HEXOSE FERMENTATION BY THE FISSION YEAST *Schizosaccharomyces pombe* AND CHARACTERIZATION OF ITS HEXOKINASE

Thesis submitted for the degree of

DOCTOR of PHILOSOPHY

BY

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DEDICATION

To my late parents

Eleluwani Rangarirai and Samuel Fako

MPANDI

CONTENTS

SUMMARY

This thesis describes a study of some aspects of ethanol production by the fission yeast *Schizosaccharomyces pombe* and the purification and characterization of its hexokinase.

Comparisons were made between *S. pombe* MY4 (also L972) and new isolates of *S. pombe* (from molasses) and *Saccharomyces cerevisiae* (from a preparation used for industrial production of ethanol) obtained in Zimbabwe. All three strains of yeasts produced ethanol with high efficiency and although the strain of *Saccharomyces cerevisiae* was somewhat more tolerant of ethanol than the two strains of *S. pombe*, the latter were more tolerant of high concentrations of sugars, growing in up to 60% (w/v) sucrose.

Studies on *S. pombe* MY4 showed that its optimum pH for growth and ethanol production was less than 5.5, and it had a requirement for air during fermentation of glucose. When growing on sucrose the extent of growth was reduced by concentrations above 20% (w/v), probably due to inhibition by the accumulating ethanol, since ethanol increased the growth inhibiting effect of high concentrations of sucrose. This inhibition could be overcome by carrying out the fermentation under reduced pressure and removing the ethanol as it was formed.

Measurement of hexokinase activity in crude cell-free extracts by two methods — one measuring the rate of formation of hexose phosphate, the other the rate of formation of ADP — showed that the concentrations of both the hexose and the enzyme had an effect upon the measured activity.

The hexokinase activity was adsorbed from crude extract by Matrex Gel Red A and eluted as a single peak at a salt concentration higher than 0.5 M to give

a purification factor of about 150-fold. With the same system, the well-characterized hexose kinases of *Saccharomyces cerevisiae* were eluted as three separate peaks - probably corresponding to its glucokinase and the PI and PII hexokinases — at a salt concentration less than 0.25 M.

The pH optimum of the *S. pombe* hexokinase was between 8 and 10, with activity decreasing to half maximal at 5.2 and 10.5. At room temperature, the enzyme was most stable between pH 7 and 10, and was stable for at least 12 months at -70°C. It was rapidly inactivated at 50°C, but retained some activity after 30 minutes at 45°C.

Fructose was phosphorylated at a faster rate than glucose. Of 21 other sugars and related compounds, only four were significantly phosphorylated, the order being D-mannose > 2-deoxy-D-glucose > D-glucosamine > D-allose. Inhibition of enzyme activity by ethanol was about 15% at 1.7 M (10% v/v), with the inhibition increasing to about 86% at a concentration of 5 M (30% v/v).

The molecular mass of the native enzyme was found to be between 98,000 and 100,000 daltons, by chromatography on Sephacryl S-300. When enzyme denatured with sodium dodecylsulphate was analysed by polyacrylamide disc-gel electrophoresis, two distinct bands were obtained indicating unequal sub-units having molecular masses of 45,000 and 52,500 daltons. Compared with published data of amino acid composition of the three enzymes from *Saccharomyces cerevisiae*, the enzyme from *S. pombe* differed particularly in the content of lysine (less than half), glutamic acid (about two-thirds), serine and glycine (about 1.5 times higher) and histidine (about 3 times higher than the PI and PII hexokinases, but slightly lower than the glucokinase).

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I cherish memories of my late mother which encouraged me to keep going, sometimes even against odds.

DECLARATION OF AUTHENTICITY

I hereby declare that none of the results in this thesis have been submitted previously to any other University or any other Institution for the award of any other degree or diploma.

To the best of my knowledge and belief this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Ester MPANDI-KHOSA.

MPANDI-KHOSA, E., MAY, J.W., MARSHALL, J.H. & SLOAN, J. (1993). Purification and characterisation of hexokinase from *Schizosaccharomyces pombe*. In *Eleventh Australian Biotechnology Conference, (Programme & Abstracts)*, pp. 187. Edited by J. Sargeant, S. Washer, M. Jones & M.A. Borowitzka. Perth: Asian-Australian Biotechnology Cooperation.

PURIFICATION AND CHARACTERISATION OF HEXOKINASE FROM *SCHIZOSACCHAROMYCES POMBE*

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As part of a study aimed at evaluating the potential of *Schizosaccharomyces pombe* for production of ethanol for use as a fuel, we have purified and characterised hexokinase from this yeast. Previous studies of this enzyme have been carried out mainly on enzymes obtained from either mammalian tissues or from the yeast *Saccharomyces cerevisiae*. The enzyme from *S. pombe* shows significant differences from these enzymes.

Enzyme assay

Two methods were used. In method 1 the rate of phosphorylation of glucose by ATP/Mg^{++} was measured by measuring formation of glucose-6-phosphate using glucose-6-phosphate dehydrogenase and $NADP^+$ and following NADPH formation spectrophotometrically at 340 nm. This method can also be used to measure phosphorylation of fructose by adding phosphoglucose isomerase to isomerise fructose-6-phosphate. In method 2 the reaction was followed by measuring formation of ADP which was coupled to NADH oxidation by adding phospho-enol pyruvate, pyruvate kinase, lactate dehydrogenase and NADH. This method can be used for a range of substrates.

Purification of enzyme

S. pombe was grown in glucose at 30°C for 48 hr, the cells harvested by centrifuging and cell extracts prepared by disruption in a French pressure cell. Cells harvested at different stages of growth showed little variation in hexokinase activity. Of several purification methods investigated the most effective proved to be separation on Matrex Gel Red A which achieved a purification factor compared to crude extracts of about 140 and a protein with a specific activity of about 7 $\mu mol\ min^{-1}\ (mg.protein)^{-1}$.

Properties

The enzyme was stable for at least 12 months at -70°C. It is rapidly inactivated at 50°C but retains some activity after 30 min at 45°C. 2.2M glucose gave some protection against temperature inactivation but fructose gave none. The pH optimum of the enzyme is 10.0 with activity decreasing to half maximal at 5.5 and 11.0 and it is most stable between pH 7 and 10. Electrophoresis on PAGE gave a single protein band while SDS PAGE gave bands corresponding to 45,500 and 52,500 daltons.

Substrate specificity

In addition to D-glucose, D-fructose was phosphorylated at approximately the same rate though there was some variation with enzyme and substrate concentrations and fructose/glucose ratios varied between 3 and 0.3; however, there was no evidence for the presence of more than one enzyme protein. Of 20 other hexoses and related compounds tested activity was found with four, the relative rates being (glucose = 100) 2-deoxy-D-glucose 60, D-mannose 54, D-glucosamine 41, and D-allose 5. Km values were determined for each substrate.

Effect of metal ions

Mn^{2+} and Co^{2+} but not Ca^{2+} showed some activity in replacing Mg^{2+}.

Effect of inhibitors

Inhibition of phosphorylation of glucose or fructose (2.2M) was produced by 2.2M D-fructose (90%) and 2.2M D-mannose (71%), but, unlike the *Saccharomyces cerevisiae* enzyme, D-xylose was not inhibitory when glucose or fructose were the substrates, but was inhibitory with D-mannose, 2-deoxy-D-glucose and glucosamine as substrates. ADP and AMP were also inhibitory, but glucose-6-phosphate did not appear to inhibit. Ethanol shows 24% inhibition at a concentration of 10% (v/v) which increases to 90% at 40% ethanol.

sequences.

Fig. 7.11 Phylogenetic tree depicting relationships determined from partial ribosomal RNA sequences. (Kurtzman and Robnett, 1991).

ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ALD	Aldolase
ATCC	American Type Culture Collection, Rockville, Md., USA
ATP	Adenosine triphosphate
BIS	N,N'-methylenebisacrylamide
BSA	Bovine serum albumin
^0C	degrees celsius
CHA	Cyclohexylammonium
cm	centimetre
CO_2	carbon dioxide
CTP	Cytidine triphosphate
d	dalton
DHAP	Dihydroxyacetone phosphate
DEAE	Diethylaminoethyl
dH_2O	distilled water
dil	dilute
DNA	deoxy-ribonucleic acid
DTT	Dithiothreitol
EC	Enzyme Commission
EDTA	Ethylene diamine tetra acetic acid
EMM2	Edinburgh Minimal Medium No. 2
EN	Enolase

ETOH	Ethanol
FK	Fructokinase
F-1,6-bisP	Fructose-1,6-bisphosphate
F6P	Fructose-6-phosphate
g	gram; average centrifugal force
g dry wt l^{-1}	grams dry weight per litre
GAP	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-P-dehydrogenase
GK	Glucokinase
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-P-dehydrogenase
GLU	Glucose
GLY	Glycerol
GTP	Guanosine triphosphate
h	hour
H^+	Hydrogen (oxidised form)
HA	Hydroxyapatite; hydroxylapatite
HK	Hexokinase
HPLC	High Pressure Liquid Chromatography
ITP	Inosine triphosphate
k(prefix)	kilo
KDPGA	2-keto.deoxy-P-gluconate aldolase
K_m	Michaelis constant
l	litre
L	Professor U. Leupold, Institute of General

	Microbiology, University of Bern, Switzerland.
LDH	Lactate dehydrogenase
m(prefix)	milli
M	molar, mole
mA	millamperes
mag	magnification
MDH	malate dehydrogenase
mg dry wt ml^{-1}	milligrams dry weight of cells per millilitre
μ (prefix)	micro
MEA	malt extra agar
MEB	malt extract broth
min	minute
MK	Myokinase
mol wt	molecular weight
n (prefix)	nano
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCYC	National Collection of Yeast Cultures,
	Food Research Institute, Norwich, England
No.	Number
NBT	Nitroblue tetrazolium
OD_{580}	optical density at 580nm
P	product

PAGE	Poly acrylamide gel electrophoresis
%	percent
PDC	Pyruvate decarboxylase
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PGAK	Phosphoglycerate kinase
PGAM	Phosphoglycerate mutase
6PGDT	6-P-gluconate dehydratase
PGI	Phosphoglucose isomerase
6PGL	6-P-gluconolactonase
pH	negative log of H^+ concentration
Pi	phosphate
PK	pyruvate kinase
PMS	Phenazine methosulphate
PMSF	Phenylmethlysulphonyl fluoride
ppm	parts per million
QMC	Queensland Molasses Company
RNA	ribonucleic acid
rpm	revolutions per minute
S	substrate
SDS	Sodium dodecyl sulphate
Std	standard
sp	species
t_d	doubling time
TEA	Triethanolamine

TEMED	N,N,N′,N′,-Tetramethylethylene-diamine
TPI	Triosephosphate isomerase
Tris	Tris-hydroxymethyl-amino methane
TSAI	total fermentable sugar as invert
U	unit
UTP	Uridine triphosphate
V_{max}	maximum velocity of reaction
vol	volume
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
YEP	Yeast Extract Phosphate

CHAPTER 1

INTRODUCTION

1.1 SCOPE OF THE THESIS

Fossil-derived oils are the source of the vast proportion of fuels and chemicals used at present. However, the production and utilization of fossil fuel has drawbacks. This fuel is not renewable and its continued and increasing use has brought environmental pollution. Alternative sources of fuel are being actively sought in many countries, and ethanol is regarded as the most suitable substitute for fossil fuel. Ethanol is safer to handle, burns more cleanly and is environmentally friendlier (Lawford & Rousseau, 1993) since it does not cause as much atmospheric pollution as fossil fuel (Rose & Beavan, 1981; Charles, 1991). Ethanol as a fuel has two important uses: (i) in automobiles either on its own or, more usually, mixed with gasoline, and (ii) for lighting and cooking in rural, non-electrified areas (Houghton-Alico, 1982; Jager 1986; Pereira, 1986).

Ethanol produced by fermentation of sugar-cane molasses using *Saccharomyces cerevisiae* — a budding yeast — is currently used as an alternative car fuel in Zimbabwe. On the other hand, a number of strains of *Schizosaccharomyces pombe* — a fission yeast — have been isolated from molasses, cane sugar and traditionally-brewed African beers (Slooff, 1970; Bridge & May, 1984). Because of its ability to grow in the presence of high concentrations of sugars and its frequent isolation in countries with hot climates, it seemed relevant to further explore the potential of *S. pombe* for the more efficient ethanol production than *Sacch. cerevisiae* for a country such as Zimbabwe.

The overall objective of this project was to explore further the potential of *S. pombe* for ethanol production. The research consists of two parts. In the first part, the conditions affecting ethanol production by *S. pombe* are studied with the objective of improving the efficiency of production under batch-culture conditions. Although *S. pombe* can grow in initially high concentrations of sugars, one restriction to complete fermentation of the sugar is the inhibition by the accumulating ethanol. A possible solution to this problem is the continual removal of ethanol as it is produced.

In the second part, the properties of the hexokinase of *S. pombe* are investigated. This is justifiable for two reasons: (i) since hexokinase catalyzes the first step in the catabolism of hexoses, it may be the site of regulation of the pathway; therefore, it is important to understand how many forms of hexokinase are present, and their kinetic and molecular properties; and (ii) in spite of many similarities, it cannot be assumed that *S. pombe* will possess the same number and type of hexokinases as *Saccharomyces cerevisiae* (see McDonald & Tsai, 1989).

1.2 ASPECTS AND IMPLEMENTATION OF FUEL-ETHANOL PRODUCTION

1.2.1 International Ethanol Production

There is continued interest in ethanol programs in developing countries in spite of decrease in oil prices (Pereira, 1986) mainly because the programs can save foreign exchange and generate employment. Although ethanol produced by fermentation cannot be regarded as the solution to the energy crisis

brought about by price increases or scarcities of supply, it may play an important role in energy strategies aimed at diversification of fuel supplies. Such considerations have led a number of countries to initiate some production of ethanol as fuel or as material for chemical industry or both. Production of some ethanol on an industrial scale is already being carried out in countries such as Brazil, India, Kenya, the Philippines, Zimbabwe, Sri Lanka, Australia, Thailand, Indonesia, the United States, Malawi and a few others (Dyson, 1981; Houghton-Alico, 1982; Trindade, 1984; Jager, 1986; Murtagh, 1986; Pereira, 1986; Avalos Ortis, 1988; Keim & Venkatasubramanian, 1989).

In the period between 1973 and 1983 oil prices doubled, causing severe problems (both direct and indirect) for the developing countries, especially those dependent on imported oil. These were followed by secondary effects associated with the world-wide balance of payments adjustments and its aftermath (Pereira, 1986). Thus the major global economic disturbances of this period were related to energy and its cost. The decline in world oil prices from US$26-28 per barrel in 1985 to $15 in mid 1986 did not diminish the need for examining alternative energy sources from biomass (Pereira *et al.*, 1987).

Very few countries can be compared with Brazil in terms of the resources for large-scale ethanol production. The ethanol industry was mandated by the government and serves national policy by significantly reducing expenditure on petroleum (Keim & Venkatasubramanian, 1989). The projected output in 1988-89 was 13.3 x 10^9 litres. Sugar cane juice and molasses are the feedstocks and sugar cane bagasse is used for fuel. The anhydrous ethanol is blended into gasoline in amounts as high as 22% v/v while the hydrous ethanol is used in ethanol-burning

cars, which constitute some 88.4% of all the new vehicles sold by the Brazilian car industry (Laluce, 1991).

In the United States of America fuel ethanol production was also encouraged by government incentives and the industry remains economically viable largely because of governmental policies and support programs (Murtagh, 1986). The production has grown from starting point of virtually zero in 1979 to 350 x 10^6 litres in 1981 and 2875 x 10^6 litres in 1985 and thereafter levelled off at 3200 x 10^6 litres per year. The total capacity is around 4369 x 10^6 litres per year (Keim & Venkatasubramanian, 1989).

The production of fuel-ethanol in the small third-world country of Malawi needs brief mentioning. With the oil crisis of the 1970s and the doubling of fuel prices in 1978-79, three studies were carried out by international companies in 1979 concerning the use of molasses for production of fuel-ethanol in Malawi. This resulted in the incorporation of the Ethanol Company in 1980 and the building of the industry at Dwangwa. In 1982, Malawi started production of ethanol and was the first country in the world to achieve a national blend of 20% ethanol in petrol (Jager, 1986). The industry has one of the most efficient processing plants in the world for both yield and consumption of process materials and produces 55 x 10^6 litres per annum. Malawi has embarked on a program to investigate distinct areas of additional utilization of ethanol. The program is the best in Africa and probably second only to Brazil in the world. The areas of research are as follows: ethanol-petrol blends (already achieved); ethanol-diesel blends; production of synthetic diesel; ethanol as a heating, lighting and refrigeration fuel; ethanol-paraffin blends and ethanol as a solvent (Jager, 1986).

1.2.2 Zimbabwe ethanol production

Zimbabwe, like Brazil, is a sugar-producing country and has integrated the ethanol industry into the sugar mill's facilities, using sugar-cane molasses as feedstock and the sugar-cane bagasse as fuel at practically no extra cost (Wenman & Tannock, 1984).

The central feature of Zimbabwe's economy until 1980 was that, for political reasons, it faced severe constraints on the ability to conduct foreign transactions, particularly to borrow and export. A study by Suckling (1983), showed that even in the absence of the first and second oil price increases, real oil prices would have risen at a rate equal to the rate experienced in 1964-73. In fact, oil prices actually rose much faster than the trend and reduced the amount of foreign currency available for purchase of non-oil imports. The government of Zimbabwe then developed a plan to shift away from oil to hydro-power and ethanol (Pereira *et. al.*, 1987). The Triangle Sugar Estate built a locally fabricated ethanol plant with a rated annual capacity of 40×10^6 litres of anhydrous ethanol per year, from sugar-cane and molasses (Rein, 1986; Wenman & Tannock, 1984). The capital cost of this plant was much lower than the cost of ethanol plants of similar capacity in Brazil (Pereira, *et al.*, 1987).

The Triangle Plant makes use of a classical batch-fermentation without yeast re-cycle. Although there is great world-wide attention to improving the efficiency of fermentation by continuous fermentation, the method has not been able to match the yields achieved by the batch system (Guidoboni, 1984; Chen & Mou, 1990), and is not being considered in Zimbabwe at present. The average

annual production of 40 x 10^6 litres amounted to between 12-15 per cent of the national gasoline requirements for 1984. Great emphasis is placed on the need for maximum efficiency of ethanol production and the minimization or elimination of losses in fermentation and distillation (Wenman & Tannock, 1984).

1.2.3 Current research and development in ethanol production

For ethanol to realise its potential as a partial or total substitute for petroleum fuels or as a substitute chemical feedstock, it is necessary that the manufacturing process be as efficient in the use of energy as possible, in order to maximise the energy return for the amount of ethanol produced and enhance the standing of the ethanol as an economically viable replacement for petroleum based raw materials (Torrey, 1983).

The challenge to improve fuel-ethanol production can be separated into two main categories: (1) engineering factors, and (2) biological factors. Engineering factors involve process or reactor design, substrate preparation, and product recovery.

Engineering factors for improvement of ethanol production have been demonstrated in studies involving continuous fermentation alone (Fraleigh *et al.*, 1989) and its industrial application (Allen, 1984; Guidoboni, 1984), or with cell recycle (Lee *et al.*, 1983; Wang *et al.*, 1984; Kuriyama *et al.*, 1985a). Mota *et al.* (1987) illustrated a 40 times increase in productivity using cell recycle by microfiltration in yeast alcoholic fermentation in continuous operations. In particular, Vraná (1983) looked at the fission yeast *Schizosaccharomyces pombe* in continuous culture. Rogers *et al.* (1982) have developed a continuous culture

system with cell recycle to achieve a high productivity process with *Z. mobilis*. Cysewski & Wilke (1978) demonstrated high productivity using a combination of cell-recycle and vacuum fermentation. Other studies have focused on batch fermentation with cell recycle (Ghose & Tyagi, 1979; Ciftci *et al.*, 1983; Patil *et al.*, 1989), or repeated-batch fermentation (Kida *et al.*, 1990; Kida *et al.*, 1991). Others still have shown the advantages of fed-batch process for ethanol production (Hsie & Su, 1981; Toukourou *et al.*, 1989). Prolonged continuous exposure of yeasts to high ethanol concentrations results in loss of cell viability, therefore recycled cells must be continually replenished or intermittently discarded completely. Product recovery processes which are more energy-efficient than distillation are being developed for acetone-butanol-ethanol fermentation processes. These include the use of liquid-liquid extraction, solid adsorbents, gas stripping and membrane processes such as pervaporation, perstraction and reverse osmosis (Maddox, 1993). These can be applied to fermentation processes as direct or partial replacement for distillation in order to help solve the problem of product inhibition (Ennis *et al.*, 1986).

There has been interest in the use of immobilised cells for production of fermented beverages and industrial ethanol (Chibata & Tosa, 1980; Ghose & Bandyopadhyay, 1980; Wada *et al.*, 1981; Nagashima, 1990). Studies on immobilised cell reactors using a highly productive strain of *Z. mobilis* demonstrated the potential of such systems for simple, low cost ethanol production (Rogers *et al.*, 1982). A combination of continuous fermentation and immobilisation of cells illustrate yet another engineering approach for improved ethanol production (Gōdia *et al.*, 1987; Hamdy *et al.*, 1990; Hoshino *et al.*, 1990).

Detailed study of the engineering factors for improved ethanol production are beyond the scope of this research.

Biological factors involve improvement on the micro-organism with regards to its substrate range and need for pre-processing of substrate, media composition, kinetic performance, tolerance of substrate and ethanol, and ethanol yield. This may involve screening of micro-organisms for superior ethanol fermentation ability or mutants capable of yielding useful secondary products, e.g. fructose for use as a sweetener (Ueng *et al.*, 1986; Koren & Duvnjak, 1990; Lee & Fisher, 1990; Koren & Duvnjak, 1991), or glycerol (St Julian, *et al.*, 1990) thus improving the economics of ethanol production.

Much of the current research focuses on utilization of alternative and cheap biomass sources such as cellulose, lignocellulosic residues and wastes from food manufacturing industries. Research on cellulose, the most abundant organic material, includes examination and evaluation of feedstocks, involving pre-treatment, hydrolysis, fermentation kinetics and recovery of ethanol (Kosaric *et al.*, D-xylose is the main constituent of hemicellulosic xylans. ~~sist mainly of D-xylose~~. The utilization of this sugar in these materials is important in the economics of conversion of plant biomass by micro-organisms, into chemicals of some industrial importance such as ethanol (Dekker, 1986). Research for utilising wastes such as cheese-whey, coffee wastes, potato-peel wastes, pineapple wastes and spent sulphite-liquor from wood-pulp industries, for ethanol production, is being undertaken (Kosaric *et al.*, 1980, Bailey *et al.*, 1982; Terrell *et al.*, 1984, Verachtert *et al.*, 1990).

Research on ethanol production also focuses on selection of: (i) yeast and

bacterial strains capable of utilising these cheaper substrates (Ingram, 1986), (ii) high ethanol yielding strains (Esser & Schmidt, 1982), (iii) strains with high ethanol and temperature tolerance (Rogers *et al.*, 1982; Ingram, 1986; Ernandes *et al.*, 1990), and (iv) strains capable of fermentation at high sugar concentrations (Kuriyama *et al.*, 1985b; Murakami *et al.*, 1987; Bertolini *et al.*, 1991). The complexity of the inhibitory effect of ethanol on yeasts makes it difficult to design a suitable selection regime.

Research has also focused on medium composition and supplementation as a means of improving ethanol fermentations. *Sacch. uvarum* produced up to 16.2% v/v ethanol when wort was supplemented with ergosterol, oleic acid and yeast extract (Nagashima, 1990). Cachot & Pons (1991) demonstrated that the addition of chitin and yeast extract to cane molasses resulted in reduction in fermentation time and improved alcoholic fermentation, using *Sacch. cerevisiae*. Guebel *et al.* (1991) showed that trace element supplements increased ethanol volumetric productivity by 20%, using *Pichia stipitis*. In a separate study, O'Connor-Cox *et al.* (1991) showed that excess assimilable nitrogen in the form of yeast extract le d to increased rates of growth of *Sacch. cerevisiae* and increased accumulation of cell mass.

The high cost of ethanol production by fermentation is mainly due to the high energy requirements for distillation. The economics of the ethanol production could be improved if a secondary by-product was produced. In the case of cane-sugar or molasses fermentation, this could be in production of fructose for use as a sweetener. Fructose in food is preferable to sucrose because it is sweeter (therefore less is taken), and it is non-cariogenic (Doelle & Doelle, 1992). Current

research on production of fructose from sucrose is being undertaken with *Z. mobilis*, since this organism produces both a glucokinase and a fructokinase allowing the removal of one of the two enzymes by mutagenesis. Using fructose-negative mutants of *Z. mobilis,* Bringer-Meyer *et al.* (1985) and Suntinanalert *et al.* (1986) produced fructose from sucrose media. Doelle & Doelle (1992) produced fructose from sugar-cane syrup and molasses also using mutants of *Z. mobilis.*

The interest in utilisation of cellulose and lignocellulosics such as agricultural residues has led to new research areas in genetic engineering to improve ethanol production by protoplast fusion and recombinant DNA technology (Panchal *et al.*, 1982; De Figueroa *et al.*, 1985; Whitney *et al.*, 1985; Russell *et al.*, 1986; Alterthum & Ingram, 1989; Ingram *et al.*, 1987; Ingram, 1991). The advent of recombinant DNA technology makes it possible to introduce extra genetic information into the yeast or bacterium and make the micro-organism capable of directly fermenting substrates considered non-fermentable (Ho, 1980). Panchal *et al.* (1982) obtained stable fusion products of the polyploid brewing yeast *Sacch. uvarum* strain and a genetically constructed diploid *Sacch. diastaticus* strain. One of the fusion products was found to have enhanced ethanol-producing capability. In addition it was found to be more osmotolerant than the parental strain. It had glucoamylase-producing capability and could utilise dextrins in the corn mash substitute.

Escherichia coli has been shown to be able to metabolise all major sugars which are constituents of plant biomass and as such, it has been possible to genetically engineer ethanol production in this bacterium which made it able to

utilise a variety of sugars (Ingram *et al.*, 1987; Ingram & Conway, 1988; Alterthum & Ingram, 1989). The ethanol production rates observed in batch culture for glucose and lactose fermentation with recombinant *E. coli* compared favourably with those observed for yeasts (Lovitt *et al.*, 1988) and may provide the basis for possible commercial application of economic significance (Panchal & Tavares, 1990; Ingram, 1991).

1.3 MICRO-ORGANISMS IN ETHANOL PRODUCTION

There is a wide range of ethanol-producing micro-organisms covering several groups of yeasts and a broad range of mesophilic and thermophilic bacteria (Table 1.1). The industrial production of ethanol appeares to be limited to strains of *Sacch. cerevisiae*, commonly known as baker's or brewer's yeast, and its related species (Lovitt *et al.*, 1988). Although most of the research on ethanol production reviewed in this study (Section 1.4), has been carried out using this yeast, the usefulness of other micro-organisms is being studied.

1.3.1 *Schizosaccharomyces pombe*

S. pombe is a facultative anaerobic fission yeast. It was first isolated from East African millet beer that is called "pombe" in Swahili. It is also the fermenting agent in Javan "arak" and Jamaican rum (Mitchison, 1970). Some strains of *S. pombe* have been isolated from molasses, cane sugar and African traditionally brewed beers collectively termed "Bantu" beer (Slooff, 1970; Bridge & May, 1984). Some of these strains have been considered to rival *Sacch. cerevisiae* in their ability to produce ethanol (Esser & Schmidt, 1982). Rose

Table 1.1 Some micro-organisms in ethanol production (adapted from Tubb, 1984).

Organism	Advantageous feature
Yeasts:	
Saccharomyces cerevisiae	Efficient substrate conversion
Schizosaccharomyces pombe	Good ethanol tolerance
Saccharomyces diastaticus	Starch hydrolysis
Kluyveromyces lactis	Conversion of lactose to ethanol.
Candida pseudotropicalis	Conversion of D-xylose to ethanol.
Pachysolen tannophilus	Conversion of D-xylose to ethanol.
Filamentous fungi:	
Fusarium sp.	Conversion of D-xylose to ethanol.
Mucor sp.	Conversion of D-xylose and L-arabinose to ethanol.
Bacteria:	
Zymomonas mobilis	Efficient substrate conversion; high specific productivity.
Clostridium thermocellum	Hydrolysis of cellulose; thermophilic.

(1976) screened flocculating yeasts for those which were tolerant to the high concentrations of sugar in molasses and for rapid production of high yields of ethanol. Two strains of *S. pombe* gave higher yields of ethanol than *Sacch. cerevisiae* and *Sacch. carlsbergensis*, while only producing a tenth of the yield of fusel oils. Another study by Haraldson & Bjoerling (1981) in which 20 strains of yeasts for ethanol production were screened at high sugar concentrations (i.e. at low water availability) and at temperatures between 32 and 45°C showed that the fission yeasts were more tolerant to low water availability than the budding yeasts, the productivity of *S. pombe* being almost twice that of *Sacch. cerevisiae* at high sugar concentration. In a preliminary evaluation of the production of ethanol using a fission yeast, *Schizosaccharomyces pombe* MY4 (L972) grew well in 9% v/v ethanol and fermented out 15% w/v glucose with 96% efficiency (Khosa, 1990). Results of further evaluation of two other species of fission yeasts indicated that these yeasts had high conversion efficiencies and seemed to have the capacity for production of high levels of ethanol (Spies, 1992). Hariantono *et al.* (1991) showed that one strain of *S. pombe* produced more ethanol than a range of strains of *Sacch. cerevisiae* when fermenting saccharified corn starch.

Although there are many genetic and physiological differences between *Sacch. cerevisiae* and *S. pombe*, the application of technology developed in *Sacch. cerevisiae* has been successfully applied to the study of *S. pombe* (Klein & Zaworski, 1990). Genetic procedures are well developed for *S. pombe* and present opportunities for strain development for ethanol production (Tubb, 1984; Russell, 1989).

S. pombe is used commercially in some industries, primarily for production

of single-cell protein from biomass (Klein & Zaworski, 1990), in production of alcohol from molasses fermentation, as a base product for vinegar production (Greenshields, 1978) and in wine production for the degradation of malic acid (Benda, 1982). *S. pombe* can metabolize both glucose and malic acid into ethanol and carbon dioxide, approximately 70% of the malic acid in grape must (pH 2.5) being converted to ethanol (Yang, 1975). An added advantage of *S. pombe* is that it is more resistant than *Sacch. cerevisiae* to sulphur dioxide used in wine making. The sulphur dioxide is added to inhibit growth of undesirable micro-organisms. In ethanol production concentrated sulphuric acid is added to the medium in the fermentation vats for the same purpose.

Hong *et al.* (1990) have identified a potential value of *Schizosaccharomyces* species as a fodder yeast from its ability to grow rapidly on the supernatant fluid from anaerobically fermented pig-waste. The large amounts of pig wastes produced in pig farms require efficient treatment to reduce BOD before discarding.

On malt agar, the maximum temperature for growth of *S. pombe* is 40°C (Slooff, 1970). In ethanol production, a strain that can perform well at high temperatures would be desirable because it would reduce the energy required for cooling of the fermenters and reduce production costs. Haraldson & Bjoerling (1981) have identified two strains of *S. pombe* that can produce ethanol at the high sugar concentrations of 30 to 50° Brix with cane molasses. A comparison of *S. pombe* CBS 352 and *Sacch. cerevisiae* showed that the former was less sensitive than the latter yeast to a high concentration of dissolved solids and that the productivity of ethanol by *S. pombe* was almost twice that of *Saccharomyces cerevisiae* at 25% dissolved solids (Haraldson & Bjoerling 1981).

D-xylose can be converted to ethanol by enzymatic isomerization to xylulose followed by yeast fermentation (Chiang *et al.*, 1981). Schneider & Wang (1980) reported that *S. pombe* was capable of producing ethanol from xylulose at rates comparable to glucose. Most species of yeasts are not satisfactory for ethanol fermentation from xylose: indeed *Sacch. cerevisiae* can only ferment xylulose slowly. A single-stage process involving a xylose-fermenting yeast would have advantages over a multistage process involving xylose isomerization followed by xylulose fermentation by a yeast (Lovitt *et al.*, 1988). With the goal of introducing the xylose isomerase gene into *S. pombe* so as to obtain a strain that ferments xylose to ethanol, Ueng *et al.* (1985) cloned the xylose isomerase gene from *E. coli* into a plasmid which was then transformed into *S. pombe*. Several transformants were able to convert xylose to ethanol.

1.3.2 *Zymomonas mobilis*

Z. mobilis is a promising bacterium with some potential for the production of ethanol from sugar cane (Doelle & Doelle, 1989a). It can withstand ethanol concentrations of between 12-15% v/v because of the exceptionally high amounts of cis-vaccenic acids and hapanoids in the cytoplasmic membrane (Schmidt *et al.*, 1986; Buchholz *et al.*, 1987). It is capable of producing ethanol more than twice as fast, and at a higher yield than yeasts. Its anaerobic nature gives it an advantage over yeast in not requiring oxygen and is therefore a more suitable micro-organism for continuous culture fermentation (Lawford, 1988), and in molasses fermentations (Doelle & Doelle, 1989b). There is also a potential to genetically manipulate *Z. mobilis* so that it can degrade cellulose and

hemi-cellulose and utilise cheap wastes including municipal wastes and agricultural residues (Viikari, 1988; Alterthum & Ingram, 1989).

Z. mobilis has several disadvantages as a candidate in industrial ethanol production. Its range of substrates is limited to sucrose, glucose and fructose, being unable to utilize lactose, maltose and starch (Rogers *et al.*,1982). Furthermore, fermentation of sucrose by *Z. mobilis* results in the formation of sorbitol and levan, both undesirable end-products (Poosaran, 1985). Doelle & Doelle (1990) reported that a high level of glucose and fructose during fermentation of molasses promoted sorbitol production. This effect would impose problems for industrial applications using molasses or cane juice as the raw material. *Z. mobilis* is limited in its growth by the presence of CO_2 in continuous culture (Nipkow *et al.*, 1985) and it does not have a high enough temperature resistance to allow one step fermentation and distillation (Mescle & Pesche, 1986).

It has long been recognised that *Z. mobilis* ferments glucose inefficiently. Its growth yield per mole of glucose was found to be approximately half that obtained for *Sacch. cerevisiae* (Dawes *et al.*, 1966; Ingram *et al.*, 1989). This is accounted for by the production of only one mole of ATP per mole of glucose fermented (Buchholz *et al.*, 1987).

Although research has indicated the potential of *Z. mobilis* as an alternative ethanol producer, it has not replaced yeasts in industrial production. One factor in its failure to replace yeasts in industrial processes is its inability to compete with naturally occuring micro-organisms in the molasses; with contaminating micro-organisms being present it is not feasible to sterilize the very large volumes of molasses wash which are used in industrial fermentations (personal observations

at Triangle Ltd, Zimbabwe).

1.3.4 Other micro-organisms

Various other micro-organisms have been studied for their potential to produce ethanol in mixed cultures.

De Figueroa *et al.* (1985) used protoplast fusion products from *Sacch. cerevisiae* and *Sacch. diastaticus* and found that the products were capable of producing more ethanol than each parental strain, using a starch containing media.

Abouziedy & Reddy (1986) compared monoculture of *Aspergillus niger* and co-culture of *A. niger* and *Sacch. cerevisiae* in direct fermentation of unhydrolyzed starch and found that ethanol yields increased several fold in mixed cultures. In a later study Abouzied & Reddy, (1987) used synergistic co-culture of an amylolytic yeast *Saccharomycopsis fibuligera* and *Sacch. cerevisiae*, a non-amylolytic yeast, to ferment unhydrolysed starch to ethanol with conversion efficiencies over 90% of the theoretical maximum.

A flocculent yeast strain *Sacch. uvarum* showed more desirable characteristics for fermentation of cane molasses than the standard strains (Raghav *et al.*, 1989). A study by Patil & Patil (1989) demonstrated an improvement in ethanol productivity when a combination of top yeast *Sacch. cerevisiae* NCIM 3281 and bottom yeast *Sacch. uvarum* NCIM 3509 were used. Production rates improved by 32.6% and 25.2% in batch fermentation and cell-recycling fermentation, respectively.

Pentose-fermenting yeast strains such as *Pachysolen tannophilus, Candida shehatae* and *Pichia stipitis* have been examined for their ability to produce ethanol

from cellobiose and from sugars liberated by hydrolysis of lignocellulosic biomass, (Dekker, 1982; Parekh & Wayman, 1986; Sreenath & Jeffries, 1987). Laplace *et al.* (1991) demonstrated that a *Pichia stipitis* and *Z. mobilis* co-culture was most suitable for an efficient fermentation of glucose and xylose in which glucose fermentation was achieved before xylose fermentation. *Candida shehatae* has also been used in fermentation of spent liquor (Yu *et al.*, 1987) and *Sacch. ellipsoideus* has been used to produce ethanol from enzymic hydrolysates of municipal waste solids (Di Giorgio *et al.*, 1982).

Production of significant quantities of ethanol (greater than 10 g l^{-1}) has been demonstrated with *Clostridium* species, *Thermoanaerobium brockii* and *Thermobacter acetoethylicus* (Stewart *et al.*, 1984). Ethanol yield of 62% (of theoretical) was obtained with *Pachysolen tannophilus* from media containing xylose and supplemented with exogenous lipids (Dekker, 1986). Thermophilic and ethanologenic species of *Clostridia* have several advantages over yeasts, such as increased production rates and broader substrates range, but these advantages are reduced by the very low ethanol tolerance of these bacteria. *Clostridium indolis* and *Clostridium sphenoides* were used in alcohol fermentation using lignin-related compounds as carbon source (Tanaka *et al.*, 1991) showing that alcohol production from depolymerised lignins was possible.

Stewart *et al.*(1984) summed up an ideal ethanol-producing micro-organism as one that should possess the ethanol tolerance and the carbohydrate-utilizing ability of *Sacch. diastaticus*. It should also possess the cellulolytic activity and thermotolerance of *Clostridium thermocellum*, the osmotolerance of *Zygosaccharomyces rouxii*, the pentose-utilizing ability of *Pachysolen tannophilus*,

the lactose-utilizing ability of *Kluyveromyces fragilis*, and the high specific fermentation rate and near-theoretical ethanol yield of *Z. mobilis*.

1.4 EFFECT OF ENVIRONMENTAL CONDITIONS ON ETHANOL PRODUCTION

Research in industrial ethanol production is aimed at improving its efficiency which is often reduced by various inhibitory effects. Inhibitors of growth and fermentation may be introduced with the inoculum or may accumulate during the process (van Uden, 1989a). Panchal & Tavares (1990) catagorise factors that inhibit ethanol fermentation as:

1. high sugar concentration;
2. end-product ethanol;
3. high temperature;
4. effect of pH and organic acids;
5. effect of dissolved gases, and
6. effect of nutrient supplements as a result of:
    i) presence of high levels of inorganic compounds, and
    ii) depletion of assimilable nutrients.

Inhibition can cause strain instability leading to selection of mutants, or it can encourage unwanted micro-organisms to dominate, thus creating a contamination problem.

1.4.1 Inhibition due to high sugar concentration

Growth of micro-organisms is inhibited in media of high osmolarity. This is due to low water activity of these media which may be 0.90 or below. In

S. pombe and other micro-organisms, polyols such as glycerol, arabitol, sorbitol and disaccharides such as trehalose are involved in adaptive responses to osmotic stress (Nord & Weiss, 1958; D'Amore *et al.*, 1988; Jones & Gadd, 1990; Struch *et al.*, 1991). An increase in osmotic pressure may affect fermentation and may lead to an increase in the concentration of intracellular ethanol (Panchal & Tavares, 1990) which may have detrimental effects on intracellular enzymes involved in ethanol production. It may affect the specific growth rate and thus cause enhanced ethanol toxicity (van Uden 1989a).

Since the amount of ethanol produced during fermentation is directly related to the concentration of glucose in the medium it would be desirable to commence fermentation with as high a concentration of sugar as feasible. Although inhibitory effects of sugar are negligible below 10% w/v (Ciftci *et al.*, 1983), plasmolysis of yeast cells begins to occur at sugar concentrations above 14% w/v but the exact concentration is strain dependent (Jones *et al.*, 1981). When fermentation is attempted in media containing high concentrations of sugars (greater than 15% w/v) the efficiency of ethanol production drops. Osmotolerant yeasts such as *Sacch. rouxii* can grow well in media containing high concentration of sugars, but they produce very low amounts of ethanol (Panchal & Tavares, 1990). Studies by D'Amore *et al.* (1988) on the effect of osmotic pressure on fermentation by brewing yeast in media containing various concentrations of sugar, indicated that there is indeed a decrease in both the growth rate and the fermentation rate with an increase in glucose concentration.

Improvements in ethanol fermentation under high osmotic pressure can be made by sequential addition of the substrate to the fermentation broth

(Nagodawithana *et al.*, 1974; D'Amore *et al.*, 1988; Panchal & Tavares, 1990). In the foregoing studies, the cells were not subjected to an osmotic "shock" with an initial high substrate concentration. Nagodawithana *et al.* (1974) showed that yeast cell viability was high when such fermentations were carried out using the "fed-batch" approach. Physiological studies have shown that the degree of inhibition of growth by ethanol is related to the concentration of glucose in the growth medium. Struch *et al.* (1991) illustrated this by showing that the cell yield and specific growth rate of *Z. mobilis* are affected by high sugar concentration in the medium. Ingram *et al.* (1989) showed that *Z. mobilis* could just tolerate 6.5% w/v ethanol in medium containing 1% w/v glucose. Although no growth occurred if the glucose concentration was increased to 10% w/v with 6.5% w/v ethanol, growth resumed if the ethanol concentration was reduced to 3.4% w/v. However, even in the presence of this lower concentration of ethanol, growth was again inhibited if the glucose concentration was increased to 20% w/v.

