has been reported for *P. tannophilus* (Detroy *et al.*, 1982; Slininger *et al.*, 1987; Bicho *et al.*, 1988).

Strain P510-5A, defective in the glucokinase, behaved similarly to the wild type strain; D-glucose was utilised preferentially over the D-xylose. A different pattern of sugar utilisation was found with P509-3C which was

defective in hexokinase A and the double mutant P509-1B. Both these strains could utilise D-glucose and D-xylose simultaneously. D-glucose utilisation by P509-1B, although initially slow, was faster than the D-xylose utilisation, which was inhibited (Figure 10). This utilisation of D-glucose can be explained by the presence of hexokinase B, which permitted slow metabolism of the D-glucose.

When the P509-1B mutant was first isolated, it was hoped that this strain could be used as a glucose-negative strain for the fermentation of a mixture of sugars in wood hydrolysates. Such a strain could be used to ferment the pentose sugars to ethanol, leaving the hexose sugars to be fermented in a second stage by a more ethanol tolerant yeast such as *S. cerevisiae*. The results of initial trials conducted on this strain by Tom Clark and Kay Deverell at the Forest Research Institute showed that D-glucose was consumed in a D-glucose/D-xylose mixture. Several different hypotheses were proposed to explain this observation. The most significant of these hypotheses was similar to the scheme outlined in Discussion Section 6 and Figure 33. This scheme was first proposed by Hung Lee, University of Guelph, Ontario, Canada (personal communication). It was suggested that the D-glucose consumption was due to the sugar being reduced to D-sorbitol and subsequently oxidised to D-fructose. The xylose pathway enzymes, xylose reductase and xylitol dehydrogenase were responsible for the catalysis of these reactions. However, since D-xylose is required for the induction of xylose reductase and xylitol dehydrogenase

(see Discussion Section 4), this scheme did not explain the consumption of D-glucose in the mixture of D-glucose and glycerol (Results Section 1.3).

The results from trials in which D-glucose utilisation was followed at high cell density further supported the view that D-glucose utilisation in mixed sugars by strain P509-1B (Figure 10) was due to the presence of hexokinase B. The fact that no growth nor D-glucose utilisation was observed in YNB-glucose inoculated at the standard cell density, can be readily explained in terms of population size. At the inoculated cell density of 2.5×10^7 cells ml^{-1} , utilisation of D-glucose in the medium was too slow to observe any significant decrease in D-glucose concentration during the 96 hr period. Similarly, the rate of growth was too slow to observe any significant increase in optical density. However, inoculation of cells into medium containing D-glucose, at the higher cell density of 5.0×10^8 cells ml^{-1} permitted observation of a decrease in D-glucose concentration and a slow increase in optical density during the incubation period. Although the rate at which individual cells metabolise D-glucose remains the same in both cases, the larger population can collectively utilise D-glucose at a rate sufficient to be measurable.

2. HEXOSE-ATP-KINASE ENZYMES IN *P. TANNOPHILUS*

Anion-exchange chromatography has often been applied to the isolation and purification of enzymes. In this study, cell-free extracts from the wild type strain and each of

the five mutants were chromatographed on DEAE-cellulose. The chromatographic profiles confirmed the existence of three hexose-phosphorylating enzymes in *P. tannophilus*, two hexokinases and one glucokinase.

The hexokinases in *S. cerevisiae* have been shown to be extremely sensitive to proteolytic modification by proteases. However, the modified enzymes can retain a high degree of catalytic activity. Modified forms of the PI and PII isoenzymes known as SI and SII required sodium chloride for elution following DEAE-cellulose chromatography with a pH gradient (Trayser & Colowick, 1961). The S forms were subsequently shown to be derived from the P forms by N-terminal cleavage of an undeca peptide (Schmidt & Colowick, 1973). Ramel *et al.* (1971) reported the existence of a modified form of hexokinase B (PII) designated as hexokinase D, following chromatographic elution of cell-free extracts on DEAE-cellulose with a pH gradient. Rustrum *et al.* (1971) reported the presence of a protease in cell extracts which can cleave hexokinases into small fragments of 26,000 molecular weight as determined by sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis. This protease can bind in latent form to purified hexokinases and can become activated in various denaturing media such as solutions containing SDS.

Proteolytic modification of both hexokinase A and B was apparent during the chromatography of cell-free extracts in this study. Both these enzymes showed variations in the chromatographic profiles obtained. The glucokinase

appeared less susceptible to proteolysis, since the shape of the peak and position of elution on the gradient was not influenced by the method of preparation of the cell-free extract.

Chromatography was initially performed on cell-free extracts that had been dialysed overnight in buffer. Evidence of proteolytic modification of both hexokinase A and B, prompted the use of rapid filtration of cell-free extracts on Sephadex G-25. When gel filtration on Sephadex G-25 instead of dialysis was used to remove salt, the formation of hexokinase D was prevented (Ramel *et al.*, 1971). The concentration of the serine protease inhibitor routinely used in the extraction buffer was increased from 0.2 mM to 2 mM to further minimise any proteolysis. Protease inhibitors have been successfully used to inhibit some of the proteases that degrade hexokinases in *Saccharomyces cerevisiae*. Bernard (1975) reported the successful prevention of proteolytic modification, by rapidly processing French press extracts by filtration on Sephadex G-25. The protease inhibitor diisopropyl fluorophosphonate (DFP) was added to the buffers before mechanical disruption of the cells and before the filtration step. The extract was then processed immediately by chromatography on DEAE-cellulose. Ramel *et al.* (1971) also included DFP in the buffers during homogenisation of the yeast and in subsequent processing of the cell-free extracts. The protease inhibitor phenylmethanesulphonyl fluoride (PMSF) has also been reported to be successful in

preventing proteolytic modification (Schulze *et al.*, 1966) and has the advantage of being less toxic.

When the wild type strain, P444-3D, was chromatographed, three enzymes, hexokinases A and B and a glucokinase, eluted with a salt gradient (Figure 16 & 22). Separation of the glucokinase and hexokinase A was only possible when the cell-free extract was desalted by filtration. Presumably, less modification of the hexokinase A had occurred and the major portion of the enzyme eluted at a slightly higher salt concentration than the glucokinase.

