# MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF ETHANOLIC D-XYLOSE FERMENTING *Pichia stipitis*, *Candida shehatae* AND THEIR FUSANTS

by

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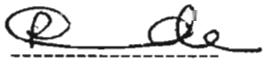
**Declaration**

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science to the University of Durban-Westville, Durban. It has not been submitted before for any degree or examination to any other University.



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# CHAPTER ONE

## INTRODUCTION AND LITERATURE REVIEW

D-xylose, the major constituent of the hemicellulose xylan, is the second most abundant renewable sugar after glucose (Jeffries, 1990). It can be fermented to ethanol by several yeasts (Jeffries, 1981a; Maleszka *et al.*, 1982a; Schneider *et al.*, 1981), but the ethanol yield is much lower than that of *Saccharomyces cerevisiae* using glucose. Most, if not all, *S. cerevisiae* strains used in commercial alcohol production are polyploid or aneuploid, suggesting that an increase in chromosome number may, in some way, be advantageous.

Intergeneric hybrids between *Pichia stipitis* and *Candida shehatae*, the two most efficient D-xylose fermenters, were constructed using the technique of protoplast fusion in an attempt to obtain more efficient D-xylose fermenting strains (Gupthar and Garnett, 1987). Although *P. stipitis*-resembling fusants were isolated, their hybrid nature was confirmed by cell volume estimation, cell DNA content, carbon utilisation and fermentation tests, nuclear DNA-DNA hybridisations as well as the isolation of recombinant phenotypes from spontaneous and induced mitotic and meiotic segregations. Preliminary tests indicated that some of the fusants fermented xylose more efficiently than their parental strains.

Further characterisation of these fusants at the molecular and biochemical levels is required in order to ascertain their genetic constitution and perhaps relate this to their

fermentation abilities.

## 1.1 D-XYLOSE

Agricultural biomass is composed of three fractions: lignin, cellulose and hemicellulose and represents one of the most abundant renewable energy sources. It could potentially be fermented to ethanol for use as a fuel extender and octane enhancer in petrol (Skoog and Hahn-Hagerdahl, 1990).

The hemicellulose component of some lignocellulosic materials can represent up to 35% of the dry biomass. The major constituent of the hemicellulose xylan is D-xylose which in some instances can constitute up to 25% of the dry biomass (Jeffries, 1990). This makes D-xylose the most abundant renewable sugar second to glucose. Hemicelluloses such as xylans have more branched, less crystalline structures than cellulose and the glycosidic linkages between anhydro-D-xylose residues in xylans are less stable and more readily hydrolysed by acid than the linkages between anhydro-D-glucose residues in cellulose (Harris, 1975 - cited by Jeffries, 1990). Xylose can therefore be recovered by dilute acid hydrolysis in yields exceeding 80 - 85%, whereas the yield of glucose from cellulose probably would not exceed 55 - 60%.

In South Africa six million tons of D-xylose could be recovered from plant refuse like sugarcane bagasse and maize residues (Dekker and Lindnar, 1979 - cited by Prior *et al.*, 1989). It could also be recovered from industrial wastes, e.g., spent sulphite liquors from pulp mills using hardwood feedstock, plants producing dissolving pulps, by autolysis

following steam treatment in the production of hardboard and fibreboard and other explosive decompression processes (Bungay *et al.*, 1983 - cited by Jeffries *et al.*, 1985). Therefore, its exploitation for ethanol production has great economic potential.

Traditionally, glucose-based substrates such as maize and molasses are fermented commercially to ethanol by *S. cerevisiae*. Although this yeast ferments glucose, it lacks the ability to ferment xylose (Batt *et al.*, 1986).

## 1.2 D-XYLOSE FERMENTING YEASTS

Originally it was thought that yeasts were incapable of fermenting xylose (Barnett, 1976). In 1959, Karczewska was the first to report the conversion of xylose by strains of *Candida tropicalis* obtained from a sulphite liquor fermentation plant (Jeffries, 1990). Several yeasts have been reported to ferment xylose (du Preez and Prior, 1985; Jeffries, 1981a; Maleszka *et al*, 1982a; and Schneider *et al.*, 1981; Toivola *et al.*, 1984) and to date, at least eight species of yeasts are known to produce significant amounts of ethanol from xylose.

Of these, three have been studied extensively: *Pachysolen tannophilus*, *C. shehatae* and *P. stipitis*. *P. tannophilus* is the best studied of all D-xylose fermenting yeasts (Dekker, 1982; James and Zahab, 1982, 1983; Jeffries, 1982; Jeffries *et al.*, 1985; Maleszka and Schneider, 1982; Schneider *et al.*, 1981; Slininger *et al.*, 1982). Its commercial exploitation, however, is constrained by its slow rate of fermentation, poor

ethanol yield and the production of significant amounts of xylitol. The next most studied yeast is *C. shehatae* (Alexander *et al.*, 1987; du Preez and van der Walt, 1983; du Preez *et al.*, 1984; Jeffries, 1985a; Sreenath *et al.*, 1986; Wayman and Parekh, 1985). *P. stipitis* CSIR-633 (=CBS 7126) has thus far been identified as the best xylose fermenter (du Preez and Prior, 1985; du Preez *et al.*, 1989).

## 1.3 D-XYLOSE METABOLISM

### 1.3.1 BIOCHEMICAL PATHWAY

In yeasts the fermentation of D-xylose proceeds by the pentose phosphate pathway (Fig. 1.1) (Jeffries, 1990). D-xylose is first reduced to xylitol by D-xylose reductase which is subsequently oxidized to D-xylulose by xylitol dehydrogenase. D-xylulose is then phosphorylated by D-xylulokinase to form D-xylulose-5-phosphate. Non-oxidative rearrangements of D-xylulose-5-phosphate by ribulose phosphate-3 epimerase, ribose phosphate isomerase, transaldolase and transketolase result in the formation of glyceraldehyde-3-phosphate and fructose-3-phosphate, which can then be converted to ethanol by the fermentative reactions of the Embden-Meyerhoff pathway. However, there is no direct evidence that this pathway functions in any D-xylose-fermenting yeast.

Alternatively, D-xylulose-5-phosphate can be split into glyceraldehyde-3-phosphate and acetyl-phosphate by phosphoketolase. This enzyme is present in *P. tannophilus* where it may be involved in acetic acid formation under anoxic conditions (Lachke and Jeffries,

1986) or an oxidative pentose bypass may be present (Bruinenberg *et al.*, 1986). A portion of the fructose-6-phosphate is oxidized to ribulose-5-phosphate via 6-phosphogluconate releasing $CO_2$ and generating 2 molecules of NADPH for each molecule of $CO_2$ released.

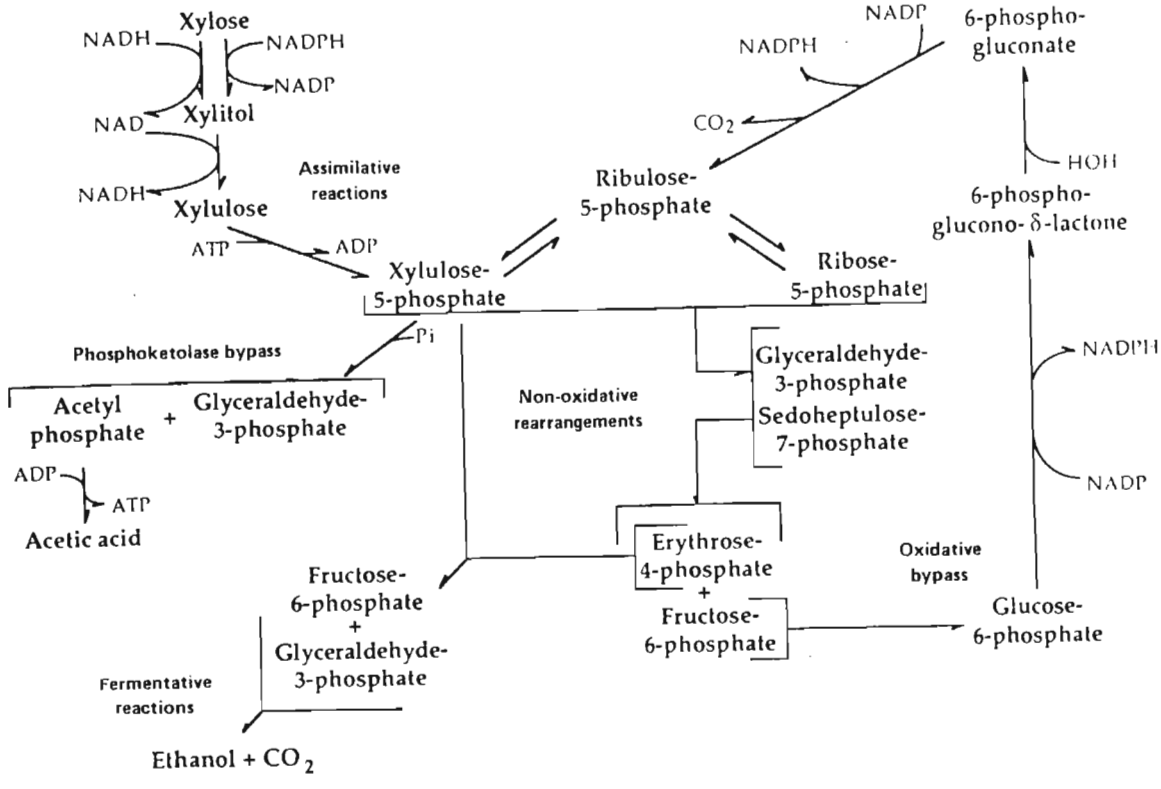



**Fig. 1.1.** Biochemical pathway of D-xylose metabolism by the xylose-fermenting yeasts (Jeffries, 1990).

The pathway or combination of pathways employed depends on the metabolic capacities of a particular yeast and the conditions under which it is grown. Fermentative yeasts generally possess both aerobic and anaerobic pathways along with adaptive regulatory mechanisms. Regardless of the carbon source employed, D-xylose fermenting

yeasts require normally functioning mitochondria and $O_2$ for growth, even if they can metabolize D-xylose anoxically (Malezska *et al.*, 1982a). This need for $O_2$ could indicate the use of a fermentation pathway that does not give rise to sufficient ATP by substrate-level phosphorylation (Evans and Ratledge, 1984).

### 1.3.2 THERMODYNAMIC CONSIDERATIONS

The conversion of D-xylose to D-xylulose is accompanied by an overall net positive free energy change ( $G^\circ$ = +1.05 kcal/mol). Xylitol is a symmetrical molecule that does not exist in ring form, and therefore should have more rotational degrees of freedom and a lower energy state. Hence, in a 2 step reduction-oxidation mechanism, the production of xylitol could be favoured.

The equilibria for D-xylose reductase and xylitol dehydrogenase from *P. tannophilus* have been studied at pH 7.0.

For D-xylose reductase, the equilibrium constant ($K_{eq}$) is as follows:

$$K_{eq} = \{[NADPH].[D\text{-}xylose].[H^+]\}/\{[NADP].[xylitol]\}$$

$$= 10^{-10}$$

(Ditzelmüller *et al.*, 1984a), and for xylitol dehydrogenase:

$$K_{eq} = \{[NADH].[D\text{-}xylulose].[H^+]\}/\{[NAD].[xylitol]\}$$

$$= 2.7 - 5.8 \times 10^{-8}$$

(Ditzelmüller *et al.*, 1984b)

These reactions favour the formation of xylitol. The actual concentration of D-xylose, D-xylulose and xylitol *in vivo* would depend on the intracellular NADPH/NADP ratio maintained by the oxidative phase of the pentose phosphate cycle and the NADH/NAD ratio obtained from respiration and/or fermentation. The NADH/NAD ratio will be lower during respiration than during fermentation.

### 1.3.3 TRANSPORT OF D-XYLOSE

For fermentation to occur, it is necessary to maintain a rate of sugar uptake in excess of that needed for growth. Also, as fermentation yields little energy, it is necessary to employ a transport system that uses very little energy.

*P. stipitis* and *C. shehatae* both possess two transport systems: a high affinity (facilitated diffusion) and a low affinity (proton symport) system, the $K_s$ for the former being ten times bigger than that of the latter (Kilian and van Uden, 1988; Lucas and van Uden, 1986). D-xylose uptake is completely inhibited at glucose concentrations of $>2$ g/l.

### 1.3.4 ASSIMILATIVE STEPS

The best studied reaction in the D-xylose metabolic pathway is the conversion of D-xylose to xylulose. In bacteria this conversion is carried out by the enzyme D-xylose

isomerase, whereas in yeasts and fungi a sequential reduction and oxidation occurs. Most yeasts and filamentous fungi use NADPH for the reduction of xylose to xylitol and NADH for the oxidation of xylitol to xylulose. In *Candida utilis* it has been pointed out by Bruinenberg *et al.* (1983) that this mode of xylose metabolism is incompatible with anaerobic utilisation of this sugar, since it leads to a net production of NADH in the overall conversion of xylose to ethanol. Bruinenberg *et al.* (1984) have suggested that anaerobic fermentation in many yeasts is blocked by a buildup of NADH. Yeasts capable of anaerobic fermentation of xylose to ethanol also possess a unique NADH-linked xylose reductase activity. This activity apparently prevents an accumulation of extra-mitochondrial NADH.

*P. stipitis* and other yeasts (e.g. *P. tannophilus*) capable of anaerobic xylose fermentation were shown to possess a xylose reductase that uses both NADPH and NADH. NADPH for the reduction of D-xylose to xylitol is provided by the oxidative portion of the pentose phosphate pathway. NAD is required for the oxidation of xylitol to xylulose. In the Embden-Meyerhoff pathway, net NAD production occurs only as a result of respiration. In the absence of respiration, xylitol cannot be oxidized and metabolism stops. This requirement was first shown for *Candida tropicalis* (Jeffries, 1981a) and later for *C. utilis* (Bruinenberg *et al.*, 1983).

Respiration is not essential for D-xylose metabolism in all yeasts. *P. tannophilus* (Slininger *et al.*, 1982), *C. shehatae* (du Preez and van der Walt, 1983) and *P. stipitis* (Toivola *et al.*, 1984) are capable of fermenting D-xylose to ethanol under anaerobic conditions.

### 1.3.4.1 D-Xylose Reductase

D-xylose reductase is the first enzyme in the metabolic pathway of D-xylose in yeasts. Its activity and regulation are therefore critical for the fermentation of xylose. Smiley and Bolen (1982) have shown that under aerobic conditions, the conversion of D-xylose to xylitol by *P. tannophilus* is accomplished primarily by an NADPH-dependant xylose reductase. Bolen *et al.* (1986 - cited by Bolen *et al.*, 1986) later reported that purified xylose reductase from *P. tannophilus* could also use NADH as a cofactor. According to Ditzelmüller *et al.* (1984a), the purified enzyme has also been shown to utilize other carbon compounds (e.g., galactose and arabinose) as substrates and has thus been characterized as an aldose reductase (EC 1.1.1.21) . The enzyme is also considered inducible since no significant aldose reductase activity is detected in cell free extracts of *P. tannophilus* prepared from cells grown on D-xylose (Smiley and Bolen, 1982).

Ditzelmüller *et al.* (1984a) reported their preparation of two *P. tannophilus* xylose reductases: one that possesses minimal NADH-linked activity ($< 0.5\%$ of the NADPH-linked activity), the other with little or no NADPH-linked activity (Ditzelmüller *et al.*, 1985). Verduyn *et al.* (1985a) have also reported the isolation of two separate xylose reductases from *P. tannophilus*: one from cells grown under aerobic conditions found to be NADPH specific (enzyme B) and the other from cells grown under anaerobic conditions found to have both NADPH and NADH linked activities (enzyme A).

The presence of an NADH-specific D-xylose reductase and its importance in the anoxic fermentation of D-xylose was first shown by Bruinenberg and co-workers (1984)

working with *P. stipitis*. *P. stipitis*, however, possesses one aldose reductase with a dual coenzyme specificity (Verduyn *et al.*, 1985b).

Enzyme A of *P. tannophilus* resembles the aldose reductase of *P. stipitis* in its use of either NADPH/NADH. Their molecular weights are 41 000 and 65 000 Da, respectively, and exhibit up to 70% of their activity with NADPH as a cofactor as with NADH. The *P. stipitis* enzyme has a lower affinity for D-xylose than the *P. tannophilus* enzyme A. (Verduyn *et al.*, 1985b).

The ratio of NADH- to NADPH- linked activity shifts under the cultivation conditions employed. Under anaerobic conditions, xylose fermentation *in vivo* by *P. stipitis* must proceed via NADH-linked xylose reduction, although kinetic studies indicate that the *in vitro* NADPH is the preferred coenzyme. Not only the relative concentrations of NADPH and NADH, but also those of $NADP^+$ and $NAD^+$ are decisive for the choice between NADPH- or NADH- linked xylose reduction *in vivo*.

#### 1.3.4.2 **Xylitol Dehydrogenase**

The conversion of xylitol to xylulose is catalyzed by xylitol dehydrogenase, the second enzyme in the D-xylose metabolic pathway. Like xylose reductase, it is inducible by D-xylose, D-galactose and L-arabinose. This suggests that the mechanism leading to expression of these two enzymes, either by new enzyme synthesis or activation of existing enzymes, is a common one for both enzymes.

Whereas xylose reductase catabolizes L-arabinose and D-galactose to L-arabitol and galactitol respectively, these products are not oxidized substrates for xylitol dehydrogenase which has an NADH-linked activity. Although it is present in L-arabinose and D-galactose-grown cells, it may serve no metabolic function but occurs as a consequence of simultaneous induction with xylose reductase (Bolen and Detroy, 1985).

Xylitol dehydrogenase from *P. tannophilus* is reported to have a molecular weight of 172 000 Da (Bolen *et al.*, 1986) and consists of four subunits. In *C. shehatae* it has a molecular weight of 82 000 Da and comprises two subunits (Yang and Jeffries, 1990) and that of *P. stipitis* 63 000 Da, with 2 subunits (Rizzi *et al.*, 1989 - cited by Yang and Jeffries, 1990).

*P. tannophilus* accumulates more xylitol than *P. stipitis* or *C. shehatae* (du Preez and van der Walt, 1983; and Slininger *et al.*, 1985). The $K_m$ for xylitol in *C. shehatae* is a quarter of that observed with the *P. tannophilus* enzyme, while the $K_m$ for xylulose is 1.7 times higher (Ditzelmüller *et al.*, 1984b). This would favour the forward reaction. A lower $K_m$ of xylitol dehydrogenase for xylitol may explain the higher ethanol yield for *P. stipitis* and *C. shehatae* (du Preez and van der Walt, 1983).

## 1.4 FACTORS AFFECTING D-XYLOSE FERMENTATION

Many different culture parameters have been examined in an attempt to improve D-xylose fermentation. The nitrogen source and the aeration rate employed are the most

important factors, but sugar and ethanol concentrations can also be significant, especially at higher temperatures. The pH optimum is broad.