1.4.2 Inhibition due to end-product ethanol

Ethanol inhibition is considered to be the principal factor restricting fermentation rate and the concentration of alcohol achievable in ethanol production processes (Pamment, 1989). This effect determines the size and cost of the fermenter required to process a given quantity of sugar and sets the alcohol concentration fed to the distillation column, thereby also determining the energy costs for ethanol separation. The effect is of major economic significance and efforts are being made to isolate yeast strains of improved ethanol tolerance and to develop processes which attempt to circumvent inhibition by removing ethanol

as it is formed (Pamment, 1989).

The rate of fermentation declines progressively as ethanol accumulates, increasing the time required to complete substrate conversion and limiting the final concentrations which are achieved. Tolerance to high concentrations of ethanol is strain dependent (Ismail & Ali, 1971). The maximum tolerated concentration is around 10% w/v for growth and 20% for ethanol production. Some strains of *Saccharomyces* species and *Schizosaccharomyces* species were found to display higher ethanol tolerance compared to other yeasts (Jones *et al.*, 1981). The biochemistry of ethanol inhibition has been the subject of a number of studies (Rose & Beavan, 1981; Stucley & Pamment, 1982; Ingram, 1986; Rose, 1987; Ingram & Dombek, 1989; Pamment, 1989; Rogers *et al.*, 1989). Results of studies with *Sacch. cerevisiae* indicate that ethanol inhibition of yeasts and the ethanol tolerance involve a number of different mechanisms. Among the specific effects which have been demonstrated are the interference by ethanol with the structure and transport properties of the cell membrane (van Uden, 1989b) and inhibition of the enzymes of the glycolytic sequence (Scopes, 1989; Lovitt *et al.*, 1988).

Since fermentation involves a linear sequence of enzymes, the maximum rate of flux through the system would depend on the rate of the slowest enzyme. Thus inhibition of only one enzyme by ethanol could slow down the overall rate of fermentation by restricting the rate of glycolysis. Such a restriction could arise from: (i) damage to the cell membrane resulting in leakage of essential cofactors and co-enzymes from the cell; or (ii) a direct inhibitory effect on the enzymes; or (iii) both of these effects together (Ingram *et al.*, 1989).

1.4.2.1 Effect on cell membrane

The primary toxic effects of ethanol are exerted on the plasma membrane. The membrane is of central importance in regulating the transport of sugar and other nutrients into the cell and in excretion of waste products, including ethanol. It is also a site for a large number of enzymes active in other cellular metabolic processes (van Uden, 1989b). The plasma membrane consists of globular proteins dispersed in a heterogeneous lipid bilayer. These lipids contain acyl residues from long chain saturated and unsaturated fatty acids. The viscosity of each fatty acid is a function of its chain length and degree of saturation. Cells alter their membrane fatty acid composition to maintain optimum fluidity with changes in environmental conditions (Ingram, 1986). Ethanol interacts directly with the lipid bilayer in membranes causing physical and chemical changes in membrane properties, which have adverse effects on cellular activity especially transport processes (van Uden, 1989b). Studies have shown that the decrease in fluidity caused by the presence of ethanol inhibits the action of one or more proteins involved in transport of sugars like glucose, maltose and glucosamine; amino acids like lysine and arginine and ammonium salts, into cells (Stucley & Pamment, 1982; Leão & van Uden, 1983; Rose, 1987; van Uden, 1989b). The molecular basis for, and the actual site of this inhibitory action remains unclear (Oliver, 1989).

Jones & Greenfield (1987) have shown that in *Sacch. cerevisiae* there is an exponential increase in plasma membrane fluidity with ethanol concentration. This increase in fluidity is a net result of a number of fundamental changes and not a simple adaptive response conferring ethanol tolerance, as was earlier proposed. It

has been demonstrated in *Sacch. cerevisiae* (Dombek & Ingram, 1987) and in *Z. mobilis* (Ingram, 1986) that addition of ethanol indirectly inhibits glycolysis by causing changes in its cytoplasmic membrane thus permitting leakage of essential cofactors and coenzymes out of the cell. Addition of ethanol to cells at early stages of fermentation induced cell leakage of magnesium and nucleotides, this leakage correlating with the extent of inhibition of fermentation. Removal of ethanol by washing did not immediately restore full fermentative activity but fermentative capacity was regained during incubation in growth medium or in phosphate buffer containing added magnesium ions. In the presence of added nucleotides and magnesium, cells were able to carry out fermentation in the presence of 20% ethanol indicating that glycolytic enzymes are functional at high ethanol concentration providing that the required intracellular concentration of cofactors and coenzymes are available (Ingram, 1986).

Using *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, Kilian *et al.* (1989) found that the final pH reached in cells increased with increasing ethanol concentration, indicating that ethanol enhances passive proton diffusion into the cells. At high specific rates of ethanol production, the ability of the membrane to transport ethanol out of the cell decreases causing an increase in the intracellular ethanol concentration (Jones *et al.*, 1981).

1.4.2.2 Effect on glycolytic enzymes

Early studies by Gray & Sova (1956), Nagodawithana *et al.* (1977) and Navarro & Finck (1982) reported the inhibition of hexokinase, glyceraldehyde

3-P dehydrogenase and pyruvate decarboxylase in extracts of *Sacch. cerevisiae* by forming reversible inhibitor-enzyme complexes. More recent studies by Millar *et al.* (1982) showed that hexokinase was less sensitive to ethanol inhibition than pyruvate decarboxylase, phosphoglycerate kinase and several other glycolytic enzymes. Ingram & Buttke (1984) reviewed some of these early studies and concluded that hexokinase was inhibited by lower concentrations of alcohol than other glycolytic enzymes. Since the enzyme acts at the beginning of the glycolytic pathway, it appeared to be a logical site for end-product inhibition.

Later studies by Scopes (1989) on glycolytic enzymes of yeasts and *Z. mobilis* showed somewhat different results. Some of the enzymes, notably phosphofructokinase, phosphoglycerate kinase, phosphoglycerate mutase and pyruvate decarboxylase were affected by presence of low concentrations of ethanol. The other enzymes were not affected until a higher level of ethanol was reached. The level of ethanol that inhibited hexokinase was found to be approximately 20% w/v, which is not likely to be of concern in industrial ethanol production. At the end of a fermentation run with *Sacch. cerevisiae*, the intracellular enzymes were bathed in a fairly high concentration of ethanol (over 10% w/v) but were still able to function fairly efficiently (Rose 1987). Ethanol inhibition may have an effect on other enzymes. It has been demonstrated that D-xylulose fermentation by *S. pombe* is inhibited by ethanol (Roman *et al.* 1984).

In living cells the enzymes may be partially protected from inactivation and replenishment of inactivated enzymes is also possible. Studies on the effect of ethanol on enzymes are often performed on purified proteins where the stabilising effects of membrane association and other proteins are not present. Thus, the

published data for ethanol inhibition may underestimate the concentrations required to inhibit enzyme activities *in situ* (Lovitt *et al.*, 1988). Few of the enzymes show any substantial change in activity at ethanol concentrations up to 5% w/v and many are not affected at 10 to 12% w/v, the normal maximum ethanol concentration achieved in industrial fermentation. Thus, the slowing and cessation of fermentation in ethanol production with increase in ethanol concentration is not primarily a result of inhibition of glycolytic enzymes by ethanol, although this may have a contributory effect on the reduction in overall production (Scopes, 1989).

1.4.3 Ethanol tolerance

The biochemical mechanisms for ethanol tolerance in micro-organisms are of interest since strains with increased ethanol tolerance ought to be more productive. Among eukaryotes, species of *Saccharomyces* appear to be the most alcohol-resistant organisms. They can grow in concentrations of 8-12% v/v, can survive when exposed to concentrations of up to 15% v/v and can ferment glucose to produce ethanol up to a concentration of around 12% v/v in normal fermentation and up to 20% v/v during sake fermentation (Ingram & Buttke, 1984). Casey & Ingledew (1986) have shown that ethanol tolerance may be genetically determined and may also be influenced by other factors like sugar level, temperature and osmotic pressure. These factors are reviewed later.

Ethanol tolerance of yeasts is associated with the presence of longer chain monounsaturated or branched fatty acids within the membrane (Lovitt *et al.*, 1988). Jones & Greenfield (1987) have demonstrated that the adaptation to ethanol in yeasts involves changes in phospholipid composition and possibly an increase in

sterol content. They presented evidence for an exponential increase in yeast plasma membrane fluidity, as measured by passive permeability to acetic acid, with increase in ethanol concentration.

Numerous investigations have examined the effects of adding various lipids to fermenting yeast cultures. Several authors have reported that the ethanol tolerance of *Sacch. cerevisiae* is improved when it is grown in medium containing unsaturated fatty acids or proteolipids (Stucley & Pamment, 1982), which promotes easier ethanol efflux (Pamment & Dasari, 1989). Ingram & Buttke (1984) reviewed the early studies that were carried out to show that membrane fatty-acyl residues were important determinants of ethanol tolerance. The benefits of adding lipids to fermentations in commercial ethanol production are known. Ethanol tolerance of strains of *Sacch. cerevisiae* used in manufacture of beer, wines and sake is sustained in part by lipids in the wort, grape juice or koji in which they are cultivated (Ingram & Buttke, 1984; Rose, 1987).

1.4.4 Intracellular ethanol accumulation

Controversy exists concerning the intracellular concentration of ethanol in yeast during fermentation (Dombek & Ingram, 1986). This controversy results from problems in the measurement of the intracellular concentration of compounds like ethanol, which are produced rapidly by metabolism and may diffuse rapidly into the medium from the cell. Results of their study indicated that the extracellular concentrations of ethanol in fermenting suspensions of *Sacch. cerevisiae* were less than or equal to those in the intracellular environment.

Novak *et al.* (1981) claimed that added ethanol was less toxic than ethanol

produced by the cell: in the range 0-72.6 g/l, ethanol produced during batch fermentation was reported to be more inhibitory than the same concentration of ethanol added to the medium. Studies on intracellular ethanol concentration and its estimation were reviewed by Pamment & Dasari (1989). They concluded that ethanol does not accumulate inside the cell, under fermenting conditions.

Guijarro & Lagunas (1984) studied the mechanism involved in accumulation of ethanol in *Sacch. cerevisiae* and found that yeast cells do not accumulate ethanol against a concentration gradient but that it passes through the plasma membrane by simple diffusion. They found very little difference between the intracellular and extracellular concentrations of ethanol.

1.4.5. Inhibition due to high temperature

Ethanol production and cell growth are biochemical processes catalysed by enzymes, whose reaction rates depend on temperature (Richter & Becker, 1987). However, high temperatures cause a decline in the growth rate and overall decrease in biomass, cellular protein, RNA, DNA and free amino acids, and in the efficiency of glycolysis and respiratory activity, resulting in formation of petites (Jones *et al.*, 1981; Aguilera & Benítez, 1989; Panchal & Tavares, 1990). Since the fermentation is exothermic, the process must be cooled, and in large plants this creates an additional operation and cost factor (Keim, 1983). The choice of operating temperatures is influenced both by physiological factors and the physical problems of evaporative loses of ethanol and foaming at high temperature (Jones *et al.*, 1981; Williams, 1983).

Most yeasts cannot grow and produce ethanol by fermentation of sugars at

temperatures above 40°C; however, some strains of *Candida*, *Hansenula*, *Kluyveromyces*, *Saccharomyces* and *S. pombe* were found to be capable of fermentation at temperatures above 40°C (Panchal & Tavares, 1990). *Z. mobilis* is much more affected by higher temperatures than the yeasts with optimum temperature for fermentation of 30°C (Ingram *et al.*, 1989).

It has been described earlier that ethanol inhibition is temperature dependent. Several studies have shown that ethanol depresses the maximum temperature for growth and enhances thermal death in some yeasts (Sá-Correia & van Uden 1983; Aguilera & Benítez, 1989). At a higher temperature, ethanol is produced more rapidly than it can be transported through the cell membrane causing a higher intracellular ethanol concentration (Amartey *et al.*, 1991). Navarro & Durand (1978) found that fermentative activity of yeast continued even when growth was inhibited by ethanol and that fermentation was only inhibited at a higher ethanol concentration, with the inhibition being accentuated by an increase in temperature.

1.4.6 Effect of pH

In industrial fermentations, hydrogen ion concentration has an effect on *Sacch. cerevisiae* growth, fermentation rates and by-product formation and also is important in controlling bacterial contamination. Starting pH values are usually in the range 4 to 5 for molasses fermentation (Jones *et al.*, 1981) and, in lightly buffered media, pH is sometimes controlled by intermittent addition of aqueous ammonia.

Both temperature and ethanol interact with pH to affect the metabolism and

ethanol yield of yeasts (Jones *et al.*, 1981; Loureiro-Dias & Santos, 1990). Pampulha & Loureiro-Dias (1989) showed that fermentation by *Sacch. cerevisiae* is completely inhibited at an internal pH of 4.8 in the absence of ethanol. This inhibition increases in the presence of 8% w/v ethanol with complete inhibition at pH 5.5. The effect may be enhanced by the presence of end-products other than ethanol, such as acetic acid and other lipophilic weak acids which may accumulate inside the yeast cells causing a decrease in internal pH (Pampulha & Loureiro-Dias, 1989). A study by Ramos & Madeira-Lopes (1990) demonstrated that in a strain of *Sacch. cerevisiae*, acetic acid at concentrations up to 1% v/v depressed the tolerance to added ethanol from 11% v/v to zero, and simultaneously narrowed the temperature range of growth from 3-42°C to 19-26°C.

1.4.7 Effect of dissolved gases

Sacch. cerevisiae is capable of growth either anaerobically by fermentation producing ethanol and CO_2, or aerobically by respiration producing CO_2 and water. When the sugar concentration in the medium is very low and oxygen concentration high, the Pasteur effect occurs and fermentation is suppressed (Oliver, 1989), respiration now occurring with a consequent higher energy yield compared with glycolysis alone. However, at high sugar concentration, the Crabtree effect occurs with consequent repression of respiration, the dominant catabolic process now being fermentation even in the presence of oxygen (Jones *et al.*, 1981; Lagunas, 1986). This does not imply that oxygen is not required during fermentation. In fact, yeast fermentation is not truly anaerobic since growing yeasts require some oxygen to produce unsaturated fatty acids and sterols

essential for membrane structure and function (Nagodawithana *et al.*, 1974; Bu'Lock, 1979; Jones *et al.*, 1981; Bajpai *et al.*, 1988; Larue & Lafon-Lafourcade, 1989).

Negation of the Crabtree effect appears to occur at high levels of dissolved oxygen tension (DOT) resulting in an increase of biomass and less ethanol production (Oura, 1969; Nagodawithana *et al.*, 1974). A study with *Sacch. cerevisiae* by Nagodawithana *et al.*, (1974) showed a progressive increase in the percentage of cells surviving as the dissolved oxygen in the medium was increased from zero to 100% dissolved oxygen tension (DOT) in rapid fermentation at 30°C, the rate of ethanol production not being affected by up to 13% DOT. At 20% DOT, fermentation was retarded and this increased with further increase of dissolved oxygen. Similar results were demonstrated with *Z. mobilis* (Tanaka *et al.*, 1990) in which cell growth rate and ethanol productivity decreased with increases in oxygen supply, ethanol productivity being more sensitive to oxygen supply than the growth rate. Guebel *et al.* (1991) showed that biomass and ethanol formation were strongly dependent on the oxygen transfer rate in *Pichia stipitis* NRRY Y-7124.

In high sugar concentration media such as molasses, it is necessary not only to encourage cell multiplication but also to retard the inhibition of surviving yeast populations until the medium contains required concentrations of ethanol. The simultaneous aeration and addition of sugar during early stages of ethanol production reduces the effects of the substrate concentration on the growth and fermentation rates (Larue & Lafon-Lafourcade, 1989).

Carbon dioxide has been shown to inhibit the growth of yeast both aerobically and during anaerobic fermentation, the inhibitory effect being enhanced

markedly by low pH values and by high concentrations of ethanol (Jones *et al.*, 1981). Chen & Gutmanis (1976) found that 50% CO_2 level in the aeration mixture, corresponding to 1.6 x 10^{-2} M of dissolved CO_2 in the fermenter broth, was significantly inhibitory to yeast growth.

1.4.8 Effect of organic and inorganic nutrient supplements

For high rates of fermentation to occur, nutrients other than sugar are important. The nitrogen content of yeasts is about 10% of the dry weight, making it an important constituent of any growth medium (Jones *et al.*, 1981). In industrial fermentations $(NH_4)_2SO_4$ and urea are widely used as nitrogen sources, the former also serving as a source of sulphur which forms 0.4% 0f the yeast dry weight (Jones *et al.*, 1981). Phosphorus is another essential factor for cell growth and fermentation. These two processes are also affected by trace elements which fall into three broad categories: macro-elements (K, Mg, Ca, Zn, Fe, Mn, Cl) required in concentrations of 0.1-1 mM; micro-elements (Co, B, Cd, Cr, Cu, I, Mo, Ni, Va) required in concentrations of 0.1-100 μM; and others (Ag, As, Hg, Li, Ni, Os, Pb, Se, Te) where concentrations greater than 10-100 μM affect growth and fermentations adversely (Jones *et al.*, 1981; Jones & Gadd, 1990).

Supplementation of fermentation media with excess assimilable nitrogen has resulted in improved ethanol yields: such sources have included hydrolysed wheat protein (Thomas & Ingledew, 1990), yeast extract (D'Armore *et al.*, 1988b; Vieira *et al.*, 1989; O'Connor-Cox *et al.*, 1991) and soya-bean flour (Bajpai *et al.*, 1988), the latter providing both proteins and lipids. Under oxygen limiting conditions (e.g. immobilised yeast cell fermentations) addition of soya-bean flour had the

effect of relieving the cells from the burden of synthesising the necessary amino acids, sterols and unsaturated fatty acids, thus enhancing ethanol productivity (Bajpai *et al.*, 1988). Mullins & Ne Smith (1987) proposed that the benefits of high-yield varieties of grain sorghum which also possess increased resistance to diseases and pests due to high levels of tannins, can be advantageously utilised in the fuel industry by addition of nitrogen or of proteolytic enzymes, which apparently release additional nitrogen from proteins in the mash. Such an explanation is consistent with the findings that the addition of 0.03% nitrogen hydrolysates of high-tannin grain sorghum accelerated the rate of ethanol production. In a separate study Patil *et al.* (1989) showed that 20-30% more ethanol was produced during fermentation of cane-sugar molasses when the wort was supplemented with chitin or fungal mycelium or both, or with skim milk.

Yeast growth and fermentation are dependent upon several ions, the absence or exhaustion of which result in inhibition or fall in fermentation rate or both. Magnesium has been shown to have a key role in relieving ethanol toxicity during yeast fermentation (D'Amore *et al.*, 1988; Vieira *et al.*, 1989; Dasari *et al.*, 1990). When ethanol is rapidly produced, there is an increased demand for magnesium at higher ethanol concentrations which cannot be met without the presence of additional magnesium and this effect is partly responsible for the fall in cell viability, although the latter may also be a result of the cell's inability to adapt quickly enough to the rising ethanol concentrations. Slaughter *et al.* (1991) demonstrated that the rate of fermentation of glucose by a polyploid strain of *Sacch. cerevisiae* growing in a defined salt medium depended on the availability of NH_4^+. Addition of NH_4^+ to nitrogen-starved medium enhanced fermentation by

stimulating both glucose utilization and 2-deoxy-D-glucose uptake. Addition of $CaCO_3$ was found to enhance ethanol production by *Z. mobilis* ZM4 at higher concentrations (200-400 g/l) of glucose (Sreekumar & Basappa, 1992). Since the pH of the medium decreases during fermentation causing inhibition of ethanol production, the presence of $CaCO_3$ or Na_2CO_3 would neutralise the acid produced in the medium and enhance production of ethanol.

In conclusion, many of the factors that contribute to the overall effect of inhibition of yeast fermentation are interdependent, as was shown by how ethanol production was affected by the interaction of glucose concentration, temperature and the ethanol tolerance of the strain of yeast (Khosa, 1990). However, although very important to the outcome of a fermentation, the interaction between these environmental factors is not well understood and research on inhibition of ethanol production is still continuing.

1.5 CARBOHYDRATE METABOLISM

The need to improve the industrial production of ethanol by microbial fermentation creates a need to understand the general metabolism of the micro-organisms, particularly in regard to the regulatory systems that determine carbohydrate utilisation. There are 2 aspects involved: uptake and catabolism of sugar.

1.5.1 Sugar uptake

The process is an important metabolic control point. The stability of sugar transport systems in yeasts is an important determinant of the time needed

to complete an industrial ethanol fermentation (Slaughter *et al.*, 1991). In fermentative yeasts, hexose-transport activity is characteristic of each yeast strain and may counteract the toxic effect of ethanol (Mauricio & Salmon, 1992).

The hexose-transport system in *Sacch. cerevisiae* has been described in considerable detail (Cirillo, 1980; Bisson & Fraenkel, 1983; Bisson, 1988). Glucose, fructose and mannose are transported by two constitutive transport systems, with high affinity under metabolising conditions and low affinity under non-metabolising conditions. In *S. pombe* the hexose transport system differs from that of *Sacch. cerevisiae.*

Study on the uptake of sugars in *S. pombe* by Hofer & Nassar (1987) showed that this yeast has a common transport system for glucose, 2-deoxy-D-glucose and glucosamine together with a facilitated - diffusion system of low affinity for the other monosaccharides tested (3-*O*-methy-D-glucose, 6-deoxy-D-glucose, D-xylose and D-arabinose). More recently a single hexose-transport system for glucose, fructose, mannose, glucosamine and 2 deoxy-D-glucose has been proposed by Hoever *et al.* (1992).

1.5.2 Fermentative pathways

The fermentation of glucose to ethanol represents a series of co-ordinated enzymatic reactions requiring functional enzymes, co-enzymes, co-factors, appropriate internal pH, a functional membrane to maintain the concentration of reactants and enzymes, and a glucose uptake system (Dombek & Ingram, 1987). Hexose molecules inside the yeast cell are catabolized via glycolysis. In the glycolytic pathway 1 mole of hexose is phosphorylated twice

to give fructose-1,6-bisphosphate which is split into 2 moles of triose-phosphate that are then oxidised to pyruvate. The metabolic steps are coupled to ATP synthesis and NAD^+ reduction. The pathway is thought to be catalysed by the same enzymes in *S. pombe* as in *Sacch. cerevisiae* although enzyme structures may differ (McDonald & Tsai, 1989). Ethanol is formed from pyruvate and two moles of ethanol per mole hexose fermented are theoretically possible with two moles of ATP being produced (Fig. 1.1). The bacterium *Z. mobilis* uses the Entner-Doudoroff pathway (Fig. 1.2) which yields two moles of ethanol from one mole of hexose but, unlike the Embden-Meyerhof pathway, only produces one mole of ATP. In practice, some of the substrate ends up as microbial biomass and some as side-products, notably glycerol, succinate and higher alcohols. Restricting formation of cell material and side-products while maintaining a high rate of fermentation is difficult. There is on-going research into means of eliminating or reducing the activity of enzymatic steps that lead to side products (Tubb, 1984; Rogers & Szostak, 1987).

Fermentation of glucose can proceed well using a reconstituted glycolytic system from purified enzymes in cell-free metabolism. Welch & Scopes (1985) demonstrated this in their study which showed that 18% glucose was totally degraded in 8 hours giving 9% ethanol, which further demonstrated that it is possible to carry out chemical transformations with multiple enzyme systems *in vitro*. Schaaff *et al.* (1989) have demonstrated that overproduction of the different glycolytic enzymes has no effect on the rate of ethanol formation, even for those enzymes that catalyse irreversible steps like hexokinase, phosphofructokinase and pyruvate kinase. Thus, the goal for increased ethanol production rates cannot be

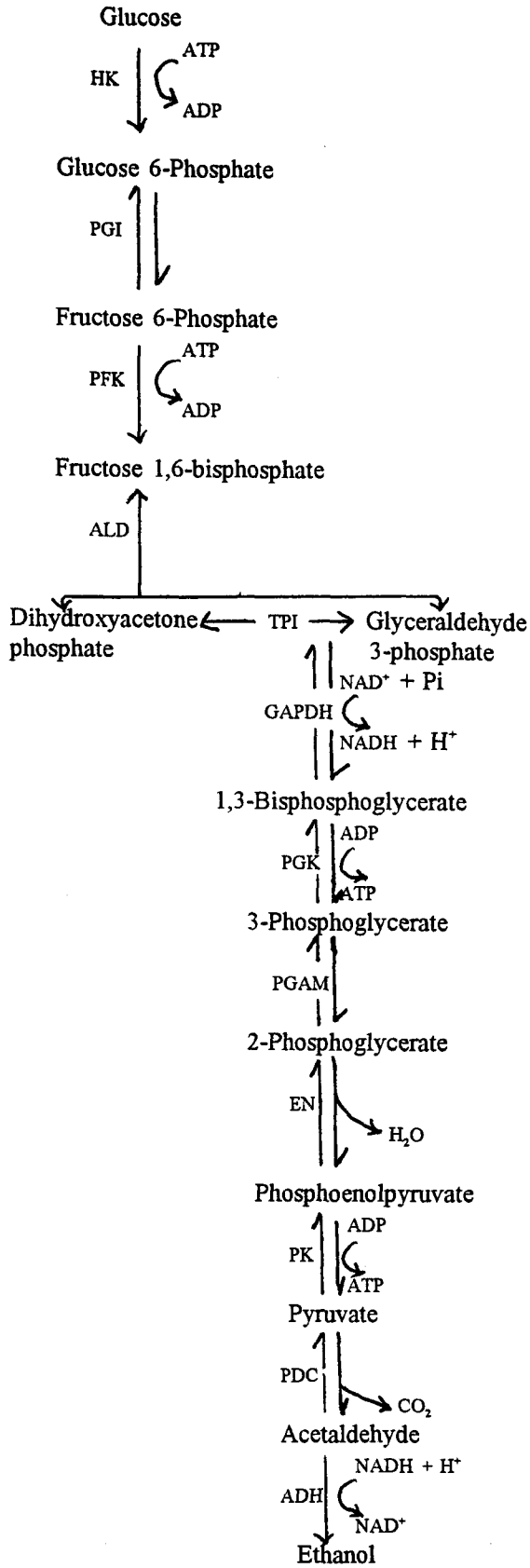


Fig. 1.1

The Embden-Meyerhof-Parnas pathway (Stryer, 1988; Wayman and Parekh, 1990).

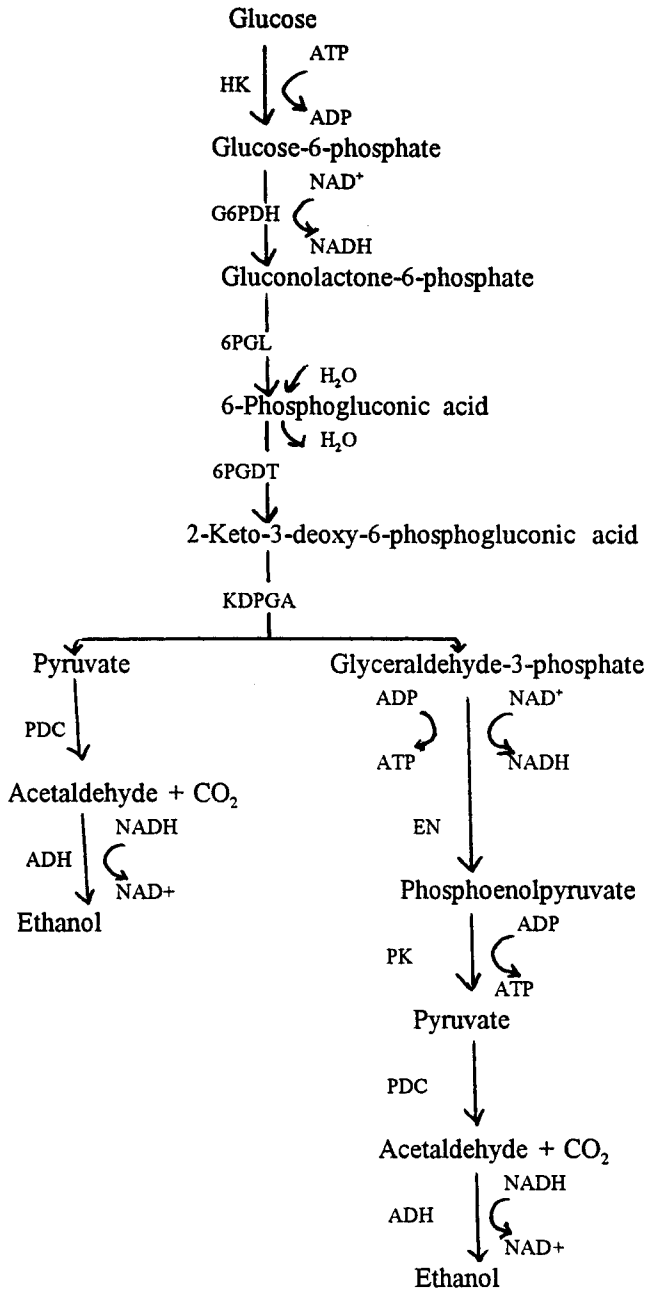


Fig. 1.2

The Entner-Doudoroff pathway (Wayman and Parekh, 1990).

achieved by simply over-producing glycolytic enzymes.

1.6 HEXOKINASES IN MICRO-ORGANISMS

1.6.1 Introduction

As with ethanol fermentation, most of the research on yeast hexokinases has been carried out using *Sacch. cerevisiae*. There are however studies that have looked at other yeasts (Sapico & Anderson, 1967; Hirai *et al.*, 1977; Delvalle & Asensio, 1978; Lagos & Ureta, 1980), and the bacterium *Z. mobilis* (Doelle, 1982a; Doelle, 1982b). Many studies have been carried out to understand the physical, kinetic and regulatory properties of *Sacch. cerevisiae* hexokinases (Maitra & Lobo, 1971; Colowick, 1973; Purich *et al.*, 1973; Barnard, 1975; Muratsubaki & Katsume, 1979; Womack & Colowick, 1979; Albig & Entian, 1988; Fernández *et al.*, 1988; Vojtek & Fraenkel, 1990).

It has been reported that ethanol has an inhibitory effect on hexokinase activity and consequently on the function of the glycolytic pathway in *Sacch. cerevisiae* (Larue *et al.*, 1984), and in *Z. mobilis* (Osman & Ingram, 1985). The studies of the hexokinases and mechanism of ethanol inhibition of fermentation are therefore an important key in understanding ethanol production in *S. pombe*.

1.6.2 Hexokinases of *Sacch. cerevisiae*

Enzymes that phosphorylate aldo- and ketohexoses (particularly glucose, mannose and fructose) with comparable specific activities are called

hexokinases. On the other hand, if a kinase is specific for glucose it is designated a glucokinase, or if specific for fructose the kinase is designated a fructokinase. In *Sacch. cerevisiae* glucose, fructose and mannose are phosphorylated by two different hexokinases called either PI and PII or A and B and a glucokinase (Kaji *et al.*, 1961; Derechin *et al.*, 1966; Colowick, 1973; Purich *et al.*, 1973; Maitra & Lobo, 1977; Muratsubaki & Katsume, 1979; Fernández *et al.*, 1988; Vojtek & Fraenkel, 1990; Zimmermann, 1992). Hexokinase PII is present in largest amounts in the cell. It phosphorylates glucose and fructose equally, while hexokinase PI is more active with fructose than with glucose. Any one of the three enzymes, hexokinases PI and PII and glucokinase allows adequate growth on glucose. They are also important for hexose uptake by the high-affinity system (Bisson & Fraenkel, 1983; Bisson, 1988). Their catalytic reaction has an indirect role in the regulation of cell metabolism (Albig & Entian, 1988). Rose *et al.* (1991) presented evidence that hexokinase PI also has some function in glucose repression.

The two native forms PI and PII - also called isozymes 1 and 2 which correspond to hexokinase A and B respectively (Colowick, 1973) - are chemically and serologically distinct and cannot be interconverted. They are distinguishable by chromatography and electrophoresis and also by catalytic properties. Both hexokinase PI and PII are homogeneous dimeric molecules which exist in monomeric forms under normal assay conditions but can dissociate into two identical monomers (sub-units) e.g. by proteolysis with trypsin. These protease-modified preparations are termed S forms (SI or SII) derived by treatment of PI or PII (Colowick, 1973). Evidence was presented (Kenkare & Colowick, 1965) for the dissociation of hexokinase into half-molecules without loss of activity,

under the influence of pH, phosphate ions and glucose.

Hexokinase PI has only about one-quarter of the specific enzyme activity of hexokinase PII, and differs from PII in substrate specificity. It is obtained as a homogenous species which has unchanged behaviour on re-chromatography. Purich *et al.* (1973) mentioned existence of a third isozyme C (PIII), which can be converted to isozyme B (PII) by high ionic strength and is said to be the predominating enzyme form in fresh lysates. This occurs during preparation. Kopetzki & Entian (1985) also reported the presence of another isozyme PII^M which had only 5-10% of the activity of hexokinase PII and which appeared to be derived from hexokinase PII by a post-translational event, since there are only two hexokinase structural genes HXK1 and HXK2.

1.6.3 Effect of substrate on relative proportions of hexokinases in fungi

Muratsubaki & Katsume (1979) found that the relative proportions of the hexokinases PI and PII in *Sacch. cerevisiae* were dependent on the carbon source in the growth medium and that levels of both enzymes were regulated, whereas glucokinase was not. Results from a different study by Fernández *et al.* (1985) contradicted the above findings in that they described the synthesis of glucokinase and hexokinase PI, but not hexokinase PII, as being regulated by the carbon source used as growth substrate. Hirai *et al.* (1977) characterised inducible forms of hexokinases I and II from *Candida tropicalis*, both of which have broad sugar-specificity and a constitutive glucokinase that is specific for glucose only. Cells of *C. tropicalis* grown under glycolytic conditions showed higher levels of

glucose-phosphorylating activity and a higher fructokinase / glucokinase (F/G) ratio, than cells grown under gluconeogenic conditions; whereas in cells of *C. lipolytica* the three hexokinases are all constitutive (Hirai *et al.*, 1977). *Neurospora crassa* has a hexokinase with four isozymes all of which can phosphorylate a wide range of sugars (Lagos & Ureta, 1980). *Rhodotorula glutinis* was found to have a hexokinase of broad sugar-specificity and a specific glucokinase (Mazón *et al.*, 1975).

1.6.4 Hexokinases in some bacteria

Although *Z. mobilis* is described as possessing two distinct and specific constitutive enzymes for glucose and fructose uptake and phosphorylation (Doelle, 1982b; Scopes *et al.*, 1985; Viikari, 1988), both these enzymes show degrees of phosphorylating activities on both glucose and fructose substrates. However, as with *Sacch. cerevisiae*, their relative proportions were found to be dependent on the carbon source. Glucokinase phosphorylates glucose only but reacts equally with ATP, CTP, GTP, ITP and UTP; however, high concentrations of these nucleotides and also ADP, AMP and G6P cause inhibition (Doelle, 1982a). Fructokinase on the other hand, is highly specific for fructose and for ATP, and is inhibited by high concentrations of glucose, G6P, F6P and ATP (Doelle, 1982a). Inhibition by G6P regulates the phosphorylation of glucose whereas fructose phosphorylation appears to be regulated by glucose, which strongly inhibits fructokinase (Viikari, 1988). Doelle (1982a) reported that neither glucokinase nor fructokinase are inhibited by 542 mM ethanol. Osman & Ingram,

(1985) showed that inhibition of fermentation by ethanol in *Z. mobilis* could be attributed to an indirect effect of ethanol on the enzymes of glycolysis.

Bacillus stearothermophilus possesses a glucokinase that is specific for glucose but phosphorylates N-acetyl-glucosamine as well (Goward *et al.*, 1986). Benziman & Rivetz (1972) showed the existence of a glucokinase and a fructokinase in *Acetobacter xylinum*. *Leuconostoc mesenteroides* has a hexokinase that is specific for mannose and fructose (Sapico & Anderson, 1967).

1.6.5 Purification of hexokinases

Early methods of extraction of hexokinases from *Saccharomyces cerevisiae* involved the use of autolysis with consequent proteolytic modification of the enzymes. Because of this, all the early work on hexokinases of *Saccharomyces cerevisiae* was carried out with these modified enzymes. Subsequent work on the unmodified enzymes has shown them to possess properties very similar to, but not identical with, the original preparations. The proteolytic modification was eventually avoided by use of mechanical disruption of cells together with a protease inhibitor such as phenylmethylsuphonyl fluoride (PMSF) (see Colowick, 1973).

Various methods for purification of hexokinases from yeasts and bacteria have been employed (Jakoby, 1971; Scopes, 1987; Ho, 1990; Voet & Voet, 1990). Treatment with protamine sulphate has been widely used for initial removal of nucleic acids from extracts (Borthwick *et al.*, 1964; Scopes, 1987). Standard methods, such as ammonium sulphate precipitation (Darrow & Colowick, 1962; Lazarus *et al.*, 1966; Purich *et al.*, 1973) and heat treatment (Maitra, 1975; Mazon

et al., 1975; Kulbe & Schuer, 1979), have been successfully used. Chromatographic methods using ion-exchange resins have been used to separate the hexokinases from *Saccharomyces cerevisiae* (Barnard, 1975; Maitra, 1970; Muratsubaki & Katsume, 1979) and from *Z. mobilis* (Doelle, 1982b).

1.6.6 Properties of *Sacch. cerevisiae* hexokinases

1.6.6.1 Kinetic properties

In *Sacch. cerevisiae* the phosphorylation of hexose to hexose-6-phosphate is greatly favoured at 30°C and pH 6.0. The sugar substrates are all of the D-series. Aldoses react in the pyranose form, ketoses in the furanose form. The rate of phosphorylation for fructose is higher than that for glucose. Mannose and glucosamine are phosphorylated at rates lower than for glucose, while 2 deoxy-D-glucose is phosphorylated at the same rate as glucose (Bergmeyer *et al.*, 1983). The phosphate donor ATP may be partially replaced by other nucleotides.

The hexokinase is inhibited by ADP and by substrate analogs which do not undergo reaction, namely xylose, lyxose and 6-deoxy-D-glucose (De La Fuente & Sols, 1963; Purich *et al.*, 1973). Non-competitive inhibitors are G6P and some related compounds namely allose-6-P, 3-deoxy-G6P, glucose 1:6-di-P and L-sorbose (Colowick, 1973; Bergmeyer *et al.*, 1983). Nonetheless, Serrano *et al.* (1973) did not find G6P to be inhibitory to hexokinase.

Of the three glucose-phosphorylating enzymes in *Sacch. cerevisiae*, glucokinase has the lowest specific activity (Gancedo *et al.*, 1977; Maitra & Lobo, 1977). It was first purified from hexokinaseless mutants derived from a haploid

strain of *Sacch. cerevisiae* It has a high rate and affinity for glucose compared to the others. Mannose, glucosamine and 2 deoxy-D-glucose are also phosphorylated but at much lower rates, while fructose is not phosphorylated. Glucokinase is inhibited by free ATP present when the ATP:Mg^{2+} ratio is high (Maitra & Lobo, 1977).

1.6.6.2 Molecular properties

Sacch. cerevisiae hexokinases exist as homogeneous dimers whose molecular weights have been reported to be within the range 93 kd to 104 kd (Derechin *et al.*, 1966; Lazarus *et al.*, 1968; Barnard, 1975; Bergmeyer *et al.*, 1983). Complete dissociation of the dimer into two monomers of 50 kd can be achieved by raising the pH, the temperature or the ionic strength, by lowering the enzyme concentration, or by addition of glucose and ADP. Glucokinase appears to exist only as a monomer and has a molecular weight of 51 kd (Maitra, 1970; Middleton, 1990).

Peptide mapping and amino acid analysis of hexokinases of *Sacch. cerevisiae* have been reported. Studies by Schmidt & Colowick (1973b) indicated that both PI and PII contain four cysteine, four tryptophan, and eleven methionine residues per mole. PI contains eight histidine residues per mole, while PII contains five. Peptide mapping indicated that there are about 27 common tryptic peptides and about 16-19 unique peptides in PI and PII. Both isozymes were found to have the same amino acid terminus valine, and the same carboxy terminus alanine.

Hexokinases PI and PII and glucokinase have been completely sequenced, with the two hexokinases showing very similar sequences (Fröhlich *et al.*, 1985; Kopetzki *et al.*, 1985; Middleton, 1990). The nucleotide sequence for hexokinase

PII (HXK2) gene corresponds to 1455 base pairs, coding for 485 amino acid residues, with a monomer molecular weight of 53.8 kd. There are five initiation regions spanning 162 base pairs and three termination sites spanning 29 base pairs. Hexokinase PI gene, coding for 484 amino acid residues, shows 76% homology with hexokinase PII, indicating a strong relation which suggests that they were derived by gene duplication.