Each of the three hexose-phosphorylating enzymes exhibited different properties, eluting at a different position on the gradient correlating with a specific buffer conductivity. Each enzyme also exhibited a different F/G ratio at the 15 mM concentration of substrate used in the enzyme assays. This latter characteristic distinguished the glucokinase and the hexokinase A which eluted as the major peaks in cell-free extracts prepared from wild type cells. Although both of these enzymes eluted closely (Figure 22) or co-eluted (Figure 16), there were large differences in the measured F/G ratios.

Mutants P510-5A and P509-3C had lost the glucokinase and hexokinase, respectively. Hence chromatography of cell-free extracts from P510-5A and P509-3C (Figures 17 & 18) revealed the presence of only hexokinase A and the glucokinase, respectively, together with smaller amounts of hexokinase B. Thus in these chromatographs, it was possible to more accurately determine the F/G ratios of

hexokinase A and the glucokinase. The glucokinase exhibited negligible activity with D-fructose as a substrate, whereas hexokinase A phosphorylated both D-fructose and D-glucose at a F/G ratio of 1.3/1.0. This later value was also obtained with the purified hexokinase A (Results Section 6.2).

In *Saccharomyces cerevisiae*, the two hexokinases, PI and PII (Colowick, 1973) and the glucokinase (Maitra, 1970) have been conveniently separated on hydroxyapatite (Gancedo *et al.*, 1977; Fernández *et al.*; 1984, Fernández *et al.*, 1985). In this study hydroxapatite was tried as a chromatographic material for separation of the enzymes from *P. tannophilus*, but higher resolution was found on DEAE-cellulose.

Results in this study showed that in *Pachysolen tannophilus*, there are also two hexokinases and one glucokinase present. Both the hexokinases phosphorylate D-fructose and D-glucose, while the glucokinase is specific for D-glucose. The two hexokinases differ widely in their F/G ratios, 3.0/1.0 and 1.3/1.0, at the substrate concentration used in the assay and in this respect are analogous to the PI and PII isoenzymes. The reported values for the F/G ratios of the hexokinases in *S. cerevisiae* are in the range 2.5-3.5/1.0 for PI and 1.0-1.5/1.0 for PII. (Ramel *et al.*, 1971; Colowick, 1973; Entian & Mecke, 1982; Fröhlich *et al.*, 1984; Fernández *et al.*, 1985).

Differences in enzyme profile were found between wild type cells that were grown on D-glucose and those grown on either D-xylose or glycerol. Hexokinase A and the glucokinase contributed the major hexose-phosphorylating activity in wild type cells grown on D-glucose (Figure 16 & 22). A lower level of hexokinase A was present in cells which had been grown on either D-xylose or glycerol (Figures 23 & 24). These results agreed with the earlier observation that both a lower hexose-phosphorylating activity and F/G value was present in cells cultivated on D-xylose in comparison to D-glucose grown cells. A similar situation was found in *Candida tropicalis* by Hirai *et al.* (1977); cells cultivated on either ethanol or acetate had lower D-glucose-phosphorylating activity and a lower F/G ratio in comparison to D-glucose grown cells. Since a greater proportion of hexokinase A was present when D-glucose was available, it seems likely that this enzyme is inducible by D-glucose. However, the enzyme was still present even in the complete absence of D-glucose, although at a reduced level. In comparison, the levels of hexokinase B and glucokinase were not affected by the carbon source on which the cells are grown. These enzymes, therefore, appear to be constitutive. In *S. cerevisiae*, the PI hexokinase and the glucokinase have been found to be constitutive, while the PII isoenzyme was inducible (Muratsubaki & Katsume, 1979; Fernández *et al.*, 1985). Similarly, hexokinase was induced in *C. tropicalis*, while a glucokinase was constitutive (Hirai *et al.*, 1977).

Hexokinase B eluted as a much smaller amount of activity in both wild type and mutants. In cells of strains P510-5A and P509-3C, defective in hexokinase A and glucokinase respectively, hexokinase B only contributed a small amount of the overall activity. If we can assume that the measured activity is an indication of the amount of enzyme present in the cell, hexokinase B was present at lower levels in comparison to the other two enzymes. Only small amounts of hexokinase B were present in P509-1B (Figure 19); higher levels being present in older cultures compared to exponential phase cells (Table VI). In order to chromatograph 5.0 units of D-glucose-phosphorylating activity, a total of 35.0 mg protein needed to be loaded onto the column. In comparison, in the wild type extract 15.0 units were present in 19.0 mg protein. Since this was the only hexose-phosphorylating enzyme present in the cell, its level was such that the cells could grow only very slowly on D-fructose and D-glucose. The faster growth on D-fructose in comparison to D-glucose correlated with the measured F/G ratio of hexokinase B, since D-fructose was phosphorylated about three times faster than D-glucose. The mechanism of control of hexokinase levels in the cell would be interesting to determine.

In *Saccharomyces cerevisiae*, mutants defective in the glucokinase and the PII hexokinase can still grow on D-glucose, D-fructose and D-mannose, due to the presence of PI (Maitra & Lobo, 1981; Lobo & Maitra, 1977b, 1977c). The PI isoenzyme was found to constitute only 9% of the total D-glucose-phosphorylating activity (Fernández *et al.*, 1985).

In a glucokinase-less and PII defective mutants, the level of PI may of course be higher. The analogous situation does not appear to be true in *P. tannophilus*, where the presence of hexokinase B alone does not permit significant growth on D-glucose. In *S. cerevisiae*, the glucokinase permits growth on D-glucose in the absence of hexokinase PI and PII; growth on D-fructose requires the presence of at least one hexokinase (Gancedo *et al.*, 1977; Lobo & Maitra, 1977b).

Hydroxyapatite chromatography of cell-free extracts from stationary phase wild type *S. cerevisiae* cultures revealed the presence of hexokinase PI and PII and the glucokinase (Gancedo *et al.*, 1977; Fernández *et al.*, 1985), whereas the PII constituted the major activity in growing cells (Gancedo *et al.*, 1977; Muratsubaki & Katsume, 1979; Fernández *et al.*, 1985). Comparisons between enzyme profiles of logarithm and stationary phase wild type *P. tannophilus* cultures showed some differences in the relative proportion of hexokinase A and glucokinase. A difference in the F/G ratio between exponential and stationary phase cells (Table VI) also suggested some differences in the enzymes between the two growth phases. However, in comparison with *S. cerevisiae*, these differences were minor.