### 1.4.1 NUTRITIONAL FACTORS

Jeffries (1983) has reported that *P. tannophilus* can grow well on either ammonium acetate or urea, but that a rich supplement such as 1% yeast extract facilitates anaerobic fermentation. Cells grown on nitrate show enhanced ethanol production under aerobic conditions. However, ethanol production is inhibited under anoxic conditions.

In the case of *C. shehatae*, Palnitkar and Lachke (1992) have reported that when organic nitrogen sources are used, there is a rapid D-xylose metabolism as well as a higher ethanol yield as compared to inorganic nitrogen sources. They also found the ratio of NADH-/NADPH-linked xylose reductase activity to be higher in cells grown in media containing organic nitrogen sources. Organic nitrogen sources also increase the levels of xylitol dehydrogenase in contrast to inorganic nitrogen sources. *C. shehatae* does not use nitrate as a nitrogen source but can produce ethanol using inorganic nitrogen sources, although fermentation is stimulated by the addition of casamino acids (Jeffries, 1985b). *P. stipitis* is also incapable of utilizing nitrate as a nitrogen source and, like *C. shehatae*, ethanol production is stimulated by yeast extract, peptone and casamino acid (Tran and Chambers, 1986 - cited by Prior *et al.*, 1989). *P. stipitis* has a lesser vitamin requirement than either *C. shehatae* or *P. tannophilus*. *P. stipitis* CBS 7126 can ferment D-xylose in the absence of any vitamins. *C. shehatae* CBS 2779 has a negligible fermentation in the

absence of biotin, thiamine or pyridoxine. Both ethanol productivity and ethanol yield of xylose fermenting yeasts are dramatically improved by the addition of biotin and thiamine (Prior *et al.*, 1989). Other vitamins, e.g., pyridoxine, myo-inositol, nicotinic acid, panthotenic acid and *p*-aminobenzoic acid have a minor influence on the D-xylose fermentation of *C. shehatae*. In the absence of thiamine and biotin, pyridoxine is essential for the complete utilization of D-xylose.

### 1.4.2 AERATION

The effect of oxygen on the fermentation of D-xylose has been examined more extensively than any other culture variable. *P. tannophilus* and *P. stipitis* show no growth under anoxic conditions (du Preez *et al.*, 1984; Skoog and Hahn-Hägerdal, 1990). In general, aeration enhances growth and ethanol productivity. *C. shehatae* maintains a higher ethanol production rate and yield over a wider range of aeration levels than other yeasts. No ethanol is produced under vigorous aeration by *P. stipitis* CBS 5773 and CBS 7126 (Bruinenberg *et al.*, 1984; du Preez *et al.*, 1989) and *C. shehatae* CBS 2779 (du Preez *et al.*, 1989). However, a finite (limited) $O_2$ supply stimulates growth as well as ethanol production by *P. stipitis* and *C. shehatae* (du Preez and van der Walt, 1983; du Preez *et al.*, 1984).

In *P. tannophilus*, ethanol accumulation appears to be associated with a transition from respiration and growth to a state of low $O_2$ consumption and fermentation (Schvester *et al.*, 1983). The dissolved $O_2$ concentration of a batch culture decreases as cell density

increases until fermentation is initiated.

The enhancement of xylose fermentation by limited aeration is believed to be due to the role of $O_2$ as terminal electron acceptor, thus relieving the partial redox imbalance in the initial two steps of D-xylose metabolism. Aeration thus has a significant effect on the xylitol and ethanol yields. The ethanol yield decreases with increasing aeration as a result of concurrent ethanol consumption (Maleszka and Schneider, 1982). The xylitol yield decreases as the specific $O_2$ uptake increases. This inverse relationship between the degree of aeration and xylitol production is also observed with *C. shehatae* and *P. stipitis* (du Preez *et al.*, 1989), and is in agreement with the hypothesis that $O_2$ relieves the partial redox imbalance in the initial two steps of D-xylose metabolism (Laplace *et al.*, 1991).

Laplace *et al.* (1991) studied the effect of $O_2$ transfer rate (OTR) on the fermentation of *P. stipitis*, *C. shehatae*, *S. cerevisiae* and *Zymomonas mobilis* and discussed the fermentative behaviour of *P. stipitis* and *C. shehatae* as a function of OTR in three steps, viz.,

a) Under anaerobic conditions (very low OTR), the electron transfer system is unable to oxidize NADH completely. Consequently, intracellular NADH increases, and this imbalance between NADH and $NAD^+$ concentration leads to xylitol excretion.

b) Xylose fermentation is enhanced by increasing OTR. It is possible to determine an OTR for which xylitol yields are the lowest and ethanol yields the highest. It is reported that *P. stipitis* requires a

lower OTR than *C. shehatae* to relieve the $NADH/NAD^+$ imbalance.

c) When $O_2$ is transferred in excess, a deviation in the pyruvate flow from the fermentative pathway to the TCA cycle is observed. This involves the production of cells at the expense of ethanol.

Skoog and Hahn-Hägerdal (1990) reported that the $O_2$ levels for maximum ethanol production are close to the detection limit (a dissolved $O_2$ of $\approx 0\%$) which corresponds to an OTR of 2 mmol/l/h. They also reported that xylose transport activity is dependant on the level of oxygenation during xylose assimilation and suggest that $O_2$ induces/activates a transport system.

### 1.4.3 pH

The growth of *P. tannophilus* is obviously favoured by a low pH environment (2.5-4.5). During fermentation maximum specific alcohol production and xylose consumption rates are attained at pH 2.5 (Slininger *et al.*, 1982). The rate of fermentation is optimal between pH 4 and 5.5 for both *C. shehatae* CBS 2779 and *P. stipitis* CBS 7126 whereas the ethanol yield is hardly affected by pH values of 2.5 and 6.5 (du Preez *et al.*, 1986). Jeffries (1985b) reported a maximum volumetric rate of ethanol production at pH 3.2 to 3.4 for *C. shehatae* ATCC 22984. Slininger *et al.* (1990) reported a broad pH range (4 - 7) that allowed optimum growth rate, specific ethanol productivity and ethanol yields for *P. stipitis* NRRL Y124.

### 1.4.4 TEMPERATURE

Slininger *et al.* (1982) reported an optimal temperature for both growth and ethanol production of 32°C for *P. tannophilus*. No xylose consumption or ethanol occurs at 40°C. Alexander (1985) suggests that the temperature used for growth affects the induction of the D-xylose reductase in this yeast. At 37°C, the activity of this enzyme is greatly diminished from that found in cells grown at 30°C. For *C. shehatae* CBS 2779 and *P. stipitis* CBS 7126, du Preez *et al.* (1986) reported a maximum rate of D-xylose fermentation and growth at 30°C. Temperatures above 30°C cause a sharp drop in ethanol production and yields as well as an increase in xylitol production while temperatures below 30°C do not affect ethanol yields but decrease the rate of production of ethanol. They also reported that *C. shehatae* is more sensitive to temperature than *P. stipitis*. Slininger *et al.* (1990) found that *P. stipitis* CSIR 633 has a maximum ethanol yield at 25°C. This is explained by growth studies that suggest that ethanol tolerance decreases with increasing temperatures (du Preez *et al.*, 1987).

### 1.4.5 SUBSTRATE CONCENTRATION

Xylose concentrations of above 5% (w/v) inhibit growth of *P. stipitis* and increase its xylitol production (Slininger *et al.*, 1985). D-glucose is used in preference to D-xylose/ D-xylulose by essentially all D-xylose fermenting yeasts studied (Hsiao *et al.*, 1982), but in *P. tannophilus*, D-glucose can have a stimulatory effect on D-xylose fermentation (Jeffries *et al.*, 1985). When D-xylose is added in small amounts to an

active fermentation of D-xylose by *P. tannophilus*, the efficiency of D-xylose utilization increases. Intermittent addition of a cellulose hydrolysate to a hemicellulose hydrolysate likewise increases the yield of ethanol, indicating that this approach may be useful for industrial applications (Beck, 1986). D-glucose addition also enhances the rate of D-xylose utilization and the final ethanol concentration achieved with *C. shehatae* (Sreenath *et al.*, 1986). However, it has little effect on the apparent yield of ethanol from D-xylose by this organism.

The growth of *C. shehatae* and *P. stipitis* shows much less sensitivity to D-xylose concentration compared to *P. tannophilus*. *C. shehatae* ferments D-xylose faster but D-glucose slower than *P. stipitis*. This difference could result from different pathways or regulatory patterns used by these yeasts. D-xylose concentrations at which maximum ethanol concentration, rates of ethanol production and yields of ethanol and xylitol occur, are generally equivalent in *P. stipitis* and *C. shehatae* strains. The marked differences observed between strains of each species and in some instances, discrepancies in the kinetic data of the same strain, reported in independent studies, could be attributed to differences in growth conditions. Xylose concentrations of $\geq$ 100 g/l produced the highest ethanol concentration and volumetric rates of ethanol production. The highest specific rate of ethanol production occurs at D-xylose concentrations of 100 g/l and lower (Prior *et al.*, 1989).

Ethanol and xylitol yields are especially sensitive to substrate concentrations. In some studies the highest ethanol yields were recorded at D-xylose concentrations as low as 5 to 10 g/l (Dellweg *et al.*, 1984).

### 1.4.6 MIXED SUBSTRATES

The hydrolysis of lignocellulosic material yields mainly sugars and lignin. The sugar component is comprised of hexoses, predominantly glucose, and small amounts of galactose, mannose as well as pentoses, predominantly xylose and small quantities of arabinose. Glucose is two to four times more abundant than xylose, although this ratio can vary depending on the wood type. Thus, for efficient fermentation of a cellulose hydrolysate to ethanol, a suitable microorganism should be capable of fermenting both glucose and xylose; overcome glucose repression of xylose utilization and be resistant to products of cellulose hydrolysis such as acetic acid and furfural.

Under aerobic conditions *P. tannophilus* ferments xylose slower and with lower ethanol yields than it does glucose. Aerobically, the glucose fermentation rate is four times higher than xylose fermentation and anaerobically it is two times higher (Jeffries *et al.*, 1985). Periodic addition of glucose (very low concentration) to aerobic fermentations of xylose enhanced the ethanol yield. However, the addition of the equivalent amounts of glucose at the beginning of fermentation has no effect. Similar additions to anaerobic fermentation also have no effect.

Since *P. tannophilus* consumes xylose and ethanol simultaneously, it is thought that because of the addition of glucose, the respiration of ethanol decreases, thus improving the yield. The addition of higher concentrations of glucose represses xylose utilization.

Strains of *C. shehatae* and *P. stipitis* show varying degrees of glucose repression

of xylose uptake. The presence of ≥ 2% glucose caused repression of xylose utilization by *P. stipitis* (2% glucose causes 20% repression). 2% glucose has a negligible effect on xylose utilization by *C. shehatae* (3% glucose has only a 10% repression). Unlike *P. tannophilus*, addition of glucose causes xylose fermentation to stop immediately (Panchal *et al.*, 1988). Sreenath *et al.* (1986) report that *C. shehatae* ferments a mixture of xylose and glucose at a rate much higher than that for either sugar alone. Grootjen *et al.* (1991) report a complete inhibition of xylose utilisation at a much lower glucose concentration (2.3 g/l) for *P. stipitis*.

### 1.4.7 ETHANOL CONCENTRATION

Ethanol accumulation is toxic to the fermentation of D-xylose. The ethanol sensitivity of *P. tannophilus* varies with the carbon source/medium employed. Slininger *et al.* (1982) report a decrease in the rate of D-xylose uptake when ethanol concentrations exceed 20 to 25 g/l. Slininger and co-workers (1987) have applied Luong's model for inhibition of growth and ethanol production to data collected with *P. tannophilus* and concluded that growth of *P. tannophilus* is inhibited at 41 g/l ethanol, but ethanol production continues at concentrations as high as 80 to 100 g/l. Cell death is significant at the highest ethanol concentrations.

Two principal hypotheses for the mechanism of ethanol toxicity are damage to cell membrane and end product inhibition of glycolytic enzymes (Ingram, 1986 - cited by Jeffries, 1990). It is also possible that ethanol inhibition results from an accumulation of

intracellular NAD(P)H. Ethanol accumulation limits growth and promotes formation of polyols (xylitol, ribitol, arabitol) and acetic acid by *P. tannophilus* (Schneider *et al.*, 1985). These shifts are similar to the kinds of changes observed when *P. tannophilus* is grown under strictly anoxic conditions. They suggest that some kind of disruption of mitochondrial function might occur with increasing ethanol concentrations. Mitochondrial activity is known to be important in determining the ethanol tolerance of *S. cerevisiae* (Aguilera and Benitez, 1985).

Ethanol concentrations and temperature are interactive variables in determining ethanol growth inhibition in yeast. Lower temperatures lead to higher ethanol concentrations with D-xylose fermentation (Lucas and van Uden, 1985). *C. shehatae* has a maximum ethanol tolerance of 47 g/l at a temperature plateau of 10 to 17.5°C. Ethanol production by *P. stipitis* CBS T126 ceases at 47 g/l whereas its ethanol tolerance limit in terms of externally added ethanol is only 33 g/l. This indicates that the toxicity of added and autogenously produced ethanol to growth of *P. stipitis* (and *C. shehatae*) is in contrast to that of *S. cerevisiae* where autogenously produced ethanol is more toxic than added ethanol (Casey *et al.*, 1985 - cited by Prior *et al.*, 1989; Jones and Greenfield, 1985; Novak *et al.*, 1981).

## 1.5 TECHNOLOGY TO IMPROVE D-XYLOSE FERMENTATION

A D-xylose fermenting yeast would need to produce 50 - 60 g/l ethanol within 36 h with a yield of at least 0.4 g/g before commercial application could be considered

(Jeffries, 1985a). To date, *C. shehatae* and *P. stipitis* are superior to all other yeast species in terms of rate and yield of ethanol production from D-xylose. However, fermentation times of > 36 h are required to achieve the desired ethanol concentrations in hydrolysates (Yu *et al.*, 1987) and pure D-xylose media (Slininger *et al.*, 1985).

Several techniques have been employed in an effort to improve D-xylose fermentation. These include an improved fermentation technology, strain manipulation by molecular techniques and protoplast fusion to produce improved or new strains of *C. shehatae* and *P. stipitis* or even strains of *S. cerevisiae* capable of fermenting D-xylose.

### 1.5.1 FERMENTATION TECHNOLOGY

Fermentation techniques to improve D-xylose fermentation have employed cell immobilization/recycling to increase densities, continuous culture, ethanol removal and coculture. Each has been beneficial in some way, but none has provided a total solution to the problem.

The relatively low fermentation rate requires either the use of large reactor volume/high cell densities. *P. stipitis* immobilized in either agar beads or on a fine nylon mesh attain ethanol concentrations of up to 40 g/l in eight days from 100 g/l D-xylose (Linko *et al.*, 1986). Dynamic cell immobilization in which cells are continuously recycled by the use of a membrane module has also proved successful. With this technique, up to 70 g/l (dry weight) of cells - or about 10 to 20 times the cell density

without recycle - can be attained. The volumetric fermentation rates increase up to 4.4 g/l/h ethanol, but the specific rates drop dramatically (Sreenath and Jeffries, 1987). Continuous fermentation without recycle has not been particularly successful. The principal problem in using continuous culture is that the biomass increases with aeration whereas ethanol yield decreases. Also, the need to produce new biomass reduces the ethanol yield that can be attained. Although single-stage continuous culture is not particularly useful for D-xylose fermentation, a multiple-stage continuous fermentation can enable *C. shehatae* to achieve considerably higher ethanol concentrations. The first stage reactor is operated at an oxygen-limited mode that will produce fermentative cells. A low dilution rate must be used because of the $O_2$ limitation. In the second stage, the cells and fresh sugar are introduced. Minimal aeration is employed in this stage and ethanol concentration is appreciably higher, so growth stops but ethanol production continues vigorously. It is important to maintain an influx of viable cells (Alexander *et al.*, 1988).

In an attempt to achieve the combined fermentation of the two sugars from lignocellulosic materials by sequential/coculture processes, Laplace *et al.* (1992) have determined the compatibility and typing of associated strains. They report the occurrence of the killer phenomenon in six *Saccharomyces* species and eleven xylose fermenting yeasts which precludes their utilization in a coculture process. However, five strains of *C. shehatae* tested did not show any inhibition on the growth of *S. cerevisiae* and a coculture process using these strains could be developed.

## 1.5.2 MOLECULAR TECHNIQUES

Strain manipulation of *C. shehatae* and *P. stipitis* in order to improve fermentation efficiency has been hampered by a poor understanding of their biology. Some success has been achieved by selecting for mutants of *C. shehatae* ATCC 22984 on a medium containing L-xylose as a carbon source and $NH_4Cl$ as nitrogen source (Jeffries, 1987 - cited by Prior *et al.*, 1989). These mutants fermented D-xylose more rapidly, possibly due to derepressed levels of assimilative enzymes. An alternative would be to manipulate *S. cerevisiae* to ferment D-xylose.

*S. cerevisiae* is used in most industrial fermentations and ferments D-glucose at a specific rate at least four times that observed in *C. shehatae* and *P. stipitis* on D-xylose (Ligthelm *et al.*, 1988). In *E. coli* the interconversion of D-xylose to D-xylulose is catalyzed by the enzyme xylose isomerase. Attempts have been made to clone the *E. coli* isomerase gene into *S. cerevisiae* (Sarthy *et al.*, 1987) to evaluate direct D-xylose fermentation. The transformation of *S. cerevisiae* by a yeast expression plasmid bearing the *E. coli* isomerase gene leads to the production of the predicted amount of protein, but the enzyme is at least a thousand fold less active in *S. cerevisiae* than in *E. coli*. Improper folding of the protein was suggested as the main reason for the reduced activity.

Recently, Kötter *et al.* (1990) and Takuma *et al.* (1991) reported the isolation of the xylitol dehydrogenase gene (*XYL2*) and the xylose reductase gene (*XYL1*) from *P. stipitis*, respectively, as well as the construction of *S. cerevisiae* transformants. Both genes are actively expressed in *S. cerevisiae* transformants. Takuma *et al.* (1991) reported

a considerable amount of enzyme activity constitutively, whereas translation and transcription in *P. stipitis* were inducible. The *S. cerevisiae* transformant could not, however, grow on D-xylose medium and could not produce ethanol from xylose. Xylose uptake and accumulation of xylitol to ethanol appeared to be limited. Kötter *et al.* (1990) successfully transformed *S. cerevisiae* with a plasmid carrying both the *XYL*1 and *XYL*2 genes. These transformants were able to grow on xylose as a sole carbon source but their rate of xylose consumption was very slow. One reason for this could be a limiting capacity of the pentose phosphate pathway. A second reason could lie in the xylose uptake. In general, monosaccharide transport in *S. cerevisiae* takes place by facilitated diffusion, whereas in *P. stipitis* xylose uptake is carried out by two proton symport systems (Kilian and van Uden, 1988).