Glucokinase is less closely related to the hexokinases. Using recombinant DNA procedures, Albig & Entian (1988) were able to compare the amino acid sequences of glucokinase to hexokinases. They found homologies of only 26% and 28% with the hexokinases PI and PII respectively. A central core spanning about 350 amino acids shows 39% homology to the hexokinases, suggesting a common ancestral protein. The physiological role of glucokinase in *Sacch. cerevisiae* has not as yet been detected from these studies.

Information on the amino acid composition of other hexokinases including wheat germ and rat enzymes, suggests that the hexokinases are closely related to one another. An interesting aspect is the presence of strong homologies over a range of 11 amino acids between *Sacch. cerevisiae* glucokinase and hexokinases, and vertebrate hexokinase III (rat hexokinase C), with 8 amino acids in common. This region is viewed as corresponding to the glucose-binding site. In these kinases, a lysine residue was found to be conserved at amino acid position 110 and possibly corresponded to the ATP-binding site (Albig & Entian, 1988). A common evolutionary origin for mammalian and *Sacch. cerevisiae* hexokinases had earlier been suggested by Marcus & Ureta (1986). They found amino acid sequences from Novikoff hepatoma hexokinase C that could be aligned within the primary

structure of *Sacch. cerevisiae* hexokinases, giving a sequence homology ranging from 30 to 57%. In rat brain hexokinase, the location of the catalytic function in the C-terminal half of the molecule is consistent with the suggestion that the present day mammalian hexokinases I, II and III, with sub-units molecular weights of approximately 100 kd, may have evolved by duplication and fusion of an ancestral gene similar to that coding for *Sacch. cerevisiae*, wheat germ and mammalian type IV hexokinases, all of which have sub-unit molecular weights of approximately 50 kd (Schirch & Wilson, 1987).

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND SOLUTIONS

Where possible, all chemicals used in this investigation were of analytical grade. The commercial sources of all chemicals are listed in Appendix 1.

Unless otherwise specified, the percentage concentration of solid solutes are expressed as % w/v and liquid solutes are expressed as % v/v.

2.2 CULTURE MEDIA

Unless otherwise stated, all media were adjusted to pH 5.5 and autoclaved at 116°C for 15 min.

2.2.1 Edinburgh Minimal Medium No. 2 (EMM2)

EMM2 (Mitchison, 1970) is a chemically-defined medium. Details of its composition and preparation are given in Appendix 2.

2.2.2 Malt Extract Medium

Liquid Malt Extract Medium (MEM) and Malt Extract Agar (MEA) were prepared according to the instructions of the manufacturer (Oxoid Ltd). When required, 50% glucose was added to MEA prior to sterilization.

2.2.3 Yeast-Extract Phosphate Sugar Media

Yeast-extract phosphate base (YEP) contained: 1% yeast extract (Difco) and 0.2% KH_2PO_4. For most experiments, the sugar added to YEP was glucose at a concentration of 1%, the medium being designated YEPG. For some experiments, the glucose concentration was increased or the glucose was replaced by sucrose. If the glucose was replaced by sucrose the medium was designated YEPS and if the sugar concentration was increased the percentage concentration was indicated in a bracket following the designation of the medium, e.g. YEP base containing 15% glucose was designated YEPG(15).

2.3 STRAINS

2.3.1 Sources of strains

Schizosaccharomyces pombe L972 (departmental catalogue No. MY4) was originally obtained from Professor J.M. Mitchison, Department of Zoology, University of Edinburgh (see Bridge & May, 1984). A new isolate of *S. pombe* (MY141) was obtained from diluted molasses at the distillery of Triangle Ltd, Zimbabwe. A strain of *Saccharomyces cerevisiae* (MY1032) was isolated from the yeast which was produced by Gweru Anchor Yeast (Pvt) Ltd, Zimbabwe, and which was used for fermentation of the diluted molasses in ethanol production by Triangle Ltd .

2.3.2 Maintenance and storage of strains

For short-term storage, the yeast cultures were maintained on slopes

of MEA kept at room temperature. For long-term storage, a dense suspension of cells was kept in glycerol medium at -70°C. The glycerol medium was prepared by mixing 30 ml of glycerol with 30 ml of a solution containing 1 g yeast extract and 0.2 g KH_2PO_4. After the pH of the mixture was adjusted to between 5 and 6, the volume was made up to 100 ml with distilled water and then dispensed in 3 ml volumes into small-screw capped vials before autoclaving at 121°C for 15 min.

2.3.3 Isolation of xerotolerant yeasts

Either 1 g or 1 ml of source material was added to 10 ml of MEM and incubated at 30° for 2 to 3 days. After mixing, serial ten-fold dilutions of the MEM cultures were prepared in 0.1% peptone and duplicate 0.1 ml samples of appropriate dilutions were spread over the surface of plates of MEA containing 50% glucose. After incubation at 30°C for 6 to 7 days, the larger colonies were picked and subcultured as patches on MEA plates. Following incubation at 30°C for 2 days, patches of growth were examined microscopically using wet mounts and any consisting of cells of appropriate morphology were picked and subcultured on MEA plates.

2.3.4 Identification of yeast isolates

The methods described by Barnett *et al.* (1983) were used to characterise new isolates of yeasts, the characters covering structural, fermentative and assimilative properties. [For fermentation and assimilation tests 1% of each substrate was used in place of the 0.05 M used by Barnett *et al.* (1983).] Each isolate was identified by entering its character profile into the computer program of Barnett *et al.* (1985).

2.4 PREPARATION OF STARTER CULTURES

A primary starter culture was prepared by transferring cells from a stock culture on an MEA slope to 10 ml of YEPG in a screw-capped bottle. Following incubation at 30°C for 3 days, the culture served as a source of inocula for the following 7 days. A secondary starter culture was prepared by transferring 1 ml of a primary starter culture to a 250 ml Erlenmeyer flask containing 100 ml of YEP medium containing 1% of the carbon source on which the cells were subsequently to be grown. The culture was used after incubation at 30°C for 2 days on a rotary shaker (model G-52, New Brunswick Scientific Co. Inc.) at 150 rpm.

2.5 ASSESSMENT OF TOLERANCE TO SUGARS OR ETHANOL

For sugar-tolerance tests, 5 ml samples of medium containing the appropriate concentration of sugar were added to 16 x 150 mm, screw-capped tubes and autoclaved at 116°C for 10 min.

For ethanol-tolerance tests, each of the concentrations of ethanol to be tested was prepared by adding the appropriate volume of ethanol to a sterile volumetric flask and then adding sufficient YEPG to give a total volume of 250 ml. Samples (5 ml) of the medium were then aseptically dispensed into sterile 16 x 150 mm, screw-capped tubes.

For each concentration of sugar or ethanol, duplicate tubes were inoculated with 0.05 ml of secondary starter culture. The contents of each tube was mixed daily during the period of incubation.

2.6 ASSESSMENT OF GROWTH AND ETHANOL PRODUCTION BY YEASTS

Unless otherwise stated, all tests were carried out at 30°C. In general, each batch of medium was inoculated with 1% of its volume of secondary starter culture. The specific growth rate (μ) of a culture was calculated using the equation:

$$\mu = 0.693\ \delta$$

where

δ = exponential doubling rate

$$= \frac{\log_{10} N_{t1} \times \log_{10} N_{t2}}{0.301\ (t_2 - t_1)}$$

where

N_{t1} = cell concentration at time t_1

N_{t2} = cell concentration at time t_2

The yield co-efficient of a product (biomass or ethanol) was calculated as:

$$\text{Yield co-efficient} = \frac{\text{Product (g)}}{\text{Substrate consumed (g)}}$$

The productivity (for biomass or ethanol) was calculated as:

$$\text{Productivity} = \frac{\text{Product concentration (g l}^{-1})}{\text{time (h)}}$$

2.6.1 Shaken cultures

For small-scale fermentation tests, cells were grown in Erlenmeyer flasks (about 110 ml of medium in 125 ml flasks and 200 ml medium in 250 ml flasks), the mouths being covered with pads of gauze and cotton wool. Following inoculation, the flasks were incubated on a rotary shaker (model G-52, New Brunswick Scientific Co. Inc.) at 150 rpm.

2.6.2 Stirred cultures

For larger-scale fermentation tests, cells were grown in stirred fermenter vessels, having 7 or 14 litre nominal capacity (models F7-100 and F14-100 respectively, New Brunswick Scientific Co Inc.), containing a volume of medium equal to about 70% of their nominal capacity. Each fermenter vessel had a stainless-steel top plate which was fitted with ports for addition of inoculum and removal of samples of the culture. The contents were stirred by three sets of impeller blades which were evenly-spaced on a central shaft rotating at 200 rpm. To improve mixing of the contents, each vessel was fitted with four evenly-spaced vertical baffles which were adjacent to the wall of the vessel. Sterile gas (either air or nitrogen) could be provided to each vessel by means of: (i) a vent in the head-plate to the head-space; or (ii) a tube below the bottom impeller of the stirrer so as to produce dispersed bubbles in the fluid phase. For incubation, up to three vessels were placed in a fermenter-drive assembly (model FS-300, New Brunswick Scientific Co. Inc.) which provided: (i) temperature control by means of a thermostated water bath; (ii) variable drive for the stirrers; and (iii) controlled air flow. When required, the pH of a culture could be monitored by a pH electrode

which was fitted through the head plate of the vessel and which was connected to a pH measuring and control unit (model pH-22, New Brunswick Scientific Co. Inc.). The control unit was provided with pumps for the addition of either acid or alkali to a vessel so as the maintain the pH at a pre-set value.

2.6.3 'Stirred' culture under reduced pressure

Fermentation under reduced pressure was carried out in a rotary-film evaporator (model 'Rotavapor R', Büchi). The 'sample' flask which was half immersed in a water bath at 30°C and which was rotated at 100 rpm contained 250 ml of culture. The receiving flask was immersed in a water bath at about 5°C. The system was operated at a pressure of about 70 mm, with air being admitted at a rate of 100 ml per min. Before sampling, the volume of the culture was adjusted to 250 ml with sterile distilled water to make-up losses due to evaporation.

2.7 GROWTH OF CELLS FOR PURIFICATION OF HEXOKINASE

Small batches of cells were grown in 2 litre Erlenmeyer flasks each containing 400 ml of medium. After inoculation (4 ml of secondary starter culture per flask) the flasks were incubated at 30°C on a rotary shaker (model G-52, New Brunswick Scientific Co. Inc.) at 150 rpm. Larger batches of cells were grown in 14 litre stirred vessels each containing 10 l of medium which was sparged with air at 400 ml min^{-1}. (See Section 2.6.2 for other conditions applying to growth in stirred vessels.)

2.8 COLLECTION OF CELLS FROM CULTURES

Cells were deposited from samples of cultures (up to 200 ml) in a refrigerated centrifuge ('Mistral' model L4, MSE) operating at 700 *g* . For larger volumes of culture (up to 10 litres), cells were harvested in a continuous-flow centrifuge (Gyro-Tester, De Laval Separator Co.)

2.9 MEASUREMENT OF THE BIOMASS CONCENTRATION OF CULTURES

Cells in a 10 ml sample of culture were deposited by centrifuging in a conical glass tube at 700 *g* for 15 min. The supernatant fluid was discarded, the cells were resuspended in distilled water and then redeposited by centrifuging. This washing process was repeated twice, then the final washed pellet of cells was stored overnight at -70°C. The cells were then freeze-dried by subjecting to vacuum for at least 24 hours in an oil-pump vacuum system (model FDA/16 BM, Dynavac). Following drying, the tubes containing the dried cells were weighed immediately and the mass of the cells obtained by subtracting the weight of the glass tube.

2.10 MEASUREMENT OF THE TURBIDITY OF CULTURES

The optical density (OD) was measured at 580 nm using a single beam spectrophotometer ('Spectronic 20', Milton Roy Co.). Measurements were made on samples of cultures in 13 x 100 mm test tubes. When the OD of samples of cultures were greater than 0.5, the samples and blanks which consisted of the corresponding uninoculated medium were appropriately diluted with water.

The OD of cultures growing in 16 x 150 mm screw-capped culture tubes

was measured *in situ.*

2.11 MEASUREMENT OF pH OF CULTURES

The pH of samples of cultures was measured using a combined electrode in conjunction with a pH meter (model 611, Orion Research).

2.12 RAPID DETECTION OF GLUCOSE IN CULTURES

For the rapid detection of glucose in samples of cultures, 'Clinistix' reagent strips (Miles Australia Pty Ltd) were used. A positive reaction was given by as little as 0.01% glucose.

2.13 MEASUREMENT OF SUBSTRATES AND FERMENTATION PRODUCTS IN CULTURES

Samples of culture from which the cells had been removed by centrifugation were passed through membrane filters with an average pore diameter of 0.45 μm (type HV, Millipore Corp.) and the filtrates were analysed for glucose, ethanol and glycerol by HPLC. The HPLC system consisted of a pump (model 6000A, Waters Associates Inc.), injection system (model U6K, Waters Associates Inc.), a resin-based ion-exchange column and a differential refractometer (model R401, Waters Associates Inc.). For samples from glucose-based media, a 7.8 x 300 mm hydrogen-form column (type HPX-87H, Bio-Rad Laboratories) was used at 55°C with 0.005 M H_2SO_4 as the mobile phase; whereas, with samples from sucrose-based media, a 4.6 x 220 mm calcium-form column (Polypore CA, Brownlee Labs Inc.) was used at room temperature with water as the mobile phase. The sample injected was usually 5 μl and the flow-rate of the mobile phase was

usually 0.3 ml per min.. The output from the refractometer was connected to an IBM-compatible computer loaded with a data acquisition and analysis program (DAPA Version 1.40, DAPA Scientific Software). Appendix 3 shows for each solute the plot relating the 'area under the curve' to concentration.

2.14 PREPARATION OF CELL EXTRACTS

Following separation from culture medium by centrifugation, the deposited cells were washed three times with Tris-HCl buffer (50 mM, pH 7.6). The final pellet was either used immediately or stored at -20°C until required. Freshly deposited or thawed pellets were suspended in the above buffer and were disrupted by passing the suspension through a pre-cooled French pressure cell (model 4-3396, American Instrument Co.) operated at 138 mPa. The effluent from the press was collected in an ice-cooled centrifuge tube and, after the third passage, the debris was deposited by centrifuging for 30 minutes at 27000 *g* and 4°C (SS34 rotor and centrifuge model RC5-C, Sorvall Instruments). The resulting clear supernatant fluid was retained as the crude extract.

2.15 MEASUREMENT OF PROTEIN CONCENTRATION

The concentration of protein in a crude extract was measured by the biuret reaction as described by Koch & Putnam (1971); this reaction is unaffected by the presence of high concentrations of nucleic acids. The concentration of protein in preparations of semi-purified or purified enzyme was measured by the method of Lowry *et al.* (1951). Solutions of bovine serum albumin were used as standards

for both methods (see Appendix 4).

2.16 MEASUREMENT OF HEXOKINASE ACTIVITY

Two methods, both described by Bergmeyer *et al.* (1974), were used for measuring hexokinase activity. The reactions which were coupled to pyridine nucleotide-dependent dehydrogenases were followed by measuring the change in extinction at 340 nm using a double-beam, recording spectrophotometer (model 552, Perkin-Elmer). The reactions were carried out at about 22°C in semi-micro cuvettes (optical path of 1 cm), the activity in a particular preparation being measured at least twice. A value of 6.22×10^{-3} l mol^{-1} cm^{-1} was used for the molar extinction coefficients of NADH and NADPH at 340 nm. Total enzyme activity is expressed as μmol product formed min^{-1} and specific enzyme activity is expressed as μM product formed min^{-1} (mg protein)$^{-1}$.

2.16.1 Method 1

This method depended on measuring the rate of formation of glucose-6-phosphate by coupling with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) to measure the rate of formation of NADPH from $NADP^+$. When this method was used for measuring the rate of phosphorylation of fructose, then excess phosphoglucose isomerase (EC 5.3.1.9) was added to convert the fructose-6-phosphate to glucose-6-phosphate.

The assay mixture contained 20 mM triethanolamine-HCl (pH 7.6), 8 mM $MgCl_2$, 0.64 mM ATP, 2.22 mM glucose, 0.7 units of glucoses-6-phosphate dehydrogenase and 0.91 mM $NADP^+$ in a final volume of 1 ml. When fructose

replaced glucose as the substrate, 3.4 units of phosphoglucose isomerase was included in the assay mixture. The reaction was initiated by the addition of the ATP.

2.16.2 Method 2

This method depended on measuring the rate of formation of ADP by coupling through pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) to measure the rate of formation of NAD^+ from NADH. This method can be used for any substrate capable of being phosphorylated by ATP.

The assay mixture contained 20 mM triethanolamine-HCl (pH 7.6), 8 mM $MgCl_2$, 1.8 mM KCl, 0.64 mM ATP, 2.22 mM glucose (or alternative substrate), 0.43 mM phospho-enol-pyruvate, 10 units each of pyruvate kinase and lactate dehydrogenase, and 0.28 mM NADH in a final volume of 1 ml. The reaction was initiated by the addition of the ATP.

2.17 PURIFICATION OF HEXOKINASE

Details of the procedures for the purification of hexokinases are given in Chapter 6.

2.18 DISC-GEL ELECTROPHORESIS

Discontinuous electrophoresis under non-denaturing conditions was carried out in gels consisting of 7% acrylamide cross-linked with N,N'-methylenebisacrylamide, contained in glass tubes (0.5 x 9.0 cm), following the

general procedure described by Davis (1964) and outlined in Appendix 5. The enzyme preparation was diluted with distilled water so as to contain 5-20 μg protein in a total of 40 μl. Sucrose (10 μl of 60% solution) and bromophenol blue tracking dye (5 μl of 0.25% solution) were added to the enzyme preparation, the mixture then being centrifuged. Each disc gel was loaded with 55 μl of the mixture. Electrophoresis was carried out using a power pack (model GPS 200/400, Pharmacia) delivering a current of 5 mA per gel and was stopped when the tracking dye had moved to within 1 cm of the end of the tubes.

Detection of glucokinase and fructokinase was done using a modification of the methods of Shaw & Prasad (1970 and Muratsubaki & Katsume (1979). The method involves electron transfer from NADPH to a tetrazolium (*p*-iodonitrotetrazolium violet or nitro-blue tetrazolium) via phenazine methosulphate, to give a coloured formazan band. For detecting glucokinase, the reaction mixture was made up of 20 mM triethanolamine buffer (pH 7.6), 2.2 mM glucose, 8.0 mM $MgCl_2$, 0.64 mM ATP, 0.9 mM NADP, 0.55 units ml^{-1} glucose-6-phosphate dehydrogenase, 60 mg ml^{-1} phenazine methosulphate and 60 mg ml^{-1} *p*-iodonitrotetrazolium. For detecting fructokinase, the glucose was replaced by fructose and the reaction mixture also contained 1.1 units ml^{-1} phosphoglucose isomerase.

Proteins were located by staining with Coomassie Brilliant Blue R. The staining solution consisted of 0.25% w/v Coomassie Brilliant Blue R, 45.4% v/v methanol and 9.2% v/v glacial acetic acid in distilled water. The gels were totally immersed in the staining solution which was gently agitated on an orbital mixer at 30 rpm for 60 min, during which time the gels and solution were protected from light. Destaining of protein gels was performed electrophoretically in a solution

consisting of 18% v/v methanol and 9% v/v acetic acid for at least 1 hour. The stained gels were washed in distilled water, followed by 7.5% v/v acetic acid in which they were then stored. Photographs were taken with a 35 mm camera (XR-10, Ricoh), using black and white film (FP4, Ilford).

Relative electrophoretic mobility (R_m) is the ratio of the distance of the enzyme or protein band from the origin over the distance of the tracking dye from the origin.

2.19 SYSTEM FOR COLUMN CHROMATOGRAPHIC SEPARATION OF PROTEINS

The system used to separate proteins by column chromatography consisted of a peristaltic pump (model P3, Pharmacia), an appropriate chromatographic column, a UV detector operating at 280 nm (model EM-1, Bio-Rad) and chart recorder (model 1325, Bio-Rad), and a fraction collector (model 2110, Bio-Rad).

2.20 CONDUCTIVITY MEASUREMENTS ON FRACTIONS FROM COLUMNS

The conductivities of fractions from a chromatographic column were measured using a probe (type PW 9513, Philips) and conductivity meter (model PW9501/01, Philips).

2.21 DETERMINATION OF MOLECULAR WEIGHT

The molecular weight of hexokinase was determined by comparing its elution volume factor (K_{av}) with those obtained for known proteins. The K_{av} represents the fraction of the stationary gel volume which is available for diffusion

of a given solute species.

Sephacryl S-300 superfine was packed in a column (model K26/100, Pharmacia) according to the manufacturer's specifications to give a bed volume of 189 ml. The column was equilibrated with 50 mM Tris-HCl buffer (pH 7.6) and operated at a flow-rate of 10 ml h^{-1}.

Using a mixture of proteins of known molecular weight, a plot of K_{av} against molecular weight was prepared and used to calculate the molecular weight of the purified enzyme protein. The K_{av} for a particular protein was calculated from the following relationship.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume for a protein

V_t = total bed volume

V_o = column void volume

The column void volume was determined with Blue Dextran 2000.

2.22 DETERMINATION OF MOLECULAR WEIGHT OF SUB-UNITS

The size of sub-units of hexokinase were determined by comparing their electrophoretic mobilities to those of polypeptides of known molecular weights. Polyacrylamide gel electrophoresis under denaturing conditions using sodium dodecylsulphate (SDS) was carried out following the procedure described by Laemmli (1970) and Weber *et al.* (1972) (see Appendix 6 for details). Samples

(0.5 ml) of hexokinase (5-20 μg protein) or mixtures of proteins of known molecular weight were added to a solution made by dissolving 75 mg dithiothreitol in a mixture of 0.1 ml 10% w/v sodium dodecylsulphate and 0.06 ml Tris-HCl buffer (0.5 M, pH 6.8). Each mixture was then boiled for 5-6 min, after which 100 μl of 60% w/v solution of sucrose and 5 μl of 0.25% w/v solution of bromophenol blue were added. After centrifuging, 55 μl of each mixture was loaded on to a separate disc gel for electrophoresis.

Gels were stained for protein with Coomassie brilliant blue R as described in Section 2.19. The molecular weight of an 'unknown' was ascertained from a plot of the molecular weights of the reference proteins against their relative electrophoretic mobilities.

2.23 DETERMINATION OF AMINO ACID COMPOSITION

The amino acid composition of hexokinase was carried out at the Department of Biochemistry, Monash University, using the 'PICO.TAG' method (Waters Associates Inc.). The three steps involved in the method were:

(1) Hydrolysis of the protein in 8 ml 3 N HCl containing 40 mg of phenol at 110°C for 22 hours, after which the cooled solution of amino acids was dried *in vacuo* for about 1 hour;

(2) Preparation of phenthiocarbamyl derivatives of the amino acids by reacting with phenylisothiocyanate;

(3) Analysis by reverse-phase HPLC using triethanolamine/ sodium acetate/methyl cyanide buffer (pH 6.4) as mobile phase A and 60% methyl cyanide as mobile phase B, a prepared mixture of amino acids (cat. no. 890614094, Pierce Chemical Co.) being used to

calibrate the system.

2.24 STATISTICAL AND NUMERICAL METHODS

Statistical analyses were performed using the computer program 'SYSTAT' (Systat Inc.).

Grouping of hexose kinases on the basis of amino acid composition was performed using the computer program NTSYS-PC version 1.6 (Applied Biostatistics Inc.).

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF YEAST STRAINS

Industrial fermentation of molasses is usually carried out at 20% or less fermentable sugar. However, to reduce distillation costs it is desirable to start with a higher sugar concentration so as to obtain a higher ethanol concentration at the end of fermentation (Bertolini, 1991). Consequently, tolerance to high concentrations of sugars — a property shown by strains of *S. pombe* — would seem to be a desirable characteristic of a yeast to be used in the industrial production of ethanol. During an earlier study of ethanol production by *S. pombe* (Khosa, 1990), *S. pombe* strain MY4 emerged as a likely candidate for use in industry. However, this strain has been in culture collections for many years (see Gutz, *et al.*, 1974) and may have lost some of its desirable properties during subculture. Consequently, it seemed justifiable to isolate new strains of *S. pombe* to compare them with *S. pombe* MY4. Furthermore, it seemed necessary to compare *S. pombe* with a strain of *Saccharomyces cerevisiae* which was currently being used for industrial fermentation of molasses.

In assessing a yeast strain for industrial production of ethanol, specific properties are required. Some of these are: (i) efficient conversion of sugar to ethanol, (ii) the need to tolerate high concentrations of sugars, and (ii) the need to tolerate a corresponding concentration of ethanol. The latter property is one of the most important yeast properties in the industrial production of ethanol by conventional batch processes; since, during fermentation, the ethanol concentration

increases and the ability of the yeast to adapt to the change influences the rate of production and the final yield of ethanol.

3.1 ISOLATION AND IDENTIFICATION OF YEAST STRAINS

3.1.1 Isolation of yeasts in Zimbabwe

Of fourteen xerotolerant yeasts isolated from a variety of sources in Zimbabwe (see Appendix 9), thirteen were found to be budding yeasts and only one isolated from diluted molasses at the ethanol distillery of Triangle Ltd was found to be a fission yeast. This latter isolate was designated MY141,

From the yeast produced by the Gweru Anchor Yeast (Pvt) Ltd, Zimbabwe, a typical colony was selected and designated MY1032.

3.1.2 Identification of the new isolates

The character profiles of the two new isolates which are shown in Appendices 7 and 8 were entered into the identification program of Barnett *et al.* (1985). Strain MY 141 was identified as *S. pombe* with only 1 discrepant result in 52 tests. [The discrepant result was the utilization of glycerol by MY141; however, this result is in agreement with that of Bridge & May (1984) who found that 93% of strains of *S. pombe* could utilize glycerol.] Strain MY1032 was identified as *Sacch. cerevisiae* with no discrepant result in 54 tests.

3.2 PROPERTIES OF THE THREE YEAST STRAINS

3.2.1 Tolerance to high concentrations of sugars

The growth of the three strains of yeasts in glucose and sucrose concentrations up to 60% are shown in Tables 3.1 and 3.2 respectively. With glucose as the sugar, *Sacch. cerevisiae* MY1032 showed more growth over the 7 day period of incubation than either *S. pombe* MY4 or MY141 when the concentration of sugar in the YEP medium was 0.5%. However, over the glucose range 10% to 30%, although the growth decreased with increasing glucose concentration, the three strains showed similar amounts of growth. As the concentration of glucose was further increased (40% and 50%), the two strains of *S. pombe* grew better than the strain of *Saccharomyces cerevisiae*, until at 60% glucose the latter strain failed to grow whereas the two strains of *S. pombe* still showed some growth. With sucrose as the sugar, the patterns of growth were similar, although more growth generally occurred at the higher concentrations than with glucose. This effect was due to higher water activity produced by solutions of sucrose having the same percentage concentration as solutions of glucose. However, it should be noted that the strain of *S. pombe* (i.e. MY141) which was recently isolated from molasses was no more tolerant of high concentrations of sugars than the strain (MY4) which had been isolated many years ago.

A more detailed comparison was made between *S. pombe* MY4 and *Saccharomyces cerevisiae* MY1032 by comparing the cell yield in various concentrations of sucrose after 5 days incubation (Table 3.3). In relation to the growth shown by *S. pombe* MY4, *Saccharomyces cerevisiae* MY1032 showed less growth at all concentrations of sucrose: the relative biomass concentration decreased from 90% at 20% (w/v) sucrose to 84% at 40% (w/v) sucrose, with a

Table 3.1 Effect of glucose concentration on the growth of *S. pombe* MY4, *S. pombe* MY141 and *Sacch. cerevisiae* MY1032

Yeast were grown in tubes with the specified concentration of glucose in YEP medium. The turbidity of each culture was measured after incubation at 30°C incubation for 7 days. The extent of growth was scored as follows:

OD_{580}	SCORE
≥ 1.5	++++
1.0 - 1.49	+++
0.5 - 0.99	++
0.2 - 0.49	+
≤ 0.19	±
no growth	-

Yeast	Amount of growth when glucose concentration (% w/v) in the medium was:						
	0.5	10	20	30	40	50	60
S. pombe MY4	++	+++	+++	++	++	+	+
S. pombe MY141	++	+++	+++	++	++	+	+
Sacch. cerevisiae MY1032	++++	+++	+++	++	+	±	-

Table 3.2 Effect of sucrose concentration on the growth of *S. pombe* MY4, *S. pombe* MY141 and *Sacch. cerevisiae* MY1032

Yeasts were grown in tubes with the specified concentration of sucrose in YEP medium. The turbidity of each culture was measured after incubation at 30°C incubation for 7 days. The extent of growth was scored as follows:

OD_{580}	SCORE
≥ 1.5	++++
1.0 - 1.49	+++
0.5 - 0.99	++
0.2 - 0.49	+
≤ 0.19	±
no growth	-

Yeast	Amount of growth when sucrose concentration (% w/v) in the medium was:					
	0.5	10	20	30	40	50
S. pombe MY4	++	+++	+++	+++	++	+
S. pombe MY141	++	++++	+++	+++	++	+
Sacch. cerevisiae MY1032	++++	++++	+++	++	++	±

Table 3.3 Effect of sucrose concentration on the growth of *S. pombe* MY4 and *Sacch. cerevisiae* MY1032

The yeasts were grown in 100 ml of YEP medium with the specified concentrations of sucrose. The turbidity of each culture was measured after incubation at 30°C incubation for 5 days.

Yeast	Relative amount of growth when sucrose concentration (w/v) in the medium was:				
	20	30	40	50	60
S. pombe MY4	100	89	63	45	22
Sacch. cerevisiae MY1032	90	78	53	14	0.6

more marked decrease to 31% at 50% (w/v) sucrose and finally to less than 3% at 60% (w/v) sucrose.

The limited tolerance to high concentrations of sugars which was shown by the recently isolated *Sacch. cerevisiae* MY141 is consistent with the previously described properties of most strains of *Sacch. cerevisiae* (Tilbury, 1980), yeasts which are normally used in industrial ethanol production. On the other hand, the ability of the two strains of *S. pombe* to grow at high concentrations of sugars is in line with the well known ability of the species to tolerate low levels of water activity (Tilbury, 1980). This ability is also consistent with the many isolations of these yeasts which have been made from molasses and cane juice (Bridge & May, 1984), materials naturally containing a high concentration of sugars.

3.2.2 Tolerance to ethanol

The effect of ethanol concentration on the growth of *S. pombe* MY4, *S. pombe* MY141 and *Sacch. cerevisiae* MY1032 is shown in Table 3.4 At 6% (v/v) ethanol, the lowest concentration tested, the extent of growth by the three strains was similar; however, this represented a reduction in the amount of growth by the *Saccharomyces cerevisiae* MY1032 in the absence of added ethanol, but not for the two strains of *S. pombe*. At each of the three higher concentrations tested (8, 10 and 12% v/v) all three strains were capable of initiating growth, although MY1032 showed a somewhat higher yield than either MY4 or MY141.

3.2.3 Production of ethanol from sugars

Tables 3.5 and 3.6 show the relative efficiency of ethanol production

Table 3.4 Effect of ethanol concentration on the growth of *S. pombe* MY4, *S. pombe* MY141 and *Sacch. cerevisiae* MY1032

Yeasts were grown in tubes with the specified concentration of ethanol in YEPG medium. The turbidity of each culture was measured after incubation at 30°C for 7 days. The extent of growth was scored as follows:

OD_{580}	SCORE
≥ 1.5	+++
1.0 - 1.49	++
0.1 - 0.99	+
< 0.1	±
no growth	-

Yeast	Amount of growth when ethanol concentration (% v/v) in the medium was:				
	0	6	8	10	12
S. pombe MY4	++	++	+	±	±
S. pombe MY141	++	++	+	±	±
Sacch. cerevisiae MY1032	+++	++	++	+	+

Table 3.5 Biomass and ethanol yields for *S. pombe* MY4, *S. pombe* MY141 and *Sacch. cerevisiae* MY1032 when growing in medium with 15% glucose

The yeasts were grown in 200 ml of YEP medium containing 15% (w/v) glucose. The concentrations of biomass and ethanol were measured after incubation at 30°C for 36 hours, after which there was no residual glucose in any of the cultures.

Yield	Final concentration in culture of:		Yield of:		Yield of ethanol as a % of the theoretical yield
	Biomass ($g\ l^{-1}$)	Ethanol ($g\ l^{-1}$)	Biomass ($g\ (g\ glucose)^{-1}$)	Ethanol ($g\ (g\ glucose)^{-1}$)	
S. pombe MY 4	10.9	67.7	0.073	0.451	88
S. pombe MY 141	10.5	71.9	0.070	0.479	94
Sacch. cerevisiae MY 1032	9.8	65.4	0.065	0.436	85

Table 3.6 Biomass and ethanol yields for *S. pombe* MY4, *S. pombe* MY141 and *Sacch. cerevisiae* MY1032 when growing in medium with 15% sucrose

The yeasts were grown in 200 ml of YEP medium containing 15% (w/v) sucrose. The concentrations of biomass and ethanol were measured after incubation at 30°C for 36 hours, after which there was no residual sucrose in any of the cultures.

Yield	Final concentration in culture of:		Yield of:		Yield of ethanol as a % of the theoretical yield
	Biomass ($g\ l^{-1}$)	Ethanol ($g\ l^{-1}$)	Biomass ($g\ (g\ sucrose)^{-1}$)	Ethanol ($g\ (g\ sucrose)^{-1}$)	
S. pombe MY 4	10.8	75.1	0.072	0.50	93
S. pombe MY 141	10.7	62.7	0.071	0.42	78
Sacch. cerevisiae MY 1032	8.9	76.9	0.059	0.51	95

by the three strains when fermenting glucose and sucrose respectively.

When using 15% w/v glucose as the substrate, each of the three strains completed the fermentation within 36 hours. *S. pombe* MY141 showed the highest yield of ethanol (94% of theoretical), followed by *S. pombe* MY4 (88%) and *Saccharomyces cerevisiae* MY1032 (86%) (see Table 3.5).

When using 15% w/v sucrose as the substrate, each of the three strains again completed the fermentation within 36 hours. *Saccharomyces cerevisiae* MY1032 and *S. pombe* MY4 showed very similar high yields of ethanol (100% and 98% respectively of theoretical) followed by *S. pombe* MY141 (82%) (see Table 3.6).

3.3 CONCLUSIONS

Each of the three yeasts showed a high efficiency of conversion of glucose to ethanol and although *Saccharomyces cerevisiae* was somewhat more tolerant to ethanol than the two fission yeasts, the latter were clearly more tolerant to high concentrations of sugar. Of the two strains of *S. pombe*, the more recent isolate (MY141) did not possess any obvious advantages over strain MY4, and because of the greater knowledge of its genetic background and its ability to be manipulated by gene cloning (see Russell, 1989), *S. pombe* MY4 was chosen for further study.

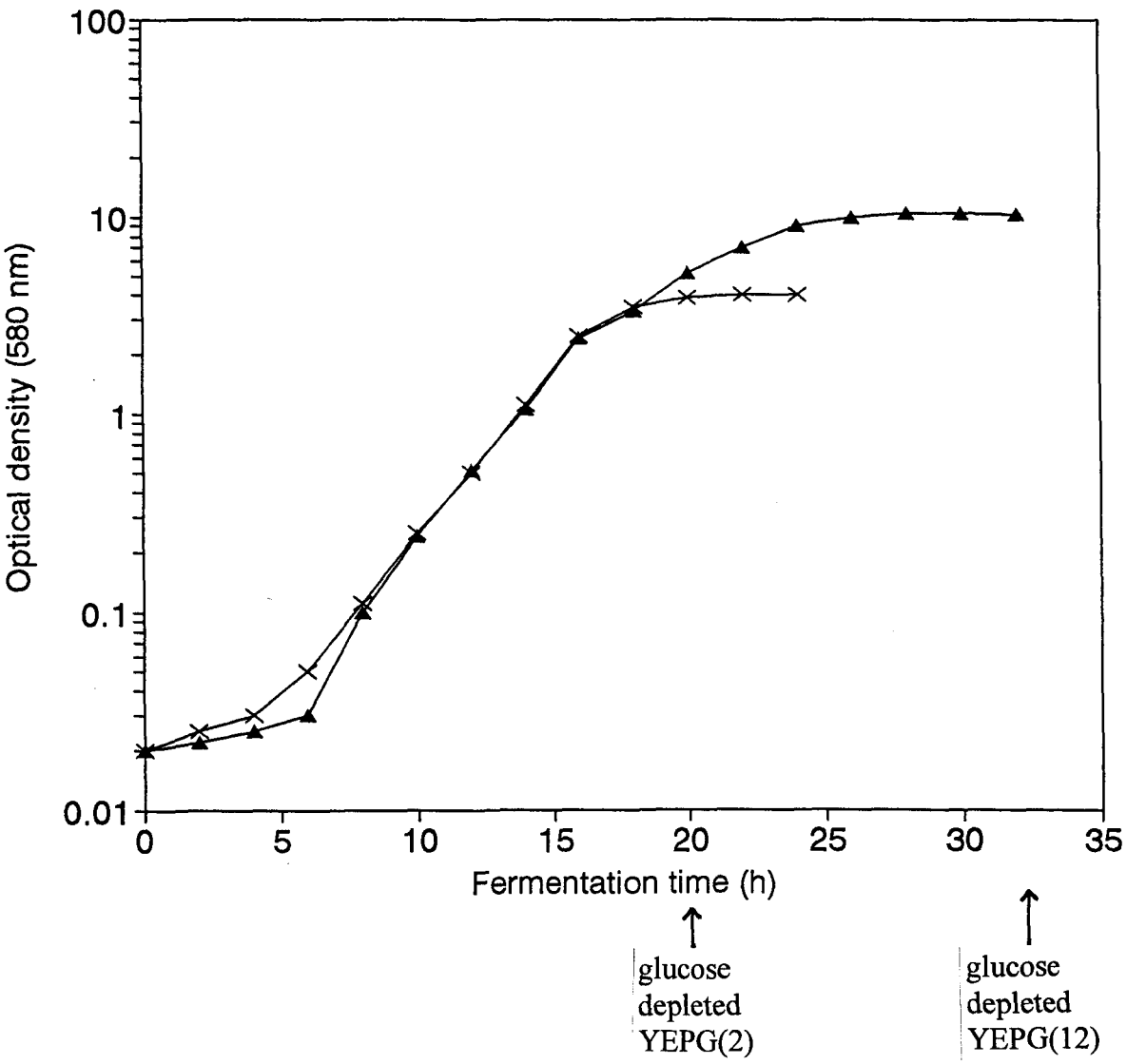
Fig. 4.1 Effect of glucose concentration upon the growth of *S. pombe* MY4

The yeast was grown in 200 ml batches of YEPG(2) and YEPG(12) at 30°C on a rotary shaker. The final sample of each culture coincided with the disappearance of glucose from the culture.

Key to plots:

x————x growth in YEPG(2)

▲————▲ growth in YEPG(12)


100
10
1
0.1
0.01
Optical density (580 nm)
0
5
10
15
20
25
30
35
Fermentation time (h)
glucose depleted YEPG(2)
glucose depleted YEPG(12)

due to the exhaustion of the glucose. In contrast, the culture in YEPG(15) showed a much more extended retardation phase which lasted about 10 hours before entering the stationary phase at about 26 hours, again due to exhaustion of glucose. The commencement of the extended retardation phase in the YEPG(15) at 16 hours could not have been due to limitation of glucose, since the culture more than doubled its biomass concentration during the subsequent 10 hours. Alternative causes for the premature commencement of this retardation phase include exhaustion of a micro-nutrient, exhaustion of oxygen, a change in pH of the medium and the build-up of toxic levels of ethanol.

4.2 EFFECT OF pH ON GROWTH AND ETHANOL PRODUCTION

The effect of the initial pH of the medium on the final cell yield of *S. pombe* MY4 was tested in 200 ml batches of YEPG(2). The cultures were incubated at 30°C for 20 hours, the time normally taken by the yeast to deplete 2% glucose at the normal starting pH of 5.5. As seen in Table 4.1, the yeast can initiate growth over the pH range of 3 to 7, the initial pH not having a major influence on the final biomass concentration (as measured by OD_{580}) over the pH range tested. The mean and standard deviation of the OD_{580} values for the eight sets of cultures is 3.6 ± 0.23, there being no significant difference between the OD_{580} values for the group as a whole. (Using analysis of variance, $P = 0.19$ for the eight sets being drawn from the same population.) However, since the cell density in the cultures was only measured after 20 hours incubation, some cultures could have exhausted the glucose (there was no residual glucose in any of the cultures) and reached stationary phase before the others.

Table 4.1 Effect of the initial pH of medium upon growth of *S. pombe* MY4

The yeast was grown in 200 ml batches of YEPG(2), the initial pH of different batches being adjusted to specific values between 3.0 and 7.0. The cultures were sampled after 20 hours incubation at 30°C on a rotary shaker. The results are mean values of triplicate cultures.

Initial pH of medium	Final pH of culture	Final OD of culture ^{580}
3.0	2.68	3.37
3.5	3.07	3.75
4.0	3.49	3.65
4.5	3.89	3.80
5.0	4.18	3.57
5.5	4.41	3.63
6.0	4.77	3.73
7.0	5.61	3.35

In order to examine the effect of controlling the pH at 5.5, cultures were grown in 5 litre batches of YEPG(15) in stirred vessels, the pH being controlled at 5.5 in one and not controlled in the other. The results for biomass concentration and ethanol concentration over a period of 43 hours are shown in Fig. 4.2 and 4.3 respectively and for specific growth rate and pH over the final 21 hours of the run are shown in Fig. 4.4.