The close similarity between P509-1B and P509-1B #6 suggested that the mutation introduced into P509-1B abolishing growth on D-fructose, did not significantly affect hexokinase B activity. The lower hexose-phosphorylating activity in the cell-free extract of P509-

1B #6 (Results Section 4.1) could account for the absence of growth on D-fructose. The nature of this lesion, designated as *fru*, has yet to be elucidated, but may be regulatory affecting hexokinase B levels within the cell.

3. KINETIC PROPERTIES OF HEXOKINASE A

Hexokinase A from a mutant of *Pachysolen tannophilus* defective in the glucokinase was partially purified and the K_m values determined (Results Section 6). Disc gel electrophoresis confirmed that the enzyme had been partially purified, only minor additional protein bands were visible even after 45 μ g of protein was electrophoresed (Plate 13). The F/G ratio at 15 mM concentration of substrate used in the assay reaction mixture was 1.32/1.00 and the K_m values were 0.36 for D-glucose and 2.26 for D-fructose (Table IX). Entian & Mecke (1982) and Fröhlich *et al* (1984) reported the F/G ratio of the *S. cerevisiae* PII hexokinase as between 1.2 and 1.5. Earlier reports determined the F/G ratio as 1.1 (Ramel *et al*, 1971) and 1.2 (Colowick, 1973). Entian *et al.* (1985) determined the K_m values for the PII hexokinase as 0.23 mM for D-glucose and 1.93 mM for D-fructose, while Maitra & Lobo (1981) reported a value of 0.2 mM for D-glucose and a somewhat higher F/G value of 1.9. Hence the purified hexokinase A from *Pachysolen tannophilus* has fairly similar kinetic properties to that of the PII isozyme. Hexokinase A is also similar in this respect to hexokinase I from *Candida tropicalis*, which has a k_m of 0.34 mM for D-

glucose and 2.2 mM for D-fructose and an F/G ratio of 1.8 (Hirai *et al.*, 1977).

The purified hexokinase A lost activity when incubated with D-xylose in the presence of MgCl₂ and ATP (Figure 26). Inactivation of the three hexose-phosphorylating enzymes of *S. cerevisiae* has been reported (Fernández *et al.*, 1984). Inactivation of PII was rapid, with almost complete loss of activity when the enzyme was incubated with 100 mM D-xylose for 60 min. (Fernández *et al.*, 1984). This inactivation, which was irreversible, was dependent on the presence of MgATP and involved phosphorylation of the protein (Fernández *et al.*, 1986). In comparison, the inactivation of hexokinase A found in this study was less rapid. Whether this reflects a difference between an enzyme that is present in a cell which has a D-xylose catabolic pathway and an enzyme present in cells which lack such a pathway, is unknown.

4. CATABOLITE REPRESSION IN *P. TANNOPHILUS*

Glucose or catabolite repression in yeast is a phenomenon in which D-glucose or closely related carbon sources such as D-fructose and D-mannose represses the synthesis of certain other enzymes (Gancedo & Gancedo, 1986), including those in the tricarboxylic acid cycle and glyoxylate bypass (Polakis & Bartley, 1965) and respiratory enzymes (Polakis *et al.*, 1965). Evidence suggests that catabolite repression in yeast differs from that in *Escherichia coli* and cyclic adenosine monophosphate (AMP) is not a direct

effector (Matsumoto *et al.*, 1983). The mechanism involved is complex in that genes subject to repression are controlled by several regulatory genes (Entian & Zimmermann, 1982; Gancedo & Gancedo, 1986).

In wild type *Pachysolen tannophilus* cells, D-xylose has been found by several researchers to be necessary for the induction of both xylose reductase and xylitol dehydrogenase (Smiley & Bolen, 1982; Maleszka *et al.*, 1983b; Bolen & Detroy, 1985; Bolen & Shepherd, 1985; Bicho *et al.*, 1988; this study, Results Section 5). In addition, both enzymes are repressed by D-glucose (Maleszka *et al.*, 1983b; 1985; Bicho *et al.*, 1988; this study, Results Section 5).

Xylose reductase and xylitol dehydrogenase were assayed in cell-free extracts prepared from wild type and mutant cells grown on either D-xylose or a mixture of D-glucose and D-xylose. D-Xylose was required for the induction of both enzymes, since little activity was found in cells grown on either D-glucose or glycerol (Table VII). Enzyme activity was repressed in the wild type cells when they were growing on media containing both D-glucose and D-xylose. Mutants, defective in hexokinase A, exhibited almost as much activity when grown in the sugar mixture as when cultured on D-xylose alone. Thus repression of these two enzymes was released. Strain P510-5A, although defective in the glucokinase, still maintained an active hexokinase A (Figure 17). Much lower xylose reductase and xylitol dehydrogenase activities were found in D-glucose/xylose grown cells of this strain compared to D-

xylose grown cells (Table VII). Hence it appeared that in this strain D-glucose repression of these two enzymes still operated. The 'co-utilisation' of D-glucose with D-xylose which was observed with mutants P509-3C, P509-1B and D/X A, presumably occurred because glucose repression no longer acted on the D-xylose catabolic pathway enzymes.

There is accumulating evidence to assign a role of the PII hexokinase of *Saccharomyces cerevisiae* in catabolite repression. Several classes of mutants, defective in glucose repression, have been isolated (Zimmermann & Scheel, 1977). The first class called *hex1* showed reduced hexokinase activity, D-glucose-phosphorylating activity was reduced by 50-60% (Entian *et al.*, 1977; Entian & Zimmermann, 1980). These mutants were no longer glucose repressible for invertase and maltase and only partially repressible for malate dehydrogenase and the respiratory enzymes (Entian *et al.*, 1977). The mutation responsible for loss of catabolite repression was found to be allelic to the structural gene for hexokinase PII (*HXK2*) (Entian, 1980). Further genetic and biochemical evidence supported the involvement of the PII isoenzyme with catabolite repression (Entian, 1980; Entian & Mecke, 1982). Gel electrophoresis of crude extracts demonstrated that the PII isoenzyme was absent in the *hex1* mutant. Genetic crosses between the mutant and the wild type confirmed that the inactive PII isoenzyme and the defect in carbon catabolite repression always co-segregated. (Entian & Mecke, 1982).