### 1.5.3 PROTOPLAST FUSION

Most, if not all, strains of *S. cerevisiae* used in commercial alcohol production are polyploid or aneuploid, suggesting that an increase in chromosome number may be advantageous. Scheda (1963) [cited by Gupthar, 1987] reported a systematic increase in the rate of ethanol production from D-glucose with increasing ploidy in *S. cerevisiae*. The technique of protoplast fusion was used to construct series of *P. tannophilus* (Maleszka *et al.*, 1983), *C. shehatae* (Johannsen *et al.*, 1985) and *P. stipitis* (Gupthar, 1987) with increased ploidy. Maleszka *et al.* (1983) reported systematic increases in rate and yields of ethanol production from D-xylose with increasing chromosome number in *P. tannophilus*. The isogenic fusant strains of *C. shehatae* and *P. stipitis* showed a very

slight increase in ethanol production and no relationship between ploidy and ethanol production. Since the *P. tannophilus* ploidy series was derived from hybridization between different strains (Maleszka *et al.*, 1983), it was thought that the ploidy series was due largely to heterosis.

During the protoplast fusion process, the cytoplasms of both cells come into contact with each other and there is mixing of the organelles of both the cytoplasms. If there is no nuclear interaction, before destabilisation of the heterokaryon, the resultant cells are known as cybrids (Fig. 1.2a). However, if nuclear interaction occurs, the resultant cells would be true hybrids with a combination of nuclear and cytoplasmic characters of both the cells (Fig. 1.2b). Another possibility that exists, is that a heterokaryon may form and partial gene exchange may occur before the heterokaryon destabilises into two partial hybrids that possess the genome of one cell with a few characters of the other.

Considering the presumed taxonomic relationship between *C. shehatae* and *P. stipitis* and their fermentative capacities, Gupthar and Garnett (1987) constructed intergeneric hybrids between these strains by protoplast fusion. *Pichia*-resembling fusants were isolated, and their hybrid nature confirmed by cell volume estimation, analysis of nuclear condition and the isolation of a variety of mutant recombinant phenotypic segregants by meiotic as well as induced and spontaneous mitotic segregation. Preliminary studies have indicated that several of the fusants ferment xylose rapidly as compared to the parental strains (Gupthar and Garnett, 1987).

Previously, classical genetic and biochemical techniques were used to differentiate

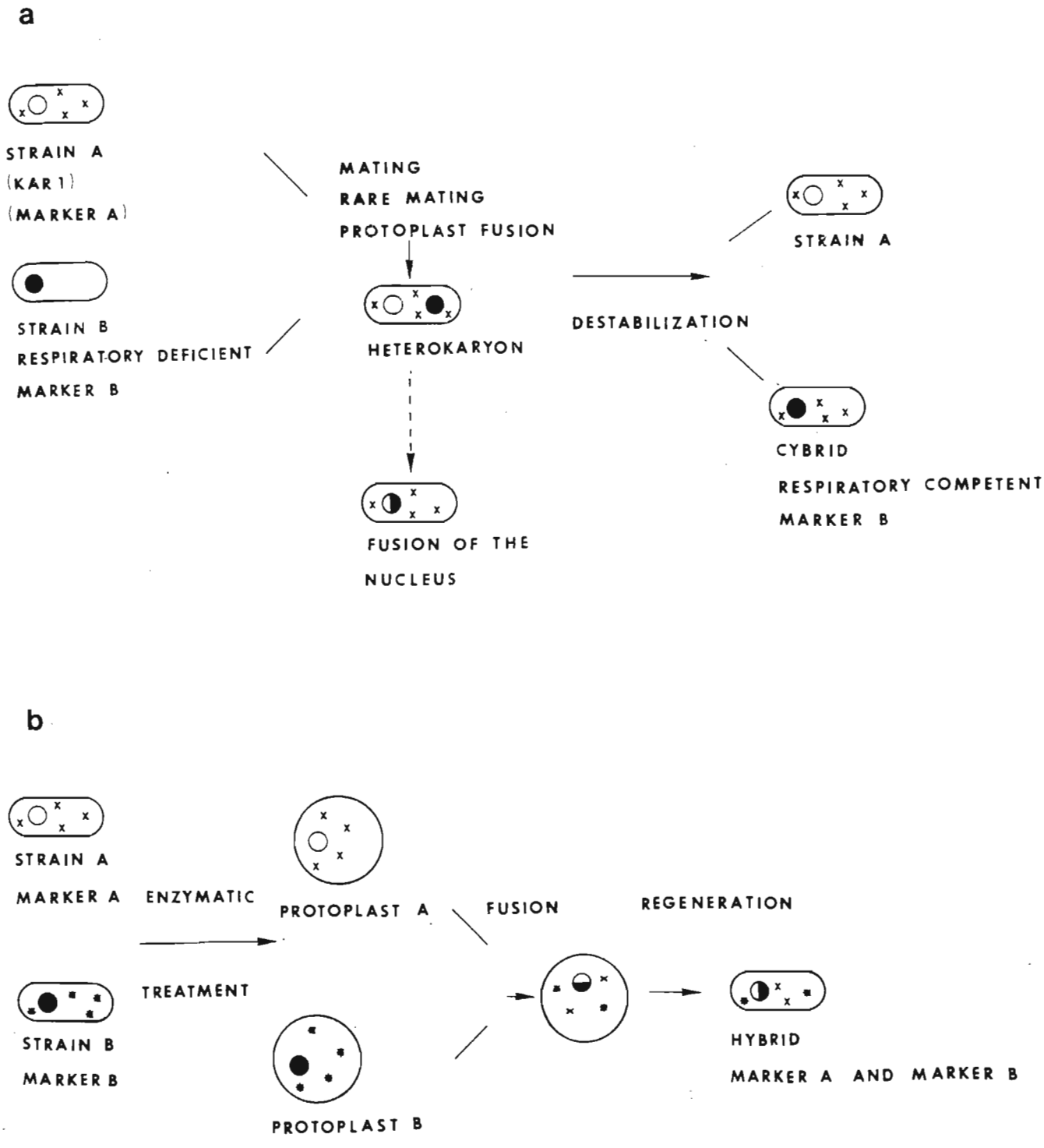



Fig. 1.2. Schematic representations of the nuclear and cytoplasmic interactions that may occur during mating or protoplast fusion to form (a) cybrids or (b) hybrids. Cytoplasmic characteristics (x and *), nuclei and their chromosomal characteristics (○ and ●) [Iserentant, 1990].

between parental strains and their hybrids or progeny, especially in the baking, brewing and wine industries. Most of the strains in the wine industry belong to the same species, viz., *S. cerevisiae*. Their identification cannot be carried out by conventional methods, and several techniques, based on molecular polymorphisms have been recently used for strain characterization. These techniques include electrophoretic fingerprinting of proteins and chromosomes, as well as restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA.

### 1.5.3.1 Protein Electrophoresis

Owing to its very high resolution, the separation of proteins on polyacrylamide gels has over the last two decades become the most commonly used technique for their characterization and analysis. The most widely employed variant of this is sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins are exposed to the anionic detergent SDS before electrophoresis on a slab gel. The extensive binding which results swamps out the intrinsic charge of most proteins and the negative charge per unit mass is approximately constant for all protein-SDS complexes. These complexes can be sieved on the gel matrix according to molecular size. The utility of standard PAGE has been greatly enhanced by the development over the last decade of two-dimensional techniques (O'Farrel *et al.*, 1977) and Western blotting (Burnette, 1981).

The first electrophoretic analyses were carried out on specific yeast enzymes in the early 1980's (Yamazaki and Komagata, 1981; Yamazaki *et al.*, 1983). van Vuuren and

van der Meer (1987) subjected the total soluble cell proteins of several wine and beer yeasts to PAGE found the fingerprints generated to be a reliable and useful tool in the identification of closely related yeast strains. The banding pattern of total soluble cell proteins of a micro-organism is a fingerprint of a large part of its genome.

### 1.5.3.2 Electrophoretic Karyotyping

The development of pulsed field gel electrophoresis (PFGE) technology for the separation of intact chromosomal DNA molecules of lower eukaryotes, has provided a novel means of characterising the chromosome sets of these organisms. (Carle and Olson, 1985; Schwartz and Cantor, 1984). Since its advent, this technique has been widely used to characterise fungal and yeast strains. The small sizes and poorly defined mitotic and meiotic morphologies of yeast chromosomes have precluded the development of a useful karyotype by light microscopy. In the case of *S. cerevisiae*, the ability to enumerate and distinguish between the chromosomes has rested almost entirely on linkage analysis.

In conventional agarose gel electrophoresis, a constant electric field is applied uniformly across the gel width. Resolution extends from $< 100$ bp to an upper limit of 20 kb, ideal for separating fragments produced by most restriction enzymes, but too low to examine intact chromosomes. In 1984, it was discovered that by applying a non-uniform electric field at different angles to the plane of the gel, very large molecules could be induced to separate on a standard agarose gel (Schwartz and Cantor, 1984). Since the electric field is alternately switched from one or more sets of electrodes to another, the

general name for the technique is PFGE. Since its original description, a number of variants have arisen, each with its own acronym. Thus we now have orthogonal field alternating gel electrophoresis (OFAGE), field inversion gel electrophoresis (FIGE), rotational field electrophoresis (RFE), contour-clamped homogenous electric field electrophoresis (CHEF) and transverse alternating field electrophoresis (TAFE) (Gardiner and Patterson, 1988; Hyde, 1990).

In PFGE, the effect of the alternating field is to force the molecules to continually change the direction in which they are moving, while inducing a net motion down the gel. The rate at which the DNA molecule can re-orient is highly sensitive to molecular weight. The key to obtaining good resolution is to match approximately the pulse time with the re-orientation time at a particular field strength (Smith and Cantor, 1989).

The introduction by Schwartz and Cantor (1984) of a technique for releasing DNA from yeast spheroplasts embedded in agarose, thereby preserving intact chromosomes, together with the pulsed field electrophoresis technique, permits the separation of chromosomes over their entire size range.

Carle and Olson (1985) used OFAGE to resolve all 16 chromosomes of *S. cerevisiae*. Vezinhet *et al.* (1990) have reported 20 different TAFE karyotypes for 22 wine yeast strains. Van der Westhuizen and Pretorius (1992) reported eight different CHEF karyotypes for 10 wine yeast strains. Miller *et al.* (1989), have identified 3 chromosomes for *P. stipitis* using OFAGE, whereas Passoth *et al.* (1992) reported 6 chromosomes each for *P. stipitis* and *C. shehatae* using OFAGE and TAFE.

### 1.5.3.3 Restriction Fragment Length Polymorphisms of Mitochondrial DNA

Mitochondrial DNA (mtDNA) is an appealing molecule for evolutionary study because it is relatively small and can be studied in its entirety. The coding potential of mitochondrial genomes is highly conserved. Both fungal and animal mtDNAs carry nearly the same genes, coding for enzymes or their subunits involved in electron transport and phosphorylation to produce ATP, RNAs and proteins required for protein synthesis as well as a number of unidentified open reading frames (Grossman and Hudspeth, 1985 -cited by Taylor, 1986).

In animals, evolution proceeds faster in the mitochondrial genome than in the nuclear genome and the circular mtDNA is inherited maternally, i.e., without recombination (Wilson *et al.*, 1985 - cited by Taylor, 1986). Fungal mitochondrial genomes are also usually circular, although a few linear genomes have been reported. However, fungal mitochondria are not always inherited uniparentally and recombination is known to occur. Animal mitochondrial genomes are usually 16 - 19 kb (Avise and Lansman, 1983 -cited by Taylor, 1986). Fungal mtDNAs are more variable (18.9 - 176 kb). The difference between the size of animal and fungal mtDNAs is apparently due to deletions and insertions (length mutations).

The highly conserved coding potential of mitochondrial genomes contrasts greatly with the large size variation observed among mtDNA molecules of different yeast species (Clark-Walker, 1985 - cited by Piškur, 1989). This can be explained by 2 hypotheses.

The skeletal DNA hypothesis suggests that DNA possesses quantitative non-genic functions in addition to its qualitative genic functions. This proposes that the non-coding sequences of the yeast mitochondrial genome specifically participate in the genome organisation and metabolism (Bernardi, 1982 - cited by Piškur, 1989; Bernardi and Bernardi, 1986). Alternatively, an extension of the selfish DNA hypothesis proposes that much DNA in the yeast mitochondrial genome is a genetic symbiont which accumulates and is actively maintained by intracellular selection (Orgel *et al.*, 1980).

The mode of transmission (from cell to cell and generation to generation) is an important aspect of mitochondrial inheritance. When 2 homoplasmic yeast cells with different mitochondrial genotypes fuse, a heteroplasmic state of mitochondrial pools occurs. Such a heteroplasmic diploid cell always gives rise to a mixed progeny composed of homoplasmic cells of different mitochondrial genotypes. However, due to its "aberrant" structure (Ehrlich *et al.*, 1972; Piškur, 1988) the yeast mtDNA is very prone to spontaneous deletions and rearrangements leading to mutant genomes characteristic of petite mutants.

DeZamaroczy and Bernardi (1985) reported that intergenic regions represent about 60% of the total genome and that these regions especially the ori/rep elements have a biological role in the mitochondrial genome. Piškur (1988) showed that mitochondrial intergenic sequences influence the transmission of nearby loci to progeny.

The widespread presence of mitochondrial plasmids has also been reported. It can be speculated that such plasmids represent means by which sequences may transfer from

one cellular compartment to another, or even between species. Some of these plasmids are unrelated to mitochondrial genomic sequences, and in a few cases where the test has been performed, no relation to nuclear sequences has been found.

RFLP analysis of mtDNA of yeast strains in the wine and brewing industry, together with electrophoretic karyotyping, has contributed enormously to the characterization of these strains.

## 1.6 SCOPE OF THIS STUDY

The objectives of the study were, firstly, to characterise and compare electrophoretic profiles of chromosomes, restricted mitochondrial and chromosomal DNA as well as proteins of the fusant and parental strains in order to determine the genetic contribution of the parental strains to the fusants. The second objective was to assess the fermentative ability of the fusants in relation to the parental strains and correlate this with the genetic constitution of the fusants to ascertain whether a relationship between ploidy or even gene copy number and ethanol production exists for these fusants.

The use of molecular techniques such as PFGE, RFLP analysis of mtDNA and chromosomal DNA and PAGE were envisaged in order to establish the degree of nuclear and cytoplasmic inheritance of the fusant strains. Batch fermentations and calculation of fermentation parameters would enable the evaluation of fermentative ability of the parental and fusant strains.

# CHAPTER TWO

## CHROMOSOMAL INHERITANCE OF THE FUSANTS OF *Pichia stipitis* AND *Candida shehatae*

### 2.1 INTRODUCTION

Improvement in product yield or process efficiency in industrial processes involving micro-organisms can be achieved by genetic manipulation of the production organisms. According to Oliver (1991), selection of desirable traits have been unintentionally/ unwittingly carried out by brewers and breadmakers over the millennia. Modern strain development has a wide range of techniques available for use with yeasts, including recombinant DNA technology, rare mating and protoplast fusion.

Recombinant DNA technology involves cloning of specific genes of interest and transformation of the production organisms with these genes. The use of recombinant DNA technology to improve the range and efficiency of yeasts' activities in classical biotechnological processes has so far been limited to the recruitment of just one or two genes which specify novel activity, e.g., *DEX*1 and *STA*1 genes of *Saccharomyces diastaticus* encoding amylolytic enzymes cloned into *S. cerevisiae* (Meaden *et al.*, 1985 and Tamaki, 1978, respectively - cited by Oliver, 1991). However, the technology currently available for yeasts should permit the wholesale genetic engineering of the organism by the addition of complete, novel metabolic pathways. According to Oliver (1991), Nederberg *et al.* (1984) and Prasad *et al.* (1987) have assembled all the yeast

genes required for the complete tryptophan biosynthetic pathway on a single plasmid. This technique can only be successful if enough is known about the genes involved in the biosynthetic pathways of interest. If this information is not available then other techniques are explored.

Most industrial yeast strains are incapable of mating since they have a polyploid genetic constitution. However, mass matings of laboratory haploids with these strains produce occasional hybrid organisms as a result of rare-mating events (Spencer and Spencer, 1977 - cited by Oliver, 1991)

Protoplast fusion is a technique used to produce hybrids between strains that do not normally mate. A number of workers have employed protoplast fusion to confer amylolytic activity on either brewery or distillery yeasts: Hockney and Freeman (1980 - cited by Oliver, 1991) hybridised *S. distaticus* and *S. cerevisiae*; Wilson *et al.* (1982) used complementation of auxotrophs between two haploid strains in a fusion of *Schwanniomyces alluvius* and *S. uvarum*; and Galembeck *et al.* (1982 - cited by Oliver, 1991) as well as Echeverrigaray (1983 - cited by Oliver, 1991) formed hybrids between *Lipomyces konoenkoae* and *S. cerevisiae*. While successful fusants capable of converting starch to ethanol were obtained in all cases, the instability of the hybrids in the absence of selection was a universal problem. Protoplast fusion of several D-xylose fermenting yeasts was also attempted. Intrageneric fusions of *C. shehatae* (Johanssen *et al.*, 1985), *P. tannophilus* (James and Zahab, 1983) and *P. stipitis* (Gupthar, 1987), as well as intergeneric fusions between *P. stipitis* and *C. shehatae* (Gupthar and Garnett, 1987) and *P. stipitis* and *S. cerevisiae* (Gupthar, 1992) were performed. However, only slight improvements in

ethanol production were reported for the *C. shehatae* and *P. tannophilus* intrageneric fusants and one *P. stipitis-C. shehatae* intergeneric fusant.

Although protoplast fusion has proved to be very useful for the construction of yeast strains with novel gene combinations, very little is known of the molecular events that occur during and after cell fusion. This is especially true in the case of intergeneric fusions where it is rarely possible to characterise the hybrids by classical genetic techniques. Even when meiotic segregation analysis can be realised, it only reveals the genetic arrangement of a few marker genes (Hoffman *et al.*, 1987).

Recently, several workers have resorted to electrophoretic karyotyping and DNA fingerprinting to study the genetic constitution of hybrids and to identify strains of industrial importance (Hoffman *et al.*, 1987; Miller *et al.*, 1989 and Smith *et al.*, 1991). These techniques are also widely used in the identification and characterisation of oenological strains of *S. cerevisiae* (van der Westhuizen and Pretorius, 1992; Vezinhet *et al.*, 1990).