Since both cultures started at the same pH, it is not surprising that the biomass increase over the initial phase of growth was very similar. However, as the uncontrolled culture increased in biomass, the pH decreased so that by 30 hours it was about 3.7 (Fig. 4.4). Over the same period, the specific growth rate of the culture which was controlled to a value of 5.5 was consistently lower than that of the culture which was not controlled. Furthermore, at 43 hours, the amount of glucose remaining in the uncontrolled culture was negligible(0.4%), whereas a substantial amount (4.0%) still remained in the controlled culture even though it had reached stationary phase. At this time, the productivity of ethanol in the controlled culture was only 0.88 g l^{-1} h^{-1} compared with a value of 1.48 g l^{-1} h^{-1} in the uncontrolled culture.

On the basis of these results and since on an industrial scale pH control is both difficult and expensive, it was decided not to control the pH of cultures.

4.3 EFFECT OF AERATION ON GROWTH AND ETHANOL PRODUCTION

The effect of aeration upon growth and ethanol production of *S. pombe* MY4 in YEPG(11) was studied in 4 litre batches of the medium in stirred vessels. The three conditions of 'aeration' provided were: (1) air sparged - i.e. air admitted

Fig. 4.2 Effect of controlling pH on biomass production during growth of *S. pombe* MY4

The yeast was grown in 5 litre batches of YEPG(12) in 7 litre stirred vessels at 30°C, the initial pH of the medium being 5.5. In one vessel the pH was maintained at 5.5 by the automatic addition of either acid or alkali; in the other vessel, the pH was measured, but was not controlled. The cultures were sparged with air at 1000 ml min^{-1}.

Key to plots:

+———+ biomass when pH not controlled

▲———▲ biomass when pH controlled at 5.5

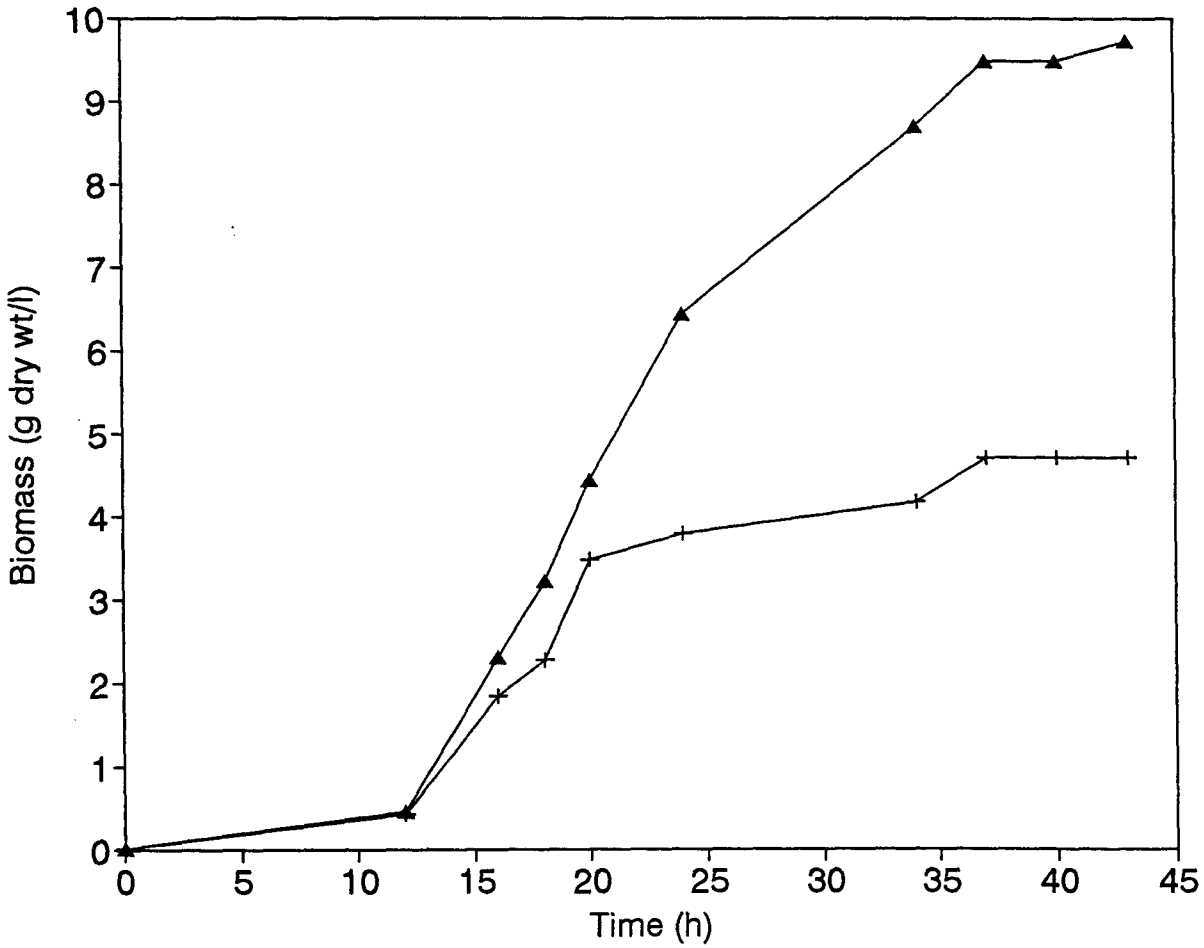

Biomass (g dry wt/l)
Time (h)
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
35
40
45

Fig. 4.3 Effect of controlling pH on ethanol production during growth of *S. pombe* MY4

The yeast was grown in 5 litre batches of YEPG(12) in 7 litre stirred vessels at 30°C, the initial pH of the medium being 5.5. In one vessel the pH was maintained at 5.5 by the automatic addition of either acid or alkali; in the other vessel, the pH was measured, but was not controlled. The cultures were sparged with air at 1000 ml min^{-1}

Key to plots:

+———+ ethanol when pH controlled at 5.5

▲———▲ ethanol when pH not controlled

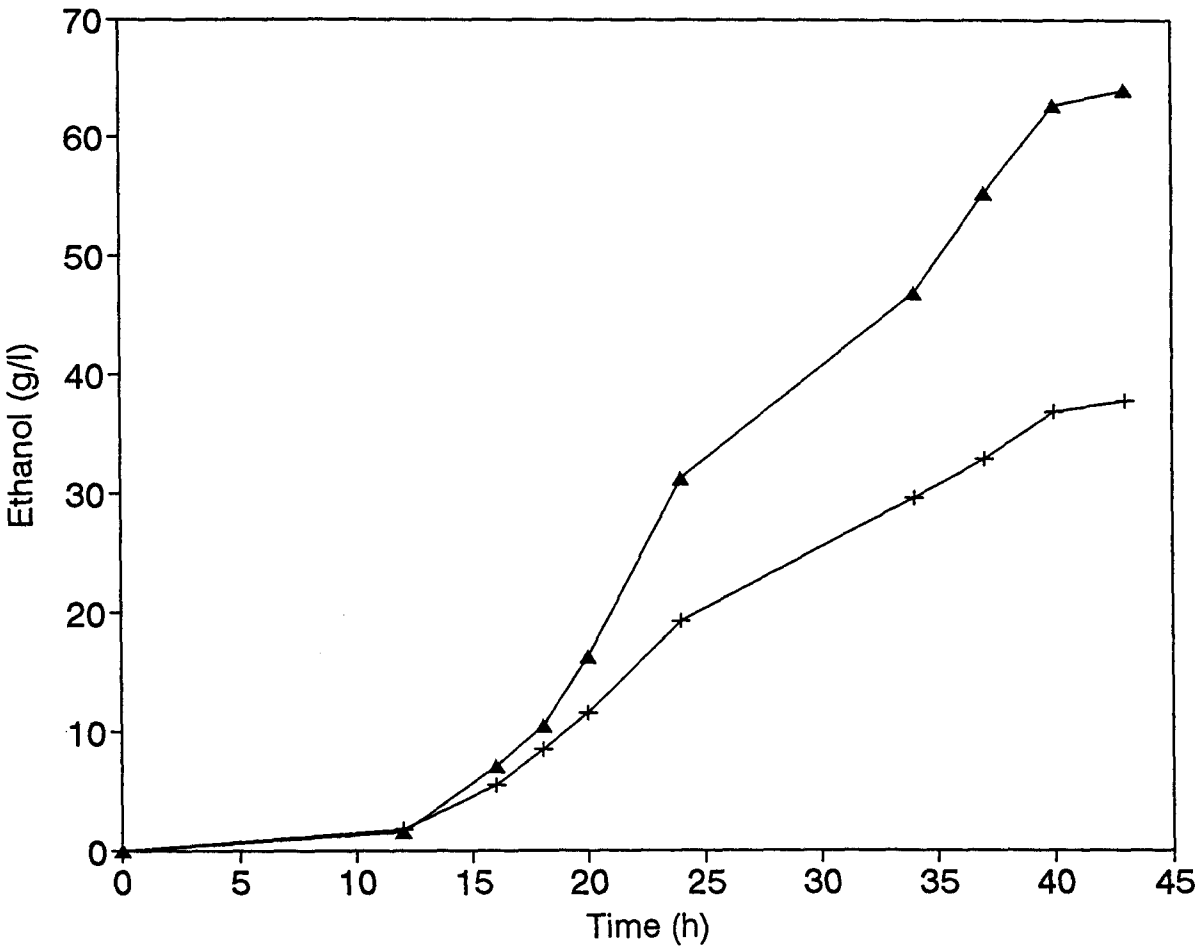

70
60
50
40
30
20
10
0
Ethanol (g/l)
0
5
10
15
20
25
30
35
40
45
Time (h)

Fig. 4.4 Changes in specific growth rate and pH in a culture of *S. pombe* MY4 when pH is not controlled and the change in specific growth rate when the pH of the culture is controlled to a value of 5.5

The yeast was grown in 5 litre batches of YEPG(12) in 7 litre stirred vessels at 30°C, the initial pH of the medium being 5.5. In one vessel the pH was maintained at 5.5 by the automatic addition of either acid or alkali; in the other vessel, the pH was measured, but was not controlled. The cultures were sparged with air at 1000 ml min^{-1}

Key to plots:

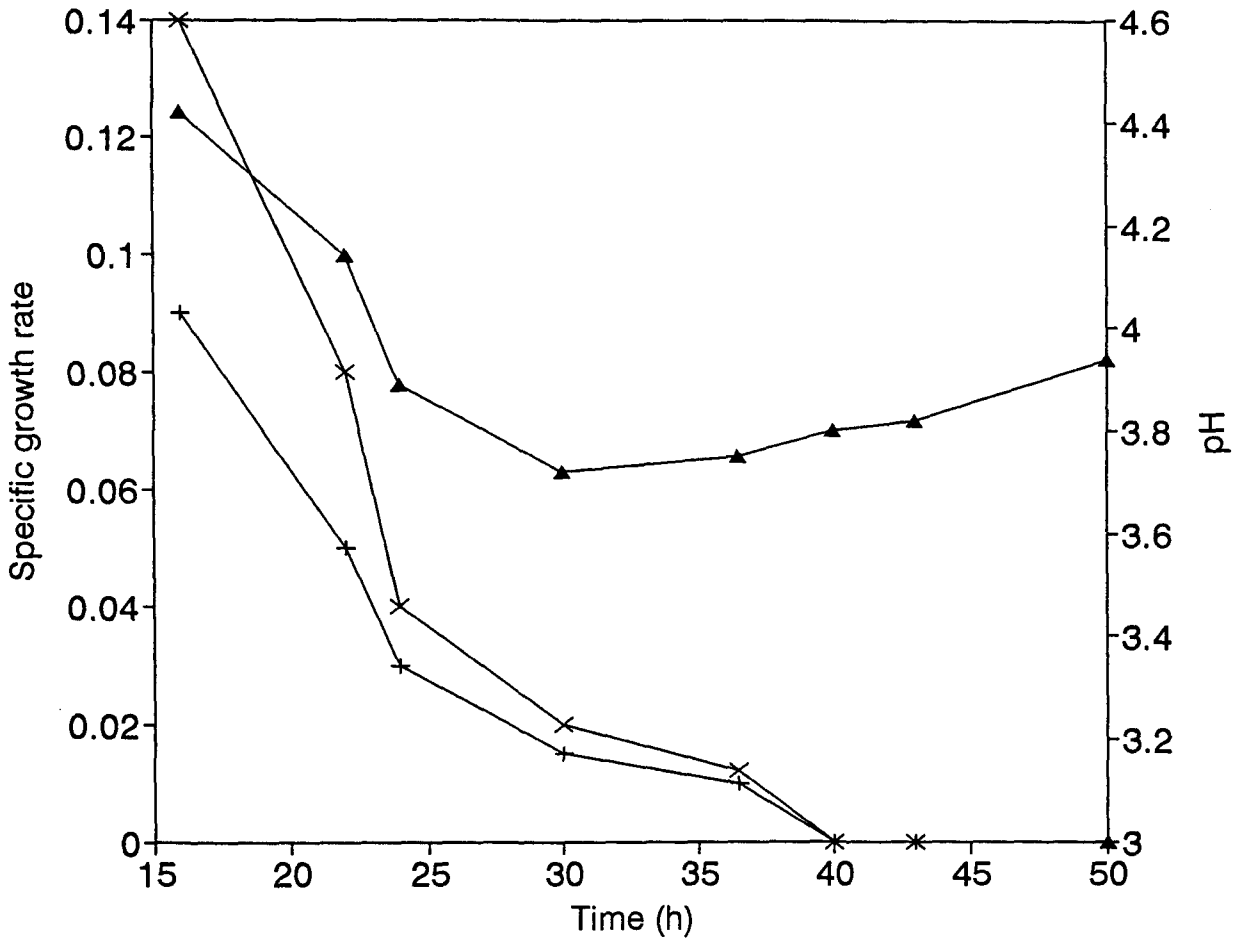
(1) culture in which pH was controlled at 5.5-

+————+ specific growth rate

(1) culture in which pH was not controlled-

x————x specific growth rate

▲————▲ pH


0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Specific growth rate
4.6
4.4
4.2
4
3.8
3.6
3.4
3.2
3
pH
15
20
25
30
35
40
45
50
Time (h)

below the bottom impeller at a rate of 1000 ml min^{-1}; (ii) air to head-space - i.e. air admitted to the head-space at a rate of 1000 ml min^{-1}; and (iii) nitrogen sparged - nitrogen admitted below the bottom impeller at a rate of 100 ml min^{-1}. The initial pH of each of the three batches of medium was 5.5.

The results of a typical run using air-sparge are shown in Fig. 4.5, the results for a typical run using nitrogen-sparge being shown in Fig. 4.6. Results for cultures to which air was supplied only to the head-space are not illustrated since they were very similar in most details to those for air-sparged cultures. A summary of biomass and ethanol yields for the three types of cultures is given in Table 4.2.

Due to the fewer samples in the early part of these experiments, the biomass is plotted on an arithmetic scale, the air-sparged culture (Fig. 4.5) showing an extended linear section corresponding to the retardation phase shown in Fig. 4.1. Over the period 16 hours to 36.5 hours, the specific growth rate of the nitrogen-sparged culture remained virtually constant at a very low value (mean and standard deviation = 0.0519 ± 0.0026); whereas over the same period the specific growth rate of the air-sparged culture declined from 0.23 to 0.009 (see Fig. 4.7). This reduction in specific growth rate of the air-sparged culture could not have been due to limitation of glucose which at 30 hours was still present at a concentration of about 2.5%, a concentration which is sufficient to maintain maximum specific growth rate (see Section 4.1). Nor was the reduction likely to be due to the fall in pH since, although the pH reached a low of about 3.9 at 24 hours, the subsequent rise in pH was not accompanied by a corresponding rise in specific growth rate (see Fig. 4.7). It is possible that the fall in specific growth

Table 4.2 Biomass and ethanol yields with *S. pombe* MY4 grown under three different gas-phase conditions

The yeast was grown in 4 litre batches of YEPG(12) in 7 litre stirred vessels at 30°C. The three gas-phase conditions were:

(1) air sparged at 1000 ml min^{-1}

(2) air to head space at 1000 ml min^{-1}

(3) nitrogen sparged at 100 ml min^{-1}

Type of gas supply to growth vessel	Time of sampling (hours)	Concentration in culture of:		Production rate of:		Yield of:		Yield of ethanol as % of theoretical yield
		Biomass ($g\ l^{-1}$)	Ethanol ($g\ l^{-1}$)	Biomass ($g\ l^{-1}\ h^{-1}$)	Ethanol ($g\ l^{-1}\ h^{-1}$)	Biomass ($g\ (g\ GLU)^{-1}$)	Ethanol ($g\ (g\ GLU)^{-1}$)	
Air sparged	36[a]	8.8	48.4	0.244	1.34	0.073	0.403	79.0
Air to head space	40[a]	6.8	50.4	0.170	1.26	0.056	0.418	82.0
N_2 sparged	53[b]	1.4	38.2	0.026	0.72	*	*	*

[a] First sample at which glucose was not detected in culture

[b] Glucose was present in the culture at 53 hours and was still present after 96 hours

* These values were not determined since glucose was not completely exhausted

Fig. 4.5 Growth and product formation by *S. pombe* MY4 under aerobic conditions

The yeast was grown in 4 litre batches of YEPG(12) in 7 litre stirred vessels at 30°C. Air was supplied to the vessel below the bottom impeller of the stirrer at a rate of 1000 ml min^{-1}.

Key to plots:

□——□	biomass
+——+	glucose
x——x	ethanol
▲——▲	glycerol

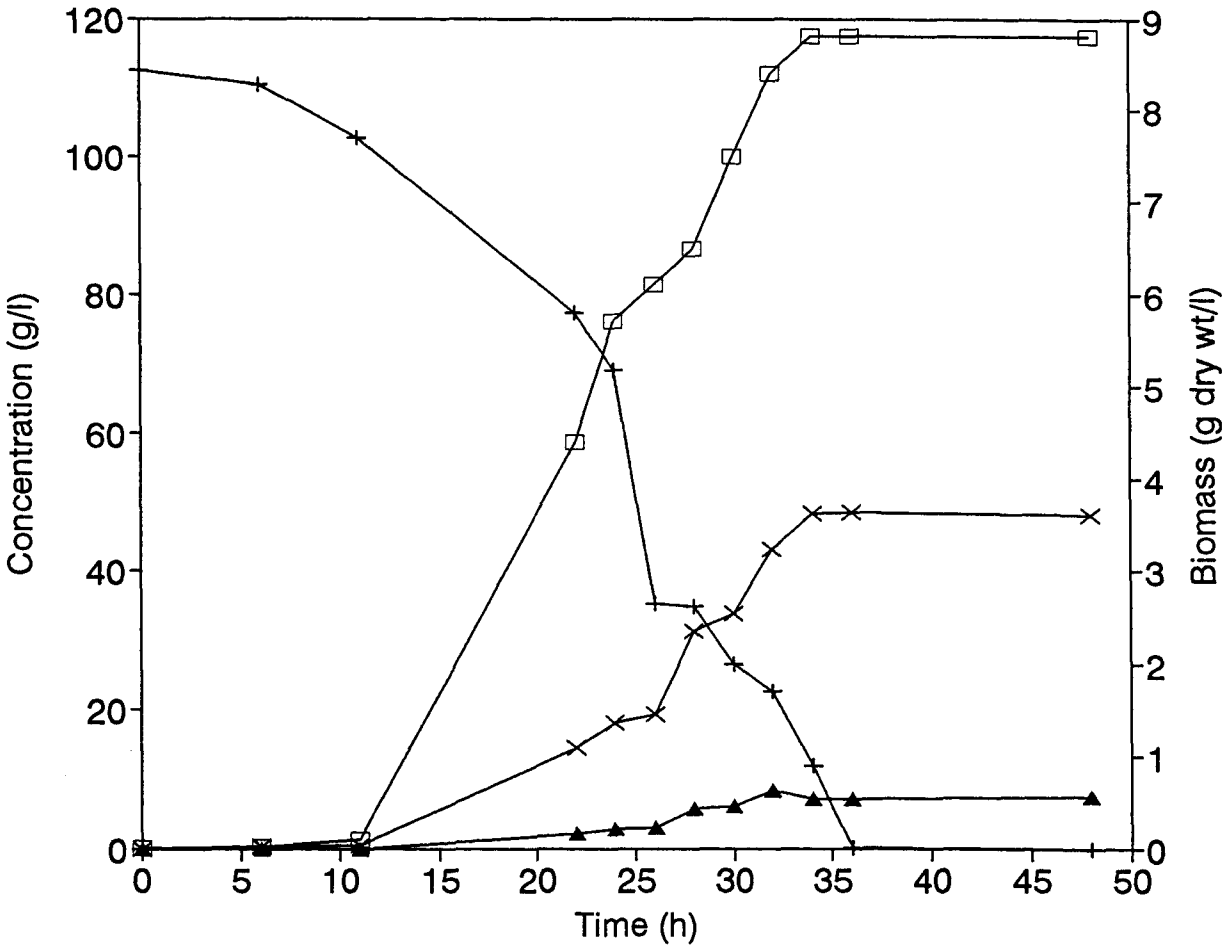
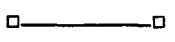

120
100
80
60
40
20
0
Concentration (g/l)
9
8
7
6
5
4
3
2
1
0
Biomass (g dry wt/l)
0
5
10
15
20
25
30
35
40
45
50
Time (h)

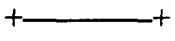
Fig. 4.6 Growth and product formation by *S. pombe* MY4 under anaerobic conditions

The yeast was grown in 4 litre batches of YEPG(12) in 7 litre stirred vessels at 30°C. Nitrogen was supplied to the vessel below the bottom impeller of the stirrer at a rate of 100 ml min^{-1}.

Key to plots:



biomass



glucose

ethanol

glycerol

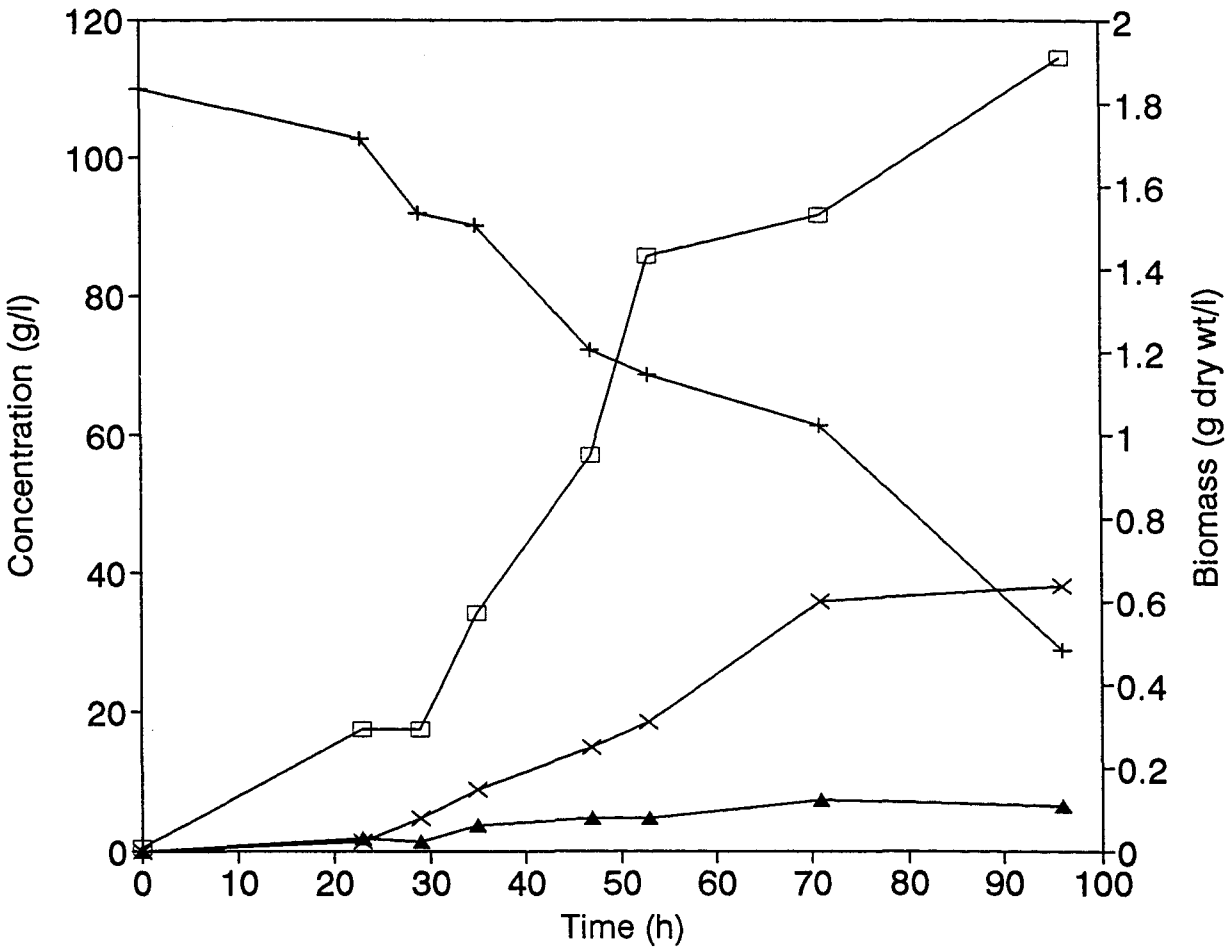

120
100
80
60
40
20
0
Concentration (g/l)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Biomass (g dry wt/l)
0
10
20
30
40
50
60
70
80
90
100
Time (h)

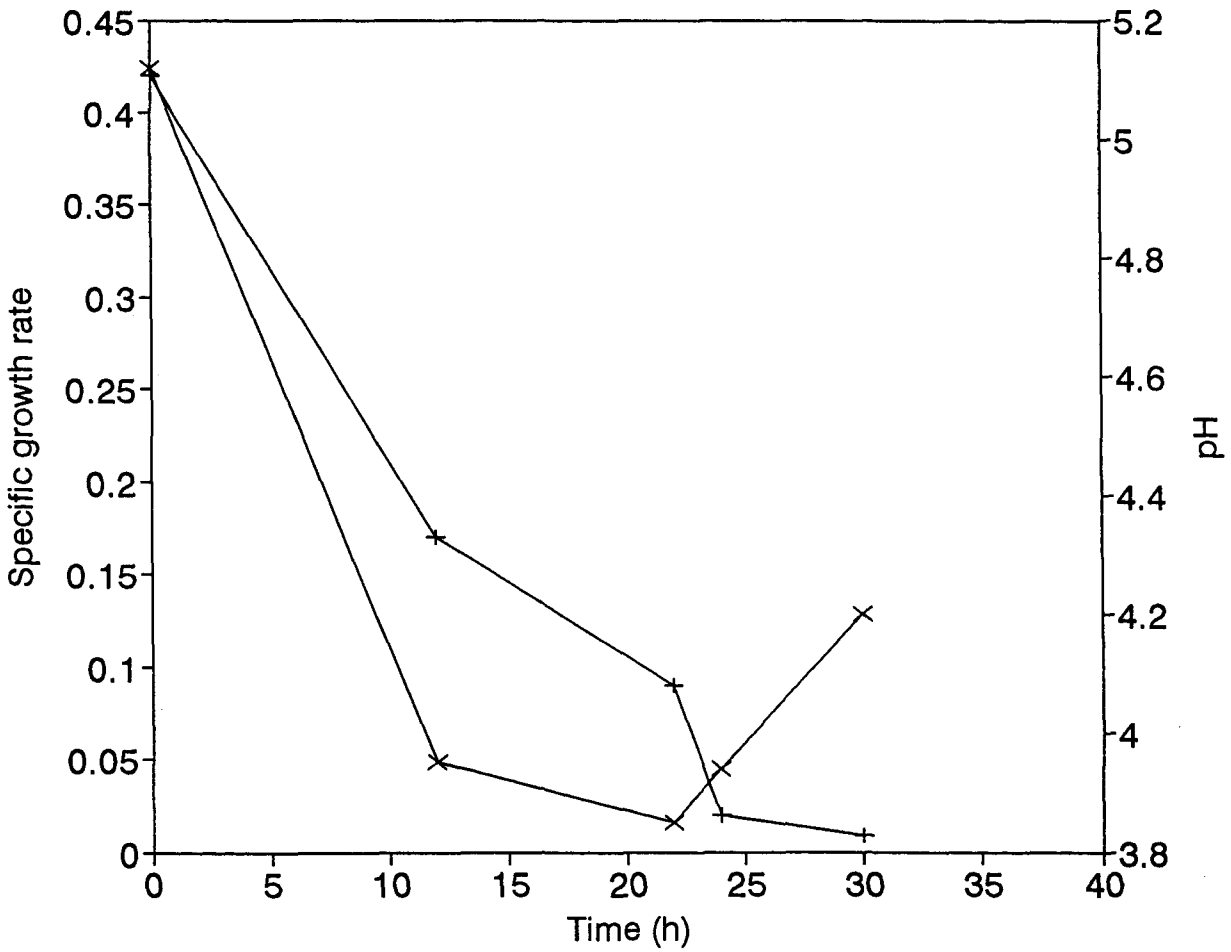
Fig. 4.7 Specific growth rate and pH of a culture of *S. pombe* MY4 growing under aerobic conditions

The yeast was grown in a 4 litre batch of YEPG(12) in 7 litre stirred vessels at 30°C. Air was supplied to the vessel below the bottom impeller of the stirrer at a rate of 1000 ml min^{-1}.

Key to plots:

+————+ specific growth rate

x————x pH


0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Specific growth rate
5.2
5
4.8
4.6
4.4
4.2
4
3.8
pH
0
5
10
15
20
25
30
35
40
Time (h)

rate may have been due to a limitation of oxygen at the high biomass concentration; however, cultures which were supplied with air to the head-space only and which presumably contained a lower concentration of dissolved oxygen showed very similar patterns of biomass and product formation.

In addition to ethanol, the major fermentation end-product, a small amount of glycerol was also formed.

4.4 EFFECT OF SUCROSE CONCENTRATION ON GROWTH

The effect of sucrose concentration on growth of the yeast was tested in 100 ml batches of YEP medium containing sucrose at concentrations over the range 20% to 60% (see Fig. 4.8). As the sucrose concentration in the medium increased from 20% to 50%, the time required to reach stationary phase increased from 2 days to 4 days, the culture in medium containing 60% sucrose showing no substantial growth over the 6-day period of incubation. In addition, the cell density in stationary phase was inversely related to the initial sucrose concentration. It is unlikely that the premature cessation of growth at the higher concentrations of sucrose was directly due to the initial sucrose concentration; however, the effect may have been due to an increase in the sensitivity to the high solute concentration (attributable to the remaining sucrose) caused by the ethanol produced as a result of fermentation. Cell viability was not determined.

4.5 THE COMBINED EFFECT OF SUCROSE AND ETHANOL ON GROWTH

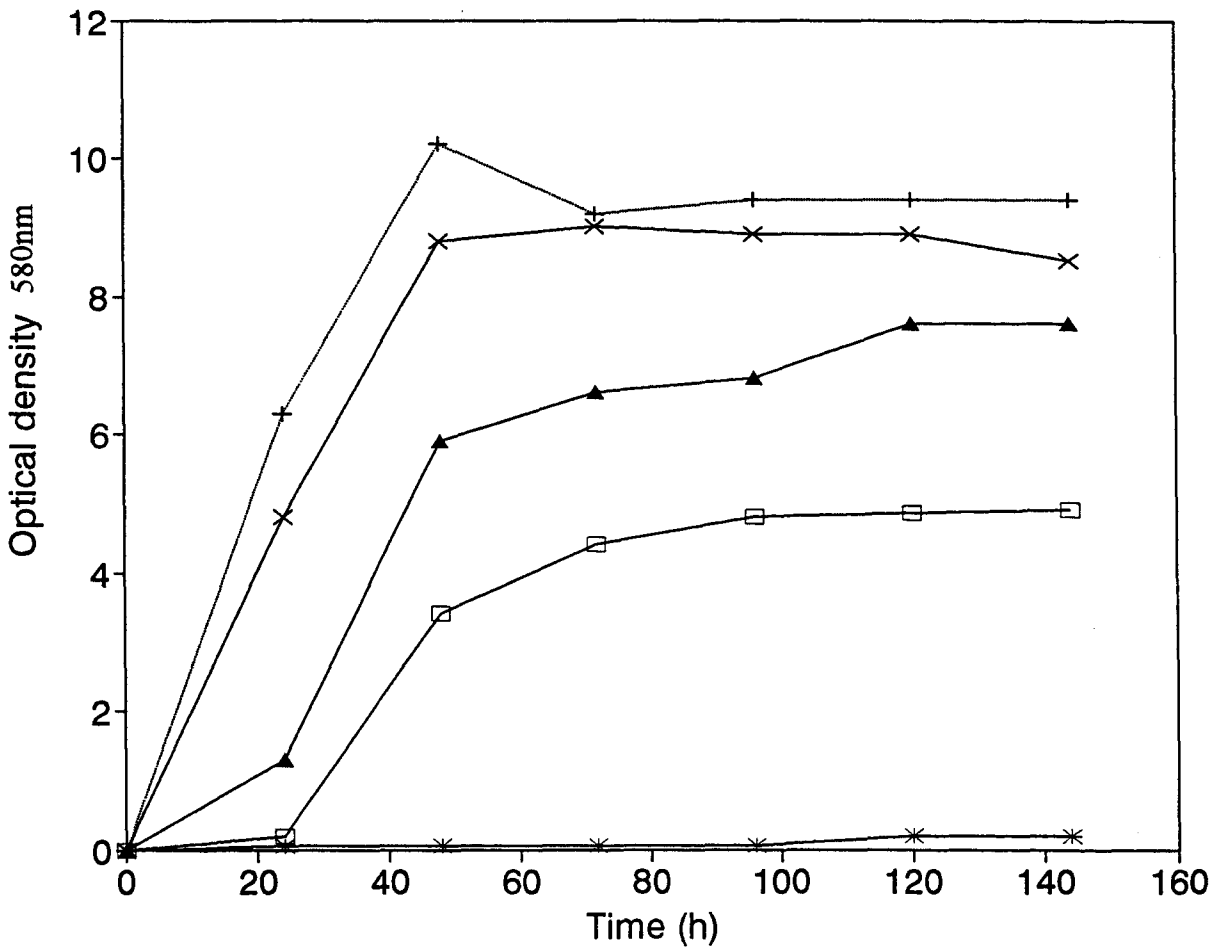
To test the effect of ethanol upon the susceptibility of the yeast to high concentrations of sucrose, a 'checker-board' experiment was set up in which the

Fig. 4.8 Effect of sucrose concentration on growth of *S. pombe* MY4

The yeast was grown in 100 ml batches of YEP medium containing the specified concentrations of sucrose and incubated at 30°C on a rotary shaker for 6 days. Results for each sucrose concentration are means of duplicate cultures.

Key to plots:

+————+	20% sucrose
x————x	30% sucrose
▲————▲	40% sucrose
□————□	50% sucrose
✱————✱	60% sucrose


Optical density 580nm
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
Time (h)

two variables were the concentrations of sucrose and ethanol. The yeast was grown in 5 ml volumes of YEP containing specified concentrations of sucrose (2% to 60%) and ethanol (0% to 12%). After incubation at 30°C for 24 hours, a clear pattern of inter-action of sucrose and ethanol concentrations was evident (Fig. 4.9.1).

With 2% sucrose in the medium, there was a gradual decline in the extent of growth as the ethanol concentration increased from 1% to 6%, with a much more marked decline from 6% to 8% ethanol, the latter being the highest concentration to permit growth. As the concentration of sucrose in the medium increased, not only did the extent of growth in the absence of ethanol decrease (at 30% sucrose, there was only 30% of the growth at 2% sucrose), but the highest concentration of ethanol to permit growth also decreased. For example, the highest concentration of ethanol to permit growth was 7% at 20% sucrose, 6% at 30% sucrose, 5% at 40% sucrose and less than 1% at 50% sucrose.

After an additional 48 hours incubation, there was a general increase in the extent of growth, although the overall pattern remained very similar to that found after 24 hours incubation. However, substantial growth had now occurred at the combination of 2% sucrose and 9% ethanol; furthermore, at 50% sucrose growth had now occurred up to a concentration of 5% ethanol (Fig. 4.9.2).

4.6 EFFECT OF REMOVING ETHANOL DURING FERMENTATION

Since the premature cessation of fermentation of sucrose by *S. pombe* MY4 appeared to be due to the combined action of the high concentration of sucrose and the increasing concentration of ethanol, it was decided to attempt to overcome the

Fig. 4.9 Combined effect of sucrose and ethanol on the growth of *S. pombe* MY4

The yeast was grown in 5 ml volumes of YEP medium containing the specified concentrations of sucrose and ethanol. The tubes containing the inoculated media were incubated at 30°C for 3 days, the OD_{580} of each tube being measured after 24 hours and 72 hours incubation. Each value in the diagrams represents the mean of duplicate cultures and is expressed as the percentage of an arbitrary maximum OD_{580} of 1.5.

Key to Figures:

Fig. 4.9.1 pattern of growth after 24 hours

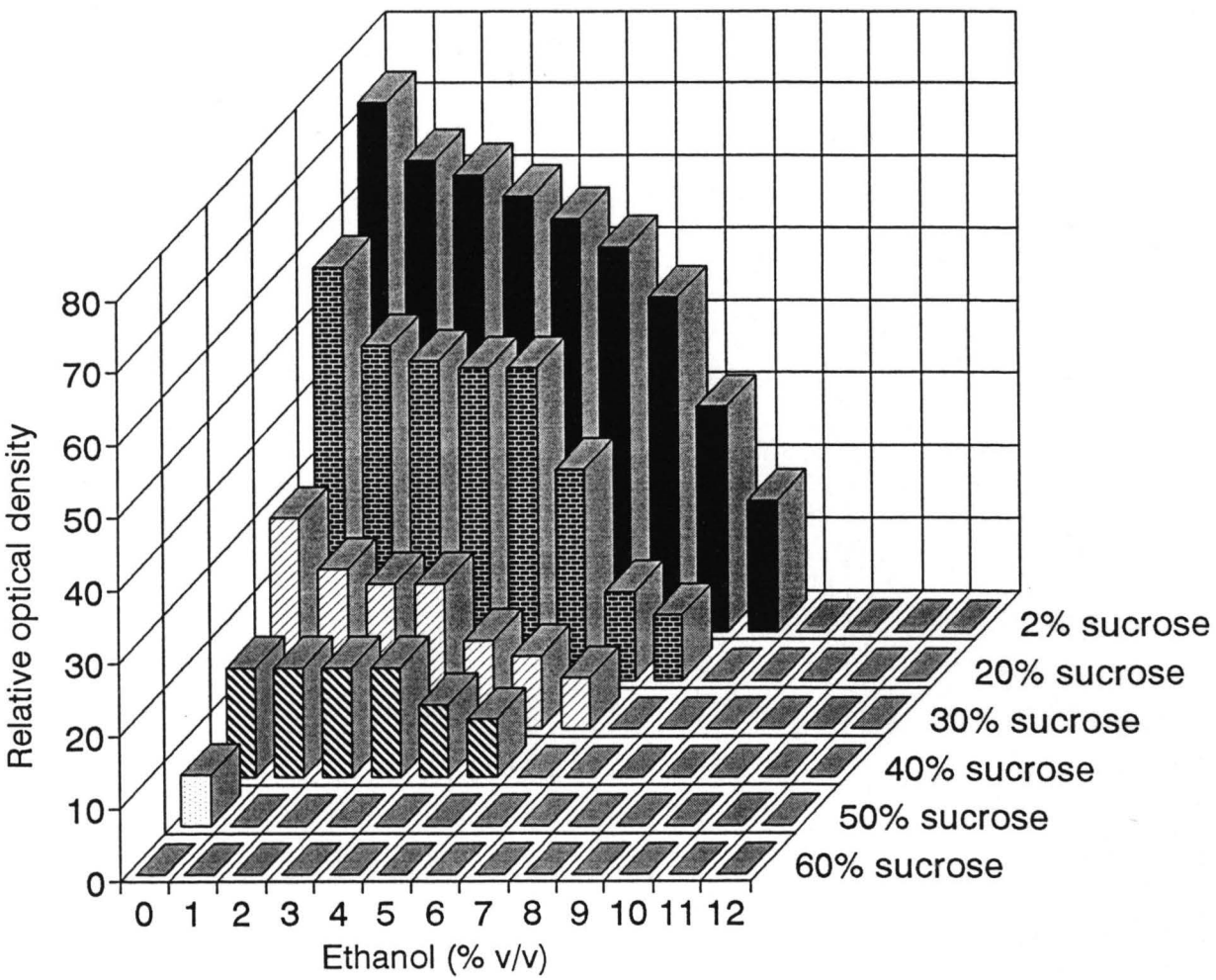
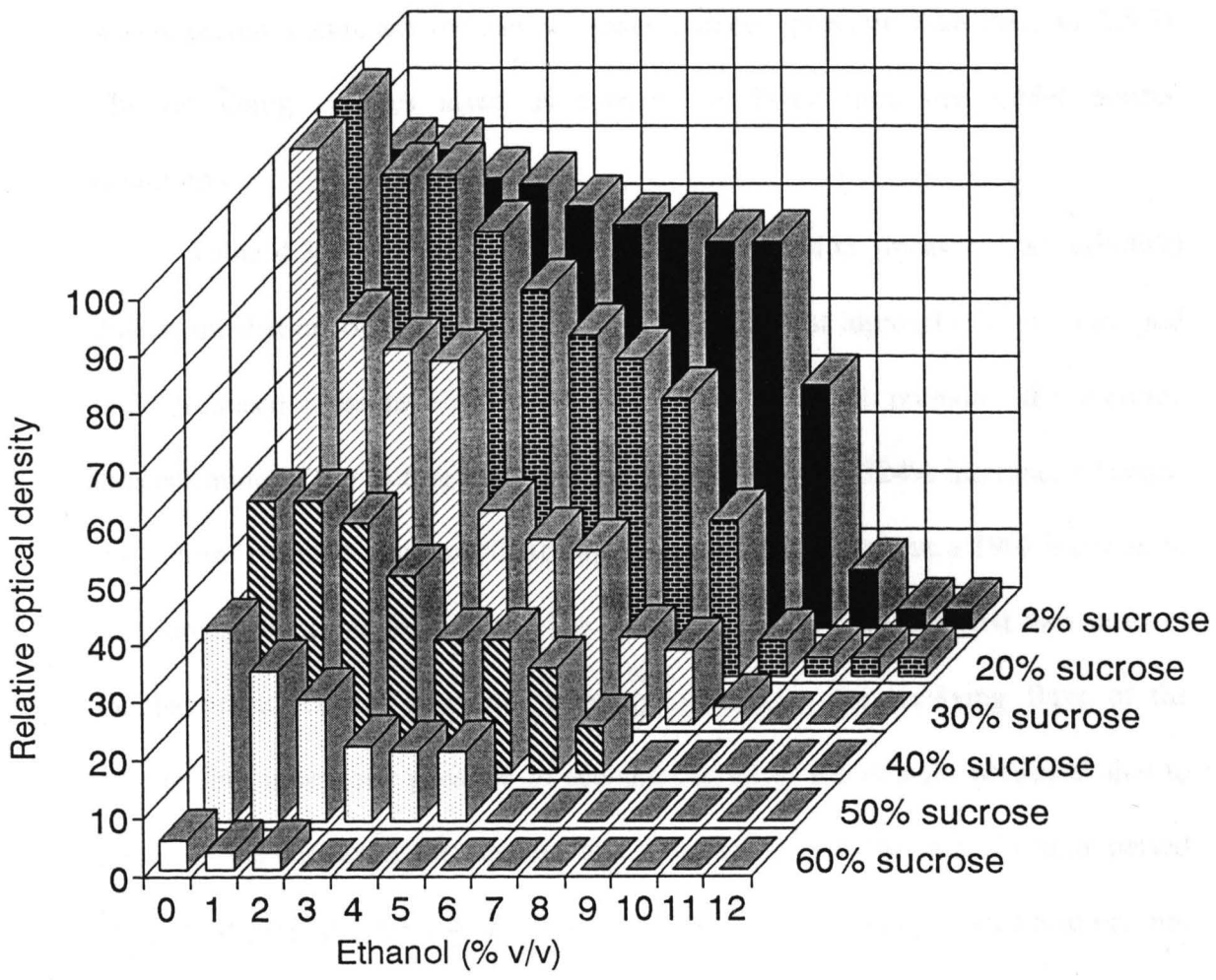

Relative optical density
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Ethanol (% v/v)
2% sucrose
20% sucrose
30% sucrose
40% sucrose
50% sucrose
60% sucrose

Fig. 4.9 Combined effect of sucrose and ethanol on the growth of *S. pombe* MY4

The yeast was grown in 5 ml volumes of YEP medium containing the specified concentrations of sucrose and ethanol. The tubes containing the inoculated media were incubated at 30°C for 3 days, the OD_{580} of each tube being measured after 24 hours and 72 hours incubation. Each value in the diagrams represents the mean of duplicate cultures and is expressed as the percentage of an arbitrary maximum OD_{580} of 1.5.