A second class of mutants designated *hex2*, had elevated PII isoenzyme activity due to increased synthesis (Entian &

Zimmermann, 1980). These mutants were no longer subject to carbon catabolite repression. The mutation responsible was shown to be a regulatory mutation in an allele controlling hexokinase PII, the synthesis of glucose-repressible enzymes and maltose uptake (Entian, 1981).

Another class of mutants were defective in the *CAT80* gene and showed normal PII activity (Entian & Zimmermann, 1980) but were defective in repression of invertase, maltase and malate dehydrogenase (Zimmermann & Scheel, 1977). A functional *CAT80* gene was found necessary for increased PII synthesis in the *hex2* mutants (Entian & Zimmermann 1982), indicating that the *CAT80* gene is another regulatory element involved in PII synthesis.

Entian (1981) and Entian & Mecke (1982) have postulated that hexokinase PII is the 'recognition site' of glucose repression which gives the triggering signal. This enzyme may be bi-functional with both a catalytic site and a regulatory domain (Entian & Fröhlich, 1984; Entian *et al.*, 1985). Mutants with defects in hexokinase PII (*hxx1^r*) have been isolated which were not subject to glucose-repression but still retained enzyme activity (Entian & Fröhlich, 1984). A modified hexokinase PII isoenzyme, PII^M, has been isolated and characterised (Kopetzki & Entian, 1985) and presumably is involved in catabolite repression. The introduction of null mutations into the PII gene (*HXX2*) has been shown to abolish glucose repression in three systems examined; the *SUC2* gene encoding invertase, the *CYC1* gene for iso-1-cytochrome c

and *GAL10* coding for an epimerase in the galactose-metabolising pathway (Hong & Botstein, 1986).

The complete role of the PII hexokinase in glucose repression has yet to be elucidated and may be more complex than expected. When D-xylose was added to *S. cerevisiae* cells in a chemostat, a loss of 98% hexokinase PII and 50% of the PI isoenzyme occurred (Fernández *et al.*, 1984). Simultaneously, invertase was derepressed. Fernández *et al.* (1987) concluded that the D-xylose induced phosphorylation of the PII hexokinase responsible for the loss of activity, produced a conformational change in the enzyme which may indirectly act as the triggering signal for derepression. A gene (*SNF1*) coding for a protein kinase has been found necessary for the expression of glucose-repressible genes such as *SUC2* (invertase) (Celenza & Carlson, 1986). This gene was shown to be the same as the *CCR1* gene (Ciriacy, 1977). Mutants defective in the *CCR1* gene were defective in derepression of isocitrate lyase, fructose-1,6-diphosphatase, alcohol dehydrogenase II and possibly cytoplasmic malate dehydrogenase (Ciriacy, 1977). Since this gene codes for a protein kinase, it has been suggested that protein phosphorylation is important in regulation of gene expression by carbon catabolite repression in yeast (Celenza & Carlson, 1986).

The results from enzyme assays conducted in this study suggested that, in *P. tannophilus*, hexokinase A might also play a role in catabolite repression. Mutants defective in the catalytic activity of this enzyme were no longer subject to D-glucose repression of either xylose reductase

or xylitol dehydrogenase. The hexokinase A isoenzyme has similar kinetic properties to the PII isoenzyme from *S. cerevisiae* as discussed previously and would appear to also have analogous functions.

Both the PI (Entian *et al.*, 1984; Kopetzki *et al.*, 1985; Stachelek *et al.*, 1986) and the PII hexokinase genes (Entian *et al.*, 1985; Fröhlich *et al.*, 1984, 1985; Stachelek *et al.*, 1986) from *Saccharomyces cerevisiae* have been cloned and sequenced. A further extension of this section of the work in this thesis might be to clone the hexokinase A gene and obtain sequence data, which then could be compared with the PI and PII sequences. Elucidation of DNA sequences essential for the regulatory role of hexokinase A in catabolite repression might be possible.

5. TRANSFORMATION OF *P. TANNOPHILUS*

Techniques have been recently developed for the transformation of yeast which do not necessitate the need to sphaeroplast (Iimura *et al.*, 1983; Ito *et al.*, 1983; Klebe *et al.*, 1983; Bröker *et al.*, 1987). The D/X A mutant, defective in all three hexose-ATP-kinases, was selected as a suitable strain for transformation with the YRp7 plasmid carrying the cloned PII hexokinase gene. This plasmid has the bacterial pBR322 ampicillin resistance gene and the yeast *TRP1* gene and *ARS1* sequence.

The attempts to transform *Pachysoles tannophilus* with this plasmid succeeded at a low frequency (Results Section 7). Exposure of the cells to LiCl for 2 1/2 hr gave better

yields compared to exposure for 1 hr. More experimentation with the conditions for transformation is required to maximise the yield of transformants. The use of more highly purified DNA or other plasmids may also give improved transformation frequencies. However this method for the transformation of *P. tannophilus* looks promising.

Successful transformation was supported by both genetic and biochemical evidence. All transformants obtained could grow on D-fructose and D-mannose as well as D-glucose (Results Section 7), growth being more rapid on D-fructose and D-glucose. This was the expected pattern of growth if the PII isoenzyme was present within the cells. One class of transformant was unstable; inheritance of the plasmid and the ability to grow on D-glucose was readily lost. Instability of the plasmid may also explain the relatively slow growth of the transformants on the hexose sugars. A second class was stable and may have arisen by integration of the plasmid into the genome. Transformation was confirmed by the presence of plasmid bands on an agarose gel following extraction of DNA from two of the transformants. This DNA was isolated using the convenient rapid boiling method of Holmes & Quigley (1981), after the cells had been sphaeroplasted. DNA from one of the transformants was then used to transform *E. coli* to ampicillin resistance, with subsequent isolation of plasmid DNA. This DNA showed both identical mobility with the YRp/HXK2-8 plasmid used in the initial transformation of *P. tannophilus* and the expected restriction pattern with *EcoRI* (Plate 10).

Chromatography of cell-free extracts from the yeast transformants revealed the presence of a single hexokinase with a 1.2/1.0 F/G ratio (Figure 29). This value was within the range of 1.2-1.5 reported for the PII hexokinase (Entian & Mecke, 1982; Fröhlich *et al.*, 1984). The activity of the enzyme on each substrate correlated with the growth of the transformants on the hexose sugars.