Electrophoretic karyotyping is performed using the technique of PFGE developed by Schwartz and Cantor (1984). Conventional agarose gel electrophoresis techniques fail to resolve DNA molecules greater than 20 kb in size. The principle behind the technique of PFGE is that DNA molecules are subjected to an electric field which periodically changes its orientation. DNA molecules are therefore constantly changing the direction of their migration in the gel according to the changing electric field. According to Schwartz and Cantor (1984), the time taken for the reorientation of the DNA molecule is

proportional to its size. The larger the DNA molecule, the longer it takes to realign itself. Therefore, DNA can be separated by size. Several variations of this technique are widely used: FIGE, OFAGE, CHEF and most recently, TAFE. TAFE was developed by Gardiner and Patterson (1988) and is unique in that the gel is vertical and the two sets of electrodes force the DNA to move in a zig-zag fashion down the width of the gel rather than across the face of horizontal gels as for the other variants of PFGE.

RFLP analysis of chromosomal DNA has been found to be another useful tool to characterise yeast strains. Morace *et al.* (1992) performed RFLP analysis of chromosomal DNA and showed that in the case of *Pichia* isolates, certain DNA molecules are species specific. It has also been reported to be a useful tool to differentiate hybrid and parental strains (van der Westhuizen and Pretorius, 1992). It was less successful in the characterisation of twenty two Bavarian lager and ale strains, since all of them displayed identical electrophoretic profiles when cleaved with *Eco*RI (Pederson, 1985 - cited by van der Westhuizen and Pretorius, 1992). However, Panchal *et al.* (1987) reported minor differences in the profiles of lager and ale strains restricted with *Hpa*I.

In this study, three parental and ten fusant strains were investigated. The parental strains comprised a triauxotrophic *P. stipitis* strain and two diauxotrophic *C. shehatae* strains (Table 2.1). Fusants were hybrids constructed by the polyethylene glycol-induced protoplast fusion technique. These fusants resembled the *P. stipitis* parent morphologically. However, preliminary characterisation using induced and spontaneous mitotic and meiotic segregation analysis, DNA-DNA hybridisations, nuclear volume, assimilation and fermentation tests of different carbon sources as well as cell DNA content

confirmed the genetic constitution of these fusants as being at least partial hybrids and not *P. stipitis* parental dissociates (Gupthar and Garnett, 1987). In order to determine the genetic contribution of each parent to the fusants, the techniques of electrophoretic karyotyping and RFLP analysis were used in this study. The objective was to generate TAFE profiles and establish the karyotypes of parental and fusant strains and to compare the karyotypes of the fusants to those of the parental strains to determine chromosomal inheritance of the fusants as well as to ascertain if any chromosome length polymorphisms (CLPs) existed. In addition, electrophoretic profiles of proteins and RFLP profiles of chromosomal DNA of the parental and fusant strains were generated to provide a more detailed characterisation of the fusants. Finally, DNA probes of two genes involved in D-xylose metabolism viz., xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*), were hybridised against the TAFE and RFLP profiles of chromosomal DNA to determine copy number of these genes. This information could be linked to the fermentative abilities of these fusants, which was studied in a subsequent chapter, to determine whether there exists some correlation between gene copy number and fermentative ability.

## 2.2 MATERIALS AND METHODS

### 2.2.1 YEAST AND BACTERIAL STRAINS

Parental and fusant strains of *P. stipitis* and *C. shehatae* were obtained from Dr. A.S. Gupthar, Department of Biochemistry, University of Durban-Westville. The parental strains were a triauxotrophic strain of *P. stipitis* and two diauxotrophic strains of *C.*

*shehatae*. These strains as well as all other yeast and bacterial strains used in this study are listed in Table 2.1. The auxotrophic parental strains were the result of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis of prototrophic strains that had been obtained from the Council for Scientific and Industrial Research in Pretoria, SA.

TABLE 2.1. Yeast and bacterial strains used in this study

| | Strain/Designation[a] | Characteristics/Source[b] |
|---|---|---|
| **Yeasts** | | |
| Parental | | |
| *P. stipitis* | PsY 633 *leu lys ala* | mutant of Y633 : CSIR |
| *C. shehatae* | CsY 117 A/1 *cys met* | mutant of Y117 A/1 : CSIR |
| *C. shehatae* | CsY 492 *met his* | mutant of Y492 : CSIR |
| Fusant | PC1 - PC5 | prototrophic fusants of PsY 633 *leu lys ala* × CsY 492 *met his* |
| | PCA1 - PCA5 | prototrophic fusants of PsY 633 *leu lys ala* × CsY 117 A/1 *cys met* |
| *S. cerevisiae* | Sc | UDW |
| *S. cerevisiae* | Y48 | CSIR |
| **Bacteria** | | |
| *Escherichia coli* | DH5$\alpha$F′ | BRL |

[a] *leu lys ala* : auxotrophic for the amino acids leucine, lysine and adenine; *cys met* : auxotrophic for the amino acids cysteine and methionine; *met his* : auxotrophic for the amino acids methionine and histidine

[b] CSIR : Council for Scientific and Industrial Research, Pretoria; BRL : Bethesda Research Laboratories, USA; UDW : Department of Microbiology, University of Durban-Westville.

## 2.2.2 GROWTH AND MAINTENANCE OF CULTURES

Yeast strains were grown on YMA (yeast-malt extract agar: 3 g yeast extract, 3 g malt extract, 5 g glucose and 15 g agar per litre) plates at 30°C for two to three days. Working stocks were stored at 4°C and subcultured every four to six weeks. For long term stock cultures, yeasts were grown in YMB (yeast-malt extract broth) at 30°C in an orbital shaker (Certomat U, Braun, Germany) at 155 rpm and equal volumes of the broth culture and sterile 30% glycerol were mixed, snap frozen in liquid nitrogen and stored at -70°C and -20°C. The *E. coli* strain was grown on YT agar (8 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar per litre) plates at 37°C overnight (O/N), then stored at 4°C. Subculturing was performed every 4 weeks. Log phase broth (YT broth) cultures were used to prepare stock cultures as described for the yeast strains.

## 2.2.3 PULSED FIELD GEL ELECTROPHORESIS

### 2.2.3.1 **Agarose Plug Preparation**

Agarose plugs were prepared using a modification of the method of Schwartz and Cantor (1984).

SCE Buffer (pH 8.0) : 1 M sorbitol

0.1 M sodium citrate

60 mM EDTA

| | | |
|---|---|---|
| Solution I | : | 0.2 M sorbitol |
| | | 20 mM sodium citrate |
| | | 12 mM EDTA |
| | | 1% 2-mercaptoethanol |
| | | 2 mg/ml novozyme |
| Solution II | : | 46 mM EDTA (pH 6.0) |
| | | 1 mM Tris.HCl (pH 8.0) |
| | | 8% 2-mercaptoethanol |
| Solution III | : | 45 mM EDTA (pH 6.0) |
| | | 1 mM Tris.HCl (pH 8.0) |
| | | 1% N-lauroylsarcosine (Na salt) |
| | | 2 mg/ml novozyme |
| LGT agarose | : | 1% low gelling temperature agarose |
| | | in 0.125 M EDTA (pH 7.5) |

Cells were grown to late log phase in YMB medium at 30°C with shaking (155 rpm on an orbital shaker). The *C. shehatae* strains were grown to early log phase (12 h) since the cell wall of yeasts become more complex and difficult to lyse with age, and this was especially true for the *C. shehatae* strains. Cells were harvested by centrifugation at 10 000 × *g* for 10 min followed by two washing steps in 50 mM EDTA (pH 7.5). The pellets were resuspended in SCE buffer (pH 7.0), cell density adjusted to 8 absorbance units at 600 nm (Ultrospec II, LKB, Sweden) and the cell suspension left to equilibrate in SCE buffer at room temperature (RT). One ml of the suspension was mixed with 350 $\mu$l solution I and 1.8 ml of molten (42°C) 1% LGT agarose (Sigma, Type VII, USA),

vortexed immediately, dispensed into a mould and allowed to solidify at 4°C. After 10 min agarose plugs were removed from the mould and incubated with gentle agitation at 37°C O/N in 5 ml solution II. During this step, the cell walls were digested by novozyme to form sphaeroplasts. Both the *C. shehatae* strains required double the enzyme concentration to achieve effective lysis of the cells, viz., 4 mg/ml novozyme. Solution II was replaced with 5 ml solution III and incubated as above. Solution III contained a detergent that lysed the sphaeroplasts and novozyme was responsible for the degradation of cellular proteins. After lysis of the cells, plugs were stored at 4°C in 0.5 M EDTA (pH 9.0). Plugs could be used for up to 5 months with 1-2 changes of the storage buffer.

#### 2.2.3.2 **Electrophoresis Conditions**

Tris-Borate-EDTA (TBE) Buffer (5×) : 45 mM Tris base
45 mM boric acid
1 mM EDTA

Agarose plugs were equilibrated in 0.25 × TBE buffer for 2 h before being loaded into the wells of a 1% low endosmosis (LE) agarose gel (Beckman, USA) in 0.25 × TBE. The wells were then sealed with 1% LGT agarose. TAFE was performed with the Geneline II DNA Mapper (Beckman, USA). Run conditions were varied in an attempt to optimise the resolution of the yeast chromosomes.

**Programme 1** (Run conditions for *S. cerevisiae* supplied by Beckman, USA)

Stage 1 : 12 h; 350 mA; 1 min

Stage 2 : 12 h; 370 mA; 2 min

**Programme 2**

Stage 1 : 5 h; 400 mA; 45 s

Stage 2 : 7 h; 350 mA; 1 min

Stage 3 : 9 h; 370 mA; 2 min

Stage 4 : 9 h; 390 mA; 3 min

**Programme 3** (Run conditions for *Schizosaccharomyces pombe* supplied by Beckman, USA)

Stage 1 : 12 h, 80 V; 60 min

Stage 2 : 24 h; 80 V; 45 min

Stage 3 : 24 h; 80 V; 30 min

**Programme 4**

Stage 1 : 12 h; 200 V; 200 min

Stage 2 : 12 h; 80 V; 60 min

Stage 3 : 24 h; 80 V; 45 min

Stage 4 : 24 h; 80 V; 30 min

**Programme 5** (Passoth *et al.*, 1992)

Stage 1 : 72 h; 200 V; 200 min

All runs were carried out at 15°C. At the end of each run, gels were stained for 45 min in ethidium bromide (2 $\mu$g/ml) and destained in distilled water for 2 h. DNA bands were visualised using a UV transilluminator (UVP Inc., USA) and photographed using a Minolta camera loaded with Ilford FP4 Plus film and fitted with a UV filter and a Vivitar number 25 (red) filter.

### 2.2.3.3 Southern Blots

Chromosomal DNA was transferred from the agarose gels to nylon membranes using a vacuum blotting unit (Omeg Scientific, South Africa).

| | | |
|---|---|---|
| Transfer solution | : | 0.4 M NaOH |
| | | 0.6 M NaCl |
| Depurination solution | : | 0.25 M HCl |

Two pieces of filter paper (Whatman 4MM) were cut to exactly the size of the agarose gel and the nylon membrane (Hybond-N Nylon 0.45 $\mu$m, Amersham, USA) was cut 4 - 5 mm bigger than the gel. The filter paper and nylon membrane were pre-wet in deionised water for 15 - 20 min. After placing the filter paper on the porous support of the vacuum blotter, the nylon membrane was centred on the filter paper. The gel was carefully placed on the membrane and a pre-cut mask was placed around the gel ensuring that at least 2 mm of the membrane was under the mask on all sides which contributed to a good vacuum seal. Blotting was carried out at a vacuum of 15 cm Hg. Approximately

15 - 25 ml of depurination solution was poured over the gel and left on for 20 - 30 min. This solution breaks the chromosomal DNA into fragments thus enhancing the transfer process. The excess depurination solution was removed and transfer solution poured onto the gel and left on for 60 - 90 min. Both the depurination and transfer solutions were regularly topped up to prevent the gel from drying up. At the end of the procedure, the vacuum pump was turned off and the gel and filter paper were removed. The position of the wells were marked onto their corresponding positions on the nylon membrane which was then air-dried. The DNA was cross-linked onto the membrane by exposure to UV light for 3 min, the membrane sealed in a plastic bag and stored at 4°C.

### 2.2.4 PLASMID DNA PROBES

Plasmids carrying the *XYL*1 and *XYL*2 genes coding for xylose reductase and xylitol dehydrogenase, respectively, were obtained from Dr. Peter Kötter of the Institut für Mikrobiologie der Johann Wolfgang Goethe-Universität, Frankfurt, Germany. Plasmids, pT7T3-X/B and pCT1, are based on the *E. coli* vector pT7T3-18V (Pharmacia, Sweden). Plasmid pT7T3-X/B contains a 1.95 kb *Xba*I/*Bam*HI fragment from *P. stipitis* which includes the *XYL*2 gene. Plasmid pCT1 contains the *XYL*1 gene of *P. stipitis* in a 2 kb *Bam*HI fragment. Both plasmids can be selected by growth in the presence of ampicillin. Restriction maps of these plasmids are shown in Fig 2.1.

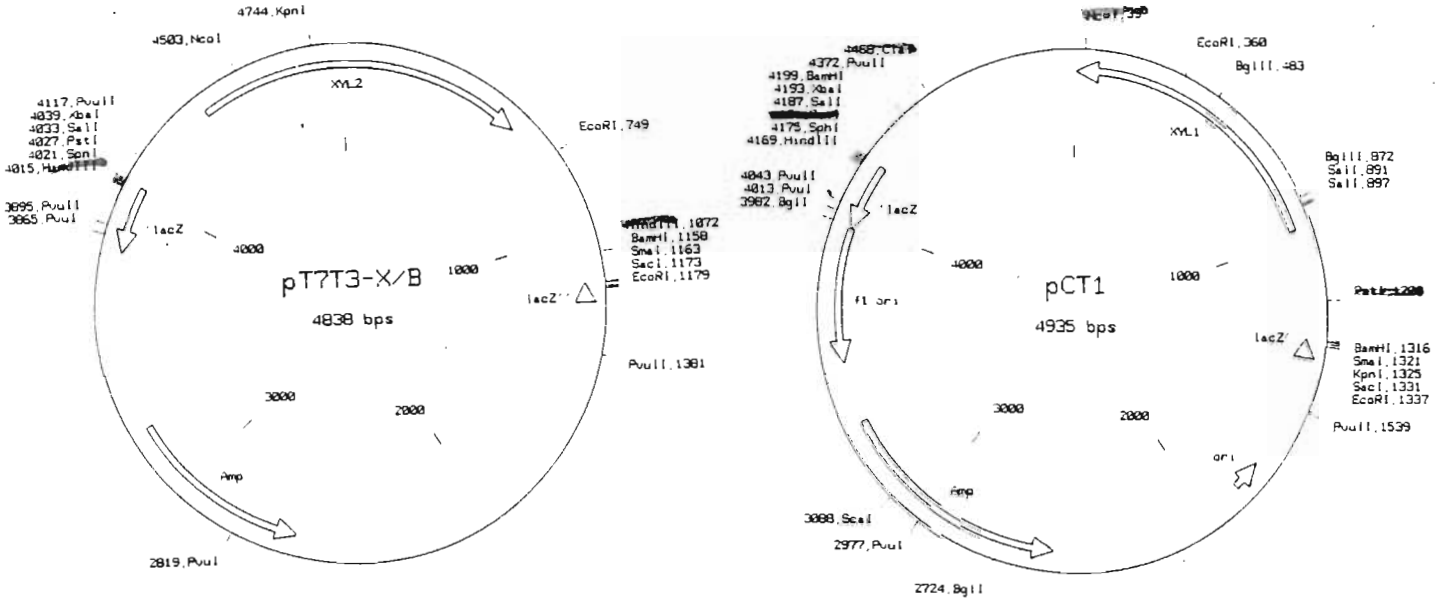



**Fig. 2.1.** Restriction maps of the plasmids pT7T3-X/B and pCT1 which harbour the *XYL2* and *XYL1* genes, respectively (Pharmacia).

### 2.2.4.1 Transformation

| | | |
|---|---|---|
| $CaCl_2$ solution | : | 50 mM $CaCl_2$ |
| | | 10 mM Tris.HCl (pH 8.0) |
| $CaCl_2$-glycerol solution | : | 50 mM $CaCl_2$ |
| | | 10 mM Tris.HCl (pH 8.0) |
| | | 15% glycerol |
| TE (pH 8.0) | : | 10 mM Tris.HCl |
| | | 1 mM EDTA |

#### 2.2.4.1.1 Preparation of Competent Cells for Transformation

One ml of an overnight culture of *E. coli* DH5$\alpha$F´ was added to 29 ml of YT medium and incubated with shaking at 37°C until the cell density was 0.375 absorbance units at 590 nm. The cells were at an optimal age for transformation at this optical density. Therefore the culture was immediately placed on ice to stop further growth. Cells were pelleted by centrifugion for 10 min at 10 000 × *g* at 4°C. The pellet was gently resuspended in 10 ml cold $CaCl_2$ solution, centrifuged as above, resuspended in 10 ml cold $CaCl_2$ solution incubated on ice for 20 min to become competent and centrifuged as above. Exposure to $Ca^{2+}$ ions lets the cell take up DNA (become competent). The pellet was finally resuspended in 2.5 ml cold $CaCl_2$-glycerol solution. 100 $\mu$l aliquots were dispensed into Eppendorf tubes and stored at 4°C for 24 h. Following this incubation step which increases the competency of the cells (Ausubel *et al.*, 1989), they were stored at -70°C.

#### 2.2.4.1.2 Transformation of Competent Cells

One $\mu$l (1 - 2 ng) of plasmid DNA was added to 100 $\mu$l of competent cells, gently mixed and incubated for 30 min on ice to allow the DNA to bind to the cells. The mixture was then placed at 42°C for 2 min. This heat shock treatment permits the entry of naked plasmid DNA into the cells. The mixture was immediately placed on ice for 15 min after heat shock, followed by the addition of 900 $\mu$l of YT medium and incubation

with shaking at 37°C for 1 h to allow expression of the ampicillin resistance genes. 100 $\mu$l of this mixture was plated onto YT agar plates supplemented with ampicillin (50 $\mu$g/ml) and incubated O/N at 37°C.

#### 2.2.4.1.3 **Screening of Transformants**

Numerous transformants were obtained on YT-ampicillin plates. Three colonies for each of the plasmids transformed were grown in YT broth containing ampicillin (50 $\mu$g/ml). Plasmid DNA was isolated using a modified version of the alkaline lysis method of Birnboim and Doly (1979) to verify the presence of plasmids.