Key to Figures:

Fig. 4.9.2 pattern of growth after 72 hours


Relative optical density
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Ethanol (% v/v)
2% sucrose
20% sucrose
30% sucrose
40% sucrose
50% sucrose
60% sucrose

problem by removal of the ethanol as it was being formed by distillation under reduced pressure.

The yeast was grown in several 250 ml batches of YEPS(55). When the OD of the culture reached 2.2, the cell density at which further growth usually ceased, one of the 250 ml batches of culture was transferred to a system which would permit continued incubation under reduced pressure (see Section 2.5.3). The remaining cultures acted as controls by being incubated under normal conditions.

Table 4.3 shows the increase in cell concentration (measured as turbidity) during incubation of the cultures of *S. pombe* MY4 at atmospheric pressure and under reduced pressure. After two days at the reduced pressure, the biomass concentration of the culture more than doubled, showing a 124% increase; whereas, the culture retained at atmospheric pressure showed only about a 19% increase in its biomass concentration during the same period. During the first two days of incubation at reduced pressure, ethanol was collected in the receiving flask of the system, but it was not possible to account for all of the ethanol produced due to losses as vapour down the vacuum line. However, over the third 24-hour period at reduced pressure, the culture showed no increase in biomass concentration, nor did a significant amount of ethanol accumulate in the receiving flask.* It seems unlikely that depletion of major nutrients was the cause for the cessation of growth after the first 2 days at reduced pressure, since cultures of this yeast reached nearly twice the biomass concentration under normal conditions of culture in the same base medium (YEP) with only 20% sucrose (see Section 4.4).

* Data not shown.

Table 4.3 Effect of reduced pressure on growth of *S. pombe* MY4 at high sucrose concentration

The yeast was grown in 250 ml batches of YEPS(55) on a rotary shaker at 30°C for 3 days. After 3 days incubation 250 ml of culture was transferred to a stirred vessel immersed in a water bath at 30°C and maintained at a reduced pressure (about 70 mm). Incubation of a duplicate culture was continued under normal conditions.

Time from commencement of incubation at reduced pressure (days)	Optical density of culture: 580nm retained at atmospheric pressure	transferred to reduced pressure
0	2.23	2.23
1	2.60	3.20
2	2.65	5.00
3	2.80	5.00

4.7 CONCLUSIONS

In this study, *S. pombe* was found to be a good ethanol producer. It grew well at low pH levels and tolerated high concentrations of glucose and of sucrose (up to 60%) and was able to initiate growth in 12% ethanol (v/v). In spite of this, the yeast was found to have increased sensitivity to ethanol in the presence of high levels of sucrose. The use of fermentation at reduced pressure was shown to be an option. It has been possible to ferment 60% sucrose at reduced pressure using *S. pombe* MY4. Nonetheless, the fermentation stopped after 3 days possibly due to an inadequate supply of sterols and fatty acids in the yeast extract medium, or of oxygen (since air and not oxygen was supplied to the yeast cells).

Further developments would be to determine what precise requirements are needed in terms of fatty acids, sterols and oxygen.

CHAPTER 5

METHODS FOR MEASUREMENT OF HEXOKINASE ACTIVITY

5.1 ASSAY PROCEDURES

In studying the purification and characterization of hexokinase it is essential to have a reliable and reproducible assay method. Hexokinase can be assayed by measuring the rate of formation of either of the two products, G6P or ADP. Two enzyme-coupled assay methods based on those described by Bergmeyer *et al.* (1983) were investigated. Method 1 measures the production of G6P by coupling to reduction of $NADP^+$ (Fig. 5.1.1) while Method 2 measures the production of ADP by coupling to oxidation of NADH (Fig. 5.1.2).

5.1.1 Variation of activity in the procedures

The specific activities of cell-free extract and purified enzyme were determined using the two procedures. The concentrations of the reagents in the assay mixtures were identical in both procedures, except for the different coupling enzymes and coenzymes used in each method. ATP was the last reagent added used in both assays. The results, illustrated in Table 5.1, revealed differences in the velocities of sugar phosphorylation between Method 1 and Method 2.

Firstly, in Method 1, under these conditions the rates of glucose and fructose phosphorylation were equal. The fructokinase/glucokinase activity (F/G) ratio was equal to 1.0 in both cell-free extract and purified enzyme, but in contrast, the rates of glucose and fructose phosphorylation were not equal in Method 2, with

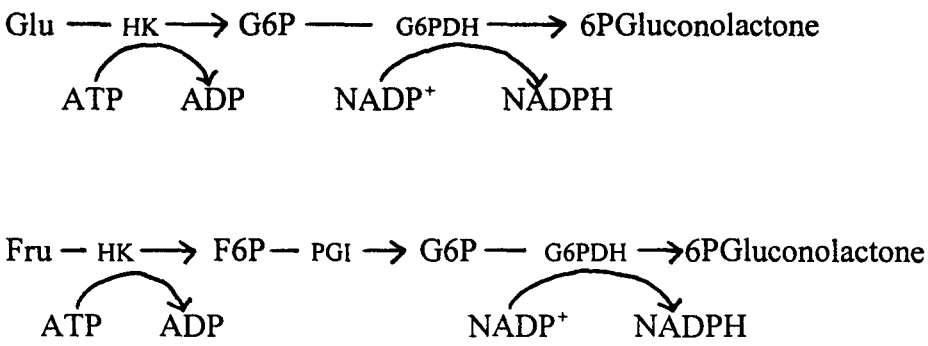


Fig. 5.1.1

Method 1: Measurement of G6P production by coupling to reduction of $NADP^+$

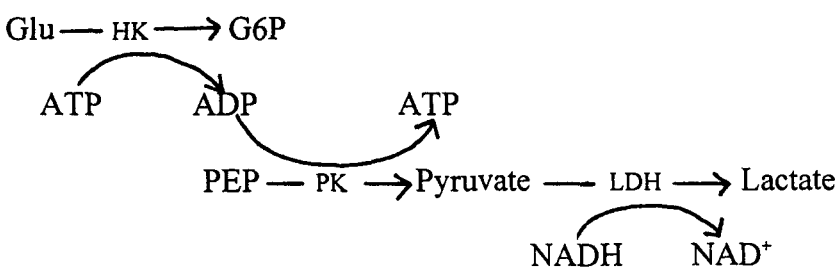


Fig. 5.1.2

Method 2: Measurement of ADP production by coupling to oxidation of NADH

Table 5.1 Determination of hexokinase activity using two procedures

The enzyme was assayed using the two methods described in the text, under identical conditions, but using different enzyme preparations both in the crude and purified states.

Protein mg ml^{-1}	Method 1			Method 2		
	specific activity mU mg^{-1}		F/G ratio	specific activity mU mg^{-1}		F/G ratio
	on glucose	on fructose		on glucose	on fructose	
crude: 67.5	85	89	1.1	203	217	1.1
crude: 20.7	99	100	1.0	187	203	1.1
purified: 0.50	6210	6130	1.0	7220	9580	1.3
purified: 0.44	7530	7710	1.0	9710	13610	1.4

fructose being phosphorylated more rapidly than glucose. With the purified enzyme, the fructokinase activity was almost 1.4 times greater than that of glucokinase, but only slightly more (1.1) in the crude extract.

Secondly, the rates were higher in Method 2 than in Method 1 for both glucokinase and fructokinase activities. G6P and ADP are both products of hexose phosphorylation. G6P was found to be non-inhibitory while ADP inhibited hexokinase activity (Section 8.3.1). In Method 1, G6P is converted to 6P-gluconate while ADP accumulates. Addition of ADP did not affect hexokinase activity when using Method 1. In Method 2, G6P accumulates but ADP is recycled to ATP. Fig. 5.2 illustrates the reaction involving formation of a ternary complex of enzyme, phosphate donor and phosphate acceptor, prior to the phosphoryl transfer reaction. It has been demonstrated with *Sacch. cerevisiae* (Colowick, 1973) that the pathway that appears to predominate is the one shown in bold arrows, with glucose being the first substrate on and G6P the last product off. The broken arrows represent an alternative pathway in the presence of PEP and PK in which ADP is converted to ATP, thus maintaining a high concentration of ATP in the system. Under these conditions, this alternative pathway becomes the favoured one, and goes at a faster rate than the earlier pathway due to this recycling of ADP. An assumption can be made that the same reactions occur in *S. pombe*, and may be the explanation for the different rates of hexokinase activity obtained in Methods 1 and 2. However, in spite of extensive studies of the hexokinase reaction there still appear to be some aspects which have not yet been satisfactorily explained.

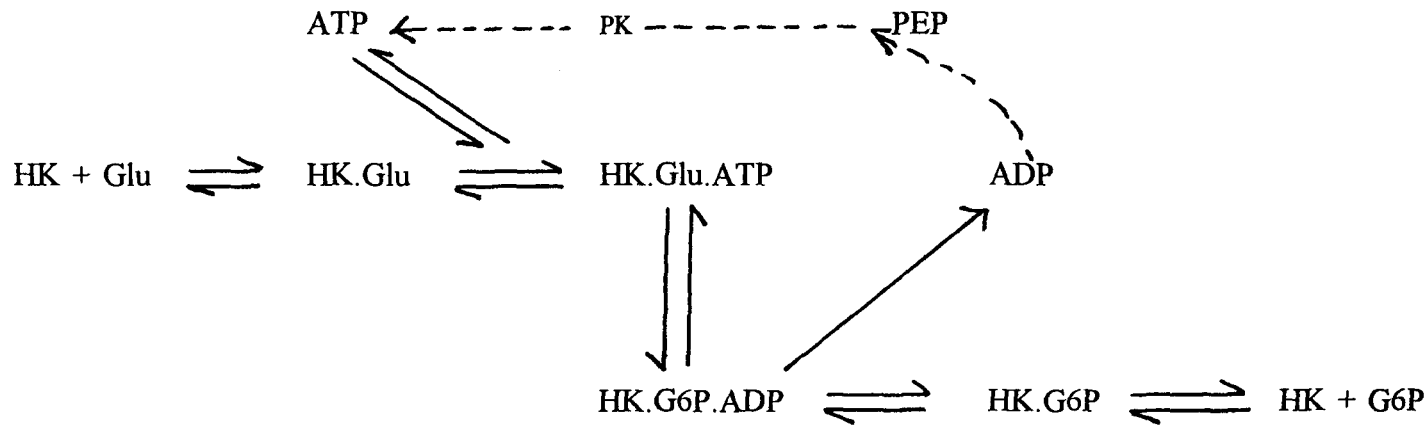


Fig. 5.2

Proposed reaction involving formation of ternary complex of hexokinase, phosphate donor, and phosphate acceptor

5.1.2 Applications

Method 1 was used for studying enzyme activity on glucose under different environmental conditions eg. pH, temperature and presence of inhibitors. It was also used for determining the substrate specificity of hexokinase in the presence of nucleotides. Method 2 was used for assay of hexokinase in the presence of intermediate products of glycolysis (G6P, F6P, F1,6-bisP) and of ethanol. It was also used for determining activity with various substrates where the phosphorylated product was not G6P. In both assay methods any factors that affect the coupling enzymes (G6PDH, PK and LDH) will invalidate the assay. To exclude such effects, appropriate control tests were done on the coupling system.

Different workers have used different concentrations of assay components and Tables 5.2 and 5.3 summarise some of these assay procedures. In view of the different versions of the assay methods described by different workers it seemed important to investigate the effect of variations in the assay method, particularly substrate concentration and enzyme concentration, and its applicability both to cell-free extracts and to purified enzyme preparations.

5.2 EFFECT OF ENZYME CONCENTRATION

Cell extracts were prepared from cells grown in YEPG(2) and harvested during the stationary phase. Hexokinase activity was assayed by measuring G6P production at pH 7.6, unless otherwise stated. The terms glucokinase activity and fructokinase activity are used in this study to represent glucose-phosphorylation and fructose-phosphorylation respectively and do not imply enzymes specific for glucose or fructose.

Table 5.2 Some variations of the hexokinase assay system using Method 1 (measurement of G6P).

Organism	Glucose mM	$MgCl_2$ mM	ATP mM	NADP mM	G6PDH U	References
Sacch. cerevisiae	222	8	0.64	0.91	0.55	Bergmeyer *et al.*, 1983
Acetobacter xylinum	50	2	10	0.5	0.55	Benziman & Rivetz, 1972
Sacch. cerevisiae	10	10	2	0.5	1.4	Maitra, 1970
Candida sp.	25	10	2	1	0.5	Hirai *et al.*, 1977
Sacch. cerevisiae	30	10	2	0.2	1	Muratsubaki & Katsume, 1979
Neurospora crassa	10	12.6	5	0.5	0.1	Lagos & Ureta, 1980
Sacch. cerevisiae	5	10	3	0.7	3	Clonis *et al.*, 1981
Z. mobilis	0.5	0.5	0.25	0.05	0.14	Doelle, 1982a
Z. mobilis	10	5	1	1	2	Scopes *et al.*, 1985
Sacch. cerevisiae	4	7	10	0.65	2	Fernández *et al.*, 1985
B. stearothermophilus	2.55	5.4	1.9	0.32	3.5	Goward *et al.*, 1988
Sacch. cerevisiae	10	10	1	0.1	0.55	van Doorn *et al.*, 1988
Sacch. cerevisiae	10	9	0.6	0.5	NI	Rose *et al.*, 1991

NI = not indicated

Table 5.3 Some variations of the kinase assay system using Method 2 (measurement of ADP).

Organism	Glucose mM	$MgCl_2$ mM	ATP mM	NADH mM	PEP mM	LDH/ PK U	References
Rabbit	2.22	8.0	0.64	0.28	0.43	10/10	Bergmeyer *et al.*, 1983
L. mesenteroides	6.67	6.67	3.33	0.33	2.67	1.7/0.03	Sapico & Anderson, 1967
Rana catesbeiana	100	1	1	0.15	2.5	1/1	Balinsky & Fromm, 1978
B. stearothermophilus	2.4	5	5	0.3	6.5	8/8	Goward *et al.*, 1986
Z. mobilis	NI	2	0.5	0.15	5	0.5/0.5	Reyes & Scopes, 1990
Sacch. cerevisiae	1.5	1.5	0.5	0.225	1	3/3	Montero-Lomeli, 1992

NI = not indicated

Results of the investigations showed that the rate of enzyme activity depended on the amount of cell-extract protein. Fig. 5.3 shows the effect of diluting the cell-extract on the enzyme activity of *S. pombe* hexokinase. At each hexose concentration tested a consistent range of activity was found between 15 and 50 mg protein/ml at which the F/G ratio remained constant (Fig. 5.4).

In a coupled enzyme system in which one enzyme is in excess and not rate-limiting, the rate of reaction in the steady-state is directly proportional to the concentration of the other enzyme. This was found not to be so in the case of *S. pombe* hexokinase where the curve of hexokinase activity against enzyme concentration is markedly non-linear. The inactivation of *Sacch. cerevisiae* hexokinases with dilution has been demonstrated with glucokinase (Maitra & Lobo, 1977) and hexokinase A (Rustum *et al.*, 1971; Purich *et al.*, 1973). Rustum *et al.* (1971) showed a decrease in the ratio of the rate of fructose phosphorylation to glucose phosphorylation with decrease in protein concentration. Inclusion of an inert protein such as bovine serum albumin in the dilution buffer prevents this inactivation (Scopes, 1987). In *S. pombe* hexokinase the non-linear relationship of rate versus enzyme concentration is similar to that demonstrated in mammalian hexokinase (Jenkins & Thompson, 1989), and suggests some change in binding of substrates to the enzyme upon dilution. This may indicate that a dilution-induced dissociation affects sugar binding. The rates of both fructose and glucose phosphorylation of *S. pombe* hexokinase appear to depend on the protein level, as both decrease with dilution. The effect appears to be greater on glucose-binding than on fructose-binding, hence the increase in F/G ratio with dilution. At lower enzyme concentrations, the substrate either does not bind or binds without

Fig. 5.3 Effect of the amount of cell-extract protein on hexokinase activity.

Cell-free extract was prepared as described in the text, and then diluted in 50 mM Tris-HCl buffer (pH 7.6). The specific activity of hexokinase was determined using Method 1.

Data shows mean values of duplicate tests.

Key to plots:

+———+ specific activity on 2.22 mM glucose

▲———▲ specific activity on 2.22 mM fructose

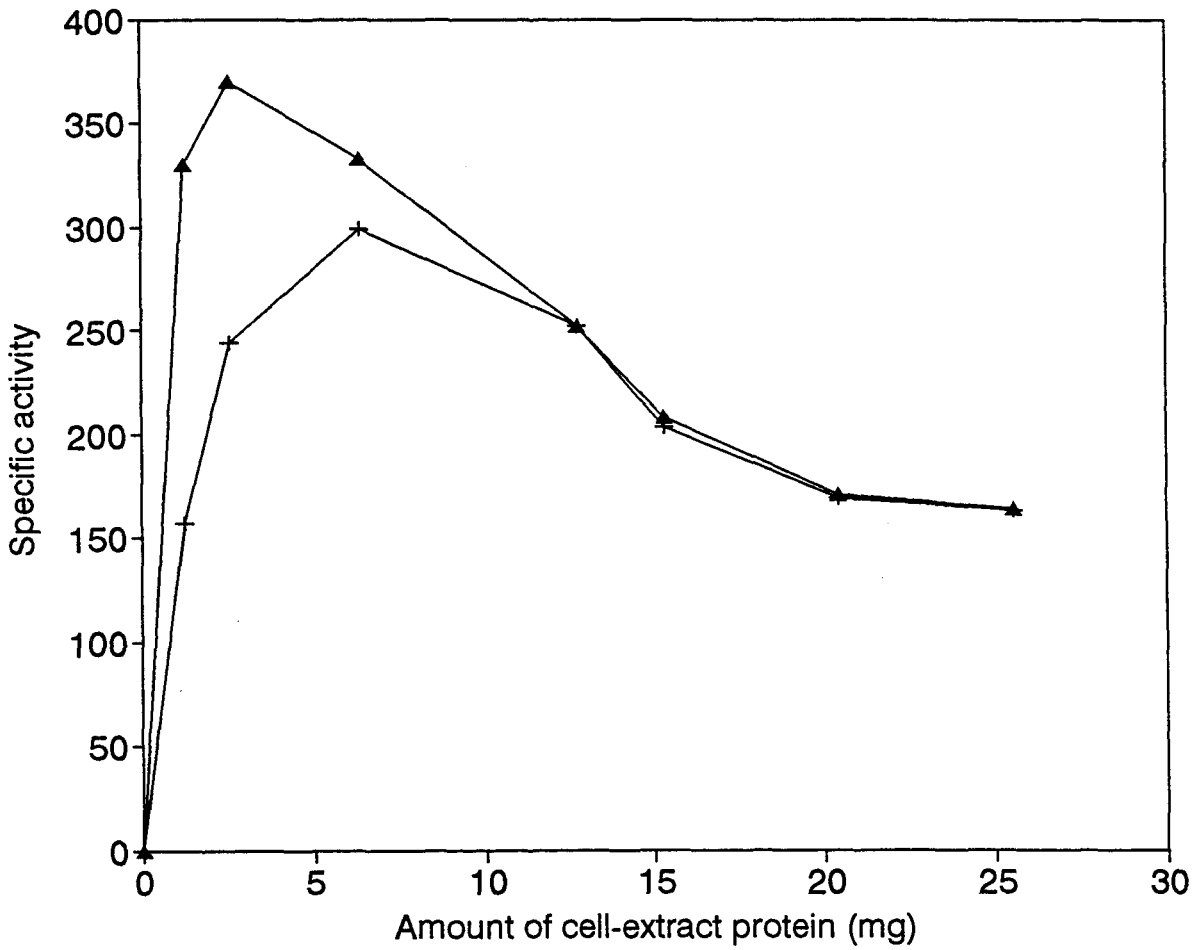
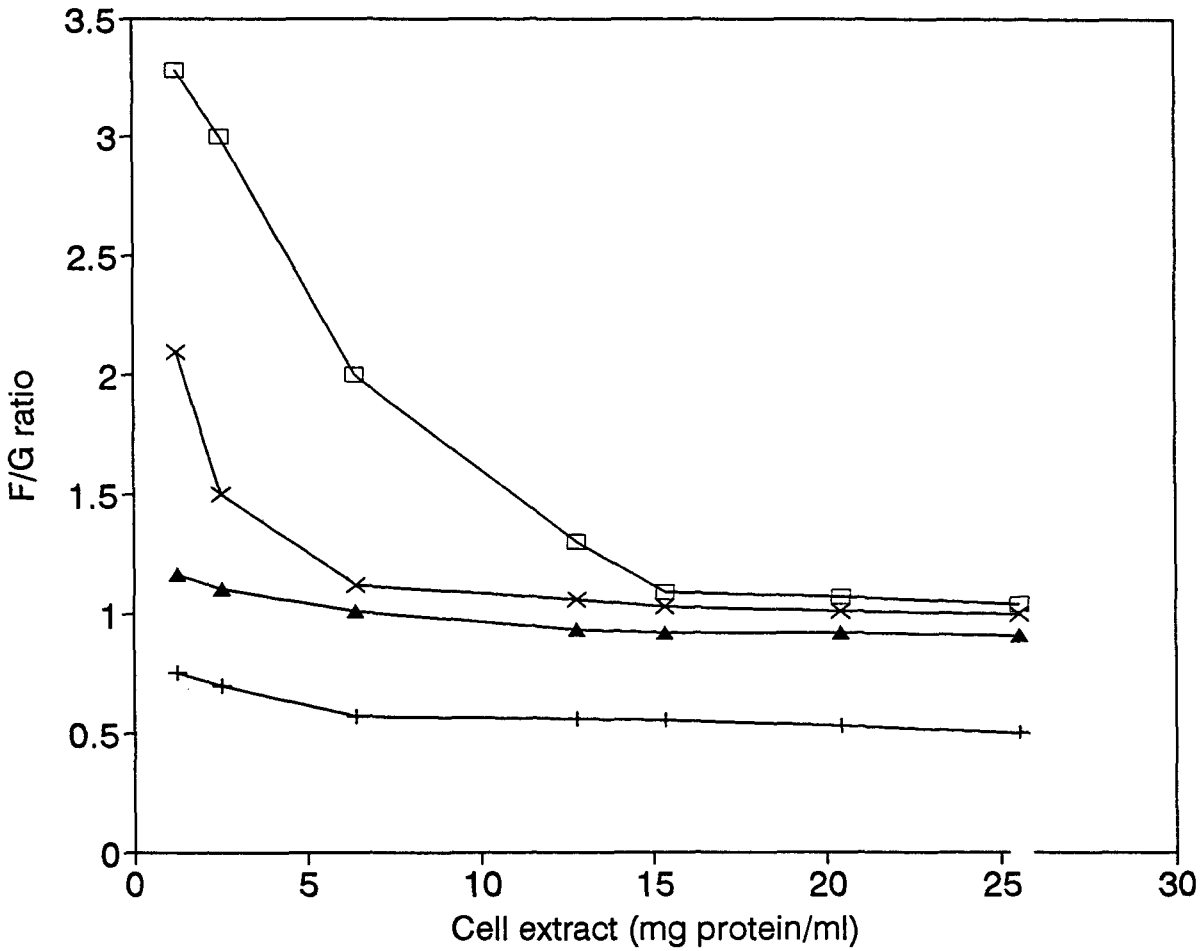

400
350
300
250
200
150
100
50
0
Specific activity
0
5
10
15
20
25
30
Amount of cell-extract protein (mg)

Fig. 5.4 Effect of dilution of cell extract on fructokinase/glucokinase (F/G) activity ratio.

Cell-free extract was diluted in 50 mM Tris-HCl buffer (pH 7.6) and hexokinase activity was determined using Method 1 in which the concentrations of glucose and fructose was changed to give final concentrations of:

+———+	0.222 mM
▲———▲	0.74 mM
x———x	2.22 mM
□———□	222 mM

Data shows mean values of duplicate tests.


3.5
3
2.5
2
1.5
1
0.5
0
F/G ratio
0
5
10
15
20
25
30
Cell extract (mg protein/ml)

inducing the full structural change necessary for the catalytic activity.

Dilution of the enzyme affects the protein-protein interactions and introduces artefacts in measurements of hexokinase activity. The protein concentration in the assay system is likely to be much lower than that found in cells. This is especially so during purification where the protein concentration of the cell-free extract decreases. In the course of purification the protein content of the purified enzyme may be lower by a factor of over 100 compared with the cell-free extract.

5.3 EFFECT OF SUBSTRATE CONCENTRATION

Fig. 5.5 illustrates the effect of hexose concentration in the assay mixture on glucose-phosphorylation and fructose-phosphorylation. When the hexose concentrations was increased from 0.022mM to 222 mM, both fructokinase activity and glucokinase activity of the cell-free extract increased. When the enzyme was diluted and both its activity and protein concentration lowered, the fructokinase activity was higher than glucokinase activity. Hexose concentration of 2.22 mM was adopted as the standard concentration in all assay procedures in order to avoid changes in the hexokinase activity with substrate concentration.

Several studies on different forms of hexokinase (Rustum *et al.*, 1971; Hirai *et al.*, 1977; Muratsubaki & Katsume, 1979; Doelle, 1982b; Rose *et al.*, 1991) used changes in the F/G ratio as evidence of more than one form of hexokinase. The results of this study show that F/G ratio is not necessarily a good indication of the distribution and separation of more than one form of hexokinase in *S. pombe*. Indeed, evidence presented later (Chapter 7) concludes that *S. pombe* produces only

Fig. 5.5 Variation in specific activity of hexokinase with glucose or fructose concentration.

Hexokinase activity was determined using Method 1 with glucose or fructose concentration range 0.022 mM to 222 mM.

Key to plots:

+————+ specific activity on glucose

x————x specific activity on fructose

Fig. 5.5.1: cell-free extract: protein concentration 36 mg ml^{-1}

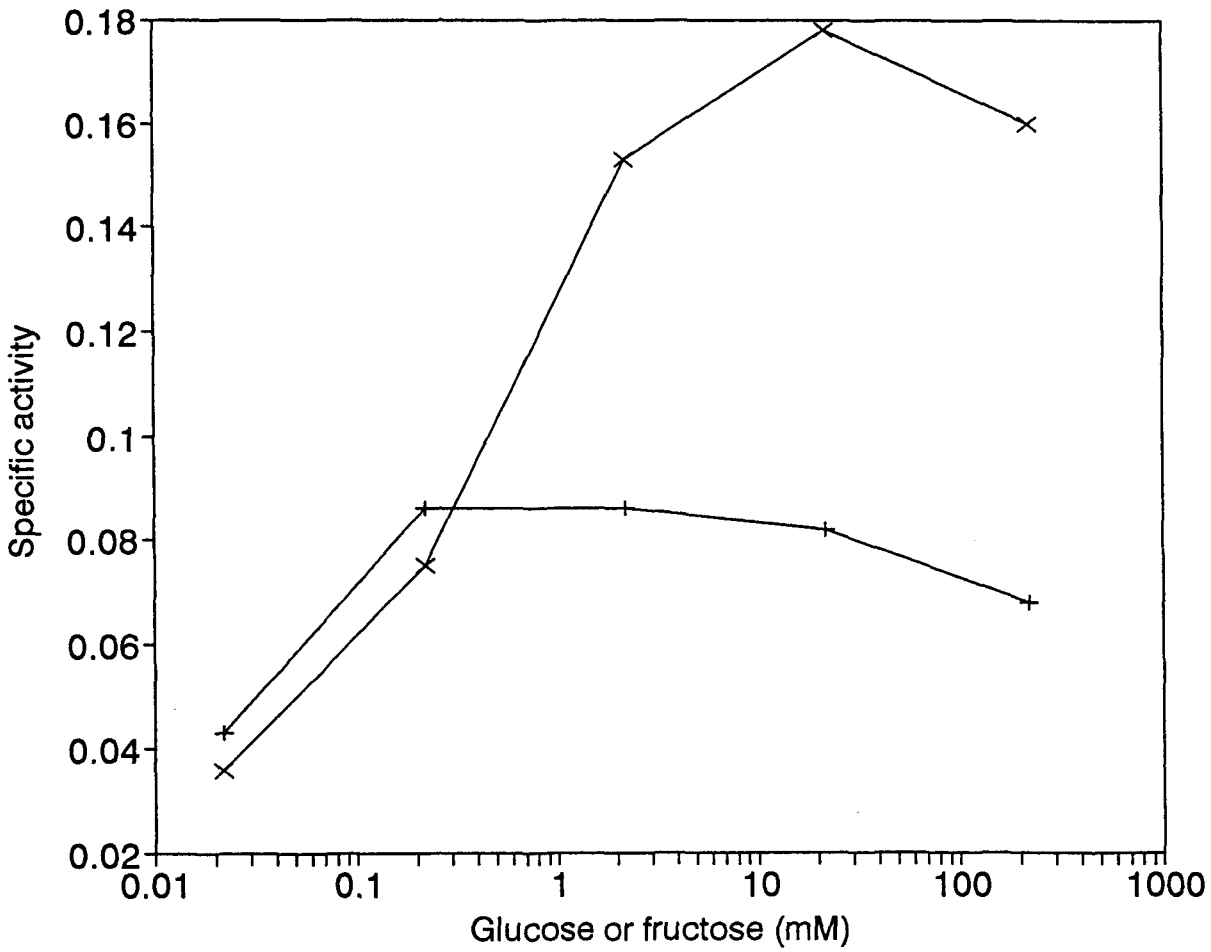

0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
Specific activity
0.01
0.1
1
10
100
1000
Glucose or fructose (mM)

Fig. 5.5 Variation in specific activity of hexokinase with glucose or fructose concentration.

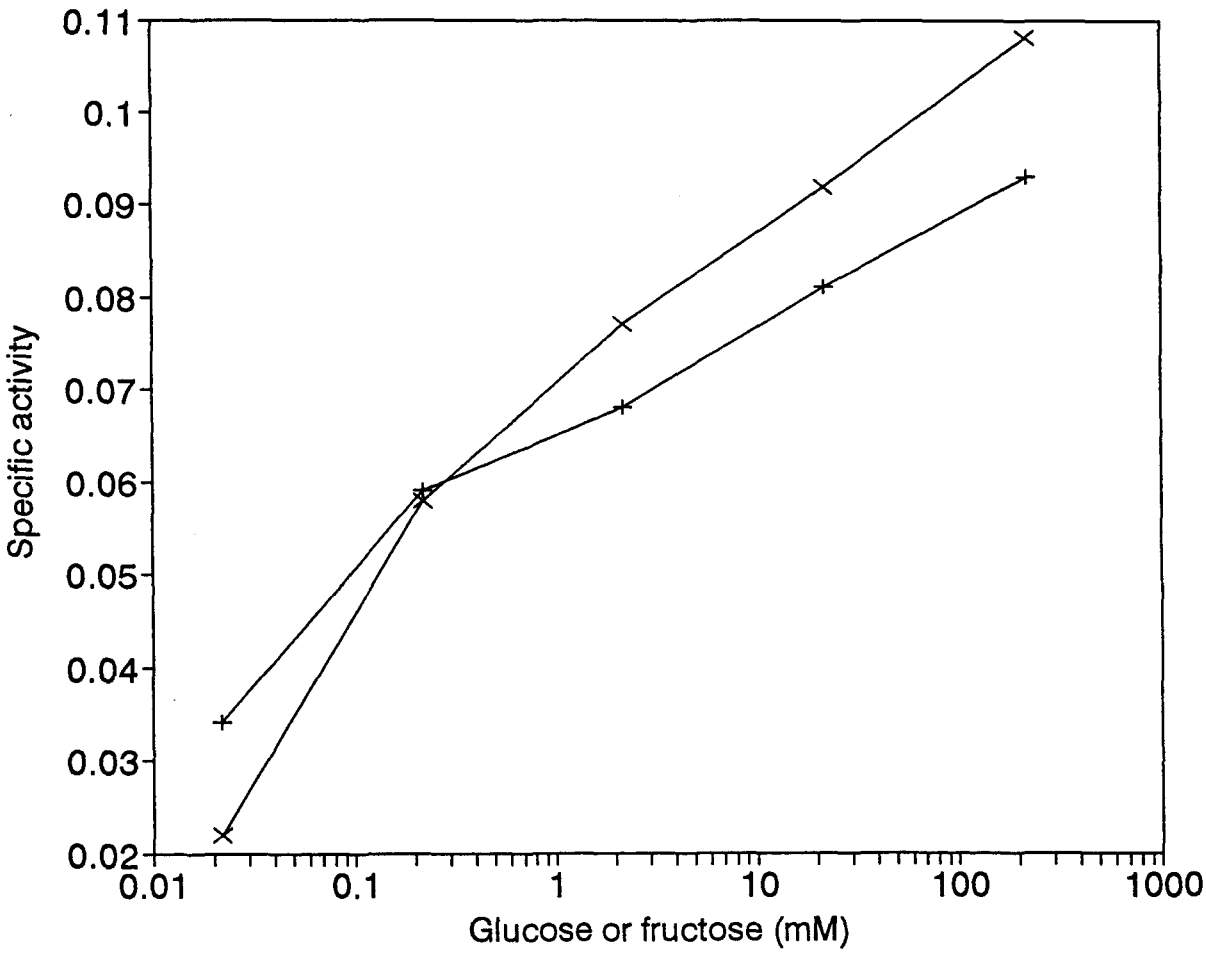
Hexokinase activity was determined using Method 1 with glucose or fructose concentration range 0.022 mM to 222 mM.

Key to plots:

+————+ specific activity on glucose

x————x specific activity on fructose

Fig. 5.5.2: cell-free extract: protein concentration 1.1 mg ml^{-1}


0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
Specific activity
0.01
0.1
1
10
100
1000
Glucose or fructose (mM)

one hexokinase.

Glucose appears to exert an inhibitory effect on hexokinase. This is further enhanced by enzyme dilution and creates the non-linear relationships illustrated in Fig. 5.3. The inhibition appears to be on the glucose-hexokinase binding itself rather than due to the formation of G6P since addition of up to 222 mM G6P to the assay mixture was found to cause no significant inhibition of hexokinase activity (Chapter 7). This property may be a regulatory mechanism whereby the inhibition of hexokinase by excess glucose is necessary for the control of sugar utilisation in *S. pombe*.

5.4 STANDARD ASSAY PROCEDURE

As a result of these studies the assay procedures described in Chapter 2 were adopted as standard for further work.

CHAPTER 6

PURIFICATION OF HEXOKINASE FROM *SCHIZOSACCHAROMYCES POMBE*

Several methods of purification of the hexokinases from *Saccharomyces cerevisiae* that were reviewed in Chapter 1 were tried with *S. pombe* hexokinase. There are no previous reports of studies of purification of hexokinase in the fission yeast *S. pombe*. This study describes and evaluates several procedures for the purification of its hexokinase.

6.1 PRELIMINARY INVESTIGATIONS

6.1.1 Growth of cells

In order to examine if there are changes in the level of hexokinase in relation to stage of growth and carbon source used for growth, *S. pombe* was grown on different substrates and harvested during either exponential or stationary phase (Table 6.1). The cell-free extracts prepared after growth on the different substrates did not differ in their F/G ratios. It appears therefore, that the glucose utilising system is of a constitutive nature and is present irrespective of the substrate on which *S. pombe* is grown, but growth on glucose to stationary phase gave the best yields of hexokinase. Consequently cells for hexokinase purification were grown in YEPG(2) and harvested during stationary phase, before disruption in a French pressure cell as described under Materials and Methods (Chapter 2).

Table 6.1 Levels of hexokinase activity in *S. pombe* grown on various carbon sources and harvested during the exponential or stationary phase.

Cells were grown in YEP (medium) containing 2% of carbon source, then harvested either during the exponential phase (OD_{580} less than 0.5) or during the stationary phase (OD_{580} above 0.5). In each case, the cell-free extract was prepared as described under Methods.

Results shown are mean values of duplicate tests.

Carbon source added to YEP	Cells harvested during exponential growth			Cells harvested in stationary phase		
	specific activity mU min^{-1} mg^{-1} substrate		F/G activity ratio	specific activity mU min^{-1} mg^{-1} substrate		F/G activity ratio
	fructose	glucose		fructose	glucose	
glucose	75	76	1.01	93	93	1.00
fructose	68	73	1.07	47	51	1.08
sucrose	81	87	1.08	60	61	1.01
glycerol	74	85	1.14	67	70	1.03
gluconate	68	70	1.02	54	58	1.06
glucose + ethanol	43	47	1.09	47	49	1.04

6.1.2 Investigation of heat inactivation

One possible approach to enzyme purification is by heat inactivation of unwanted protein. Table 6.2 shows the results of a series of experiments in which cell-free extract was heated rapidly in a constant temperature water bath. After heating for 10 minutes the enzyme was rapidly cooled to 0°C on ice. The precipitated protein was removed by centrifugation and the supernatant was assayed for hexokinase activity. There was no separation of glucokinase and fructokinase activities when the cell-free extract was heated to 60°C, at which all activity stopped. Since no improvement in the purification was observed, thereafter heat inactivation was not included in further purification procedures.

6.2 PURIFICATION PROCEDURE 1

This involved 4 steps (Table 6.3); all operations were carried out at 4°C. An overall recovery of 10% enzyme activity and 121-fold purification was achieved in 5-6 days.

6.2.1 Step 1: Protamine sulphate treatment

The cell-free extract was treated with protamine sulphate to precipitate nucleic acids. One mg of protamine sulphate was gradually added for every 10 mg of protein of freshly prepared extract. This was stirred for 15 minutes before being centrifuged at 15000 rpm (27000 g) and the precipitate was discarded.

Table 6.2 Thermal inactivation of hexokinase activity in a cell-free extract.

Cell-free extract was heated rapidly in a constant temperature bath 10 minutes before being rapidly cooled to 0°C on ice. Hexokinase activity was assayed using Method 1.