There was some evidence for catabolite repression in the transformants as the consequence of the presence of the PII isoenzyme within the cells. The utilisation of D-xylose in a D-glucose/D-xylose mixture was slower than the rate of D-xylose utilisation when present as a sole carbon source (Figure 28). This may be due to glucose repression of the xylose pathway enzymes.

6. ALTERNATIVE PATHWAY FOR GLUCOSE UTILISATION

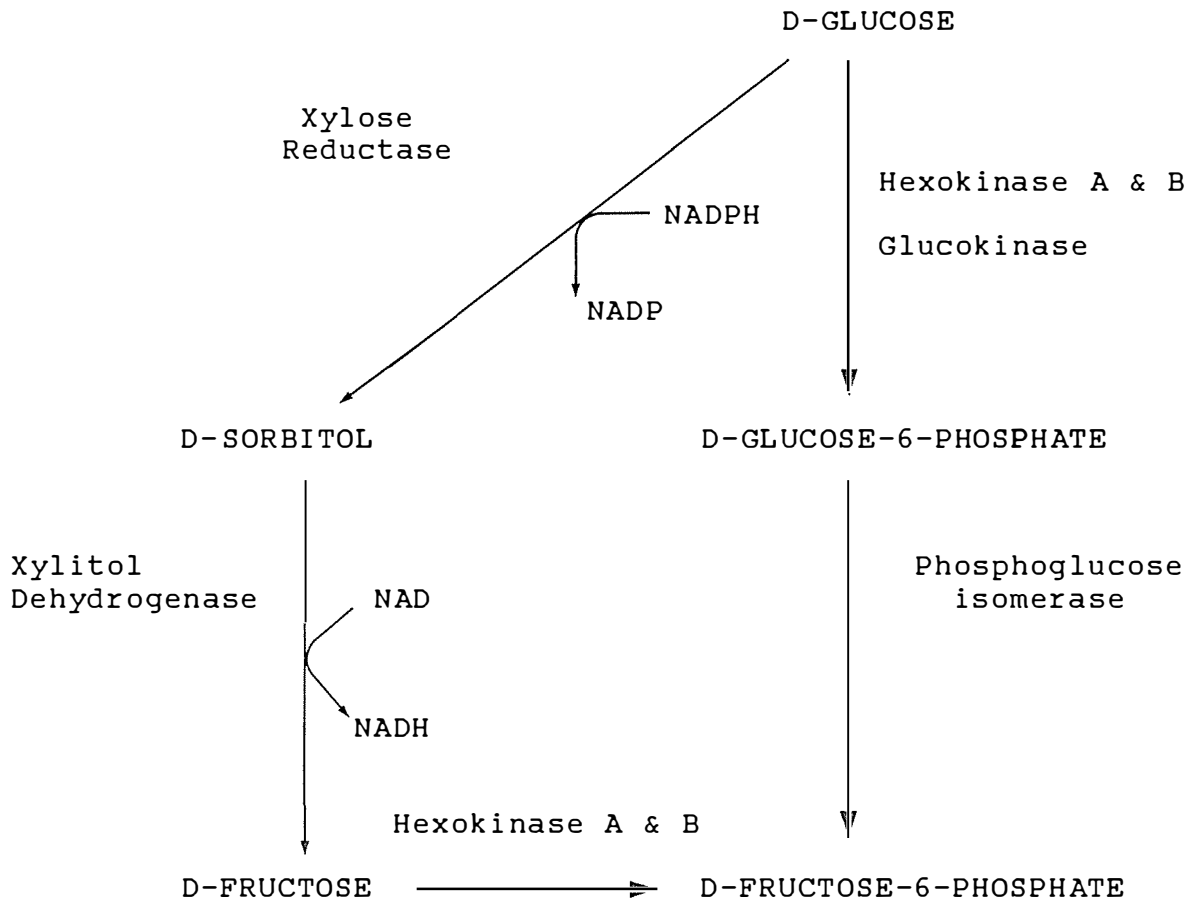
The complete elimination of all hexose-phosphorylating activity was expected to result in a strain unable to utilise D-glucose in a mixture of D-glucose and D-xylose, whilst retaining fermentative ability on D-xylose. The strain D/X A was defective in all three enzymes but surprisingly both this strain and its derivative F/G 2 could utilise D-glucose. A slow conversion of D-glucose to D-fructose was apparent in media inoculated at the higher cell density (Figure 15). The D-glucose consumption by both strains did not support cellular growth and no ethanol was produced. Therefore it was unlikely that catabolism of the D-glucose as such occurred. The rate of conversion of

D-glucose to D-fructose by both strains was slow. An even slower rate of conversion was shown by these strains when the cells were pre-cultured on glycerol instead of D-xylose (Paul Bicho, personal communication).

A possible pathway for the conversion of D-glucose to D-fructose by these strains is summarised in Figure 33. By this pathway, the D-glucose is reduced to D-sorbitol, catalysed by xylose reductase, and the D-sorbitol subsequently oxidised to D-fructose, catalysed by xylitol dehydrogenase. Both enzymes are known to have broad substrate specificities. The NADPH specific xylose reductase in *Pachysolea tannophilus*, in addition to D-xylose, can use L-glyceraldehyde, L-arabinose, D-ribose, (Ditzelmüller, 1984a; Morimoto, 1987) and D-erythrose, 2-deoxy-D-glucose, propionaldehyde, D-glyceraldehyde, valeraldehyde (Morimoto, 1987). A lower level of activity was found with several other substrates including D-glucose, D-mannose and D-galactose (Morimoto, 1987). The NAD specific xylitol dehydrogenase, in addition to xylitol, used D-sorbitol (Ditzelmüller, 1984b; Morimoto, 1987) and ribitol (Morimoto, 1987).

In this study, only very low xylose reductase activity was found with D-glucose as substrate. However D-sorbitol was found to be a good substrate for xylitol dehydrogenase, with 71% of the activity measured with xylitol. The requirement for D-xylose induction of xylose reductase and xylitol dehydrogenase could explain why there was poor conversion of D-glucose to D-fructose by D/X A and F/G 2

Fig. 33 Proposed Alternative Pathway for D-glucose Utilisation in *Pachysolen tannophilus*



—————> Known pathway
 - - - - -> Alternative pathway

when the cells were precultured in glycerol, since glycerol did not induce these enzymes.