Solution A : 25 mM Tris.HCl (pH 8.0)
50 mM glucose
10 mM EDTA
Just before use add : RNase (100 $\mu$g/ml)
Lysozyme (5 mg/ml)

Solution B : 0.2 M NaOH
1% SDS
(prepared fresh)

Solution C : 3 M sodium acetate (pH 4.8)

Phenol equilibrated with 0.1 M Tris.HCl (pH 6.8), final pH 7.6

Phenol/chloroform (1:1)

Chloroform (Chloroform:isoamyl alcohol, 24:1)

Ethanol 100%, 70%

TE (pH 8.0)

Selected colonies were grown overnight at 37°C in 5 ml YT broth supplemented with ampicillin, with vigorous aeration. A 1.5 ml aliquot of the bacterial culture was pelleted in an Eppendorf tube by centrifugation at 15 800 × *g* for 1 min in a microcentrifuge (Microfuge II, Beckman, USA). The pellets were resuspended in 100 µl solution A and incubated for 5 min at RT. During this incubation step, the cell wall was degraded by lysozyme. Protoplasts that formed were lysed by the addition of 200 µl of solution B and incubation for 5 min at RT. The RNA liberated when cell lysis occured was degraded by RNase. Denatured chromosomal DNA and proteins were precipitated by the addition of 150 µl of cold solution C and incubation on ice for 5 min. Cell debris was pelleted by centrifugation for 10 min at 15 800 × *g* at 4°C. The supernatant containing the plasmid DNA was extracted once with an equal volume of phenol/chloroform in order to remove residual proteins. Eppendorf tubes were repeatedly inverted to ensure maximum extraction, then centrifuged for 3 min as above to separate the aqueous and organic phases. The upper aqueous phase was collected carefully, without disturbing the protein layer that forms between the organic and aqueous phases. The aqueous phase was then extracted with an equal volume of chloroform to remove all traces of phenol. Plasmid DNA was precipitated by the addition of 0.1 volume of cold solution C and two volumes 100% ethanol and incubation at -70°C for 30 min. Plasmid DNA was pelleted by centrifugation at 15 800 × *g* for 15 min at 4°C. The pellet was washed with 70% ethanol, vacuum dried, resuspended in 20 µl TE and stored at -20°C.

### 2.2.4.2 Isolation of DNA Probes

The entire *XYL*1 and *XYL2* genes were chosen as probes for further work. In order to obtain these probes, plasmid DNA isolated in section 2.2.4.1 was cleaved with appropriate restriction endonucleases and electrophoresed on LGT agarose gels. DNA bands representing the *XYL*1 and *XYL2* genes were excised from the gel and purified using the Geneclean kit (Bio 101, USA). The physical maps of the plasmids indicate that cleavage of pCT1 with *Nco*I and *Pst*I and pT7T3-X/B with *Hin*dIII produces three and two fragments, respectively. The 1 160 bp *Nco*I-*Pst*I fragment contains the *XYL*1 gene and the 1 895 bp *Hin*dIII fragment contains the *XYL2* gene.

#### 2.2.4.2.1 Restriction and Electrophoresis of Plasmid DNA

| | | |
|---|---|---|
| Tris Acetate EDTA (TAE) Buffer (50×) | : | 40 mM Tris base |
| | | 20 mM glacial acetic acid |
| | | 2 mM EDTA |
| Gel loading buffer (6×) | : | 40% sucrose |
| | | 0.25% bromophenol blue |

All restriction endonucleases and their corresponding buffers were obtained from Boehringer Mannheim, Germany. A typical restriction reaction contained the following components in a total volume of 15 $\mu$l: $\approx$ 500 ng DNA, 1 × restriction buffer and 4-5 U restriction endonuclease. Following incubation for 1.5 h at 37°C, the restricted DNA was

run on a 1% LGT agarose gel in 1 × TAE electrophoresis buffer at 80 V for 3 h. The gel was stained for 15 min in ethidium bromide and viewed on a UV transilluminator. Bands corresponding to the 1 160 bp *Nco*I-*Pst*I fragment of pCT1 and the 1 895 bp *Hin*dIII fragment of pT7T3-X/B were excised from the gel and purified as described below.

#### 2.2.4.2.2 **Isolation of DNA Fragments**

DNA fragments in the agarose gel slices were purified using the Geneclean kit.

6 M NaI

NEW Wash

Glassmilk

The approximate volumes of the gel slices were determined (1 g ≈ 1 ml) and ≈ 0.4 g gel slices were placed in 1.5 ml Eppendorf tubes. Three volumes of NaI was added to each tube (final concentration of NaI ≈ 4.5 M) and incubated in a water bath at 50°C until the agarose dissolved completely. Five μl of glassmilk was added to the tubes and incubated on ice for 10 min, mixing every 1 - 2 min. During this incubation step, DNA fragments in the solution bind to the silica beads in the glassmilk. The silica matrix with the bound DNA was pelleted by centrifugation in a microcentrifuge for approximately 30 s at 15 800 × *g* at 4°C. The supernatant was discarded and the pellet washed three times with 500 μl of ice cold NEW wash to remove contaminants. The pellet was washed by repeated pipetting and 5 s centrifugation steps. After removing the supernatant from the

last wash, tubes were spun for a few seconds and the last drops of liquid removed with a fine tipped pipette to avoid diluting the eluate. DNA was eluted from the glassmilk by the addition of 12 $\mu$l TE buffer and incubation at 55°C for 2 - 3 min. The mixture was centrifuged for 30 s and the supernatant containing the eluted DNA carefully removed and placed in an Eppendorf tube. This elution step removes $\approx$ 80% of the bound DNA. A second elution step using 8 $\mu$l of TE buffer was carried out. Both eluates were combined and stored at -20°C. An aliquot of this DNA was run on a 0.8% agarose gel in order to estimate the concentration of the DNA fragments.

#### 2.2.4.3 **Labelling of Probes**

*XYL*1 and *XYL*2 genes were labelled using the nonradioactive Dig DNA Labelling and Detection Kit (Boehringer Mannheim, Germany). Digoxigenin is a hapten which is bound via a spacer arm to uridine nucleotides. It becomes incorporated enzymatically into nucleic acid probes by random primed DNA labelling.

Hexanucleotide mixture (10×)

dNTP labelling mix (10×) : 1 mM dNTP (N = C, G, T, A)

0.35 mM Dig-dUTP

Klenow enzyme (2 U/$\mu$l)

200 mM EDTA (pH 8.0)

4 M LiCl

DNA fragments were denatured in a boiling water bath for 10 min and immediately cooled on ice. A typical labelling reaction had the following components in a total volume of 20 $\mu$l: 10 - 1 $\mu$g freshly denatured DNA, 2 $\mu$l hexanucleotide mixture, 2 $\mu$l dNTP labelling mixture and 1 $\mu$l Klenow enzyme.

These components were added to an Eppendorf tube on ice, mixed well, centrifuged briefly and incubated for 20 h at 37°C. The labelling reaction was stopped by the addition of 2 $\mu$l EDTA solution. Labelled DNA was precipitated with 2.5 $\mu$l LiCl and 75 $\mu$l cold 100% ethanol at -70°C for 30 min. Precipitated DNA was pelleted by centrifugation at 15 800 × *g* for 30 min, washed with 70% ethanol and vacuum dried. The labelled DNA was dissolved in 50 $\mu$l TE buffer and stored at -20°C.

#### 2.2.4.4 **Hybridisation**

Pre-hybridisation solution : 5 × SSC

1% blocking reagent

0.1% N-lauroylsarcosine (Na salt)

0.02% SDS

All volumes indicated are for 100 cm$^2$ of membrane. Membranes were pre-hybridised in sealed plastic bags with 20 ml of pre-hybridisation solution at 45°C for 4 h with gentle agitation. The pre-hybridisation solution was replaced with 2.5 ml of pre-hybridisation solution containing ≈ 30 ng of freshly denatured labelled DNA and incubated

at 45°C with gentle agitation O/N. Following hybridisation, membranes were washed twice for 5 min at RT with 50 ml of 2 × SSC/0.1% SDS and twice for 15 min at 45°C with 0.1 × SSC/0.1% SDS. Membranes were used immediately for the detection of hybrid DNA.

### 2.2.4.5 Detection of Hybrid DNA

After hybridisation to target nucleic acids, bound probes were detected by an enzyme-linked immunoassay using high affinity antibody Fab fragments coupled to alkaline phosphatase and visualisation using either the colour substrate nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate (X-phosphate) or the chemiluminescent substrate Lumigen PPD followed by exposure to X-ray film.

| | | |
|---|---|---|
| Buffer 1 | : | 100 mM maleic acid |
| | | 150 mM NaCl |
| | | pH 7.5 |
| Buffer 2 | : | 1% blocking reagent in buffer 1 |
| Buffer 3 (pH 9.5) | : | 100 mM Tris.HCl |
| | | 100 mM NaCl |
| | | 50 mM $MgCl_2$ |
| Buffer 4 | : | TE (pH 8.0) |

Anti-digoxigenin alkaline phosphatase conjugate

| | | |
|---|---|---|
| Colour solution | : | 35 $\mu$l NBT |

45 $\mu$l X-phosphate

10 ml buffer 3

Lumigen solution : Lumigen PPD (10 mg/ml)

10 ml buffer 3

All volumes indicated are for 100 $cm^2$ of membrane and all reactions occurred at RT. Membranes were washed briefly (1 min) in buffer 1 and incubated for 30 min with 100 ml of buffer 2. The anti-digoxigenin alkaline phosphatase conjugate was diluted to 150 mU/ml in buffer 2. Membranes were incubated with 20 ml of this diluted antibody conjugate for 30 min with gentle agitation. Unbound antibody conjugate was removed by washing twice for 15 min with 100 ml of buffer 1. Membranes were equilibrated for 2 min in 20 ml buffer 3 and sealed in plastic bags with 10 ml of colour solution. The colour reaction was allowed to proceed O/N in the dark. Membranes were then washed for 5 min with 50 ml buffer 4 and stored in the same buffer solution. The procedure for chemiluminescent detection is the same up to the point before the addition of the colour solution. Membranes were incubated for 5 min in the substrate solution and the excess substrate solution blotted off on 3MM Whatman paper. Membranes were sealed in plastic bags, incubated at 37°C for 30 min to activate the alkaline phosphatase and exposed for 30 min at RT to X-ray film.

### 2.2.5 RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

In order to study restriction fragment length polymorphisms (RFLPs), chromosomal

DNA embedded in agarose plugs were cleaved with various restriction endonucleases and analysed by agarose gel electrophoresis.

26 mg/ml bovine serum albumin (BSA)

1 M dithiothreitol (DTT)

Agarose plugs were prepared as previously described in section 2.2.3.1. Prior to electrophoresis, plugs were equilibrated, first in 2 × 500 $\mu$l aliquots of sterile deionised water for 24 h, then for 5 h in 155 $\mu$l of 1 × restriction buffer. Chromosomal DNA in the plugs was cleaved for 20 h using the following restriction endonucleases: *Dra*I, *Eco*RI, *Hin*dIII and *Xba*I. A typical restriction reaction contained the following components in a total volume of 155 $\mu$l: 1 × restriction buffer, 0.13 mg/ml BSA, 1.3 mM DTT and 15-18 U of enzyme.

DNA in the plugs was subjected to electrophoresis on 0.8% agarose gels in 0.5× TBE buffer at 35 V for 20 h. The gel was stained in ethidium bromide, viewed on a UV transilluminator, vacuum blotted as in section 2.2.3.3 and hybridised as in section 2.2.4.4 to the DIG-labelled probes described in section 2.2.4.3.

### 2.2.6 SDS-POLYCARYLAMIDE GEL ELECTROPHORESIS

The total soluble cell proteins of the fusants were electrophoresed on SDS-

polyacrylamide gels and compared to the protein profiles of the parental strains. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Havana Screening Electrophoresis System (Desaga) in a discontinuous buffer system according to the procedure of Laemmli (1970).

### 2.2.6.1 Preparation of Polyacrylamide Gels

| | | |
|---|---|---|
| Acrylamide/bisacrylamide 30% | : | 30 g acrylamide |
| | | 0.8 g N,N´-methylenebisacrylamide |
| | | $H_2O$ to 100 ml |
| Separation buffer (5×) | : | 1.875 M Tris.HCl |
| | | 0.5% SDS |
| | | pH 8.8 |
| Stacking buffer (10×) | : | 1.25 M Tris.HCl |
| | | 1% SDS |
| | | pH 6.8 |
| Electrode buffer (10×) | : | 0.25 M Tris.HCl |
| | | 1.92 M glycine |
| | | 1% SDS |
| | | pH 8.3 |

1% N,N,N´,N´-tetramethylenediamine (TEMED)

8% $(NH_4)_2S_2O_8$

Staining solution : 0.25% Coomassie brilliant blue

4.5 volumes methanol

4.5 volumes $H_2O$

1 volume glacial acetic acid

A separation gel was prepared by mixing 10% acrylamide/bisacrylamide, 1 × separation buffer and 0.15% TEMED. After degassing the mixture for 30 s, $(NH_4)_2S_2O_8$ was added to a final concentration of 3%, mixed rapidly and poured into the gel mould with the aid of a glass syringe. Enough space was left at the top of the mould for a 1 cm stacking gel. To prevent $O_2$ from diffusing into the gel and inhibiting polymerisation, a 20% ethanol solution was used to overlay the gel which was left at RT for 1 h. The ethanol was poured off once polymerisation was complete and the gel rinsed thoroughly with deionised water to remove unpolymerised acrylamide. Residual drops of water were removed by blotting with paper towel. The stacking gel comprised 4% acrylamide/bisacrylamide, 1 × stacking buffer, 0.05% TEMED and 0.12% $(NH_4)_2S_2O_8$ and was prepared as the separation gel. The mixture was poured directly onto the separation gel, a comb was inserted into the stacking gel which was left to polymerise at RT for 1 h. The comb was removed after polymerisation was complete, the wells rinsed thoroughly with deionised water to remove unpolymerised acrylamide and the gel was mounted in the electrophoresis chamber.

### 2.2.6.2 Preparation of Soluble Cell Proteins

SDS gel loading buffer : 50 mM Tris.HCl, pH 6.8

2% SDS

0.1% bromophenol blue

100 mM DTT added just before use

Cells were inoculated into 5 ml of CA medium and grown O/N at 30°C on an orbital shaker at 155 rpm. A 1.5 ml aliquot of the O/N culture was centrifuged at 15 800 × *g* for 2 min. The pellet was washed once in distilled water and frozen in liquid nitrogen. The frozen pellets were ground using a mortar and pestle, with three cycles of freezing and grinding per pellet. The resulting cell paste was resuspended in 500 $\mu$l of 1 × SDS gel loading buffer, boiled for 5 min in a water bath and centrifuged briefly at 15 800 × *g* to pellet cell debris. The supernatant was decanted and used to load the gels with a Hamilton microsyringe.

Electrophoresis was carried out at 75 mA O/N. Once the dye front reached the end of the gel, electrophoresis was stopped, gels removed from the mould and simultaneously fixed and stained for at least 4 h in staining solution. Gels were destained in the staining solution lacking Coomassie blue dye. This solution was replaced several times to speed up the destaining process. Gels were stored in 20% glycerol until photographed.

## 2.3 RESULTS

### 2.3.1 AGAROSE PLUG PREPARATION

Agarose plugs contained chromosomal DNA of high molecular weight. Very little or no shearing occurred during the preparation (Lane 1 in Figs. 2.8 to 2.11).

### 2.3.2 ELECTROPHORETIC KARYOTYPING

The electrophoretic banding patterns of chromosomal DNA were used to characterise parental and fusant strains. The three parental strains showed unique profiles. All fusant strains displayed an identical banding pattern to that of the *P. stipitis* parent.

It was found that run conditions were extremely important to obtain optimal resolution of the chromosomes (Figs. 2.2 to 2.7). The run conditions of programme 1 produced three bands for the *P. stipitis* parent and the fusant strains and two for both *C. shehatae* parental strains (Fig. 2.2). It is generally accepted that under optimal electrophoretic conditions bands represent individual chromosomes. Chromosomes are usually numbered from the lightest band upwards. The brightness of the band representing chromosome III indicated that it contained at least two co-migrating chromosomes that appeared unresolved under these run conditions. Programme 2, having a slightly longer run time and consisting of two more stages, effectively resolved this band into two chromosomes (Fig. 2.3). The bands representing the heavier chromosomes of both *C.*

*shehatae* parents in Fig 2.2 were also resolved to produce two and three bands for CsY 492 *met his* and CsY 117 A/1 *cys met*, respectively. The middle band for CsY 492 *met his* appeared to contain more than one chromosome. This was verified by subsequent electrophoretic separations. Chromosome sizes in *P. stipitis* were estimated, using the SW5000 Gel Documentation System (U.V.P. Inc., U.K.) and the chromosomes of *S. cerevisiae* as size markers, to be approximately 0.93 Mb, 1.00 Mb, 1.71 Mb and 1.86 Mb; in *C. shehatae* CsY 117 A/1 *cys met* 1.34 Mb, 1.52 Mb, 1.69 Mb and 1.85 Mb; and in *C. shehatae* 492 *met his* 1.38 Mb, 1.68 Mb, 1.72 Mb and 1.86 Mb.

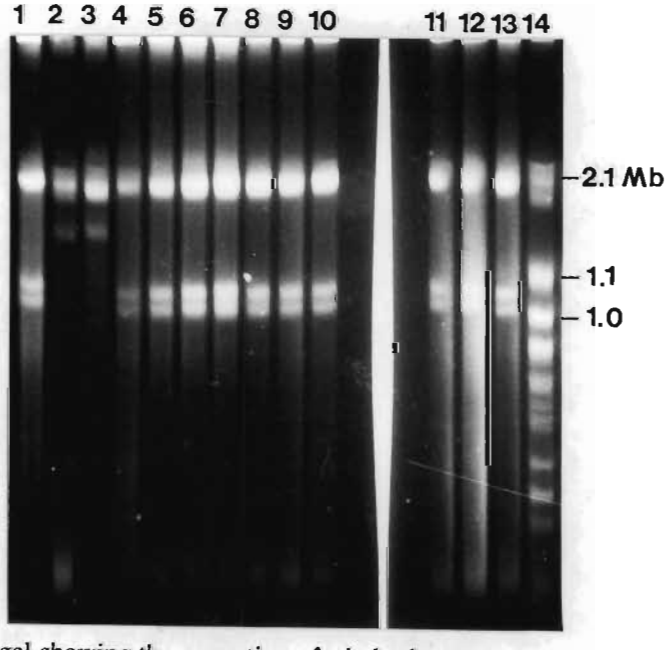



**Fig. 2.2.** TAFE gel showing the separation of whole chromosomes of *P. stipitis, C. shehatae* and their fusants using the run conditions of Programme 1. Lane 1: *P. stipitis* 633 *leu lys ala*, lane 2: *C. shehatae* 117 A/1 *cys met*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5, lanes 9-13: PCA1-PCA5 and lane 14: *S. cerevisiae* marker.