Temperature	% Relative rate		F/G activity ratio
°C	on glucose	on fructose	
20	100	100	1.0
40	89	87	0.98
45	88	87	0.99
50	74	81	1.09
55	14	14	1.0
60	0	0	0

Table 6.3 Summary of purification procedure 1.

The cells were grown and samples prepared as described in Methods. The results shown represent a typical purification procedure.

Step	Vol. ml	Protein mg ml^{-1}	Total protein mg	Total activity mU	F/G	Specific activity* mmol min^{-1} mg^{-1}	Purification factor	Yield %
Cell-free extract	18.0	65	1170	101000	1.09	0.09	1	100
Protamine sulphate	15.5	48	744	86000	1.07	0.12	1.4	85
1.8 M $(NH_4)SO_4$	7.8	18	140	21000	1.04	0.15	3	20
2.4 M $(NH_4)_2SO_4$	15.1	1.8	27	15000	1.03	0.5	6	15
DEAE cellulose	6.0	0.16	1	10000	1.05	10	121	10

* represents hexokinase activity on glucose.

6.2.2 Step 2: Ammonium sulphate fractionation

The supernatant from protamine sulphate treatment (pH 5-6) was adjusted to 46% saturation (1.8 M) by gradual addition of 26.7 g NH_4 $(SO_4)_2$ per 100 ml of supernatant, with efficient stirring (Wood, 1976). After about 90 minutes the precipitate was removed by centrifugation at 27000 g for 30 minutes; enzyme activity remained in the supernatant. The salt saturation was increased to 62% (2.4 M) by addition of 9.57 g $NH_4(SO_4)_2$ per 100 ml. The precipitate so formed was collected by centrifugation at 27000 g for 30 minutes, then dissolved in a small volume of 50 mM Tris-HCl buffer (pH 7.6). The solution was desalted on a Sephadex G-25 column equilibrated with 50 mM Tris-HCl buffer (pH 7.6). Fractions that were enzymatically active were pooled and pH re-adjusted to 7.6. Ammonium sulphate fractionation did not separate hexokinase activity into more than one component.

6.2.3 Step 3: Ultra-filtration

The enzyme solution eluted from the Sephadex G-25 column was concentrated by ultra-filtration. A stirred ultra-filtration cell (Model 202, 75 psi; Amicon Corporation, Massachusetts, USA) with a PM 30 membrane of retentivity greater than 30000 molecular weight, was used following manufacturer's recommended method.

6.2.4 Step 4: Diethylaminoethyl (DEAE)-cellulose ion-exchange chromatography

A 30 by 1.5 cm column was packed with DEAE-cellulose (Whatman DE 22) and equilibrated with 50 mM Tris-HCl buffer (pH 7.6) according to the

method recommended by the manufacturers using a flow-rate of 100 ml hr^{-1}. The flow-rate was then decreased to 58 ml hr^{-1}, which was maintained throughout the process, and the column was loaded with 80 to 100 ml of the desalted material from step 2. The column was washed with buffer to remove non-adsorbed proteins. The hexokinase was eluted from the column with a linear salt gradient of 0 to 2 M NaCl in starting buffer (total volume 400 ml). The eluate was collected in equal fractions (2 or 4 ml) and their conductivity was measured. Hexokinase was eluted at a salt concentration of 0.75 M as a single peak.

6.3 PURIFICATION PROCEDURE 2 (HYDROXYAPATITE CHROMATOGRAPHY)

Purification by chromatography on hydroxyapatite was attempted using the salt gradient elution method as outlined by Fernández *et al.*, (1985). Hydroxyapatite prepared by the method of J.H. Marshall (personal communication) was packed in a 1.6 cm diameter column (30 cm long) at 4°C and equilibrated by washing for at least 4 hours with 25 mM phosphate buffer pH 7.6. The desalted enzyme solution was pumped onto the column and left for several hours to bind onto the hydroxyapatite. The hexokinase was eluted by a linear phosphate gradient generated by slowly running 200 ml of 500 mM phosphate buffer pH 7.6 into 200 ml of 25 mM phosphate buffer pH 7.6. Fractions were collected and assayed for activity as previously described. The hexokinase was eluted as a single peak (result not shown) at a salt concentration of 320 mM. A purification factor of 13 and a yield of 38% were achieved.

Although hydroxyapatite was capable of binding and eluting hexokinase from *S. pombe*, the use of this material presented problems. Hydroxyapatite packs

so finely that the column could only be used once before being repacked due to build up of back-pressure. The purification factor of 13 was much lower than that obtained with the DEAE-cellulose or dye-ligand chromatography. Nonetheless, it was important to try purifying and separating hexokinase using this material since it had been used successfully in other studies. Womack *et al.* (1973) obtained a better separation of *Sacch. cerevisiae* PI and PII hexokinases with hydroxyapatite than with DEAE-cellulose chromatography and Fernández *et al.*, (1985) also used hydroxyapatite which separated the *Sacch. cerevisiae* hexokinases into two peaks representing hexokinase PI and PII, whereas the hexokinase from *S. pombe* eluted as a single peak.

6.4 PURIFICATION PROCEDURE 3 (DYE-LIGAND AFFINITY CHROMATOGRAPHY)

6.4.1 Selection of dye ligand

A Mātrex™ Gel (Amicon Corp. Mass. USA.) screening kit consisting of 2 ml columns of gels (Red A, Blue A, Orange A, Green A and Blue B and a control), was used to screen for dyes that bind to hexokinase. Each column was regenerated using 8.0 M urea in 0.5 M NaOH followed by equilibration using 20 mM Tris-HCl buffer pH (7.6). This procedure was repeated before each sample application as a column regeneration step. Each tube was loaded with 0.5 ml of freshly prepared crude extract containing 11 mg ml^{-1} protein. The columns were washed with 10 ml of buffer to remove unbound proteins and eluted successively with 10 ml portions of 0.1 M up to 1.5 M KCl in equilibration buffer. Procion Red

H-3B, the dye present in Matrex Red A was found to have the most suitable binding and elution properties (Table 6.4) and was chosen for further purification of hexokinase. For similar concentration of protein, 100% of *S. pombe* hexokinase bound to the Matrex Red A gel, but only 26 % of the hexokinases of *Sacch. cerevisiae* was bound.

6.4.2 Effect of pH

The effect of pH on purification of hexokinase was investigated using 2 ml columns packed with Matrex Red A. Tris-HCL buffer (50 mM) at pH (i) 6.5; (ii) 7.0; (iii) 7.5, and (iv) 8.0. was used both for the preparation of cell-free extract and for the loading and elution of enzyme from the columns. The results showed that there was no significant difference in binding between pH 7.0 and 8.0 but slightly lower at pH 6.5 (6% lower). Tris-HCl buffer (50 mM) at pH 7.6 was adopted for use in purification of hexokinase from *S. pombe*.

6.4.3 Dye matrex Red A purification method

Dye matrex Red A was packed in a column with a bed volume of 30 ml and equilibrated with 50 mM Tris-HCl buffer (pH 7.6). The cell extract was added to the column and the column washed with 90 ml of the same buffer. Elution was carried out with a linear gradient of NaCl generated by slowly adding 200 ml of 2 M NaCl in 50 mM Tris-HCl (pH 7.6) to 200 ml 50 mM Tris-HCl (pH 7.6) at a flow rate of 33 ml per hour and collecting 5 ml fractions. The results from a typical experiment are shown in Fig. 6.1 and the purification achieved is summarised in Table 6.5.

Table 6.4 Screening dye-Matrex gels for binding of *S. pombe* hexokinase.

Each dye-Matrex gel was packed in a small column and equilibrated with 20 mM Tris-HCl buffer (pH 7.6). Each tube was loaded with 0.5 ml of freshly prepared crude extract containing 11 mg (ml protein)$^{-1}$. The columns were washed with 10 ml of buffer to remove inert proteins and eluted successively with 10 ml portions of 0.1 M up to 1.5 M KCl in equilibration buffer. Experimental conditions are described in the text.

Dye Matrex	% of enzyme bound	% of enzyme eluted
Control	0	.
Red A	100	100
Green A	64	32
Blue A	0	.
Blue B	0	.
Orange A	0	.

Fig. 6.1 Purification of *S. pombe* hexokinase using salt gradient on Red A dye-matrex column.

Cell-free extract was prepared in 50 mM Tris-HCl buffer pH 7.6 as described under Methods before being chromatographed on Red A dye-matrex column, and eluted with a linear gradient of NaCl in Tris-HCl buffer. Data represents a typical purification profile showing activity on glucose. The activity on fructose (not shown) was similar to that of glucose.

Key to plots:

+————+	specific activity (mmol min^{-1} (mg protein)$^{-1}$)
▲————▲	protein (mg ml^{-1})
————	salt gradient
	(the concentration of NaCl ranges from 0 to 1.0 M over the extent of the ordinate)

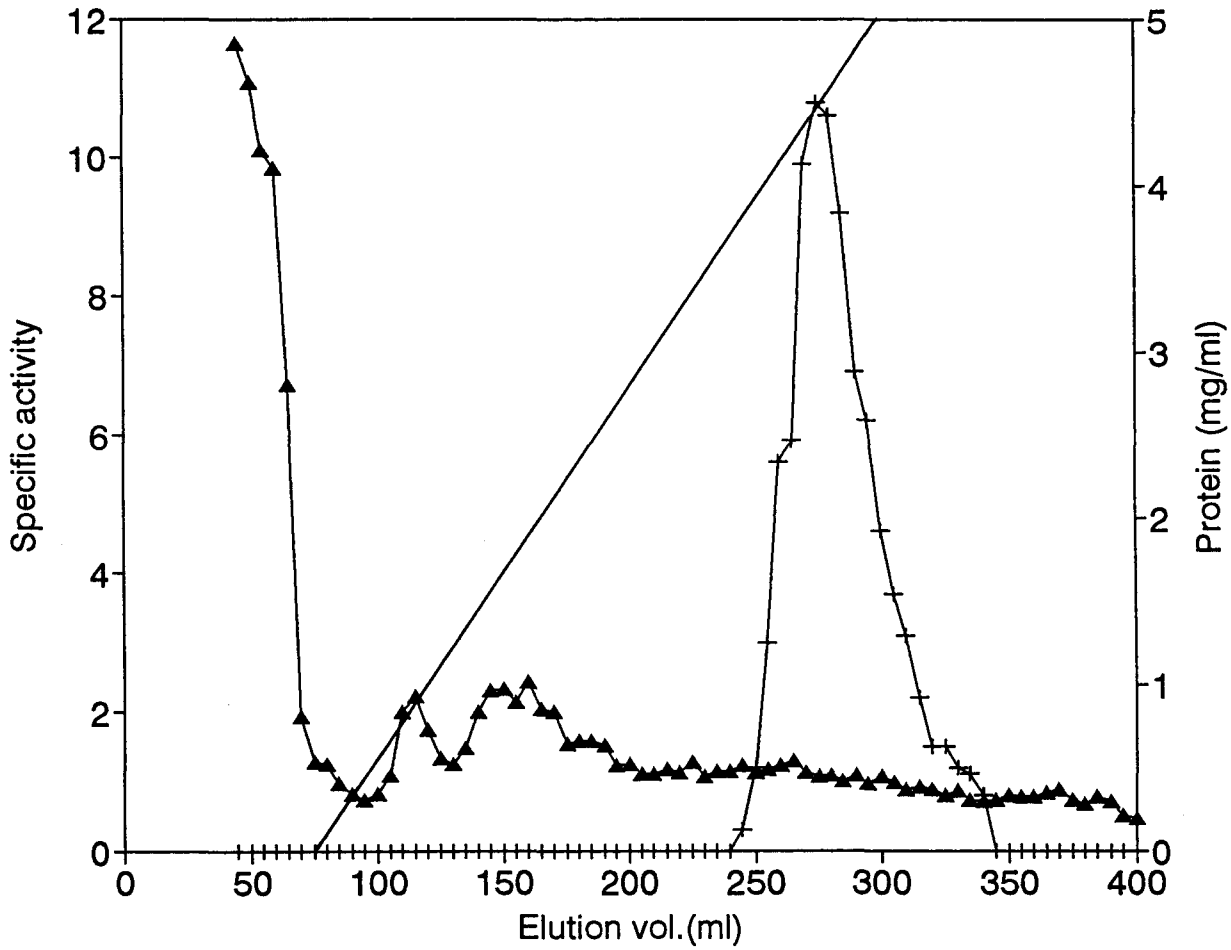

Specific activity
Protein (mg/ml)
Elution vol.(ml)
0
2
4
6
8
10
12
0
1
2
3
4
5
0
50
100
150
200
250
300
350
400

Table 6.5 Purification of *S. pombe* hexokinase on Red A dye-matrex gel.

The results shown represent a typical purification procedure starting with cell-free extract; hexokinase activity was measured with glucose.

	Vol. ml	Protein mg ml^{-1}	Total protein mg	Total activity mU	Specific activity mmol min^{-1} mg	Purification factor	Yield %
Crude extract	40	66.0	2630	120000	0.076	1.0	100
Active peak from dye-matrex gel	5	0.44	2.18	23500	10800	142	11.8

The hexokinase was eluted as a single peak at a salt concentration of 0.75 M. There was no evidence for the existence of more than one form of hexokinase in *S. pombe* and no significant change in the ratio of fructokinase/glucokinase activities was found.

Further purification of hexokinase was attempted using gel filtration on Sephacryl S-300 superfine prepared and packed into a Pharmacia K 26/100 column (bed height, 94 cm; total bed volume, 189 ml), which was also used for molecular weight determination. The column was equilibrated using 50 mM Tris-HCl buffer (pH 7.6). The most active fraction from the matrex Red A column was applied manually and the column eluted with the same buffer at a flow rate of 10 ml per hour.

The most active fraction recovered had a specific activity of 7.2, which was also that of the matrex Red A purified fraction applied. The procedure thus did not increase the purity of the hexokinase and was not used in later preparations.

The enzyme was purified to apparent homogeneity as determined by disc-gel electrophoresis (Fig. 6.2). The cell-free extract and the purified enzyme were run on polyacrylamide gel electrophoresis (PAGE) as described under Materials and Methods (Section 2.18). Hexokinase ran as a single main band with a relative electrophoretic mobility (R_m) of 0.46 giving additional confirmation to the presence of only one hexokinase in *S. pombe*. The presence of another faint band on the disc gels may be due to alcohol dehydrogenase acting on traces of ethanol present in the system (Marshall, *et al.*, 1984). The preparation had been shown to contain both alcohol dehydrogenase and myokinase (Table 6.6; see also Section 7.1.2).

Fig. 6.2 Disc-gel electrophoresis of hexokinase from *S. pombe*.

The enzyme was purified using Red A dye-matrex as described in the text. Approximately 50 μg protein were placed on each gel (specific activity at 22°C, 7.6 U (mg protein)$^{-1}$). Running pH was 8.5 at a current of 5 mA per gel-tube for about 1 hour.

The protein band (left) matches the enzyme band (right) which represents both fructokinase and glucokinase activity. The dark band at the bottom of the protein gel represents the dye-front.

Staining procedures for protein and hexokinase were as explained under Materials and Methods

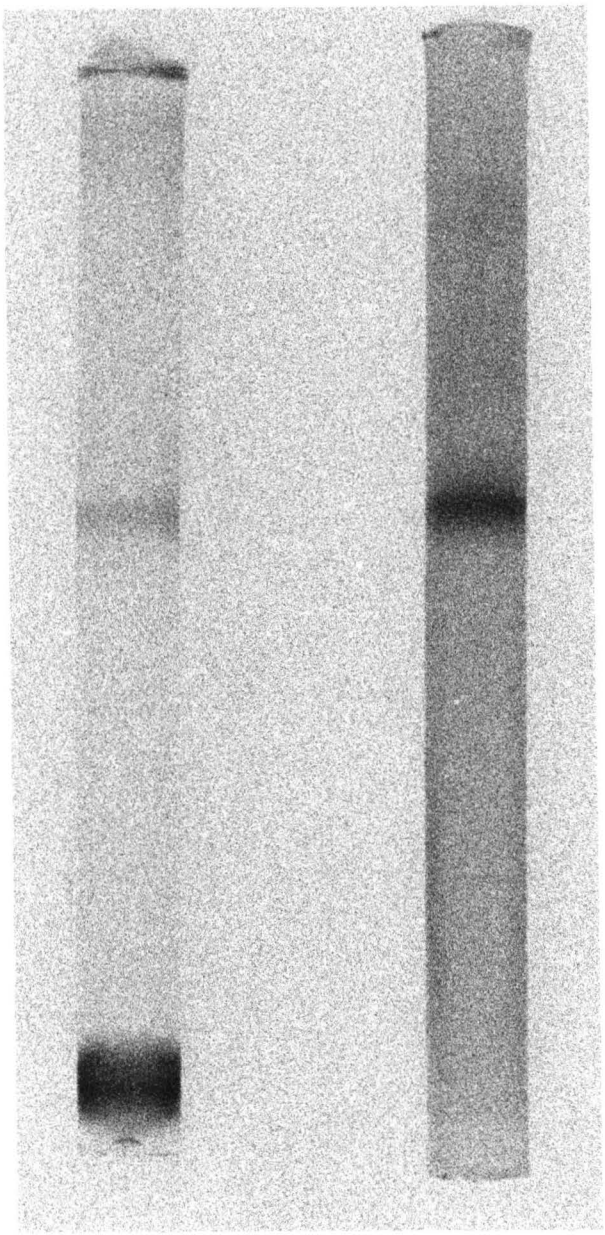


Table 6.6 Levels of some enzymes present in cell-free extract and in purified hexokinase.

Each enzyme was assayed using the method of Bergmeyer *et al.* (1974). The 'contamination' level of the enzyme relative to hexokinase, was calculated using the equation:

$$\text{Level of contamination} = \frac{\text{Specific activity of enzyme}}{\text{Specific activity of hexokinase}}$$

Enzyme	Specific activity mU min^{-1} mg^{-1}		'Contamination' level relative to HK	
	Cell-free extract	dye-matrex fraction	cell-free extract	dye-matrex fraction
hexokinase	55	7700	1	1
alcohol dehydrogenase	2800	650	52	0.1
aldolase	11	0	0.2	0
phosphofructokinase	4	0	0.8	0
glucose 6P-dehydrogenase	4	0	0.8	0
phosphoglycerate kinase	7	0	0.10	0
phosphoglucose isomerase	195	0	3.6	0
myokinase	nd	18	nd	0.002

The following enzymes were not detected in the cell-free extract:

lactate dehydrogenase, pyruvate decarboxylase, pyruvate kinase and triosephosphate isomerase.

Key:

nd = not determined

6.4.4 Does the enzyme need protection?

Some workers have considered it advisable to add appropriate protecting agents during purification of hexokinase to prevent inactivation or proteolytic degradation (Sols *et al.*, 1958; Kaji *et al.*, 1961; Gazith *et al.*, 1968; Schulze and Colowick, 1969; Schmidt and Colowick, 1973a) Several experiments were carried out in which a protective agent was added to the buffer used in preparing the cell extract and the subsequent stages of purification on the dye matrex Red A gel. Compounds tested included 1.0 mM ethylenediaminetetraacetic (EDTA) acid, 2.0 mM 2-mercaptoethanol, 1.0 mM phenylmethyl sulphonyl fluoride (PMSF), and 1.0 mM EDTA and 2.0 mM 2-mercaptoethanol. No significant improvement in activity was found.

6.4.3 Purification of *Sacch. cerevisiae* hexokinases

For comparison with *S. pombe*, a preparation of hexokinase from *Sacch. cerevisiae* was carried out by the same procedure. Using the same salt gradient elution method 3 peaks of activities were eluted (Fig. 6.3). The first peak contained hexokinase activity that did not bind and was eluted in the buffer. The other 2 peaks eluted at a salt concentration under 0.25 M, which was considerably lower than that at which the hexokinase from *S. pombe* eluted. Since it is known that *Sacch. cerevisiae* has 3 hexokinases (Colowick, 1973), it is not unreasonable to assume that the 3 peaks represent glucokinase, hexokinase PI and hexokinase PII, although no further studies were carried out to characterise them.

6.4.6 Advantages of the dye-affinity method

Fig. 6.3 Purification of *Sacch. cerevisiae* hexokinase using salt gradient on Red A dye-Matrex column.

Purification procedure as in Fig. 7.1.

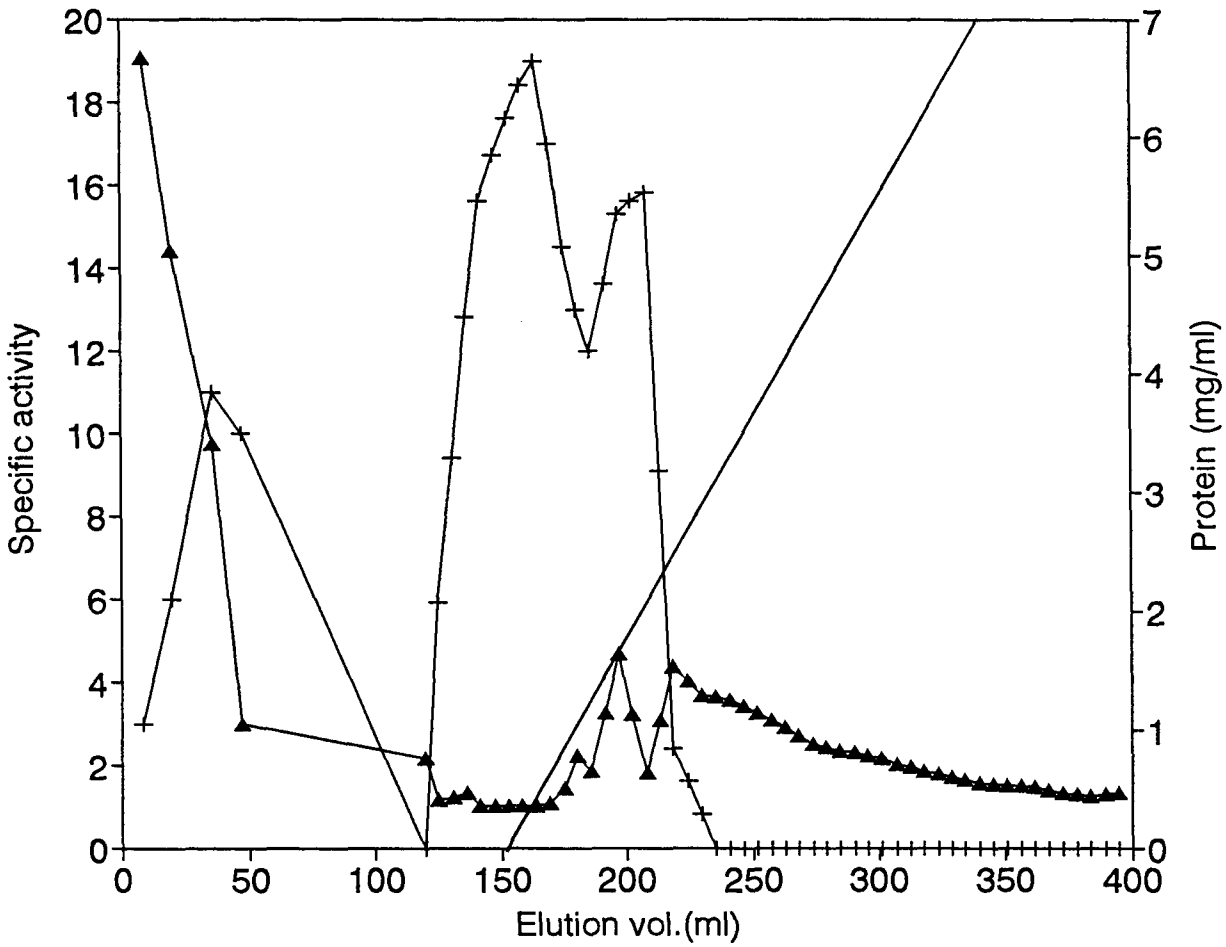
Key to plots:

+———+ specific activity (mmol min^{-1} (mg protein)$^{-1}$)

▲———▲ protein (mg ml^{-1})

——— salt gradient

(the concentration of NaCl ranges from 0 to 1.0 M over the extent of the ordinate)


Specific activity
Protein (mg/ml)
Elution vol.(ml)
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5
6
7
0
50
100
150
200
250
300
350
400

The use of dye-ligand chromatography for enzyme purification has several advantages, the most important being speed. The whole purification procedure was undertaken in 24-36 hours compared to 5 or 6 days using procedure 1. This is important not only because of less physical effort required but also because the activity of hexokinase in an impure state may be lost if the purification process takes too long. A further important benefit is the ability to apply extracts directly on to a dye-ligand column without prior processing (Scopes, 1993). Affinity elution using a salt gradient further increases the chance of obtaining a high degree of purification as is shown on Table 6.5. A 110 to 160-fold purification was achieved with recoveries of the total activity loaded of up to 40% in a salt gradient and up to 100% in batch elution. Over-loading as recommended by Scopes *et al.* (1992) was not aimed for as this tended to confuse the calculation of the exact amount of enzyme bound on to the column. The volume of enzyme applied was maintained equal to, or less than, the void volume of the column in order to ensure complete binding. The same technique was adopted for the loading of the enzyme on to other columns used in the various purification attempts in this study.

Dye-ligands in affinity chromatography were first made use of in the late 1960s when it was discovered that proteins could bind to certain triazine textile dyes coupled on to porous support adsorbents such as agarose and silica. It has been used to separate hexokinases from a variety of organisms and particularly from *Sacch. cerevisiae*. The dyes interact with the enzyme / protein just like natural ligands but have no biological relation to the protein. It has been noted that similar enzymes from different sources do not necessarily exhibit the same dye-

binding specificity and the hexokinase from *S. pombe* was found to bind on to different dye-matrex from those used for purification of other hexokinases (Scawen & Atkinson, 1987).

CHAPTER 7

CHARACTERIZATION OF HEXOKINASE FROM *SCHIZOSACCHAROMYCES POMBE*

7.1 SUBSTRATE SPECIFICITY

7.1.1 Sugars

Phosphate acceptor specificity of *S. pombe* hexokinase was determined using Method 2 (see Material and Methods). A total of 14 hexoses, 3 pentoses, 5 disaccharides and 1 trisaccharide were tested. Each substrate was made up in 50 mM Tris-HCl buffer (pH 7.6) and their rates of phosphorylation were compared at substrate concentrations of 2.22 mM, 22.2 mM and 222 mM. The results shown in Table 7.1 illustrate the variations in the rates of phosphorylation of sugars with substrate concentrations. Significant activity was found with 5 hexoses and related compounds, their relative rates at concentration of 2.22 mM being (D-fructose = 100): D-glucose 61, D-mannose 32, 2-deoxy-D-glucose 29, D-glucosamine 18 and D-allose 3. When the concentration of hexose was increased from 2.22 mM to 22.2 mM, the rate of fructose phosphorylation was increased by 28% and that of D-mannose doubled, while those for D-glucose, 2-deoxy-D-glucose and D-glucosamine decreased slightly. A further ten-fold increase in hexose concentration to 222 mM increased the rate of D-fructose phosphorylation by 45% while that of D-glucose decreased by 13%. (See also Section 6.3).

The specificity of *S. pombe* hexokinase can be explained in terms of

Table 7.1 Relative rates of *S. pombe* hexokinase on sugar substrates.

The rates were determined using Method 2 but using several concentrations of the substrate. The activity of hexokinase on each substrate is expressed relative to that of D-fructose at each substrate concentration.

Results are average values of duplicate tests.

Compound	Relative rate at		
	2.22 mM	22.2 mM	222 mM
D-fructose	100	128	145
D-glucose	61	57	48
D-mannose	32	61	22
2-deoxy-D-glucose	29	27	nd
D-glucosamine	18	15	5
D-allose	3	nd	nd

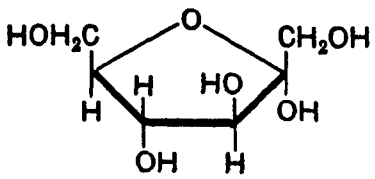
The following substrates had no measurable activity:

L-glucose, L-mannose, L-sorbose, α methyl-D-glucoside, N-acetyl-D-glucosamine, D-galactose, L-idose, 6-deoxy-D-galactose, D-xylose, D-ribose, L-arabinose, sucrose, maltose, lactose, trehalose, melibiose, raffinose.

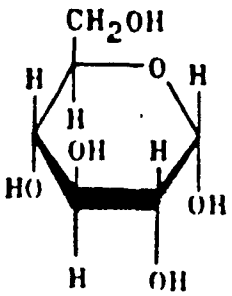
molecular structures depicted in Fig. 7.1. D-mannose, 2-deoxy-D-glucose and D-glucosamine are C(2) epimers of D-glucose differing at the second carbon, causing some reduction in hexokinase activity. N-acetyl-D-glucosamine is an analog of D-glucose containing a bulky substituent on the 2-carbon, which causes steric interference. The specificity for D-glucosamine is much lower than that for D-fructose or D-glucose while there is no activity with N-acetyl-D-glucosamine. A change on the third carbon as in D-allose, a C(3) epimer of D-glucose has a much greater effect on activity, reducing it to 3% of that shown with D-fructose (and 5% of that shown with D-glucose).

Hexokinase was found to be most active on D-fructose, a ketose, and less active on D-glucose, an aldose. D-fructose possibly is the preferred hexose form for hexokinase binding. On the other hand, hexokinase possibly binds more strongly with D-glucose than it does with D-fructose. Hence the dissociation of the hexokinase-glucose complex in the presence of ATP may be slower than that of the hexokinase-fructose complex, in a situation where product inhibition does not occur. When it is in solution, D-fructose occurs 31.6% in the furanose form, a 5-ring structure and 68.4% in the pyranose, a 6-ring structure (Budavari, 1989). D-glucose has a 6-ring structure. It can be seen that the positions of C_3, C_4, C_5 and C_6 carbon in D-glucose and D-fructose furanose form are superimposable although the positions on the fructose are skewed somewhat relative to glucose. Viewed this way, D-glucose and D-fructose bear a close structural similarity, even though fructose consists of a 5-membered ring and glucose of a 6-membered ring.

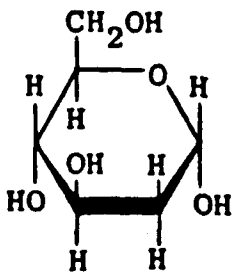
In the presence of 2.22 mM hexose, hexokinase activity with D-galactose or L-idose was less than 0.1% of that shown with D-fructose. Other hexoses and



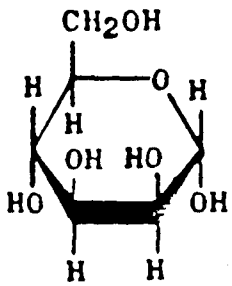
α-D-fructose



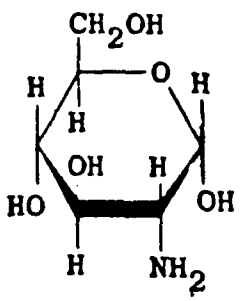
α-D-glucose



α-2-deoxy-D-glucose



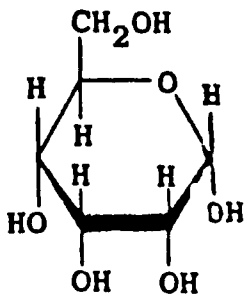
α-D-mannose



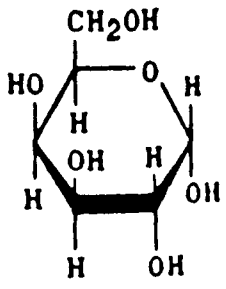
α-D-glucosamine

Fig. 7.1

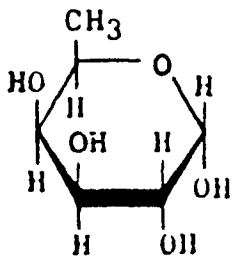
Molecular models of some hexose sugars and sugar-analogs (Budavari, 1989).



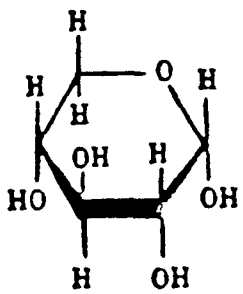
α-D-allose



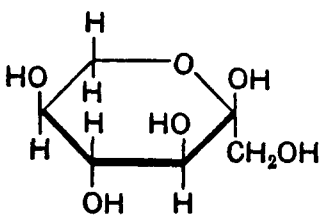
α-D-galactose



α-6-deoxy-D-galactose



α-D-xylose



α-L-sorbose

Fig. 7.1 (continued)

Molecular models of some hexose sugars and sugar-analogs (Budavari, 1989).

related compounds tested were α-methyl-D-glucoside, L-sorbose, L-glucose, L-mannose and 6-deoxy-D-galactose, all of which did not show measurable activity with hexokinase. All of these sugars differ from D-glucose either at the C_4, C_5 or C_6, suggesting that the configuration at these carbon atoms is critical for binding of the substrate. There was also no activity with the pentoses D-xylose, D-ribose and L-arabinose, or with the disaccharides and trisaccharides tested.

The specificity of *S. pombe* hexokinase for sugars was found to be similar to *Sacch. cerevisiae* hexokinase PI and PII but differed with respect to their relative affinities and relative rates (Purich *et al.*, 1973).

7.1.2 Nucleotides

The rate of hexokinase activity in increasing ATP concentration was tested using Method 1. The concentration of ATP was increased from 0.064 mM to 6.4 mM. Glucose and fructose were used as substrates at concentrations of 2.22 mM, in the presence of 8 mM $MgCl_2$ (Fig. 7.2). The rate increased up to an ATP concentration of 1.3 mM for both sugars and was then constant. Higher substrate concentrations were not investigated.

GTP was found to be markedly less active than ATP as a phosphate donor. Among the nucleotides assayed, ADP gave a 'false' phosphorylation while AMP could not act as a phosphate donor and failed to show activity with either glucose or fructose (Table 7.2). The apparent activity of ADP as a phosphate donor was very puzzling indeed until the possible explanation was traced to myokinase activity. It has been shown in Chapter 6 that there was a trace of myokinase present in the purified hexokinase (<1%). ADP converts to ATP and AMP in presence of myokinase according to the reaction:

Fig. 7.2 Effect of ATP concentration on hexokinase activity.

The rate of hexokinase activity in increasing ATP concentration was tested using Method 1. The concentration of ATP was increased from 0.064 mM to 6.4 mM. Glucose and fructose were used as substrates at concentrations of 2.22 mM, in the presence of 8 mM $MgCl_2$.

This graph is the result for glucose.

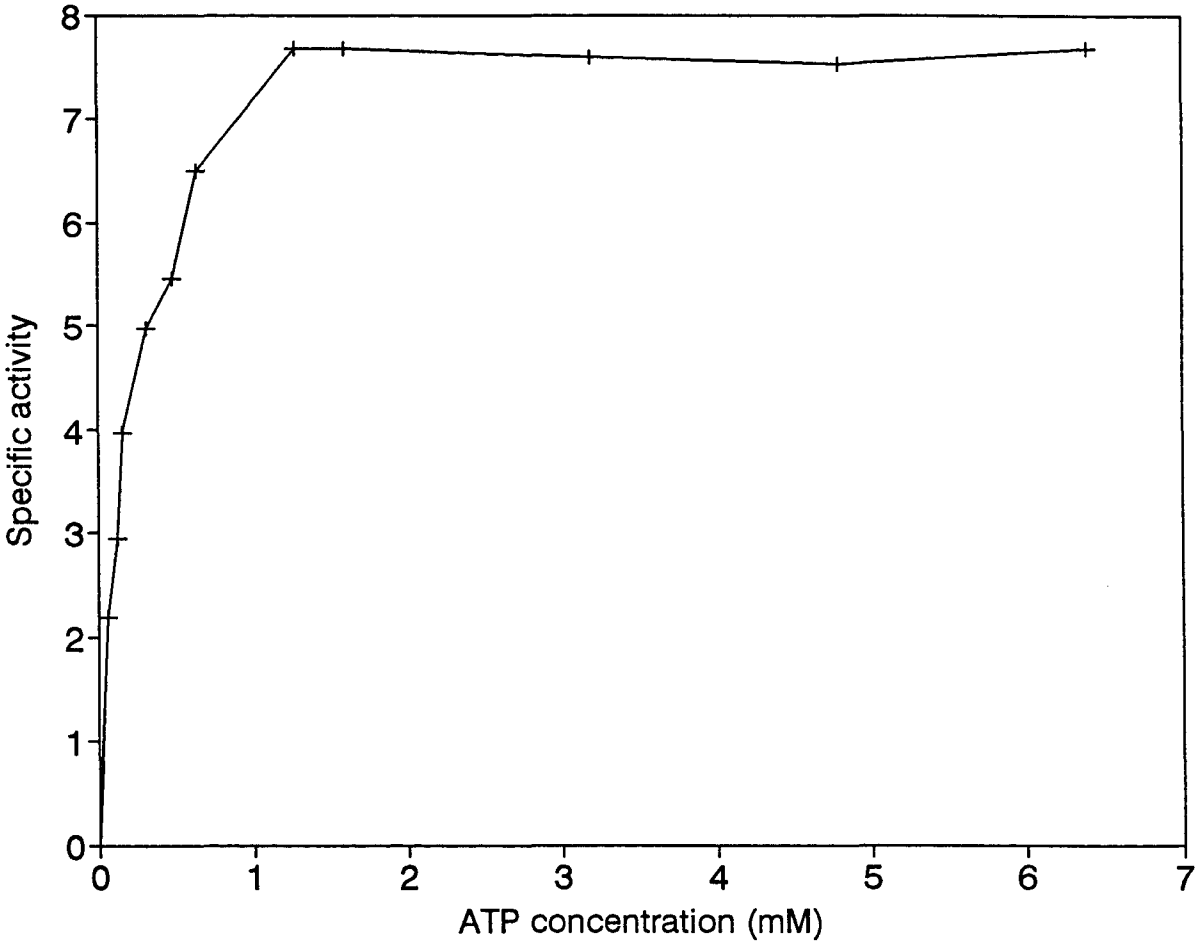

Specific activity
ATP concentration (mM)
0
1
2
3
4
5
6
7
8

Table 7.2 Relative rates of glucose phosphorylation when ATP is replaced by other nucleotides.

The rates were determined using Method 1 and 2.22 mM glucose as substrate. The activity of hexokinase in presence of 8.0 mM Mg^{2+} at room temperature, is expressed relative to that of ATP.

Results are average values of duplicate trials.

The 'false' rate obtained with ADP is explained in the text.

Nucleotide (0.64 mM)	Relative rate
ATP	100
ADP	24
AMP	0
GTP	3.4

$$AMP + ATP \longleftrightarrow 2ADP$$

With ATP available, the phosphorylation of glucose goes ahead giving a false impression of ADP activity.

7.1.3 Effect of metallic ions on activity

Hexokinase activity on glucose and fructose in increasing Mg^{2+} concentration was measured using Method 1, with Mg^{2+} concentration varied between 0.08 mM and 100 mM. Hexokinase activity required the presence of magnesium no activity being observed in the system in its absence. In the presence of 2.22 mM glucose and 0.64 mM ATP, maximum rates were obtained with Mg^{2+} concentration of 2 mM. Increasing the concentration above this resulted in decrease in hexokinase activity (Fig. 7.3).

The effect of other bivalent and monovalent cations on hexokinase activity was investigated in order to determine whether any other cation could replace Mg^{2+}. The activity of hexokinase on glucose and fructose was measured using Method 1 in which $MgCl_2$ was replaced by the same concentration of another cation. The results (Table 7.3) showed that other bivalent cations could replace Mg^{2+} partially. Mn^{2+} and Co^{2+} showed 47% and 35% hexokinase activity respectively, relative to the activity with Mg^{2+}, but Ca^{2+} showed very little activity (4%), and Zn^{2+} and Cu^{2+} none. Monovalent cations could also replace Mg^{2+} but showed a progressive reduction in hexokinase activity with size of molecule. The activities of Li^{+}, Na^{+} and K^{+} were 21%, 15% and 14% respectively, relative to the activity with Mg^{2+}.

Fig. 7.3 Effect of $MgCl_2$ concentration on hexokinase activity.

Hexokinase activity on glucose in increasing Mg^{2+} concentration was measured using Method 1, with Mg^{2+} concentration varied between 0.08 mM and 100 mM.