The results of experiments with cell-free extracts suggested that a conversion of D-sorbitol to D-fructose could occur in the cell. Over half of the D-sorbitol was converted to D-fructose within 24 hr (Plate 13). This conversion required the presence of NAD. The incomplete conversion may be a consequence of the kinetic properties of xylitol dehydrogenase, the reverse reaction of D-xylulose reduction to xylitol being favoured at pH 7.0 (Ditzelmüller *et al.*, 1984b). The increase in the 'catabolic reduction' charge (NADH/NAD + NADH) which would occur in the reaction mixture could also shift the equilibrium in favour of D-sorbitol. Bolen & Detroy (1985) followed the conversion of D-xylose, L-arabinose or D-galactose with *in vitro* cell-free extracts by HPLC. The product of NADPH-linked xylose reductase activity with D-xylose as the substrate was demonstrated to be xylitol. Independent confirmation of xylitol as the product was obtained by capillary gas-liquid chromatography of acetylated *in vitro* samples. Identification of the products of the *in vitro* reaction mixtures in this study relied on HPLC analysis alone.

Although D-sorbitol and D-fructose were detected in the cell-free extracts with D-glucose as the substrate (Plates 11 & 12), the amount of conversion was minimal. This may have been due to the low activity of NADPH-dependent xylose reductase with D-glucose. Interestingly, D-fructose was only detected when NADPH alone was present as the cofactor

and not when NAD was additionally present. The reason for this was not clear.

In this study, there was sufficient evidence to say that the pathway of D-sorbitol utilisation passes through D-fructose as shown in Figure 33. However, it is less certain whether D-glucose is converted to D-sorbitol as a first step in the conversion of D-glucose to D-fructose by the 'glucose-negative' mutants. This pathway, however, remains a distinct possibility.

Conversion of D-sorbitol to D-fructose appears to be the first step in the catabolism of this carbon source. The involvement of xylitol dehydrogenase in catalysis of this reaction was supported by the observation that the mutant P52-1C could not grow on D-sorbitol. This mutant was defective in the xylitol dehydrogenase enzyme and was unable to grow on D-xylose, although capable of normal growth on D-glucose. Mutants, defective in all three hexose-phosphorylating enzymes, had also lost the ability to grow on D-sorbitol. Although the D-sorbitol could be converted to D-fructose by these strains, subsequent metabolism of the D-fructose was blocked by the lesions within the two hexokinase genes. This could explain the reason why, when the glucose-negative strains D/X A and F/G 2 were grown on D-glucose, D-fructose accumulated in the medium. No D-sorbitol was ever detected in the media, which was not surprising, since D-sorbitol was readily converted to D-fructose by the NAD-linked xylitol dehydrogenase.

7. STRAIN IMPROVEMENT OF *P. TANNOPHILUS* BY MUTAGENESIS AND HYBRIDISATION

A series of mutations have been introduced into *Pachysolen tannophilus*, three of which have been shown to affect the hexokinase and glucokinase enzymes, resulting in a glucose-negative phenotype. Minimal deleterious effects on D-xylose utilisation were apparent; in some cases ethanol production and yields were enhanced rather than diminished (Table XIII).

Ethanol production and yields by mutants D/X A and F/G 2 were good in comparison to the wild type strain (Table XIII). Ethanol yields were higher in YEP medium compared to YNB medium. Yeast extract is known to elevate specific growth rates in *Pachysolen tannophilus* (Dellweg *et al.*, 1984) and may improve ethanol yields. Wild type yields in YEP-xylose were comparable to those determined at the Forest Research Institute (Clark *et al.*, 1986), with yields of $0.16 \text{ g (g D-xylose consumed)}^{-1}$ in YEP-xylose medium. Cultural conditions employed were similar to those used in this study, although the cells were inoculated into the medium at a higher density. Under these conditions, the best mutants, NO₃-NO₃-4 and several of the P727 segregants, produced up to 6.5 g l^{-1} ethanol with a yield of $0.35 \text{ g ethanol (g D-xylose consumed)}^{-1}$. More recent studies have found even higher ethanol yields from D-xylose for the P727 segregants, particularly P727-9B (Paul Bicho, personal communication).

In this study, the mutant F/G 2 produced a maximum ethanol concentration of 4.7 g l^{-1} with a yield of 0.25 g g^{-1} . This was promising for a strain which was essentially hexose-negative and capable of fermenting D-xylose in the presence of D-glucose. Higher yields may be possible by employing cell recycling and using a higher cell density for inoculation.

By eliminating all hexose-phosphorylating enzymes from *Pachysolen tannophilus*, a strain, D/X A, was derived which was unable to metabolise D-glucose. This strain could slowly convert the D-glucose to D-fructose as discussed above. Fermentation trials with this strain in YNB media containing both D-glucose and D-xylose as carbon sources proved disappointing (Figure 11 and Table XIII). Although utilisation of D-glucose was slow, consumption of the D-xylose was inhibited and no ethanol was produced. A similar result was obtained with YEP-glucose/xylose. The presence of residual catabolite repression on xylose reductase and xylitol dehydrogenase by the D-glucose appeared unlikely in view of the results of assays on these enzymes (Results Section 5, Table VII). A possible explanation may be competitive inhibition of D-xylose utilisation by D-glucose. This would occur if D-glucose was converted to D-fructose by the alternative pathway as previously discussed. As well as substrate competition for enzyme, D-glucose could compete with D-xylose for NADPH, while sorbitol could compete with xylitol for NAD. D-glucose may also compete with D-xylose for transport into the cell. Alternatively, D-glucose may still exert

repression on enzymes other than xylose reductase and xylitol dehydrogenase which are essential for utilisation and fermentation of D-xylose

The selection scheme described in Materials & Methods 11.2 generated a strain designated F/G 2, capable of metabolising D-xylose to ethanol in the presence of D-glucose, in YEP media (Figure 32). D-glucose was also slowly converted to D-fructose. This mutant behaved disappointingly in YNB-glucose/xylose (Figure 30); consumption of the D-xylose ceasing after 96 hr incubation and little ethanol being produced. A different situation occurred in the YEP-glucose/xylose medium. Complete utilisation of the D-xylose occurred and much of the apparent utilisation of D-glucose was accounted for by conversion to D-fructose. Ethanol was produced (4.1 g l^{-1}) with a yield of 0.19 g g^{-1} sugar consumed (Figure 32, Table XIII). Since almost all of the sugar metabolised at the point of maximum ethanol concentration in the medium was D-xylose, these values for the ethanolic fermentation of D-xylose can be viewed as improvements over the wild type strain. This strain or the #24 derivative may have potential use for the fermentation of wood hydrolysates rich in pentose sugars, such as those derived from hardwoods.