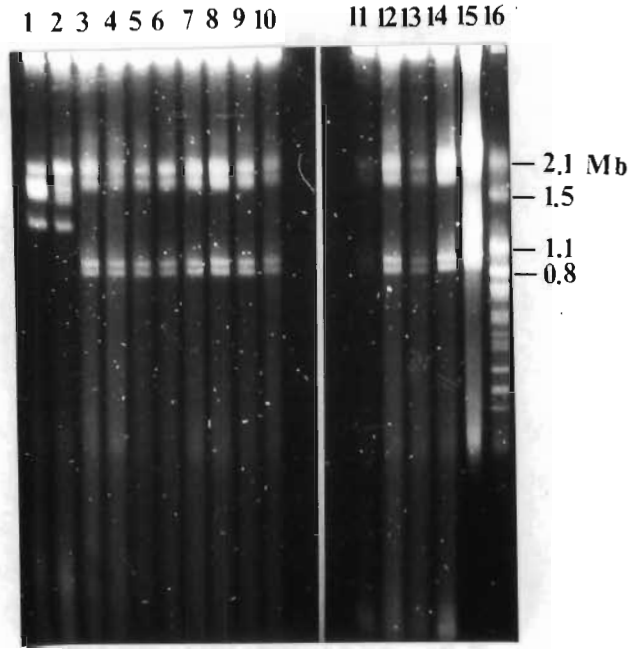



**Fig. 2.3.** TAFE gel of the chromosomes of *P. stipitis*, *C. shehatae* and their fusants using the run conditions of Programme 2. Lane 1: *C. shehatae* 492 *met his*, lane 2: *C. shehatae* 117 A/1 *cys met*, lane 3: *P. stipitis* 633 *leu lys ala*, lanes 4-8: PC1-PC5, lanes 9-13: PCA1-PCA5, lane 14: *P. stipitis* 633 *leu lys ala*, lane 15: *C. albicans* marker (overloaded) and lane 16: *S. cerevisiae* marker.

The run conditions for the optimal separation of *C. albicans* and *S. pombe* standards were also used in an attempt to improve the resolution of the heavier chromosomes. The programme for the former organism did not provide a profile containing any additional bands compared to that previously obtained (Fig. 2.3) while the programme for the latter showed an interesting pattern of separation (Fig. 2.4). This programme is designed for optimal separation of chromosomes in the 3 - 6 Mb range. Therefore, the heavier chromosomes of *P. stipitis* and the fusant strains were resolved. However, the lighter chromosomes of the yeast strains in this study were not resolved.

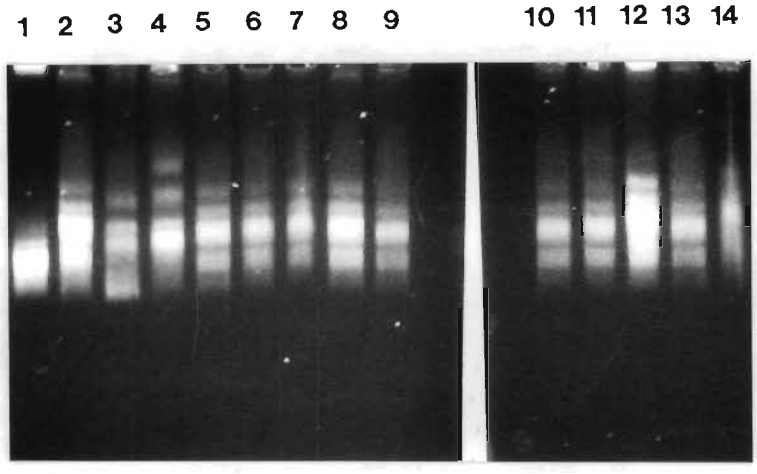



**Fig. 2.4.** TAFE gel showing the separation of whole chromosomes of *P. stipitis*, *C. shehatae* and their fusants using the run conditions of Programme 3. Lane 1: *S. cerevisiae* marker, lane 2: *P. stipitis* 633 *leu lys ala*; lane 3: *C. shehatae* 117 A/1 *cys met*, lane 4: *C. shehatae* 492 *met his*, lanes 5-9: PC1-PC5, lanes 10-14: PCA1-PCA5.

It is interesting to note that an apparent CLP exists between PCA3 and PsY (Fig. 2.4, lanes 12 and 2, respectively) compared to the rest of the fusants. The intensity and thickness of the third and fourth bands for *P. stipitis* and the fusant strains suggested that they comprised of more than one chromosome (lanes 2, and 5-13, Fig. 2.4). The *S. cerevisiae* marker appeared as one very thick band as conditions were not optimal for the separation of these chromosomes.

A combination of the electrophoretic parameters for programmes 3 and 5 was used to create programme 4. This programme effectively resolved the genome of *P. stipitis* and the fusant strains into five chromosomes (Fig. 2.5). However, separation of the heavier

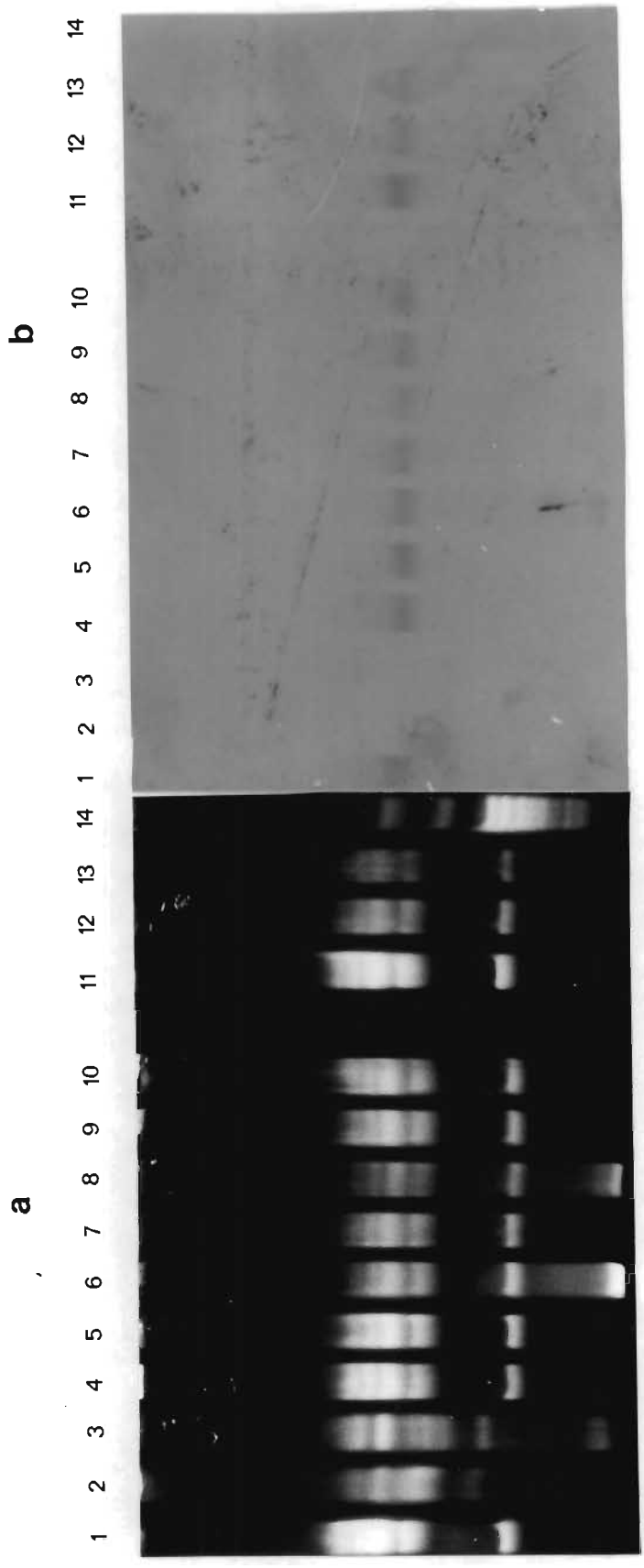



**Fig. 2.5** TAFE gel of the chromosomes of *P. stipitis*, *C. shehatae* and their fusants using the run conditions of Programme 4 (a) and hybridisation using the *XYL*1 probe (b). Lane 1: *P. stipitis* 633 *leu lys ala*, lane 2: *C. shehatae* 117 A/1 *cys met*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5, lanes 9-13: PCA1-PCA5 and lane 14: *S. cerevisiae* marker.

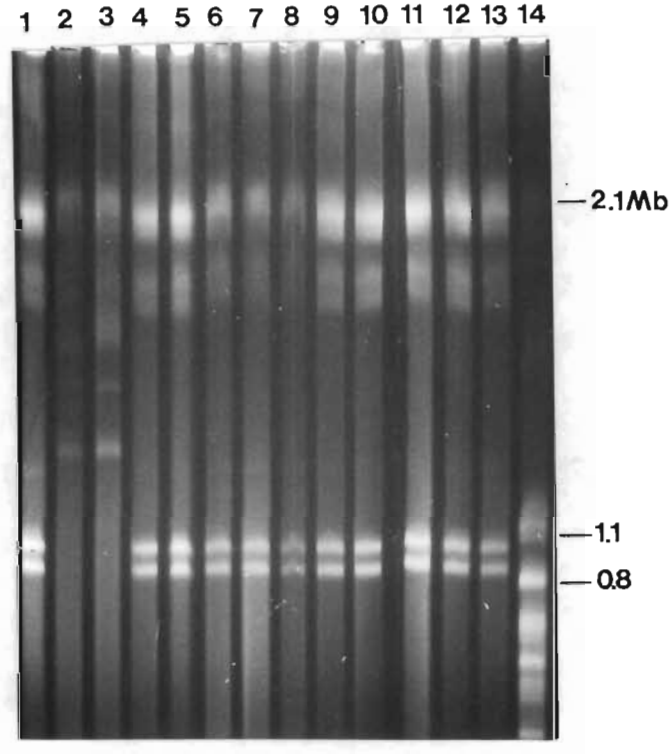



**Fig. 2.6.** TAFE gel showing the separation of whole chromosomes of *P. stipitis*, *C. shehatae* and their fusants using the run conditions of Programme 4. Lane 1: *C. shehatae* 492 *met his*, lane 2: *C. shehatae* 117 A/1 *cys met*, lane 3: *P. stipitis* 633 *leu lys ala*, lanes 4-8: PC1-PC5, lanes 9-13: PCA1-PCA5 and lane 14: *S. cerevisiae* marker.

chromosomes was unsatisfactory, i.e., the bands were not distinct. The intensity of the band that migrated the furthest suggested that it contained at least two chromosomes that were not resolved. A similar programme to the above but with a longer run time (96 h) allowed the resolution of this band into two chromosomes (Fig. 2.6). Again, the heavier chromosomes were not distinct and in some cases the DNA appeared to be diffuse and the background very high.

Passoth *et al.* (1992) reported the presence of five to six chromosomes for *P. stipitis* using TAFE. When their run conditions were used, the genomes of *P. stipitis* and the fusant strains were resolved into six distinct chromosomes, while that of CsY 117 A/1

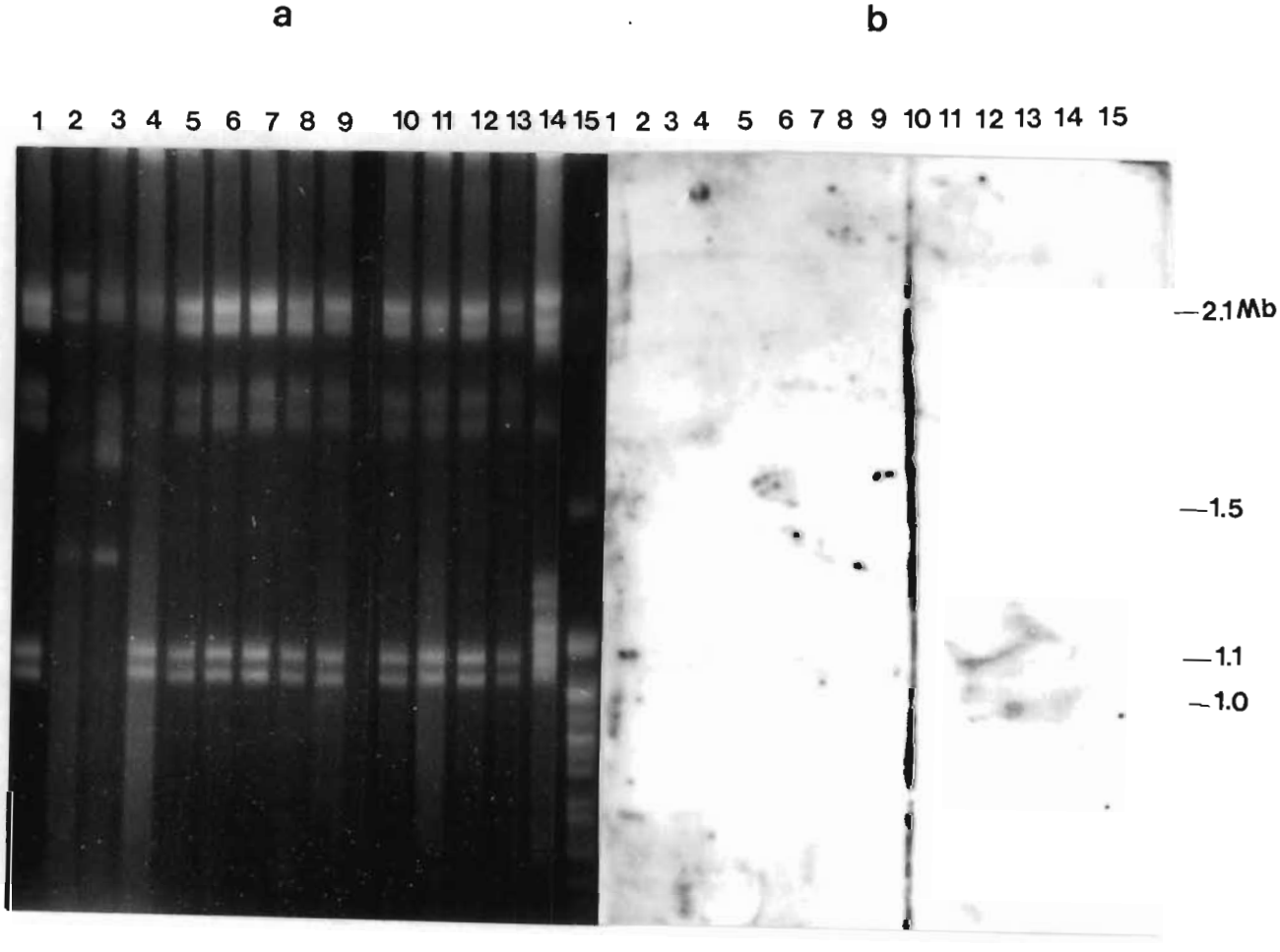



**Fig. 2.7.** TAFE gel of the chromosomes of *P. stipitis*, *C. shehatae* and their fusants using the run conditions of Programme 5 (a) and hybridisation using the *XYL2* probe (b). Lane 1: *P. stipitis* 633 *leu lys ala*, lane 2: *C. shehatae* 117 A/1 *cys met*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5, lanes 9-13: PCA1-PCA5, lane 14: *C. albicans* marker and lane 15: *S. cerevisiae* marker.

*cys met* and CsY 492 *met his* were resolved into five distinct chromosomes (Fig. 2.7). The chromosome sizes were estimated to be approximately 2.15 Mb, 1.67 Mb, 1.21 Mb, 1.15 Mb and 1.11 Mb for *C. shehatae* 117 A/1 *cys met*; 1.87 Mb, 1.81 Mb, 1.20 Mb, 1.16 Mb and 1.09 Mb for *C. shehatae* 492 *met his* and 1.90 Mb, 1.58 Mb, 1.24 Mb, 1.20 Mb, 0.93 Mb and 0.84 Mb for *P. stipitis* and the fusant strains.

Colour detection of the TAFE gel, probed with the *XYL*1 gene, indicated that this gene was located on chromosome II (Fig. 2.5) for the *P. stipitis* parent and the fusants. At this hybridisation and washing temperature of 68°C, a very faint signal was obtained for chromosome I for *C. shehatae* 492 *met his* and no signal was obtained for the *C. shehatae* 117 A/1 *cys met* parent. Two faint signals evident in lanes 6 and 8 were most probably due to binding of the probe to fragments of sheared chromosomal DNA carrying the *XYL*1 gene. A less stringent hybridisation and washing temperature of 45°C was employed for the *XYL2* gene probe in an attempt to obtain signals for the *C. shehatae* strains, since they did not have the same degree of homology with the *XYL*1 probe as *P. stipitis* and the fusants. A faint signal was obtained for the *P. stipitis* parent only (Fig. 2.7). Signals were not obtained for either the fusants or the *C. shehatae* parental strains. These hybridisation conditions were repeated using the *XYL2* gene probe against a blot of the gel in Fig. 2.6 and the same results were obtained, i.e., a faint signal for the *P. stipitis* parent only.

## 2.3.3 RFLP ANALYSIS OF CHROMOSOMAL DNA

Intact chromosomal DNA embedded in agarose plugs was cleaved with various restriction endonucleases and the resulting fragments separated on agarose gels. All of the enzymes used cleaved the chromosomal DNA to produce a smear of fragments smaller than 20 kb. However, interposed in this streak were a few high intensity bands. The general pattern that emerged was that all fusants and the *P. stipitis* parent possessed identical profiles. Both *C. shehatae* parental strains displayed almost identical profiles but differed from the fusants and *P. stipitis*.