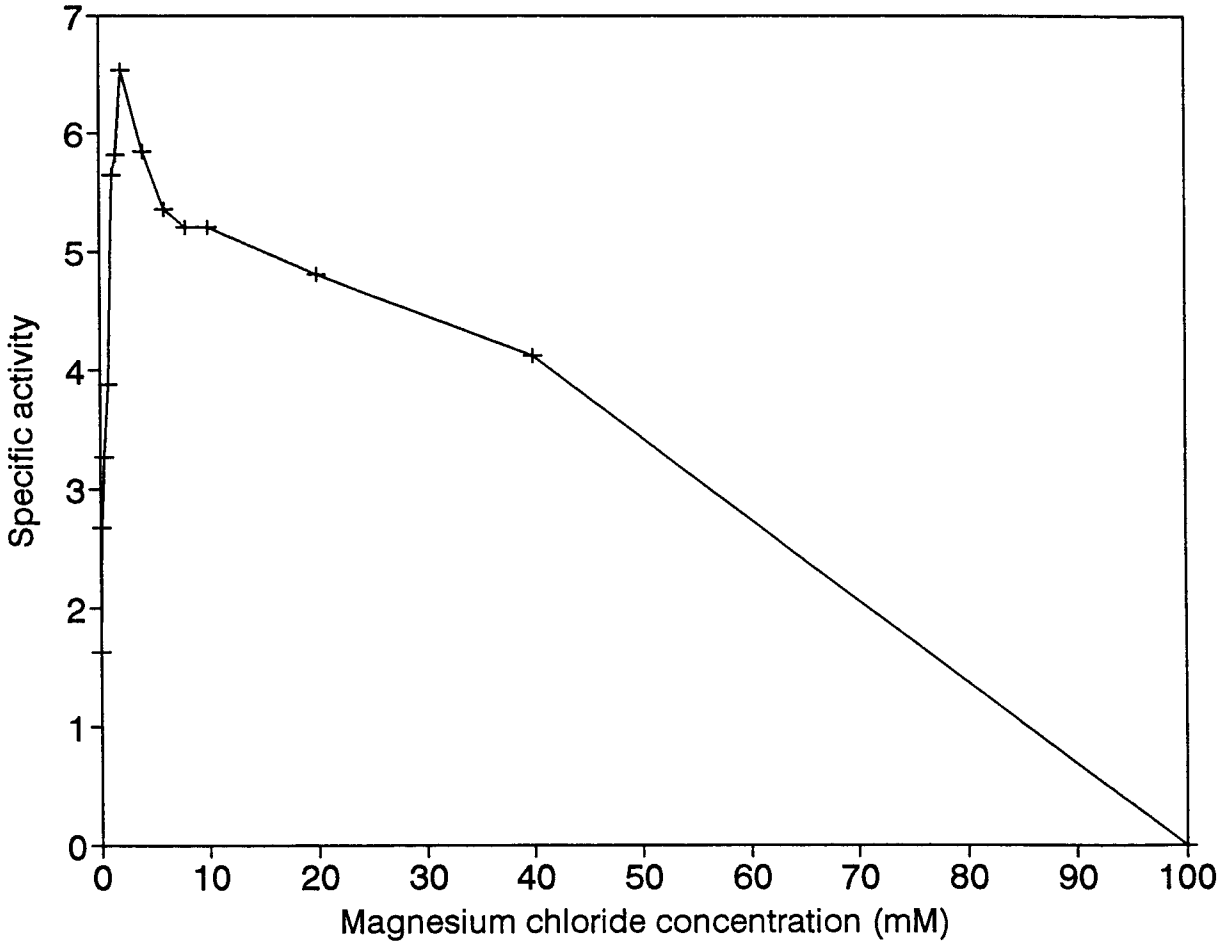

Specific activity
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
100
Magnesium chloride concentration (mM)

Table 7.3 Effect of replacing Mg^{2+} with other cations.

The rate of activity was determined using Method 1 in which $MgCl_2$ was replaced with another cation as chloride.

Cation added at 8.0 mM	Relative rate		F/G activity ratio
	with glucose	with fructose	
No addition	0	0	.
Mg^{2+}	100	100	1.02
Mn^{2+}	48	47	1.0
Co^{2+}	33	35	1.09
Li^{+}	20	21	1.06
Na^{+}	15	15	1.0
K^{+}	14	14	1.0
Ca^{2+}	4	4	1.0
Cu^{2+}	0	0	.
Zn^{2+}	0	0	.

7.2 DETERMINATION OF ENZYME KINETICS

K_m and V_{max} values (Table 7.4) were determined by varying the concentration of substrates in the assay mixture at 22°C, pH 7.6. The results were analysed using two procedures: (i) double reciprocal plot or Lineweaver-Burk, and (ii) direct linear plot described by Eisenthal & Cornish-Bowden (1974). A wide range of substrate concentrations was involved in the experimental measurements. The higher values were therefore crowded on to the left side of the double reciprocal plot leading to large errors in K_m and V_{max} values. The direct linear plot was found to be more accurate as it was simple to construct, composed entirely of straight lines requiring no calculation. The kinetic constants were read off the plot directly. For each observation (substrate concentration S, and velocity V) points were marked off with K_m = -S on the X axis, and V_{max} = V on the Y axis. A line was then drawn through the two points, extending into the first quadrant. This was repeated for all observations, with the lines intersecting at a common point, whose co-ordinates provided the values of K_m and V_{max}. This is illustrated in Fig. 7.4 with glucose as the substrate.

The values for sugar substrates were determined using concentrations ranging between 0.22 mM and 2.22 mM with 0.64 mM ATP and 8.0 mM $MgCl_2$. D-fructose had a K_m value of 0.77 mM whereas that for D-glucose was much lower (0.05 mM). The K_m and V_{max} values may further explain the difference in the rates of phosphorylation between D-glucose and D-fructose, with D-fructose having both a higher substrate concentration at the half-maximal reaction, and also a higher maximum rate of reaction that occurs when the substrate concentration is saturating (10.35 μmol l^{-1} min^{-1}) than D-glucose (5.8 μmol l^{-1} min^{-1}. D-mannose

Table 7.4 K_m and V_{max} data.

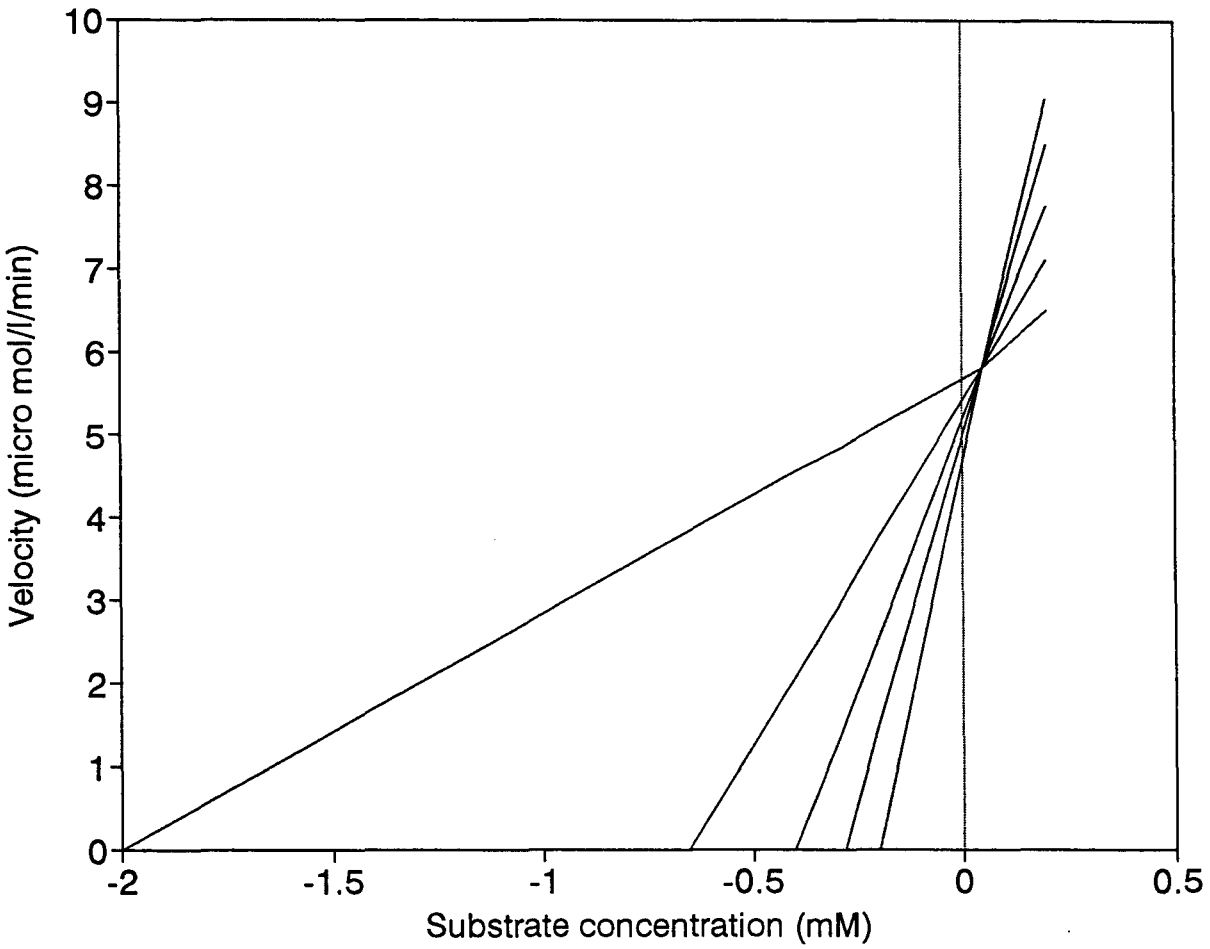
The Michaelis constants and maximum velocities were determined using the direct line plot (see text for details). In the case of sugar substrates the assays were based on ADP formation (Method 2) in which the ATP was held constant at 0.64 mM nucleotides were assayed using Method 1, in which glucose was held constant at 2.22 mM.

Compound	K_m mM	V_{max} μmol l^{-1} min^{-1}
D-fructose	0.77	10.35
D-glucose	0.05	5.8
D-mannose	0.06	3.02
2-deoxy-D-glucose	0.18	3.19
D-glucosamine	0.24	1.8
ATP	0.19	8.05
$MgCl_2$	0.32	7.3

Fig. 7.4 Determination of K_m and V_{max} by the direct linear plot.

Each line represents one observation of substrate and velocity and has intercepts on the K_m and V axes. The point of intersection of the lines gives the best-fit values for K_m and V_{max}.

For each observation (substrate concentration S, and velocity V) points were marked off with $K_m = -S$ on the X axis, and $V_{max} = V$ on the Y axis. A line was then drawn through the two points, extending into the first quadrant. This was repeated for all observations, with the lines intersecting at a common point, whose co-ordinates provided the values of K_m and V_{max}.


10
9
8
7
6
5
4
3
2
1
0
Velocity (micro mol/l/min)
-2
-1.5
-1
-0.5
0
0.5
Substrate concentration (mM)

had a K_m value of 0.06 mM while 2-deoxy-D-glucose and D-glucosamine both had values higher than D-glucose (0.18 mM and 0.24 mM respectively). In presence of 2.22 mM glucose, ATP had an apparent K_m of 0.19 mM and a V_{max} value of 8.05 μmol l^{-1} min^{-1}. Mg^{2+} had a K_m value of 0.32 mM and a V_{max} of 7.3 μmol^{-1} l^{-1} min.

7.3 EFFECT OF INHIBITORS ON ACTIVITY

An investigation of the effect of a variety of inhibitors on the hexokinase activity is shown in Table 7.5.

7.3.1 Inhibition by substrates

The inhibition of glucose and fructose phosphorylation by other hexoses was tested by assaying for hexokinase activities in the presence of each potential inhibitor. The activity of hexokinase was measured out in the presence of 2.22 mM glucose or fructose plus the same concentration of the other hexose, using Method 1. L-idose, L-glucose and D-xylose were not inhibitory but inhibition occurred with 2-deoxy-D-glucose (39%), D-mannose (23%), D-glucosamine (15%), D-allose (9%) and D-galactose (8%). This illustrates that those sugars that are substrates can also act as competitive inhibitors of glucose binding. Although D-xylose did not inhibit hexokinase activity even at a concentration 100-fold greater than either D-glucose or D-fructose as substrate, it did inhibit completely when 2-deoxy-D-glucose, D-mannose or D-glucosamine was the substrate used.

Table 7.5 Effect of inhibitors on hexokinase activity.

The activity of the enzyme in the presence of each inhibitor was determined using glucose (2.22 mM) as substrate. Details of the methods used are described in the text.

Inhibitor	Compound	% Inhibition
Carbohydrates	2 deoxy-D-glucose	39
	D-mannose	23
	D-glucosamine	15
	N-acetyl-D-glucosamine	12
	D-allose	9
	D-galactose	8
	D-xylose	0
	L-glucose	0
	L-idose	0
Nucleotides	ADP	49
	AMP	71
Cations	Zn^{2+}	100
	Cu^{2+}	100
	Mn^{2+}	89
	Co^{2+}	86
	Li^{+}	61
	Na^{+}	37
	Ca^{2+}	29
	K^{+}	22
Metabolic intermediates	F1P	52
	F6P	8
	G6P	3
	G1P	2
	F-1,6bisP	0

The inhibition of hexokinase activity by other nucleotides in the presence of ATP was tested using Method 1. The concentration of the inhibitor added to the assay mixture was equal to that of ATP. Glucose was used as the substrate at a concentration of 2.22 mM in the presence of 8 mM $MgCl_2$. Hexokinase activity was inhibited by ADP (49%) and AMP (71%). The inhibition of activity by ADP appears to be responsible for the difference in the velocities of the two assay methods (Section 6.1). In method 1 ADP accumulates (and therefore inhibits) and reduces the velocity of the reaction, while in method 2, ADP is recycled.

7.3.2 Inhibition by cations

The inhibition of hexokinase activity on glucose and fructose by other cations in the presence of Mg^{2+} was tested. The assay was carried out using Method 1 in the presence of 8.0 mM of both $MgCl_2$ and cation inhibitor. Although Mn^{2+} and Co^{2+} could replace Mg^{2+}, they inhibited the activity of hexokinase on both glucose and fructose in the presence of Mg^{2+}. The inhibition due to the presence of the ions K^+, Ca^{2+} and Na^+ (22%, 29% and 38% respectively) was lower than inhibition due to Li^+, Mn^{2+} and Co^{2+}, which gave 61%, 83% and 86% inhibition respectively. The effect of Cu^{2+} and Zn^{2+} could not be determined since they inhibited the coupling enzymes used in both detecting system assays.

7.3.3 Inhibition by metabolic intermediates

The inhibition of hexokinase activity by metabolic intermediates was tested in the presence of 2.22 mM glucose or fructose, using Method 2. Results are shown in Table 7.5. G6P in concentrations ranging from 0.22-22.2 mM had very

little effect on the hexokinase activity on glucose (3% inhibition at 22.2 mM concentration) and on fructose (6% inhibition at 22.2 mM concentration). There was also little effect on hexokinase activity in the presence of 2.22 mM of F6P and G1P. F-1,6bisP had no effect on either fructokinase or glucokinase activity but F1P was found to inhibit glucokinase activity (52%) and fructokinase activity (50%), giving values much higher than those observed with the other metabolites.

G6P has been reported as both inhibitory and non-inhibitory of hexokinase. The hexokinase from *S. pombe* resembles glucokinase and hexokinase from *Rhodotorula glutinis* in not being inhibited by G6P (Mazon *et al.*, 1975), but differs from mammalian hexokinases, where G6P is a powerful competitive inhibitor (Jenkins & Thompson, 1989), and from *Sacch. cerevisiae* hexokinases. In *Sacch. cerevisiae* G6P is an inhibitor of hexokinase (Bergmeyer *et al.*, 1983) but some studies have shown that a concentration of up to 5 mM does not inhibit hexokinase *in situ* (Serrano *et al.*, 1973). Glucokinase from pea seeds (Turner *et al.*, 1977), is inhibited by ADP but not by G6P.

7.3.4 Inhibition by end-product

The effect of ethanol on hexokinase activity was determined by adding increasing concentrations of ethanol ranging from 0 to 6.85 M (0 to 40% v/v) to the assay mixture. The rate of activity was measured using Method 1. As an alternative hexokinase was incubated in 50 mM Tris-HCl buffer (pH 7.6) for 30 minutes at room temperature, in increasing concentrations of ethanol, before being assayed for activity on glucose and fructose using the same method. The ADP assay system is unsuitable due to traces of ADH present in the purified

enzyme, which could catalyse NADH oxidation by reaction with ethanol.

The results are shown in Fig. 7.5. A concentration of 1.7 M (10% v/v) ethanol produced 13-16% inhibition, which increased to 85-87% at a concentration of 4.3 M (25% v/v) ethanol. Hexokinase activity was completely inhibited by 5 M (30% v/v) ethanol.

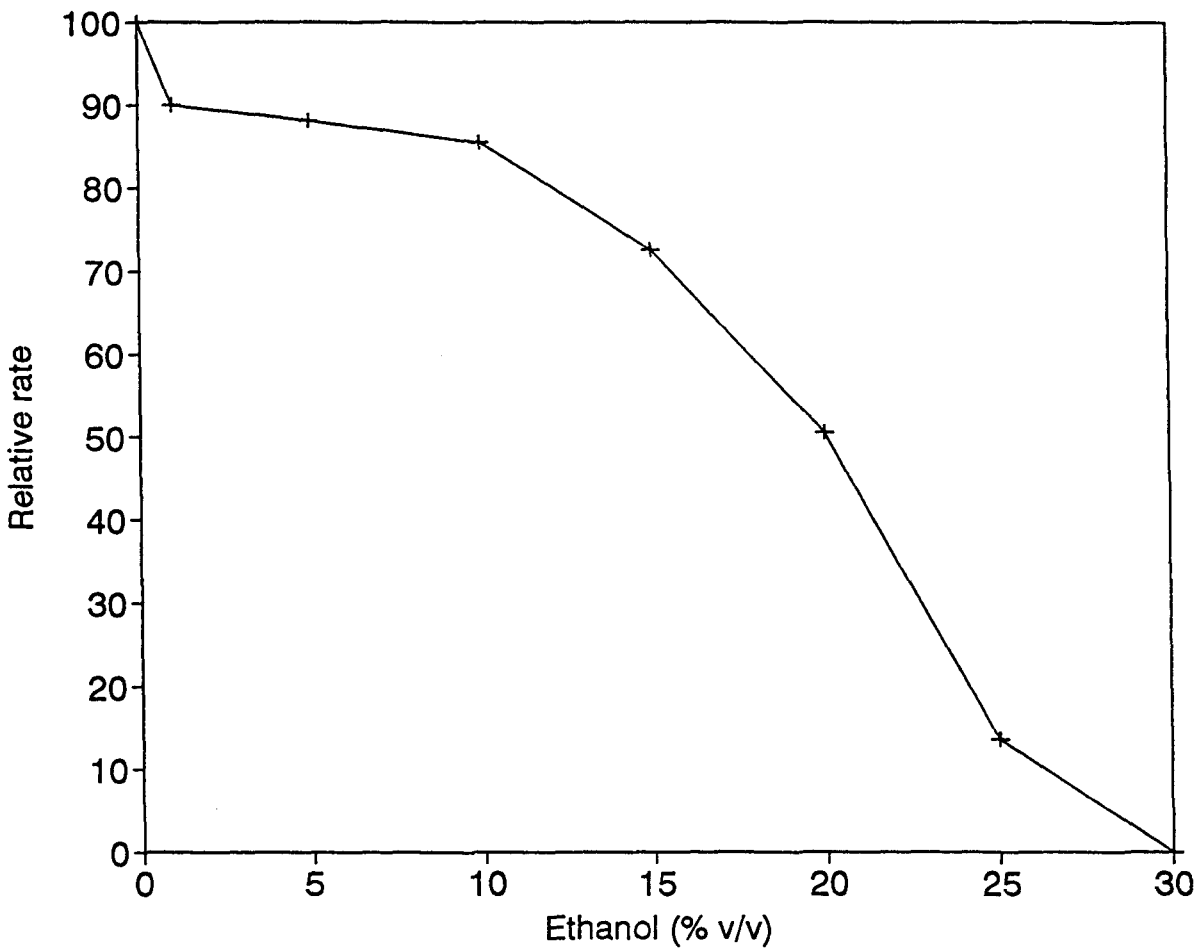
The two hexokinase isoenzymes from *Sacch. cerevisiae* have been found to have different sensitivities for ethanol. Studies by Navarro & Finck (1982) demonstrated that cells that retain high ethanol concentration (30% v/v) rapidly lose their hexokinase activity and cells that retain only 10% of ethanol have 50% of their enzymatic activity at the end of the fermentation. During growth and fermentation, *S. pombe* produces relatively low concentrations of ethanol, which are below the concentration of added ethanol that is likely to cause significant inhibition on hexokinase. It has been demonstrated with *Sacch. cerevisiae* that the cessation of fermentation does not occur as a result of inhibition of hexokinase and alcohol dehydrogenase, but is due to some other factors affecting the viability of yeast cells (Larue *et al.*, 1984). Therefore the presence of end-product ethanol would not seem to have a severe inhibitory effect on hexokinase during ethanol production using *S. pombe*.

7.3.5 Other inhibitors

EDTA (1 mM), PMSF (1 mM), and 2-mercaptoethanol (2 mM) were added separately to 50 mM Tris-HCl buffer (pH 7.6) and the buffers used to assay for activity on glucose using Method 1. There was no inhibition of hexokinase activity by these chemicals at the concentrations tested, the rates of activity being

Fig. 7.5 Effect of added ethanol on hexokinase activity.

For conditions used see details in the text.



137%, 122% and 119% respectively, relative to the standard assay. Therefore, these chemicals appear to act as activators during enzyme assay only, since they were found to have no effect during the purification stage (see Section 7.4 2.1). Addition of sodium dodecyl sulphate (SDS) at 0.3 mM concentration (0.01%) reduced hexokinase activity by 74%, and 80 mM urea by 55%. The enzyme was completely inhibited by 3.5 mM SDS and 0.8 M urea.

7.4 EFFECT OF pH ON ACTIVITY

Determination of hexokinase activity at different pH values was carried out using buffers prepared according to methods described by Dawson *et al.* (1959). Rates of hexokinase activity were measured by both methods in which the Tris-HCl buffer (pH 7.6) was replaced by a series of buffers (pH 3.7 to 12.6). The rates of hexokinase activity on glucose and on fructose at the different pH levels were similar. Hexokinase was most active in the range of pH 5-10 with the activity rapidly lost below pH 5 and above 10.0 (Fig. 7.6). Hexokinase was found to be most active at pH 10 with one-half maximal activity at 5.2 and 10.5. Activity of 90% or more relative to the peak pH activity were maintained between pH 7.0 and 10.0. The enzyme is therefore more active under alkaline conditions. Hexokinase preparations from other sources generally have maximal enzyme activity between pH 8 and 9.

7.5 EFFECT OF TEMPERATURE ON STABILITY OF ENZYME

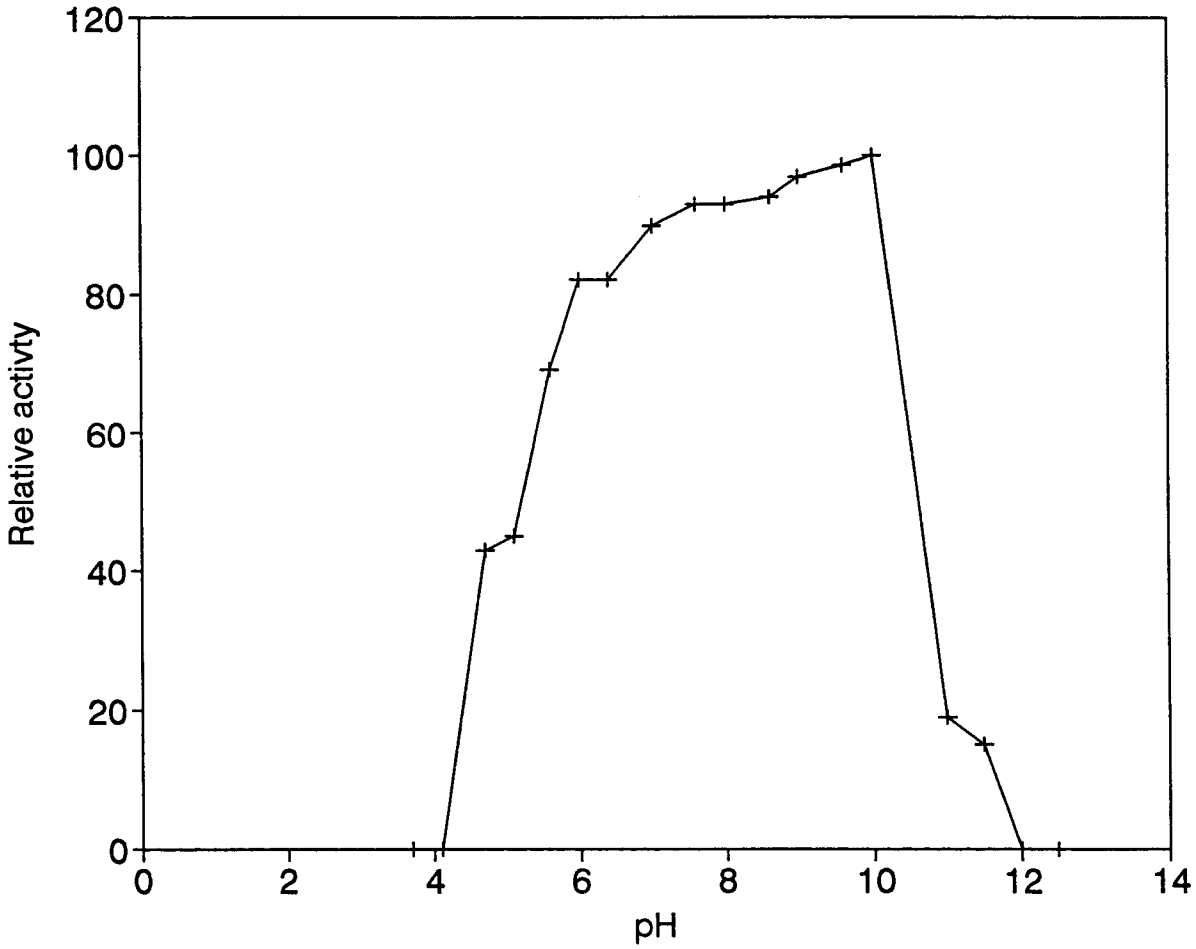
The stability of hexokinase as a function of temperature was determined by heating small tubes containing purified enzyme with either 50 mM Tris-HCl buffer

Fig. 7.6 Effect of pH on hexokinase activity.

Hexokinase activity at the different pH levels was measured using both assay methods as explained under Materials and Methods. The Tris-HCl buffer (pH 7.6) was replaced by a series of buffers (pH 3.7 to 12.6) and the measurements were made in duplicate. The pH profiles for hexokinase activity on either glucose or fructose were found to be similar. Observed rates are relative to the rate at pH 10.0.

Key:

pH 3.7 to 5.6:	KH phthalate-NaOH buffer (0.05 M)
pH 6.0 to 8.5:	triethanolamine-HCL buffer (0.05 M)
pH 9.0 to 12.5:	glycine-NaCl buffer (0.05 M)


120
100
80
60
40
20
0
Relative activty
0
2
4
6
8
10
12
14
pH

(pH 7.6) alone, or 50 mM Tris-HCl buffer (pH 7.6) plus either glucose or fructose for 10 or 30 minutes in a water bath maintained at the required temperature. At the end of the incubation period, hexokinase activity was assayed using Method 1 and using glucose as substrate for preparations heated with glucose, fructose for preparations heated with fructose and both sugars for those heated without sugar. Hexokinase activity was still present after incubating the enzyme at 45°C for 30 minutes but was completely inactivated at 50°C after 10 minutes. When the enzyme was incubated in the presence of glucose or fructose at 45°C, different results were observed. At both 2.22 mM and 222 mM concentrations, glucose gave some protection against temperature inactivation but fructose did not (Table 7.6). The activities given are relative to that of the enzyme as measured at 20°C without substrate protection. The activity of the enzyme decreased when it was incubated in the presence of fructose. The activity of hexokinase on glucose when incubated in the presence of substrate for 10 minutes, was 84% compared to 77% without. On the other hand, the activity of hexokinase on fructose when heated for 10 minutes with the substrate, was 55% compared to 79% without substrate protection. Furthermore, when the enzyme was heated for 30 minutes in the presence of glucose or fructose, the activity on glucose was 67% while that on fructose was 22%.

The difference in hexokinase activity on glucose and fructose at high temperatures may be due to the difference in stereochemistry of the two sugars which may further affect their binding capacities to the hexokinase molecules.

The stability of hexokinase was further judged by its ability to retain activity over a period of time during storage. Freshly prepared and purified hexokinase retained its activity for over 72 hours at 4°C in 50 mM Tris-HCl buffer

Table 7.6 Effect of temperature on hexokinase activity with substrate protection.

The enzyme was heated for (i) 10 minutes and (ii) 30 minutes at 45°C with and without substrate protection. Hexokinase activity was assayed using Method 1 and using glucose as substrate for preparations heated with glucose, fructose for preparations heated with fructose and both sugars for those heated without sugar.

Sugar added during heating (2.22 mM)	Time of heating (min)	Relative activity with glucose	fructose
none	0	100	100
none	10	77	79
none	30	61	68
glucose	10	84	.
glucose	30	67	.
fructose	10	.	55
fructose	30	.	22

(pH 7.6). When kept at room temperature (20°C), there was a loss of 23% of the activity in 24 hours which increased to 96% after 48 hours (Table 7.7). The enzyme was stable for at least 12 months at -70°C.

7.6 MOLECULAR PROPERTIES

The molecular weight of the native enzyme was measured by chromatography on Sephacryl S-300 as described in Section 2.5.6, and was found to be between 98000 and 100000 dalton (Fig. 7.7). The size of sub-units was determined on SDS-PAGE as described in Section 2.5.7. Two distinct bands were obtained equivalent to molecular weights of 52500 and 47500 dalton (Fig. 7.8). This size is close to that reported for the hexokinases PI and PII from *Sacch. cerevisiae*, each of which exists as a dimer with molecular weight of 93000 to 104000 dalton. *Sacch. cerevisiae* glucokinase exists only as a monomer and has a molecular weight of 51000 dalton (Middleton, 1990).

The amino acid composition of purified hexokinase was determined using an HPLC system Waters Chromatography and a PICO.TAG method, details of which are explained under Materials and Methods. Table 7.8 shows the amino acid composition of *S. pombe* and *Sacch. cerevisiae* hexokinases. The *S. pombe* differed from the hexokinases and glucokinase found in *Sacch.cerevisiae* particularly in lysine where it is less than half, glutamic acid where it is about 2/3, and in serine and glycine, both of which are 1.5 times higher. The amount of histidine in *S. pombe* hexokinase is 3 times higher than in the two *Sacch. cerevisiae* hexokinases but is slightly lower than in the glucokinase. The amino acid composition could also be used to determine the relationships between hexokinases of *S. pombe* and *Sacch. cerevisiae* as shown in the phenogram in Figure 7.9.

Table 7.7 Effect of temperature on stability of hexokinase during storage.

Purified enzyme was stored in 50 mM Tris-HCl (pH 7.6) at 3 different temperatures for periods up to 300 days. The activity was determined using Method 1 at 20°C.

Temperature °C	Period (days)	Relative rate	
		on glucose	on fructose
20	(fresh)	100	100
	1	77	82
	2	5.5	5.4
	3	0	0
4	1	100	100
	2	100	100
	3	100	100
	7	75	82
	15	62	63
	36	33	38
	56	1	1
-20	7	100	100
	56	100	100
	300	90	78

Fig. 7.7 Determination of molecular weight.

Molecular weight was determined by comparing the distance migrated by purified hexokinase on Sephacryl S-300 to that of known standards. The calibration curve is the plot of molecular weight against K_{av} (the fraction of the stationary gel volume which is available for diffusion of a given solute species.

Calibration proteins used were: catalase (240000), aldolase (158000), chymotrypsinogen A (25000) and cytochrome C (12500).

X indicates the K_{av} value at which hexokinase was eluted, giving a molecular weight of 105000 dalton.

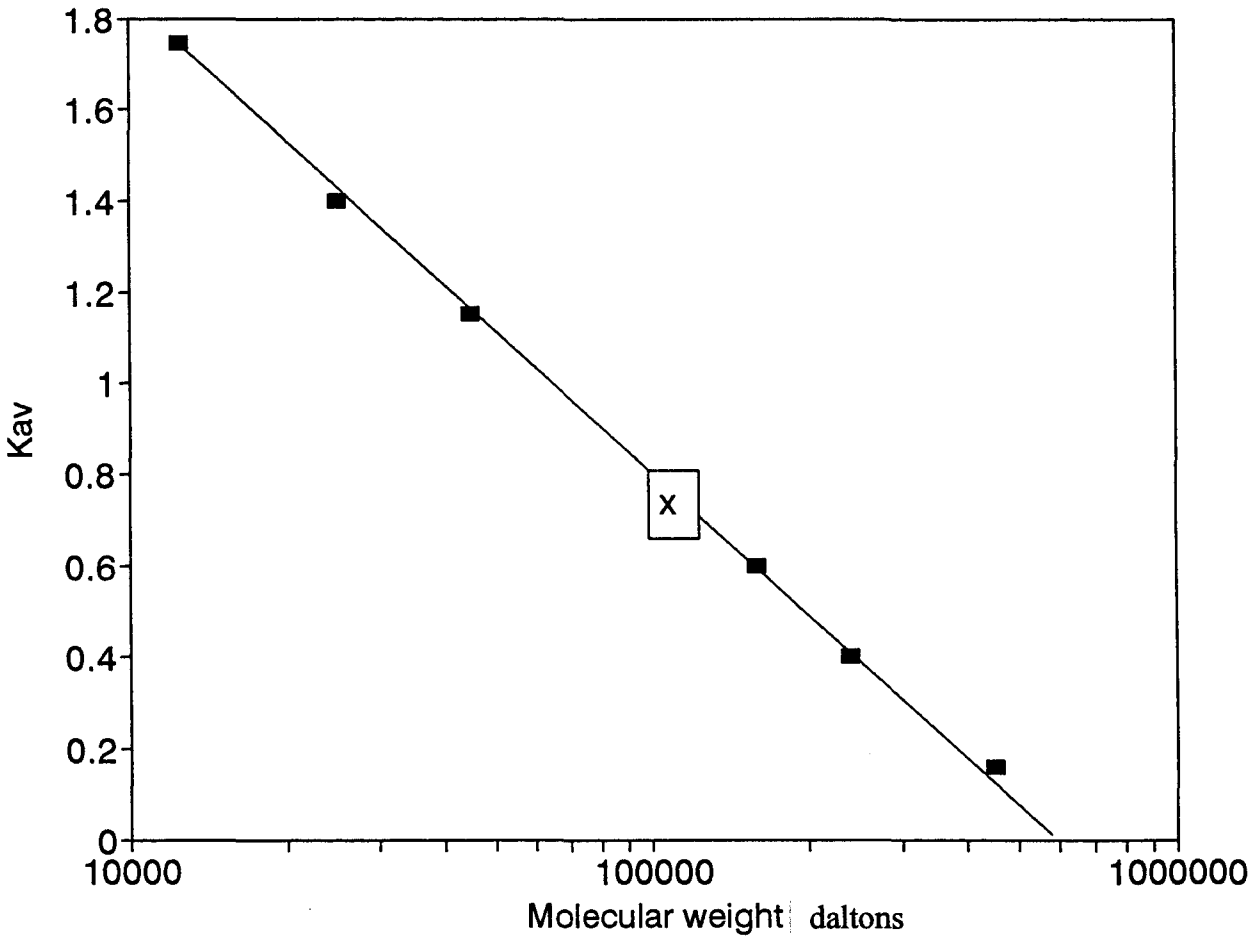

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Kav
X
10000
100000
1000000
Molecular weight daltons

Fig. 7.8 Determination of sub-units.

SDS-PAGE was prepared and carried out as described in the text (see Section 2.22). The calibration curve is the plot of molecular weight against relative electrophoretic mobilities of the proteins.

The mobility of hexokinase was compared to those of the calibration proteins used: bovine serum albumin (68000), egg albumen (45000), chymotrypsinogen A (25000) and cytochrome C 12500).

X and Y indicate the two positions at which hexokinase gave bands corresponding to molecular weights of 52500 and 45500 dalton.

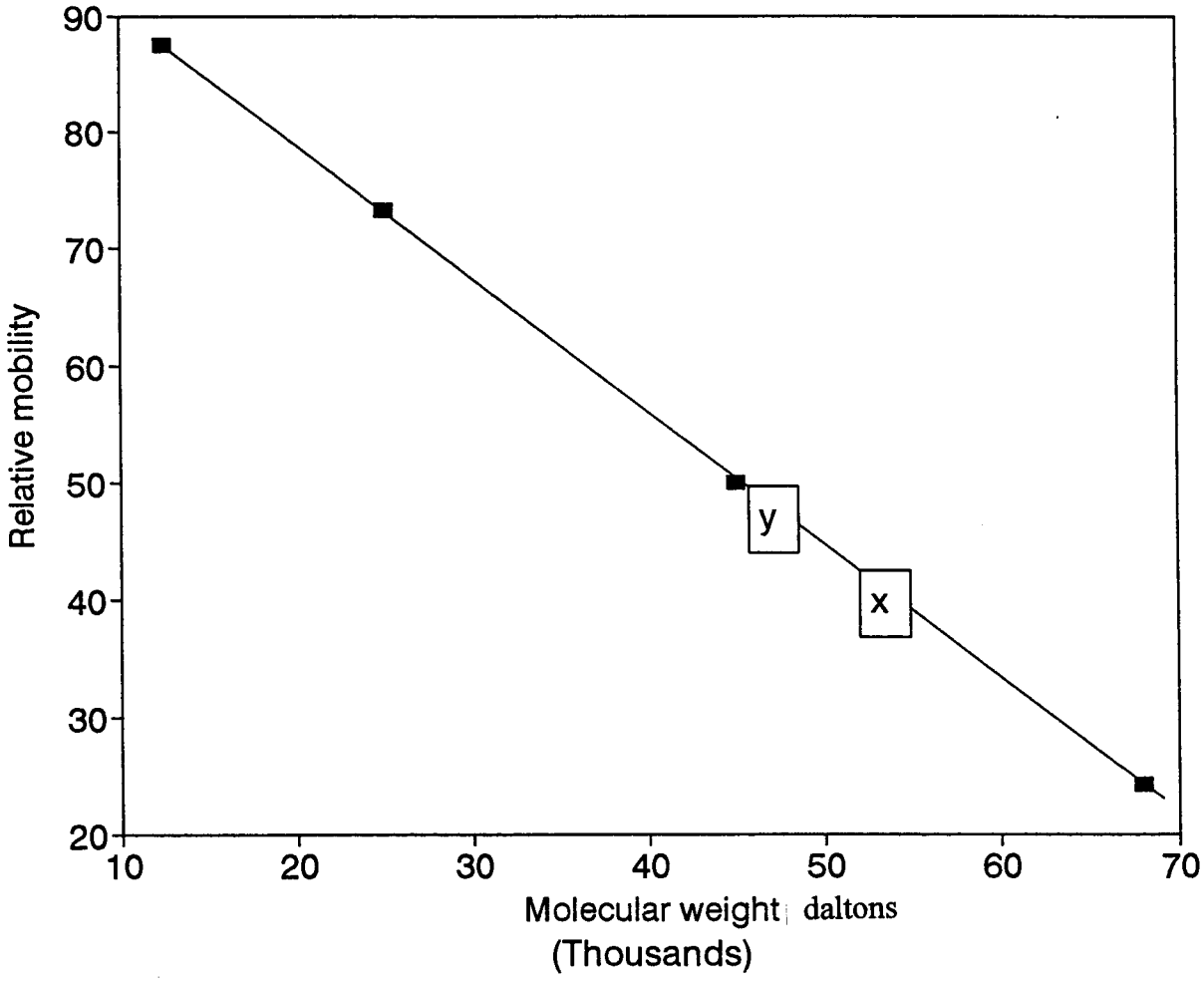

90
80
70
60
50
40
30
20
Relative mobility
y
x
10
20
30
40
50
60
70
Molecular weight daltons
(Thousands)

Table 7.8 Amino acid composition of *Sacch. cerevisiae* & *S. pombe* hexokinases.

Results show mole % values for each amino acid.

Amino Acid	*Sacch. cerevisiae*			*S. pombe*
	[a]Glucokinase	[b]Hexokinase PI	[b]Hexokinase PII	[c]Hexokinase
Lysine	7.2	8.0	7.3	3.1
Histidine	4.2	1.7	1.1	3.8
Arginine	4.6	3.8	3.9	5.0
Aspartic acid	8.1	11.8	11.3	9.3
Threonine	7.2	6.3	6.2	6.3
Serine	5.9	4.9	5.3	9.2
Glutamic acid	10.3	11.0	11.5	7.2
Proline	5.9	5.3	6.2	5.1
Glycine	8.3	8.9	8.6	12.1
Alanine	5.0	6.8	7.1	8.4
Valine	5.0	5.7	5.1	5.8
Methionine	2.6	2.5	2.4	2.1
Isoleucine	4.4	6.3	7.7	4.8
Leucine	12.5	10.3	7.5	8.2
Tyrosine	2.6	3.2	3.2	4.1
Phenylalanine	4.2	3.8	4.9	4.4
Cysteine	0.9	-	0.9	1.3

[a] Maitra & Lobo, 1977
[b] Schmidt & Colowick, 1973
[c] Amino acid composition was determined as described under Materials & Methods. Results are the average of duplicate determinations.

Fig. 7.9 UPGMA phenogram showing relationships between hexose kinases of *S. pombe* and of *Saccharomyces cerevisiae* based upon amino acid composition.