The nature of the mutation in the F/G 2 strain, designated as γ , which enabled complete utilisation of D-xylose in YEP-glucose/xylose but not in YNB-glucose/xylose, is not known at present but may be related in some way to the inclusion of yeast extract in the media. This mutant grew

similarly on D-xylose, D-glucose, D-fructose and D-mannose to the parental D/X A strain. Resistance to 2-deoxyglucose was also similar to that shown by the D/X A strain.

8. FINAL CONCLUSIONS

In this study, the characteristics of 2-deoxyglucose resistant mutants of *Pachysolen tannophilus* were investigated and our knowledge of the hexose-phosphorylating enzymes in this yeast extended. This knowledge is necessary in order to isolate suitably altered strains capable of efficient fermentation of pentose sugars in the presence of hexoses. This is especially true in view of the involvement of hexokinase A in catabolite repression. A more complete understanding of the mechanism of catabolite repression is essential to fully exploit pentose-fermenting yeasts for the fermentation of wood hydrolysates, which contain mixtures of pentose and hexose sugars.

The acquisition of resistance to 2-deoxyglucose in the mutants correlated with loss of hexose-ATP-kinase activity. Strains resistant to high concentrations of the analogue had lost virtually all activity and were unable to grow on the hexose sugars.

Pachysolen tannophilus was found to possess three hexose-phosphorylating enzymes, hexokinases A and B, with F/G ratios of 1.3/1.0 and 3.0/1.0 respectively, and a D-glucose

specific glucokinase. Hexokinase B and the glucokinase appeared to be constitutive, while hexokinase A was induced by D-glucose.

The results of enzyme assays for xylose reductase and xylitol dehydrogenase suggested that hexokinase A has a role in carbon catabolite repression in an analogous manner to the PII hexokinase from *Saccharomyces cerevisiae*.

Hexokinase A was partially purified and studied further. Both the F/G ratio and the km values of the enzyme were similar to those reported for the PII isoenzyme. The purified enzyme was inactivated by D-xylose in the presence of MgATP; a property shared with the PII hexokinase.

A mutant defective in all three hexose-ATP-kinases was transformed with the YRp/HXK2-8 plasmid. The gene was expressed and hexokinase activity demonstrated within the cell. Preliminary experiments suggested that glucose repression of D-xylose utilisation was restored within the cell.

Although a mutant defective in the two hexokinases and the glucokinase was unable to utilise D-glucose as a sole carbon source, D-xylose utilisation was severely inhibited in the sugar mixtures. Further mutants capable of relatively efficient D-xylose fermentation to ethanol in the presence of D-glucose were selected for. The genetic and biochemical nature of the mutation responsible has yet to be determined.

Mutants defective in hexose-ATP-kinase activity were capable of conversion of D-glucose to D-fructose. A possible pathway for this conversion was presented.

Two superior ethanol producing mutants were hybridised and the hybrids made available for fermentation trials at the Forest Research Institute, Rotorua.

The yeast *Pachysolen tannophilus* can be readily subjected to genetic manipulation and thus is an ideal yeast for further study in the areas of hexose and pentose metabolism and catabolite repression. Many of the original aims of this study were achieved and several new potential avenues for future research were discovered, which may lead to a greater understanding of the interaction of hexose and pentose utilisation.

Nihil Boni Sine Labore

Figure A1: Standard Curve Relating Optical Density to Viable Cell Numbers (Wild Type Cells)