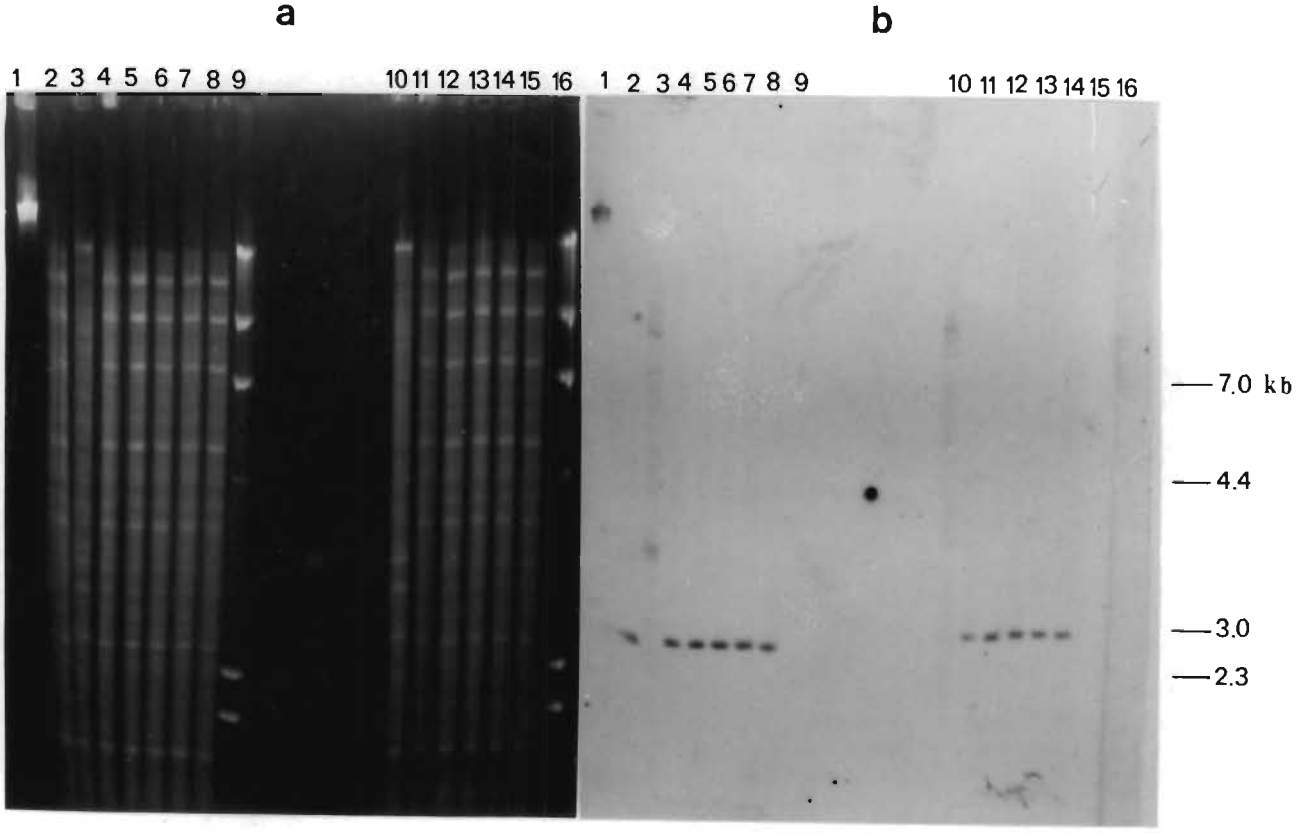



**Fig. 2.8.** RFLP profiles of *Eco*RI-digested chromosomal DNA of parental and fusant strains (a) and hybridisation with the *XYL1* probe (b). Lane 1: uncleaved DNA, lane 2: *P. stipitis* 633 *leu lys ala*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5, lanes 9 and 16: Molecular Weight Marker II (Boehringer Mannheim), lane 10: *C. shehatae* 117 A/1 *cys met* and lanes 11-15: PCA1-PCA5.

Cleavage with *Eco*RI produced seven discrete, high intensity bands for *P. stipitis* and the fusant strains (Fig. 2.8), as well as five bands for *C. shehatae* 117 A/1 *cys met* and *C. shehatae* 492 *met his*. As expected, a smear of fragments ranging in size from 20 kb to a few hundred base pairs was also evident. Restriction with *Xba*I also produced seven high intensity bands for *P. stipitis* and the fusants, but only four for both the *Candida* strains (Fig. 2.9). *Hin*dIII cleavage revealed a slight degree of polymorphism between the

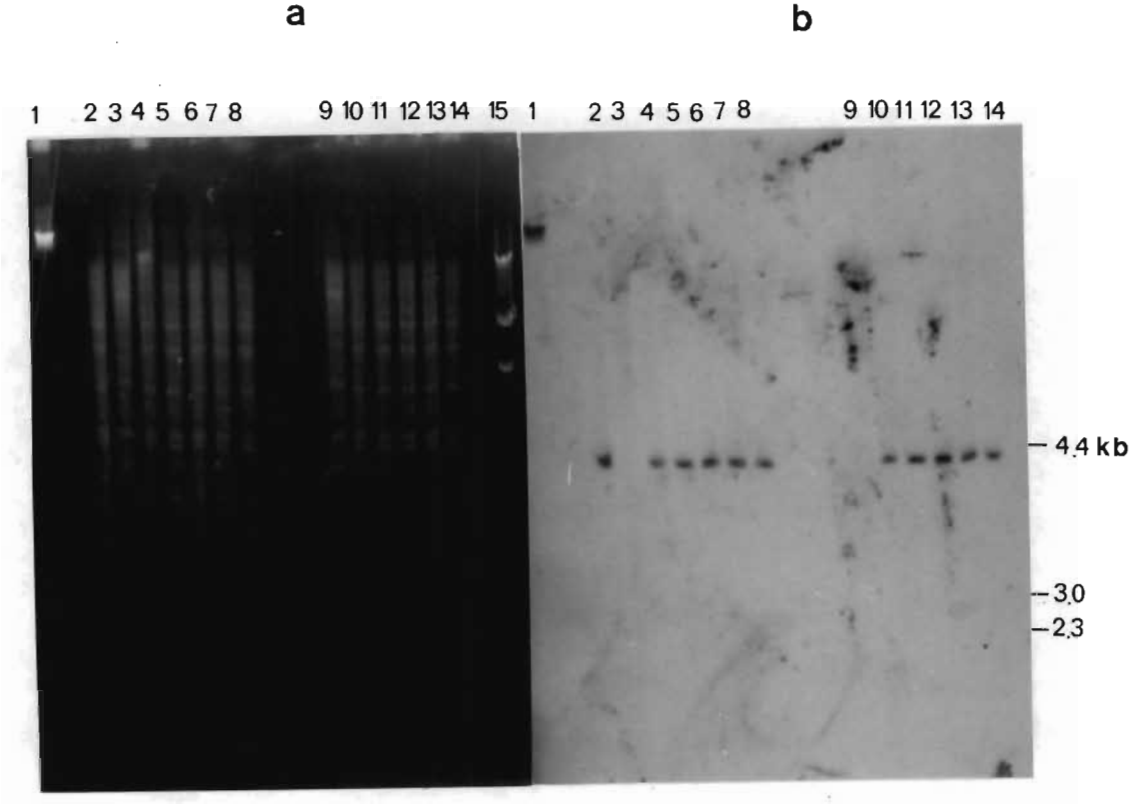



**Fig. 2.9.** RFLP profiles of *Xba*I-digested chromosomal DNA of parental and fusant strains (a) and hybridisation using the *XYL*1 probe (b). Lane 1: uncleaved DNA, lane 2: *P. stipitis* 633 *leu lys ala*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5, lane 9: *C. shehatae* 117 A/1 *cys met*, lanes 10-14: PCA1-PCA5 and lane 15: Molecular Weight Marker II (Boehringer Mannheim).

*Candida* parental strains (Fig. 2.10). With this restriction enzyme, *C. shehatae* 492 *met his* had a DNA profile that was more similar to *P. stipitis* and the fusants than to *C. shehatae* 117 A/1 *cys met*. Again, seven high intensity bands were evident for *P. stipitis* and the fusants but only three were observed for the *Candida* parents. *Dra*I cleavage of chromosomal DNA resulted in larger restriction fragments compared to fragments produced by the other three restriction endonucleases. *Dra*1 is considered to be a "rare-cutting" enzyme and would thus produce fewer and therefore larger fragments.


**Fig. 2.10.** RFLP profiles of *Hin*dIII-digested chromosomal DNA of parental and fusant strains. Lane 1: uncleaved DNA, lane 2: *P. stipitis* 633 *leu lys ala*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5, lane 9: *C. shehatae* 117 A/1 *cys met* and lanes 10-14: PCA1-PCA5.

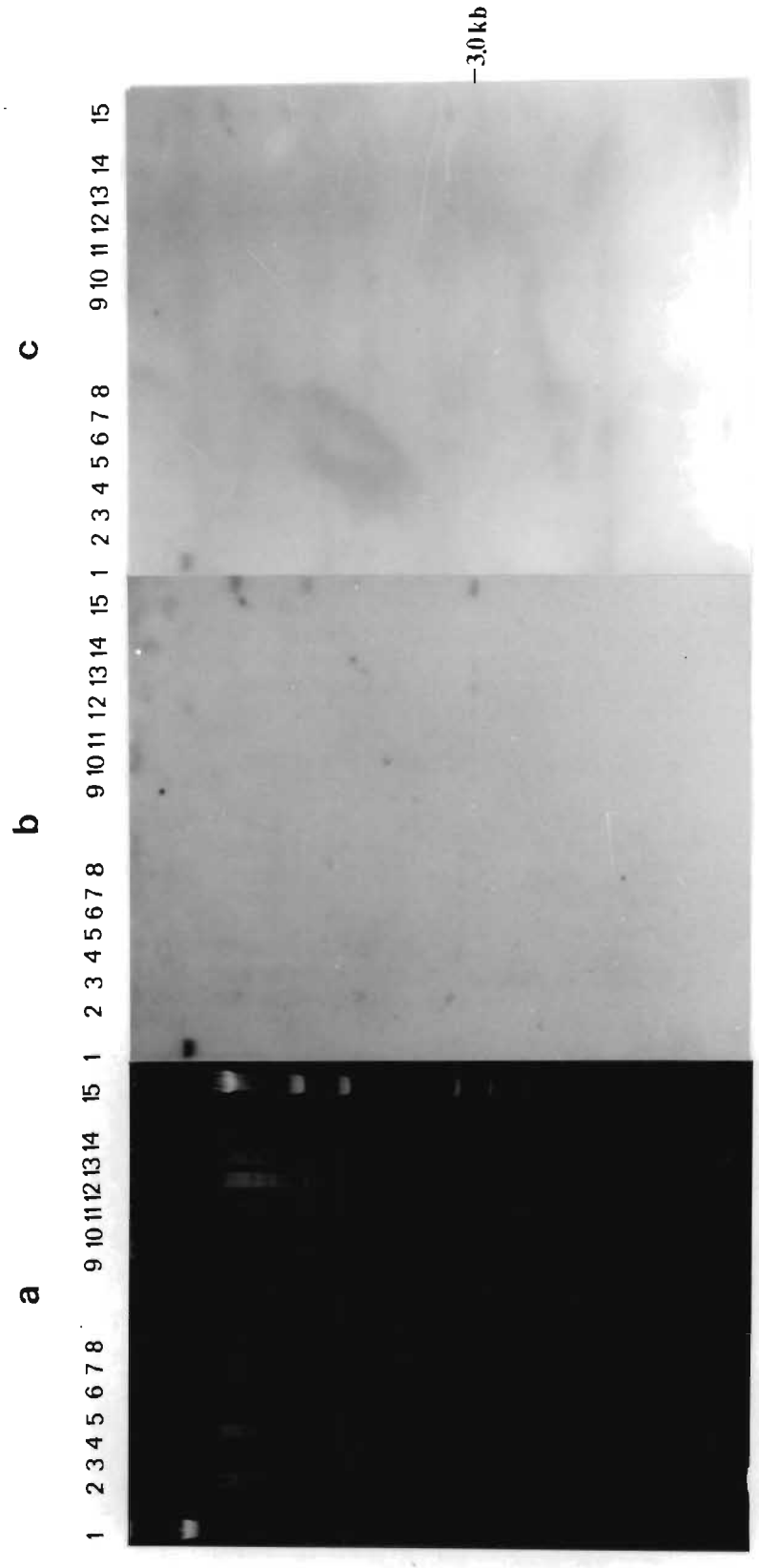



**Fig. 2.11.** RFLP profiles of *Dra*I-digested chromosomal DNA of parental and fusant strains (a) and hybridisation using the *XYL*1 (b) and *XYL2* probes (c). Lane 1: uncleaved DNA, lane 2: *P. stipitis* 633 *leu lys ala*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5, lane 9: *C. shehatae* 117 A/1 *cys met*, lanes 10-14: PCA1-PCA5 and lane 15: Molecular Weight Marker IV (Boehringer Mannheim).

The largest of the seven bright bands for *P. stipitis* and the fusants was approximately 4 kb (Fig. 2.11), which was much smaller than the largest band obtained with the other enzymes.

Hybridisation of cleaved chromosomal DNA with the *XYL*1 gene probe resulted in an identical signal for the fusants and *P. stipitis*. Only one bright signal was evident for these strains for all of the DNA profiles generated. For the RFLP profile generated by *Eco*RI, a signal occurred at approximately 3 kb for *P. stipitis* and the fusants. Both the *Candida* parental strains produced several fainter signals at 7.0, 5.8 and 5.7 kb for *C. shehatae* 492 *met his* and 7, 6.6, 5.8 and 5.7 for *C. shehatae* 117 A/1 *cys met* (Fig. 2.8). None of the signals corresponded to a high intensity band on the gel. The *Xba*I-cleaved chromosomal DNA profile produced a single signal for all strains. The signal obtained for *P. stipitis* and the fusants was very bright compared to that of the *Candida* strains indicating that the probe was more specific for the *XYL*1 gene of *P. stipitis* than that of *C. shehatae*. These signals corresponded to *Xba*I fragments of $>9.4$ kb for *C. shehatae* 117 A/1 *cys met* and *C. shehatae* 492 *met his* and approximately 4 kb for the fusants and *P. stipitis*, respectively (Fig. 2.9). Chemiluminescent signals obtained using the *XYL*1 probe for *Dra*I-cleaved (Fig. 2.11) and *Hin*dIII-cleaved (not shown) chromosomal DNA were very faint, even for *P. stipitis* and the fusants. This low level of signal intensity may be due to several successive stripping and re-probing steps performed when hybridisation conditions were being established. For both these blots, as well, only one signal was obtained for *P. stipitis* and the fusants as well as *C. shehatae* 492 *met his*.

The *XYL*2 gene only hybridised to *P. stipitis* DNA (Fig. 2.11). Signals were not

obtained, either for the fusants or the *C. shehatae* strains.

## 2.3.4 POLYACRYLAMIDE GEL ELECTROPHORESIS

The electrophoretic profiles of the total soluble cell proteins of the fusant and parental strains produced a similar trend to that observed for the profiles generated by TAFE and RFLPs of chromosomal DNA, i.e., the fusants and the *P. stipitis* strain displayed identical profiles, while the *C. shehatae* strains had similar profiles. *P. stipitis* and the fusants differed from both *C. shehatae* strains at several points on the gel (Fig. 2.12).

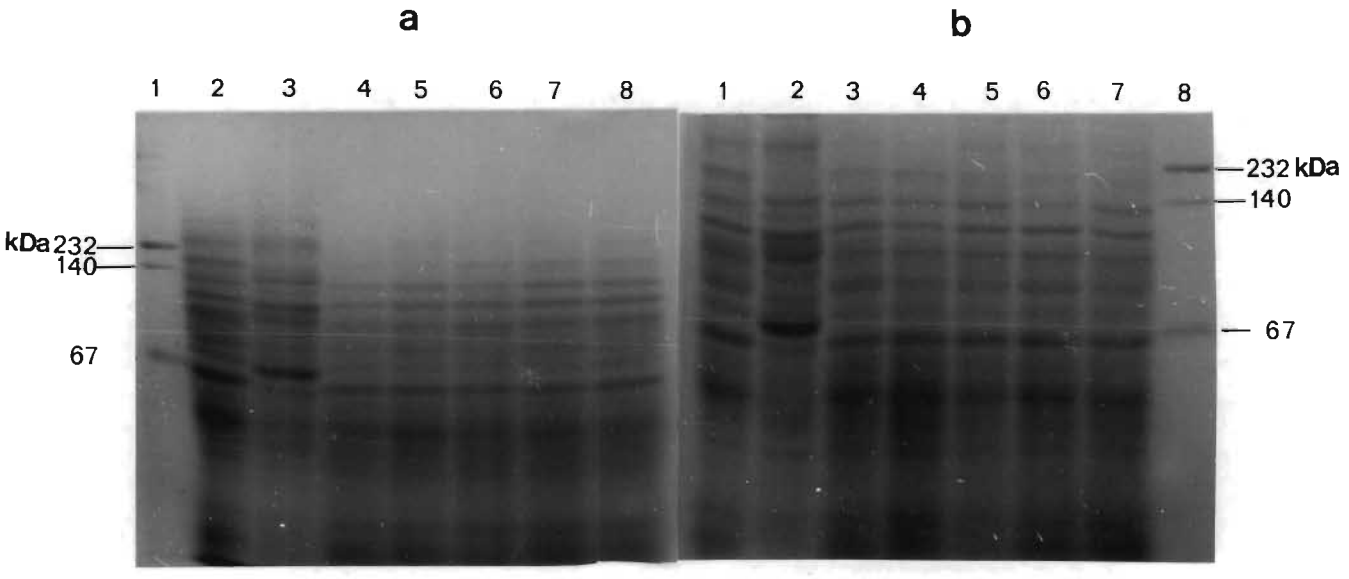



**Fig. 2.12.** SDS-PAGE profiles of total soluble cell proteins of *P. stipitis*, *C. shehatae* and their fusants. (a) Lane 1: High Molecular Weight Protein Standard (Pharmacia), lane 2: *P. stipitis* 633 *leu lys ala*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1-PC5; (b) Lane 1: *P. stipitis* 633 *leu lys ala*, lane 2: *C. shehatae* 117 A/1 *cys met*, lanes 3-7: PCA1-PCA2 and lane 8: High Molecular Weight Protein Standard (Pharmacia).

A large number of low molecular weight proteins with small size differences were evident at the bottom of the gel (dark, diffuse region), whereas fewer, discrete and relatively less abundant high molecular weight proteins were noticed at the top of the gel. Most of the protein bands were common to all strains. These bands were probably due to proteins common to yeast strains, whereas the bright band of approximately 70 Da was common to both *C. shehatae* strains and therefore appeared to be specific to these organisms.

## 2.4 DISCUSSION

Since the introduction of electrophoretic techniques for separating intact chromosomal molecules of lower eucaryotes by Schwartz and Cantor (1984), several refinements of this technique have enabled the electrophoretic karyotyping of a large number of fungi, trypanosomes, *Plasmodium* and *Giarata*. Based on the sizes of the *S. cerevisiae* chromosomes, the molecular weights of the chromosomes of both parental and fusant strains were established. *P. stipitis* possessed six chromosomes (Fig. 2.7) which corresponded to those reported by Passoth *et al.* (1992). The number and sizes of the chromosomes of the fusants was identical to those of *P. stipitis*. Analysis of TAFE gels allowed not only the differentiation of the *P. stipitis* and *Candida* parental strains, but also effectively resolved the *Candida* chromosomes to show differences between these two strains. Passoth *et al.* (1992) reported that a remarkable degree of polymorphism existed among the five strains of *C. shehatae* studied with regard to their electrophoretic karyotype. They reported the presence of five to six chromosomes for *C. shehatae* strains

ranging in size from 1.25 to 3.5 Mb. In this study, both *Candida* strains possessed five chromosomes whose sizes were estimated to be 2.15 Mb, 1.67 Mb, 1.21 Mb, 1.15 Mb and 1.11 Mb for *C. shehatae* 117 A/1 *cys met* and 1.87 Mb, 1.81 Mb, 1.20 Mb, 1.16 Mb and 1.09 Mb for *C. shehatae* 492 *met his*. For the larger chromosomes, a longer run time coupled with a lower voltage was effective for resolution of these molecules.