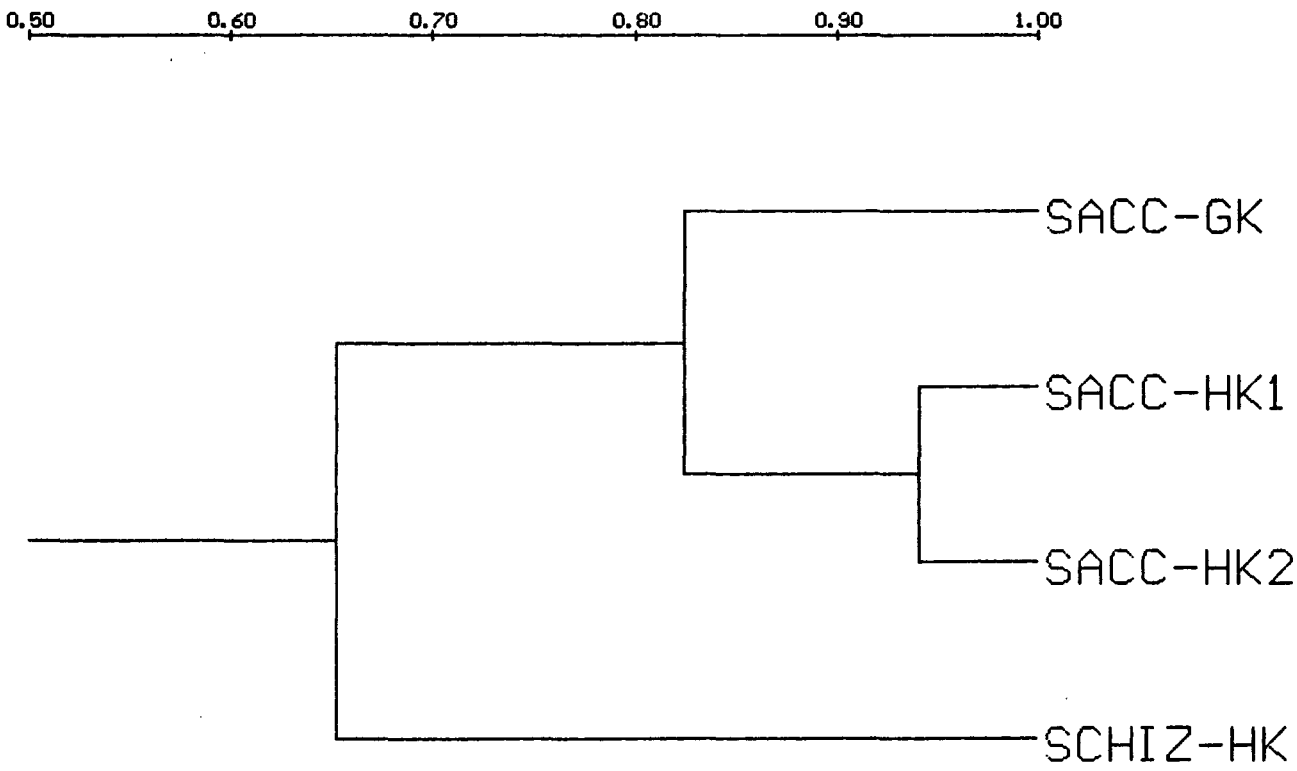
The phenogram was constructed using linkage values obtained by an average-linkage analysis (i.e. the unweighted pair-group method using arithmetic averages - UPGMA) of the similarity values which were calculated from the proportion of each amino acid present in each of the enzymes using the product-moment correlation coefficient.

When the UPGMA phenogram was constructed using average taxonomy distances, the same sequence of grouping was obtained with a matrix correlation r = 0.9827.

The values of the proportion of each amino acid present in each of the enzymes of *Sacch. cerevisiae* were obtained from Schmidt and Colowick (1973) and Maitra and Lobo (1977).

Key to plots:

SACC - GK	*Saccharomyces cerevisiae* glucokinase
SACC - HK	*Saccharomyces cerevisiae* hexokinase 1
SACC - HK2	*Saccharomyces cerevisiae* hexokinase 2
SCHIZ - HK	*Schizosaccharomyces pombe* hexokinase


0.50
0.60
0.70
0.80
0.90
1.00
SACC-GK
SACC-HK1
SACC-HK2
SCHIZ-HK

Several hexokinases are closely related to one another as indicated by amino acid composition of hexokinases from *Sacch. cerevisiae*, wheat germ and rat enzymes. *Sacch. cerevisiae* hexokinases PI and PII show very similar sequences (Middleton, 1990). Glucokinase is less related to the hexokinases. Fig. 7.10 illustrates the strong homologies between the *Sacch. cerevisiae* hexokinases and human or rat hexokinase I. The *Sacch. cerevisiae*, rat and mouse glucokinases are more closely related to themselves than to any of the hexokinases. Further studies on molecular properties of hexokinase may take the form of cloning of the hexokinase gene from *S. pombe*, which would allow sequencing of the gene and deriving of the amino acid sequence of the enzyme. For instance, what is the amino acid sequence of *S. pombe* hexokinase and how does it differ from that of *Sacch. cerevisiae* or mammalian hexokinase? Did they originate from the same ancestral gene? This may bring about interesting comparisons with other hexokinases illustrated in Fig. 7.10, and taxonomic comparisons among some of the species of yeasts based on hexokinases as was done using partial ribosomal RNA sequences illustrated in Fig. 7.11. (Kurtzman & Robnett, 1991).

The lack of indication as to what form of hexokinase the commercial 'yeast' enzyme (presumably *Sacch. cerevisiae*) from Boehringer, is, has been somewhat confusing to the author. In addition, the study by Bergmeyer *et al.* (1983) on 'yeast' hexokinase, from which the assay methods used in this study were derived, does not specify which hexokinase (PI or PII) has the properties indicated. Colowick (1973) considers the Boehringer enzyme is PI but the others may be PII. Thus, it has been difficult in some cases to compare the properties of

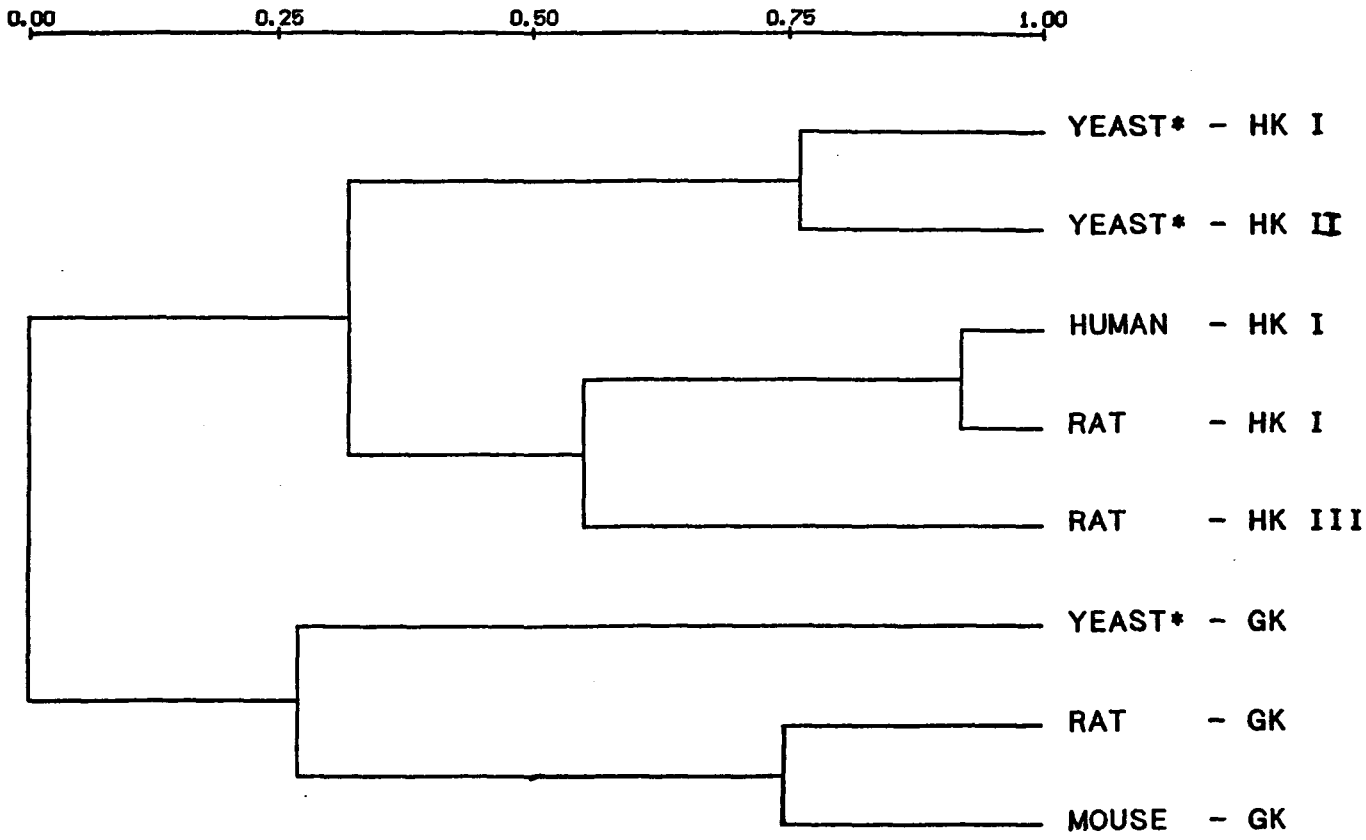
Fig. 7.10 UPGMA phenogram showing relationships between hexose kinases from different sources based on the percentage match of amino acid sequences.

The phenogram was constructed using linkage values obtained by an average-linkage analysis (i.e. the unweighted pair-group method using arithmetic averages - UPGMA) of the exact percentage match of the amino acid sequences for each of the pairs of hexose kinases. The values of percentage matches were obtained from Middleton (1990).

Key to plots:

Yeast* hexokinase I	Yeast-HKI
Yeast* hexokinase II	Yeast-HKII
Yeast* glucokinase	Yeast-GK
Human hexokinase I	Human-HKI
Rat hexokinase I	Rat-HKI
Rat hexokinase III	Rat-HKIII
Rat glucokinase	Rat-GK
Mouse glucokinase	Mouse-GK

* *Saccharomyces cerevisiae*



* *Saccharomyces cerevisiae*

Fig. 7.11 Phylogenetic tree depicting relationships determined from partial ribosomal RNA sequences. (Kurtzman and Robnett, 1991).

A phylogenetic tree depicting species of *Saccharomyces*, *Debaryomyces*, *Schwanniomyces* and *Schizosaccharomyces* calculated from the combined small and large sub-unit sequences, using a program DNAML, Version 3.11. Branch interrupted by // are shown at one-half of their computed lengths.

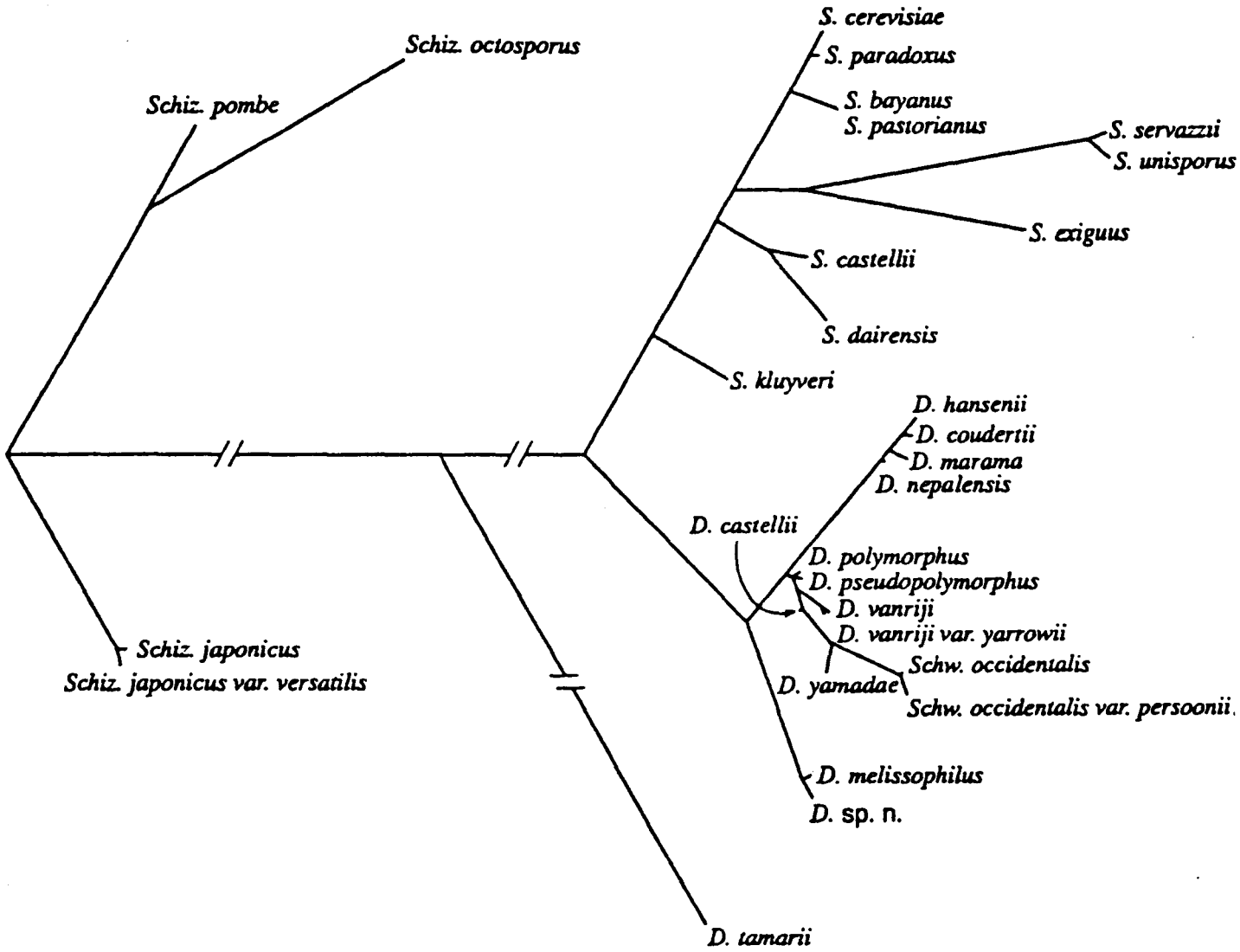
Key to abbreviations:

D. *Debaryomyces*

S. *Saccharomyces*

Schiz. *Schizosaccharomyces pombe*

Schw. *Schwanniomyces*


S. cerevisiae
S. paradoxus
S. bayanus
S. pastorianus
S. servazzii
S. unisporus
S. exiguus
S. castellii
S. dairensis
S. kluyveri
Schiz. octosporus
Schiz. pombe
D. hansenii
D. coudertii
D. marama
D. nepalensis
D. castellii
D. polymorphus
D. pseudopolymorphus
D. vanriji
D. vanriji var. yarrowii
Schw. occidentalis
D. yamadae
Schw. occidentalis var. persoonii
Schiz. japonicus
Schiz. japonicus var. versatilis
D. melissophilus
D. sp. n.
D. tamarii

S.pombe hexokinase to those of 'yeast' hexokinase from those other studies, where the form of hexokinase is not specified .

CHAPTER 8

CONCLUDING REMARKS

8.1 ETHANOL PRODUCTION BY *S. POMBE*

Further research should be focused on ways of enhancing ethanol production from *S. pombe* particularly by removal of ethanol during fermentation of initially high concentrations of sugar in order to take advantage of the inherent ability of the yeast to tolerate high concentrations of sugars. However, such research should employ appropriate sources of substrate which have potential in industrial applications and not media based on yeast extract which are not directly relevant to the industrial situation.

Attempts at performing the growth and fermentation stages at different temperatures, e.g. growing cells at 30°C and fermenting at a higher temperature, should be undertaken. Such an approach would have the practical advantage of reducing the energy for cooling the fermenters which is very relevant in countries with hot climates and assisting in the removal of ethanol from the fermentation stage by distillation at reduced pressure.

A personal interest is to find out more about the extent of the involvement of *S. pombe* in production of traditional African fermented foods. This is an important area of research which appears to be largely neglected. Since some strains of *S. pombe* were initially isolated from African millet 'beers', further investigations of their role in production and quality of such foods would seem to be warranted.

8.2 THE HEXOKINASE OF *S. POMBE*

Further studies of the hexokinase of *S. pombe* could be directed to the elucidation of the amino sequence of the enzyme either directly or indirectly by cloning of the relevant gene. Such information would allow comparisons with the sequences for hexose kinases of other organisms and throw some light on the phylogeny of *S. pombe* in relation to *Saccharomyces cerevisiae* and other eukaryotes for which such information is available. It would be of some interest to compare the relationships so derived with those revealed using partial ribosomal RNA sequences.

The cloning of the hexokinase gene of *S. pombe* would be assisted by the isolation of a hexokinaseless mutant of the yeast. Such an isolation should be straightforward since there is only one hexokinase and, presumably, only one corresponding gene. Furthermore, cloning of the glucokinase gene from, for instance, mammalian liver would give a yeast which could selectively ferment sucrose to produce ethanol and fructose, the latter having value as a non-cariogenic sweetener.

In view of the ease with *S. pombe* can be grown, the ease with which its hexokinase can be purified and the stability of the enzyme, the latter would seem to have potential commercial value. Furthermore, since it produces only a single enzyme, it would seem to have an advantage over the mixture of hexokinases normally produced from *Saccharomyces cerevisiae*.

In the words of B. Charles (1991),"ethanol is a renewable fuel whether distilled from sugar cane or sweet sorghum. Its production can be guaranteed for

as long as the sun continues to shine." Zimbabwe will continue to depend on bio-ethanol in order to "keep its wheels rolling".

APPENDIX 1

MATERIALS

1. CHROMATOGRAPHIC ADSORBENTS

Amicon Corporation, Lexington, Massachusetts, USA.

Dye Matrex Blue A, Blue B, Green A, Red A, Orange A

Pharmacia Fine Chemical Co., Uppsals, Sweden.

Sephadex G-25, Sephacryl 300

Sigma Chemicals Co., St Louis, Missouri, USA.

Hydroxyapatite

Whatman Ltd., Springfield Mill, Maidstone, Kent, England.

DEAE-cellulose DE 52

2. CALIBRATION PROTEINS

Boehringer Mannheim GmbH, Germany

albumin (bovine serum)	mol wt 68000d
albumin (hen egg)	mol wt 45000d
aldolase (rabbit muscle)	mol wt 158000d
catalase (beef liver)	mol wt 240000d
chymotrypsinogen A (bovine pancreas)	mol wt 25000d
cytochrome C (horse heart)	mol wt 12500d

Pharmacia Fine Chemical Co., Uppsals, Sweden.

catalase (bovine liver)	mol wt 232000d
ferritin (horse spleen)	mol wt 440000d
thyroglobulin (bovine thyroid)	mol wt 669000d
Blue dextran 2000	mol wt 2000000d

3. ENZYME PREPARATIONS

Boehringer Mannheim GmbH, Germany.

BSA

G-6-PDH from yeast, Grade 1, 1 mg/ml, 350 units/mg.

HK from yeast, 2 mg/ml, 140 units/mg.

LDH from rabbit muscle, 5 mg/ml, 550 units/mg.

PGI from yeast, 2 mg/ml, 350 units/mg.

MK from hog muscle, 2 mg/ml, 360 units/mg.

PK from rabbit muscle, 10 mg/ml, 200 units/mg.

4. GENERAL CHEMICALS

Ajax Chemicals, Sydney, Australia.

D-glucose, glycerol, manganese chloride, mercuric chloride, methylene blue potassium dihydrogen orthophosphate, sodium hydroxide, urea.

British Drug House, Poole, Dorset, England.

acrylamide, L-arabinose, barium chloride, bromophenol blue, copper sulphate, ethanol, D-glucosamine, hydrochloric acid, maltose, magnesium chloride, manganese sulphate, N,N′methylene bisacrylamide, potassium chloride, oleic acid, TEMED, zinc sulphate.

Calbiochem-Behring Corp., San Diego, CA., USA.

D-mannose, lactose, 2-mercaptoethanol, raffinose, SDS, sucrose.

Mallinckrodt Australia Pty., Ltd.

ferric chloride, glycine, magnesium sulphate, sodium chloride.

May & Baker Ltd., Dagenham, England.

lithium chloride, cobalt chloride.

Merck Darmstradt, Germany.

calcium chloride, potassium hydrogen phthalate.

Nutritional Biochemical Corporation, Ohio, USA.

D-ribose

Pierce Chemicals, Rockford, Illinois, USA.

6-deoxy-D-galactose, L-glucose, L-mannose

Sigma Chemicals Co., St Louis, Missouri, USA.

D-allose, Coomassie brilliant blue, 2-deoxy-D-glucose, D-fructose, D-galactose, α-methyl-D-glucoside, D-xylose, L-sorbose, melibiose, N-acetyl-D-glucosamine, nitroblue tetrazolium, phenazine methosulphate, p-iodonitrotetrazolium violet, PMSF, trehalose, triethanolamine, Tris-HCl.

5. MEDIA COMPONENTS

Becton Dickinson, Pty., Ltd., Australia.

agar (BBL).

Calbiochem Behring Corp., San Diego, CA., USA.

vitamins.

Difco Laboratories, Detroit, Michigan, USA.

agar (noble), yeast extract.

Oxoid Ltd., London, England.

bacteriological agar, malt extract, malt extract agar, mycological peptone.

6. METABOLITES

Boehringer Mannheim, Germany.

ATP (disodium salt),

F6P (disodium salt),

G6P (disodium salt),

NADH (disodium salt),

NADP (disodium salt),

Phosphoenolpyruvate.

Calbiochem Behring Corp., San Diego, CA., USA.

AMP (monosodium salt) grade A,

ADP (monosodium salt) grade A,

F-1,6-bisP (tetracyclohexy ammonium salt).

Koch-Light Laboratories, Colnbrook, Bucks, England.

G-1-P (dipotassium salt).

Sigma Chemical Co., St. Louis, Missouri, USA.

F-1-P (barium salt).

APPENDIX 2

EDINBURGH MINIMAL MEDIUM NO. 2

A.	Nitrogen source	
	Ammonium chloride	5 g
B.	Phosphate source	
	Sodium dihydrogen phosphate	300 mg
C.	Salts	
	Sodium acetate	1 g
	Potassium chloride	1 g
	Magnesium chloride	1 g
	Sodium sulphate	10 mg
	Calcium chloride	10 mg
D.	Vitamins	
	Inositol	10 mg
	Nicotinic acid	10 mg
	Calcium pantothenate	1 mg
	Biotin	10 ug
E.	Trace elements	
	H_3BO_3	500 µg
	$MnSO_4.H_2O$	400 µg
	$ZnSO_4.7H_2O$	400 µg
	$FeCl_3.6H_2O$	200 µg
	$H_2MoO_4.H_2O$	100 µg
	Kl	100 µg
	$CuSO_4.5H_2O$	40 µg
	Citric acid	1 mg
F.	Carbon Source	
	Glucose	10 g

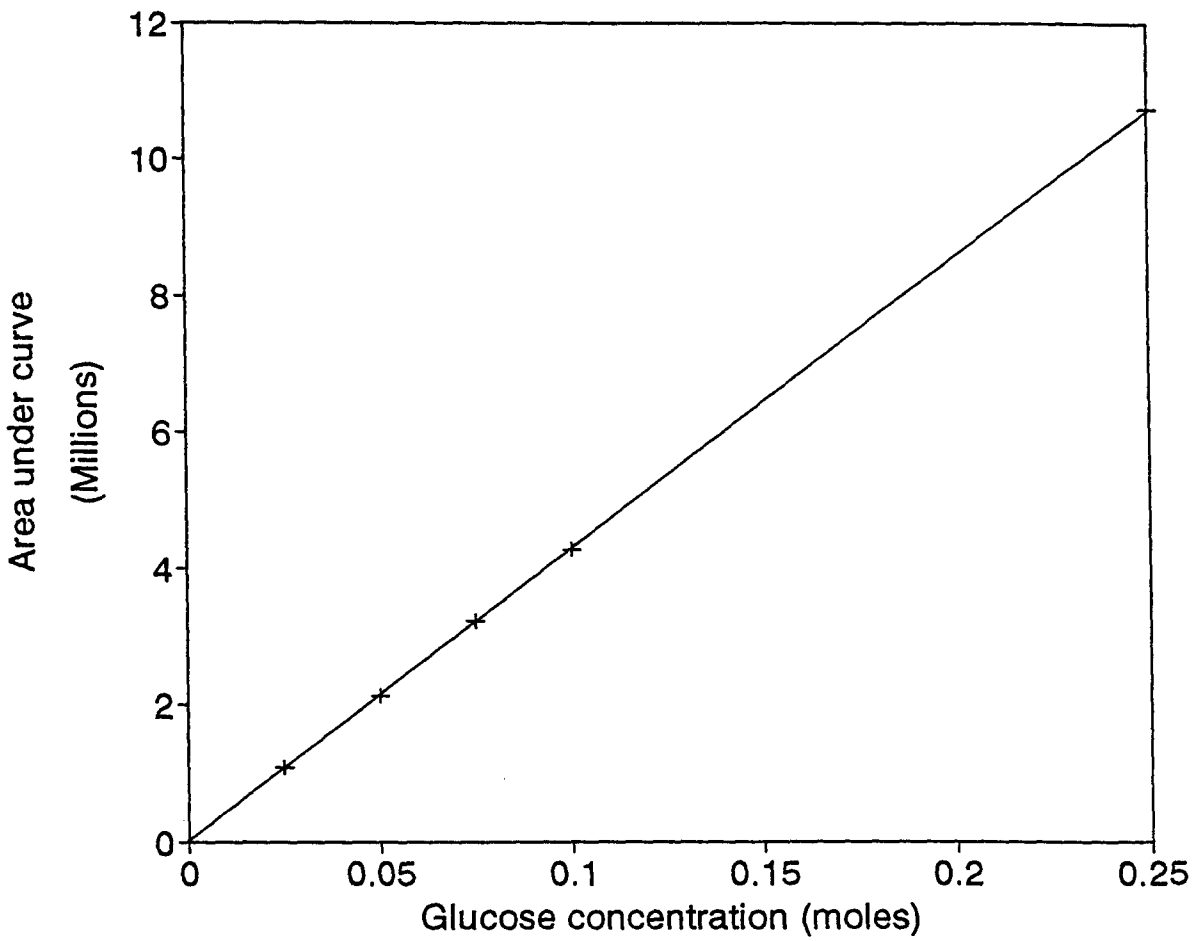
The quantities of ingredients required for the preparation of 1 litre EMM2 are shown above. Concentrated stock solutions of D and E were prepared separately and appropriate amounts of these were added to A, B and C before sterilization at 115°C for 15 min. A 10% glucose stock wasautoclavedseparately and added to the sterile basal medium just before use to give a final concentration of glucose of 1%.

APPENDIX 3

HPLC PLOTS

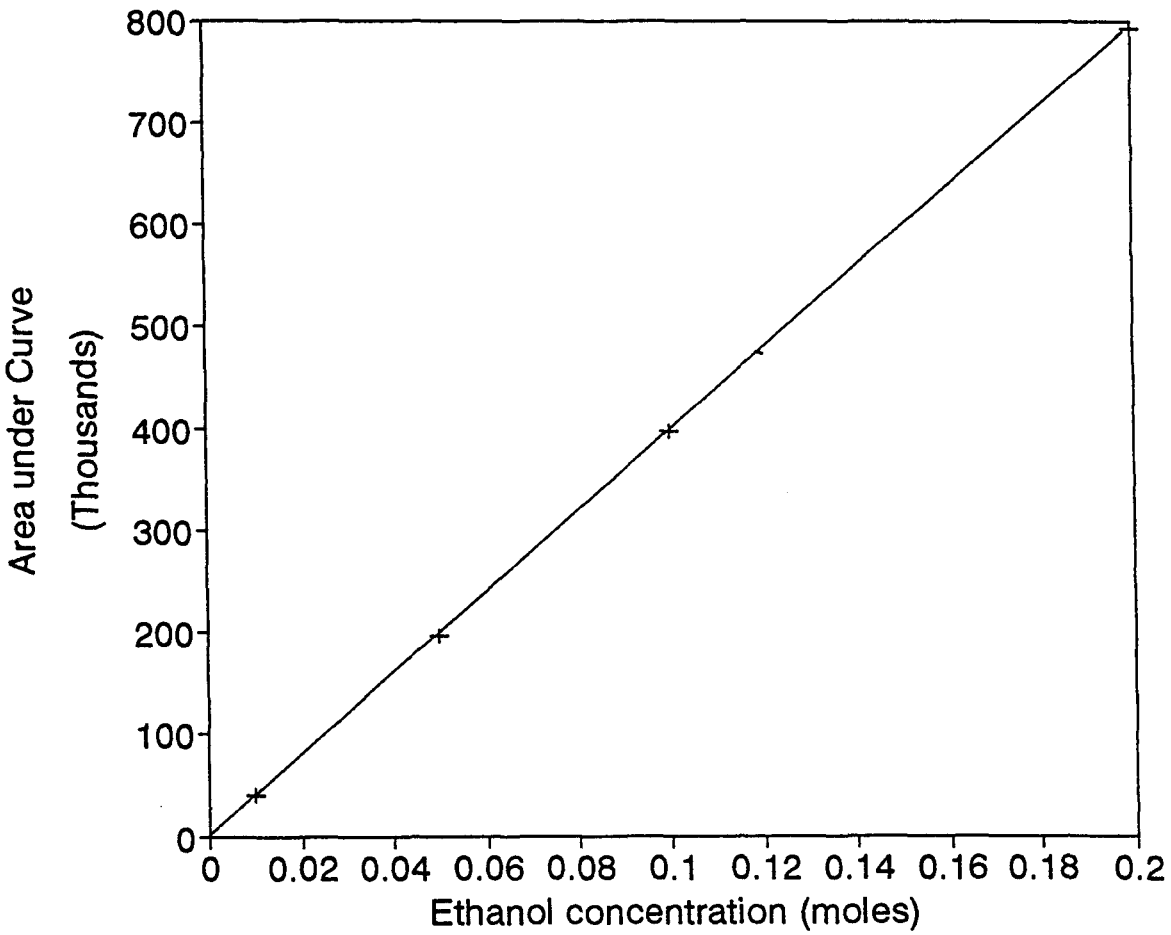
3.1 GLUCOSE

Slope = 4.25×10^7



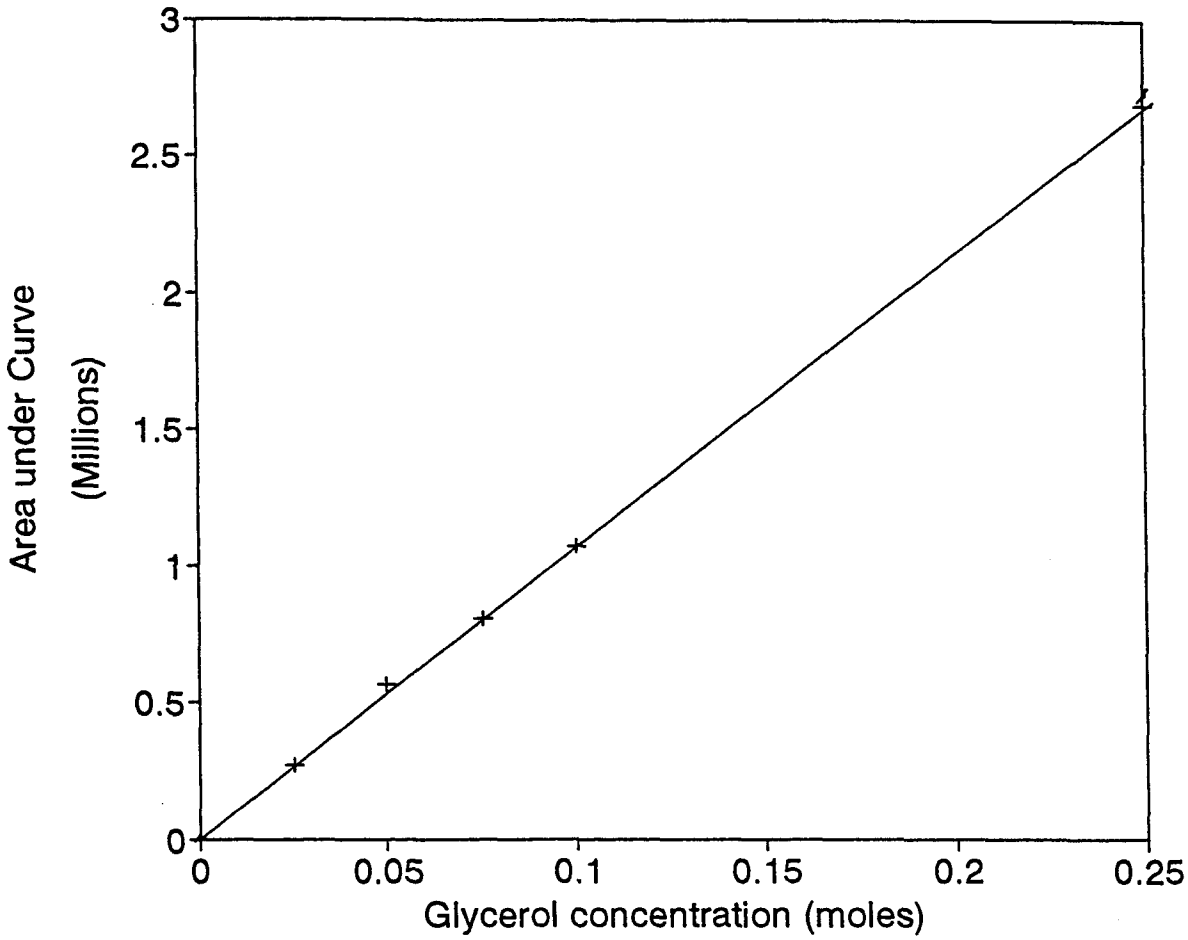
3.2 ETHANOL

Slope = 3.96×10^6



3.3 GLYCEROL

Slope = 1.52×10^7



APPENDIX 4

COLORIMETRIC DETERMINATION OF PROTEIN

Biuret Method:

Reagent A	$CuSO_4.5H_2O$	1.25 g
	dH_2O	245 ml
Reagent B	10 M NaOH	230 ml
	NH_4OH	25 ml

Reagents A and B were mixed and the reagent was stored in a glass bottle.

Procedure:
To a tube containing 1.0 ml of protein solution 4.0 ml of biuret reagent was added. Colour was allowed to develop at room temperature for 30 minutes and was read at 330 nm against a reagent-water blank in a Perkin-Elmer 552 spectrophotometer. A standard curve was prepared with crystalline BSA in the range of 0-1.0 mg /ml.

Lowry Method:

Reagent A	Na_2CO_3	20 mg
	NaOH	4 mg
	dH_2O	100 ml
Reagent B	$CuSO_4.5H_2O$	0.5 mg
	Na_3citrate	1.0 g
	dH_2O	100 ml
Reagent C	Reagent A	50.0 ml
	Reagent B	1.0 ml
Reagent D	Folin-Ciocateau reagent	2.0 ml
	dH_2O	3.0 ml

Procedure:
To 0.5 ml of enzyme solution containing up to 0.5 mg/ml protein, 2.5 ml reagent C was added, mixed and allowed to stand for 10 min. To this solution, 0.25 ml reagent D was added, mixed and allowed to stand for 20-30 minutes at room temperature. The colour was read at 750 nm wavelength against a reagent-water blank in a Varian DMS 100S UV/VIS spectrophotometer. A standard curve was prepared with crystalline BSA in the range of 0-50 μg/ml.

APPENDIX 5

DISC GEL POLYACRYLAMIDE ELECTROPHORESIS

A. Separating gel buffer

1 M HCl	48.0 ml
Tris-HCl	36.3 g
TEMED	0.23 ml
dH_2O to make up to	100.0 ml

B. Separating gel monomers

Acrylamide	28.0 g
BIS	0.735 g
dH_2O to make up to	100.0 ml

C. Separating gel catalyst

Ammonium persulphate	0.14 g
dH_2O to make up to	100.0 ml

Separating gel

Mix A, B, C, and H_2O in the ratio 1:2:4:1 by volume respectively.

D. Sample and stacking gel buffer

1 M HCl	48.0 ml
Tris-HCl	5.98 g
TEMED	0.46 ml
dH_2O to make up to	100.0 ml

E. Stacking gel monomers

Acrylamide	100.0 g
BIS	2.5 g
dH_2O to make up to	100.0 ml

Stacking gel

Mix D, E, F and H_2O in the ratio 1:2:1:4 by volume respectively.

F. Sample and stacking gel catalyst

Riboflavin	4 mg
dH_2O to make up to	100.0 ml

G. Tray buffer

Tris-HCl	3.0 g
Glycine	14.4 g
dH_2O to make up to	1000.g

Tracking dye

Bromophenol blue	0.25%
Sucrose	60%

APPENDIX 6

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

1. Preparation of separating gel: 10% Acrylamide

Acrylamide (30% w/w) + BIS (0.8% w/w)	10.0 ml
1 M Tris-HCl pH 8.8	11.25 ml
10% SDS	0.3 ml
TEMED	0.015 ml
dH_2O	8.35 ml
10% Ammonium persulphate	0.15 ml

2. Preparation of stacking gel: 3% Acrylamide

Acrylamide (30% w/w) + BIS (0.8% w/w)	1.0 ml
0.5 M Tris-HCl pH 6.8	2.5 ml
10% SDS	0.1 ml
TEMED	0.005 ml
dH_2O	6.37 ml
10% Ammonium persulphate	0.025 ml

3. Tray buffer (pH 8.3)

Tris-HCl	6.07 g
Glycine	28.27 g
SDS	2.0 g
dH_2O to make up to	2.0 l

APPENDIX 7

IDENTIFICATION OF *SCHIZOSACCHAROMYCES POMBE*

Test No. in Program	Result	Test
1	+	D-glucose fermentation
2	-	D-galactose fermentation
3	+	maltose fermentation
4	-	α-methyl-D-glucoside fermentation
5	+	sucrose fermentation
6	-	α,α-trehalose fermentation
7	-	melibiose fermentation
8	-	lactose fermentation
9	-	cellobiose fermentation
10	-	melezitose fermentation
11	+	raffinose fermentation
12	+	inulin fermentation
13	-	starch fermentation
14	-	D-galactose growth
15	-	L-sorbose growth
16	-	D-glucosamine growth
22	+	sucrose growth
23	+	maltose growth
24	-	α,α-trehalose growth
25	-	α-methyl-D-glucoside growth
26	-	cellobiose growth
29	-	melibiose growth
30	-	lactose growth
31	+	raffinose growth
32	-	melezitose growth
33	-	inulin growth
34	-	starch growth

35	+	glycerol growth
53	-	ethanol growth
61	-	growth without vitamins
71	+	growth at 25°C
72	+	growth at 30°C
73	+	growth at 35°C
74	+	growth at 37°C
75	-	growth at 42°C
78	+	50% D-glucose growth
79	+	60% D-glucose growth
80	-	starch formation
84	-	pink colonies
85	-	budding cells
86	-	polar budding
87	+	splitting cells
88	-	filamentous
89	-	pseudohyphae
90	-	septate hyphae
91	-	arthroconidia
92	-	ballistoconidia
93	-	symmetric ballistoconidia
94	+	ascospores
95	+	ascospores round, oval, conical, reniform
96	-	ascospores cap, hat, Saturn, walnut-shaped
97	-	ascospores needle-shaped or whip-like

Identification:

0 species can give the results specified

1 species differ by 1 result:

Schizosaccharomyces pombe discrepant test(s) 35*

APPENDIX 8

IDENTIFICATION OF *SACCHAROMYCES CEREVISIAE*

Test No. in Program	Result	Test
1	+	D-glucose fermentation
2	+	D-galactose fermentation
3	+	maltose fermentation
4	+	α-methyl-D-glucoside fermentation
5	+	sucrose fermentation
6	+	α,α-trehalose fermentation
7	+	melibiose fermentation
8	-	lactose fermentation
9	-	cellobiose fermentation
10	+	melezitose fermentation
11	+	raffinose fermentation
12	-	inulin fermentation
13	+	starch fermentation
14	+	D-galactose growth
15	-	L-sorbose growth
16	-	D-glucosamine growth
17	-	D-ribose growth
18	-	xylose growth
22	+	sucrose growth
23	+	maltose growth
24	+	α,α-trehalose growth
26	-	cellobiose growth
29	+	melibiose growth
30	-	lactose growth
31	+	raffinose growth
32	+	melezitose growth
33	-	inulin growth

34	+	starch growth
35	+	glycerol growth
53	+	ethanol growth
54	-	nitrate growth
71	+	growth at 25°C
72	+	growth at 30°C
73	+	growth at 35°C
74	+	growth at 37°C
75	-	growth at 42°C
78	+	50% D-glucose growth
79	-	60% D-glucose growth
80	-	starch formation
81	-	acetic acid production
84	-	pink colonies
85	+	budding cells
86	-	polar budding
87	-	splitting cells
88	-	filamentous
89	-	pseudohyphae
90	-	septate hyphae
91	-	arthroconidia
92	-	ballistoconidia
93	-	symmetric ballistoconidia
94	+	ascospores
95	+	ascospores round, oval, conical, reniform
96	-	ascospores cap, hat, Saturn, walnut-shaped
97	-	ascospores needle-shaped or whip-like

Identification:

1 species can give the results specified

Saccharomyces cerevisiae

APPENDIX 9

MATERIALS WHICH WERE SCREENED IN ZIMBABWE FOR XEROTOLERANT FISSION YEASTS

Material	Locality of origin
Commercial beer	Harare
'Malted' sorghum	Chikore
	Gutu
	Masvingo
	Mutare
'Malted' millet	Chipinge
'Tea fungus'	Bulawayo
Sweet sorghum	Masvingo
Molasses	Triangle
Diluted molasses	Triangle
Juice of fruit	Masvingo

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