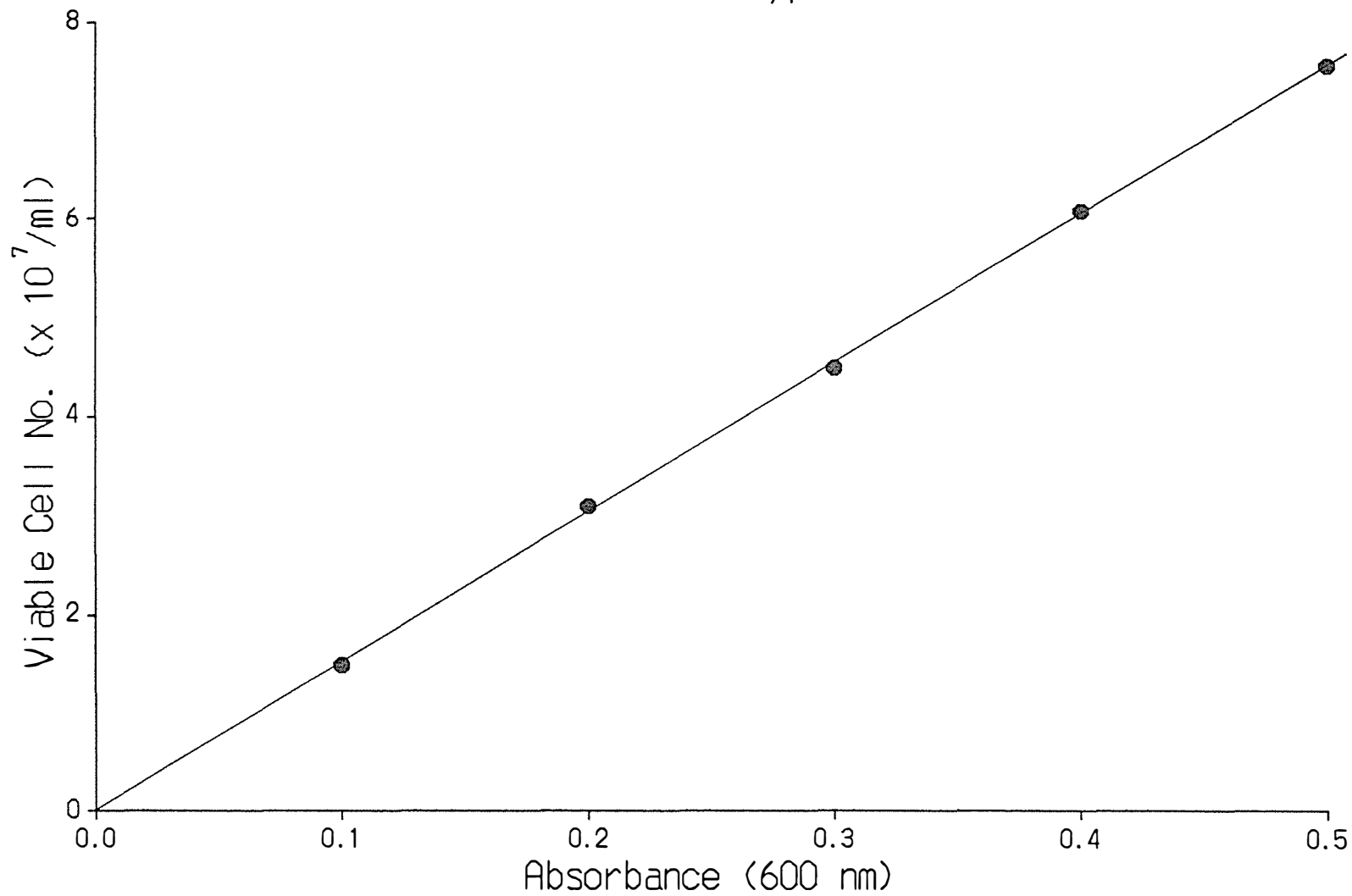
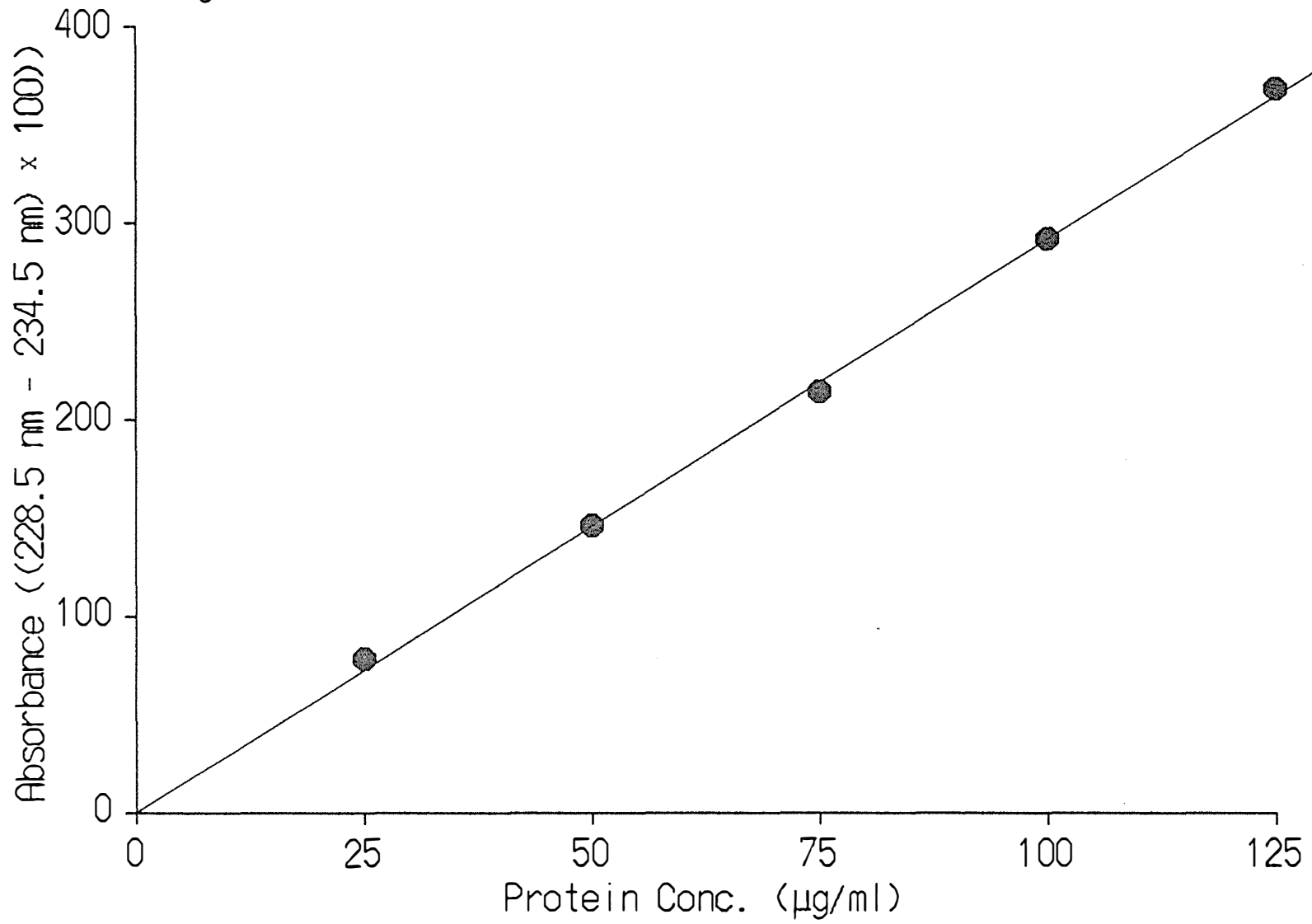


Figure A2: Protein Standard Curve (Bovine Serum Albumin)



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ERRATA

Page	Line	Number	Typos	Others
1	27	are = is		
3	4		ascophore	
3	22-23			invert: be readily an NADH-linked
11	2			
12	20	has = have		
14	9	was = were		
21	24			an NADPH
26	3			...is an...feature
41	8		acetonitrile	
41	12		acetonitrile	
73	16		mutagenise.	
86	3			in = depletion from
88	12			no = neither
98	1	was = were		
99	17	conductivities		
148	8			no = neither
151	16			<u>The</u> latter
152	4-5			<u>The</u> latter
161	14		necessary	
162	3-4			...also to have...
170	24	was = were		