Other workers (Miller *et al.*, 1989; Viljoen *et al.*, 1988) reported the separation of the *P. stipitis* genome into only three chromosomes, using shorter run times and higher voltages. During the course of this work, we have reported the presence of four chromosomes for *P. stipitis* using short run times and higher voltages (Selebano *et al.*, 1993). The increased resolution of the genome of *P. stipitis* into six chromosomes with the lengthening of run time and the decrease of voltage clearly demonstrated that these run conditions are required for the optimal resolution of large chromosomes. D'Souza *et al.* (1993) also attributed the superior resolution of large chromosomes with small size differences between them, to the use of longer run times but also suggested ramped pulse intervals instead of discrete switching times.

Several reporters used electrophoretic karyotyping successfully to analyse genetic hybrids (van der Westhuizen and Pretorius, 1992) and fusion products (Hoffman *et al.*, 1987). Provided that the parental strains involved in either protoplast fusion or single spore matings possess different electrophoretic karyotypes, it should be possible to characterise fusants/hybrids regarding the origin of their chromosomes as well as changes in chromosome lengths as compared to those of the parental strains. In this study the parental strains involved in the fusion possessed different electrophoretic karyotypes.

However, the fusants appeared to have inherited the chromosomes of *P. stipitis*. Nuclear DNA-DNA homology analysis of these fusants showed that they possessed 74% to 86% homology with the *P. stipitis* parent (Selebano *et al.*, 1993) and are therefore predominantly comprised of *Pichia* DNA. That the fusants should possess most of the chromosomes of the *P. stipitis* parent was therefore expected. However, TAFE revealed that the fusants inherited their chromosomes entirely from the *P. stipitis* parent. In the fusion between *Candida tropicalis* and *Saccharomyces fibuligera*, Provost *et al.* (1978 - cited by Gupthar and Garnett, 1987) suggested that some genes of one parent can become associated with the genome of the other. It would therefore appear that in this study, fusion led to the integration of *Candida* genes into the chromosomes of *P. stipitis*. These fusants are assumed to be the result of recombinational events and not parental dissociates since mitotic and meiotic segregation experiments of the prototrophic fusants led to the isolation of recombinant phenotypes (Gupthar and Garnett, 1987). The integration of a few genes, which would be in the order of $\approx$ 1 to 10 kb, is unlikely to drastically alter the migration of large chromosomes. Hence, in the case of partial hybrids, electrophoretic karyotyping is not sufficient to differentiate between fusant and parental strains.

It was therefore decided to employ RFLP analysis of the entire genomic DNA to visualise differences due to gene insertion in the *P. stipitis*-like fusants. Chromosomal DNA embedded in agarose plugs was used in the RFLP analysis as it was considered more accurate to use intact DNA rather than genomic DNA that would have undergone some degree of shearing during the isolation procedure. Because of its large size, yeast chromosomal DNA possesses numerous sites for most of the restriction endonucleases, even those termed "rare-cutters". When chromosomal DNA is cleaved by restriction

endonucleases, a whole range of different sized fragments are generated, producing a smear on an agarose gel. In this study, all four restriction endonucleases produced a more or less uniform smear, except *Dra*I, which produced a higher proportion of large fragments than smaller ones. This could have been the result of incomplete cleavage or that this enzyme did not have as many recognition sites on the chromosomal DNA as the other three restriction endonucleases employed. The latter conclusion is more likely since *Dra*I is considered to be a "rare-cutter". It was not possible, however, to distinguish between the fusants and the *P. stipitis* parental strain using RFLP analysis. The interpretation of electrophoretic banding patterns of chromosomal DNA restriction fragments is therefore complicated because discrete fragments are generally not apparent unless they are derived from repeated sequences (Meaden, 1990).

All restriction endonucleases employed, successfully cleaved the chromosomal DNA to produce a uniform smear with a few high intensity bands. These high intensity bands were due to fragments of the same size generated by the cleavage of repeated sequences, most likely to be ribosomal or mtDNA (Bostock *et al.*, 1993). Therefore, it was not expected that the probes should hybridise to these high intensity bands since they represent repeated ribosomal sequences previously confirmed by hybridisation using ribosomal RNA gene probes. Differentiation between the fusants and the *P. stipitis* strain was not possible based on the RFLP profiles. Thus these results confirm the conclusion arrived at by Meaden (1990) that direct analysis of DNA restriction fragments was limited in the information which it could provide. RFLP analysis did, however, permit the differentiation between the genera *Candida* and *Pichia*. Morace *et al.* (1992) performed RFLP analysis on several *Pichia* isolates and showed that in most cases the profiles were

species-specific. Van der Westhuizen and Pretorius (1992) succeeded in fingerprinting wine strains as well as differentiating hybrid and parental *S. cerevisiae* strains using RFLPs of genomic DNA.

Although Meaden (1990) concluded that any attempt to fingerprint yeast strains by DNA probing was best approached by using a variety of probes and restriction endonucleases until a combination that suited the investigator's needs was found, only two probes were used in this study. They were chosen on the basis of the amount of information such an exercise would yield with regard to gene copy number of the initial enzymes in the xylose metabolic pathway. These fusants were constructed in the hope of obtaining superior D-xylose-fermenters. The first two enzymes in the D-xylose metabolic pathway were thus chosen as probes. It was anticipated that probing with these genes would shed some light as to the ploidy of these fusants with regard to these genes.

The hybridisation experiments demonstrated that the *XYL*I gene in the fusants was identical to that of the *P. stipitis* parent. Only this gene was present, with no apparent contribution from the *Candida* parental strains. The *XYL*1 gene probe not only hybridised to the same chromosome (Fig. 2.5), but a signal of approximately the same intensity and corresponding to a fragment of an identical size was apparent for all the fusants and the *P. stipitis* parent. For all the restriction profiles, the signal appeared at a different location for the *Candida* parents. However, the restriction profile generated by *Eco*RI produced three signals for both the *Candida* strains. This was probably due to the presence of *Eco*RI sites within the *XYL*1 gene and therefore three fragments were generated giving rise to three positive signals. It is also possible that the signal

corresponding to the heaviest fragment was due to incomplete cleavage of the DNA.

The *XYL2* probe hybridised to *P. stipitis* DNA only. Both probes were derived from a strain of *P. stipitis* (Kötter *et al.*, 1990) and therefore hybridised to the *P. stipitis* DNA even under very stringent conditions. The *XYL*1 gene of *C. shehatae* only hybridised to the probe under low stringency conditions. The *XYL2* gene probe produced no signal with either the *C. shehatae* or fusant DNA. The *XYL2* gene of *C. shehatae* therefore has very little homology with the *P. stipitis XYL2* gene. If the absence of a *XYL2* signal was universal, then one could conclude that the sequence of the probe gene had very little homology with that of the strains under investigation. However, since a positive signal was obtained for *P. stipitis* only, it could be construed that the fusants possessed the *XYL2* gene originating from the *C. shehatae* parental strains. This cannot be accepted as proof that this gene originated from the *C. shehatae* parent, since very weak signals were obtained for *P. stipitis*. The *P. stipitis* stain should have produced a stronger signal since the probe was obtained from a *P. stipitis* strain.

Protein profiles generated by SDS-PAGE were not effective for differentiating the fusants from the *P. stipitis* parental strain, although they did prove useful in distinguishing between *P. stipitis* and *C. shehatae*. Van der Westhuizen and Pretorius (1992) successfully used SDS-PAGE to differentiate between hybrid and parental strains in a breeding programme for wine strains. This technique has proved to be effective in characterising true hybrid strains that possess a combination of the unique properties of either parental strain. Since both parental strains of the fusants in this study displayed very few differences, characterisation of the fusants was difficult using protein

electrophoretic profiles. Van Vuuren and van der Meer (1987) used electrophoretic profiles of total soluble cell proteins to fingerprint 29 *S. cerevisiae* strains and found this technique to be useful to group and identify closely related yeast strains.

The recent development of the random amplified polymorphic DNA (RAPD) technique has allowed rapid fingerprinting of bacterial, plant, animal and fungal species (Welsh and McClelland, 1990; Williams *et al.*, 1990). Williams *et al.* (1990) proposed that hybrid cell lines carrying deletions or additions of large chromosomal segments could be screened relative to appropriate controls to identify the region of the genome carrying the deletions or additions. Similarly, this technique could be applied to identify DNA sequences in the fusants that originate from the *C. shehatae* parent. Bostock *et al.* (1993) performed a comparative study of fingerprinting techniques using RAPDs, PFGE and RFLPs to obtain a molecular typing system for *C. albicans* and concluded that RAPD was quick, reproducible, economical and provided a degree of discrimination approaching PFGE. This technique therefore would seem the technique of choice to characterise fusant/hybrid strains.

# CHAPTER THREE

# CYTOPLASMIC INHERITANCE OF THE FUSANTS OF *Pichia stipitis* AND *Candida shehatae*

## 3.1 INTRODUCTION

mtDNA, because of its relatively small size can be studied in its entirety and is therefore an appealing molecule for evolutionary study. The mitochondrial genome of *S. cerevisiae* has been studied in great detail and the entire genome sequenced (Taylor, 1986).

Fungal mtDNAs are variable in size, ranging from 18.9 kb in *Torulopsis glabrata* (Clark-Walker and Sriprakash, 1981) to 176 kb in *Agaricus bitorquis* (Hinze *et al.*, 1985) whereas animal mitochondrial genomes are consistently small (16-19 kb). Despite their size differences both animal and fungal mtDNAs carry nearly the same genes. However the size difference between animal and fungal mtDNAs and the variability among fungal mtDNAs is due to deletions and insertions (Taylor, 1986).

Over 95% of mitochondrial proteins are encoded by nuclear genes. More than 200 complementation groups corresponding to as many nuclear genes necessary for mitochondrial function or biogenesis have been described (Bolotin-Fukuhara and Grivell, 1992). mtDNA codes for enzymes or their subunits involved in electron transport and

phosphorylation to produce ATP (cytochrome C oxidase, apocytochrome b, oligomycin-sensitive ATPase), RNAs and proteins required for protein synthesis (*var*-1 ribosomal protein, S-5 ribosomal protein, S and L- rRNA and at least 25 tRNA's) as well as a sizeable number of unidentified open reading frames (Taylor, 1986).

Fungal mitochondrial genomes are usually circular. Linear exceptions include the slime mould *Physarum polycephalum* (Kawano *et al.*, 1982 - cited by Taylor, 1986) and *Hansenula mrakii* (Fukuhara, 1981 - cited by Taylor, 1986). Recently Fukuhara *et al.* (1993) reported on the frequency, occurrence and general features of linear mtDNAs of yeasts, particularly in the genera *Pichia* and *Williopsis*. They found that the frequency of linear mtDNA was higher than that originally reported. Of 58 species studied they reported 13 that possess linear mtDNA. They used the technique of PFGE for preliminary identification of linear/circular mtDNA, later confirmed by restriction site mapping and electron microscopy. They found 16 presumed linear mtDNAs using PFGE of which 13 were later confirmed to be linear after restriction site mapping.

In animals, mtDNA is inherited maternally without recombination. In fungi uniparental mtDNA inheritance from the maternal parent has been demonstrated (Mitchell and Mitchell, 1952). This was later confirmed by other workers, notably Manella *et al.* (1979) who used mtDNA marked with different length mutations. In the interspecific cross between *Allomyces macrogynus* and *A. arbusculus*, Borkhardt and Olson (1983) used RFLPs to demonstrate that mtDNA is inherited paternally. Uniparental mitochondrial inheritance is however not the rule in fungi as it is in animals. The mitochondrial genome in *S. cerevisiae* has long been known to undergo recombination (Borst and Grivell, 1978 -

cited by Taylor, 1986). Recombination of mtDNAs in forced heteroplasmons of two varieties of *Aspergillus nidulans* was demonstrated by Earl *et al.* (1981) using RFLP analysis. Similar results were reported by Baptista-Ferreira *et al.* (1983) for *Coprinus cinereus* where hyphal fusion precedes part of the mating. They also report that recombination is not random; certain features unique to each parental molecule (variable insertions in genes and regions flanking the genes) are nearly always present in the recombinant molecules. Finally, in 1985 (Silliker, 1985 - cited by Taylor, 1986) demonstrated biparental mtDNA inheritance in the plasmodial slime mould, *Didymium iridis*.

The presence of mitochondrial plasmid DNAs have been reported by various researchers: In 1991, Meyer reported the presence of mitochondrial plasmids ranging in size from 2.0 - 4.4 kb in 8 of 12 *Trichoderma viride* isolates; in 1989, May and Taylor and in 1990, Collins and Saville reported their presence in *Neurospora crassa* isolates. The patterns of the plasmids in *T. viride* ranged from single bands to complex ladder-like patterns. Restriction endonuclease cleavage of these ladder-like patterns produced a single band, thus proving that the many bands represent different forms of a single molecule rather than many different plasmids. They also demonstated that different strains that have similar plasmids do not have similar mtDNA restriction patterns. This suggests that mtDNA and plasmids have evolved independently or that plasmids were transferred horizontally. The horizontal transfer of plasmids independent of the mtDNA has been reported to occur under laboratory conditions among *Neurospora* isolates (Collins and Saville, 1990; May and Taylor, 1989). May and Taylor (1989) also reported that plasmids are inherited paternally but mtDNA is inherited maternally in *N. crassa*.

Most plasmids have no homology with mtDNA which implies that they are not derived from mtDNA. Preliminary data indicates that there is no homology with nuclear DNA. Scazzocchio (1987) speculates that such plasmids represent means by which gene sequences may transfer from one compartment to another within a species or even between species. In *kalilo* strains of *N. intermedia* the insertion of a 9 kb element that exists as a free plasmid in the nucleus into the mitochondrial genome is directly related to the onset of senescence in these strains (Bertrand *et al.*, 1985 - cited by Scazzocchio, 1987). In *Podospora anserina*, the presence of a number of plasmids which originate from a limited number of regions of the mitochondrial genome is responsible for the onset of the senescence phenomenon (Wright *et al.*, 1982).

Our objectives in this study were to use the RFLP technique in order to generate profiles for both parental and fusant strains. A comparative study of these profiles would give an indication of the type of mitochondrial inheritance that occured during the protoplast fusion process. It would also enable us to determine whether transmission of the mitochondrial genome occurs with the absence or presence of recombinational events.

## 3.2 MATERIALS AND METHODS

### 3.2.1 ISOLATION OF MITOCHONDRIAL DNA

Several methods for the isolation of mtDNA were attempted. The first method involved the mechanical disruption (grinding, sonication, French pressure cell) of yeast

cells followed by the isolation of mitochondria on a cesium chloride-bis-benzimide gradient. The second method involved the enzymatic hydrolysis of the cell wall, isolation of mitochondria by differential centrifugation followed by lysis of the mitochondrial membrane and isolation of mtDNA. The third method involved the enzymatic hydrolysis of the cell wall followed by isolation of mitochondria on a sucrose density gradient and lysis of the mitochondria and isolation of mtDNA.

However, the following modified method was used to isolate mtDNA used in the restriction reactions. It is a combination of the methods of Defontaine *et al.* (1991) and Querol and Barrio (1990).

| | | |
|---|---|---|
| Washing solution | : | 1.2 M Sorbitol |
| | | 50 mM EDTA |
| | | 2% Mercaptoethanol (v/v) |
| Solution A | : | 0.5 M Sorbitol |
| | | 10 mM Tris-HCl (pH 7.5) |
| Solution 1 | : | 20% Sucrose |
| | | 10 mM Tris-HCl (pH 7.5) |
| | | 0.1 mM EDTA (pH 7.5) |
| Solution 2 | : | 60% Sucrose |
| | | 10 mM Tris-HCl (pH 7.5) |
| | | 0.1 mM EDTA (pH 7.5) |
| Solution 3 | : | 50% Sucrose |
| | | 10 mM Tris-HCl (pH 7.5) |

Solution 4 : 44% Sucrose

10 mM Tris-HCl (pH 7.5)

Solution 5 : 10 mM Tris-HCl (pH 7.5)

1mM EDTA

50 mM NaCl (pH 7.5)

Solution B : 100 mM NaCl (pH 7.5)

1% N-lauroyl sarcosine (w/v)

50 mM Tris-HCl (pH 7.5)

10 mM EDTA (pH 7.5)

5 M potassium acetate

mtDNA from each of the parental and fusant strains was isolated from 1 l of a 24 h culture grown in YMB at 30°C with vigorous agitation. Cells were harvested at 3 800 × *g* for 10 min at 4°C. The pellet was washed twice in sterile distilled water, once in the washing solution, resuspended in solution A (5 ml solution A/ 0.3-0.4 g wet weight) containing 2% mercaptoethanol and 0.5 mg/ml Novozyme and incubated at 37°C for 45 min with agitation (100 rpm) on an orbital shaker. Most of the formed sphaeroplasts were osmotically lysed at this step. The cellular lysate was centrifuged at 3 800 × *g* for 20 min at 4°C to pellet cell debris and nuclei. The supernatant containing mitochondria was centrifuged at 15 000 × *g* for 15 min at 4°C and the crude mitochondrial pellet washed once with 5 ml of solution 1. After centrifugation as above the mitochondrial pellet was resuspended in 2 ml of solution 2, transferred to polyallomer tubes, overlaid sequentially with 4 ml of solution 3 and 4 respectively and centrifuged for 90 min at 285 000 × *g* at 4°C in a Beckman SW 41 rotor. Mitochondria formed a discrete band at the interface of

solutions 3 and 4. Mitochondria were collected by puncturing the tubes with a syringe, diluted in 4 ml of solution 5 and the suspension was centrifuged at 15 000 × *g* for 20 min at 4°C. The mitochondrial pellet was resuspended in 2 ml solution B, allowed to lyse at RT for 30 min followed by the addition of 0.5 ml potassium acetate, incubation at -20°C for 15 min and centrifugation at 17 000 × *g* at 4°C for 20 min. The supernatant was transferred to polypropylene centrifuge tubes (JA 20.1, Beckman, USA) and the mtDNA precipitated by the addition of 1 volume isopropanol. After incubation at RT for 10 min, the mtDNA was pelleted by centrifuging at 17 000 × *g* for 10 min at RT. The DNA was washed once in 70% ethanol, vacuum dried and dissolved in 200 µl TE and stored at -20°C.

### 3.2.2 RESTRICTION OF MITOCHONDRIAL DNA

mtDNA was cleaved with restriction endonucleases as described in section 2.2.4.2.1 The following restriction endonucleases were used: *Eco*RI, *Hin*dIII, *Eco*RV, *Bam*HI, *Pst*I.

### 3.2.3 AGAROSE GEL ELECTROPHORESIS

Restriction digests were separated on a 0.7% agarose gel at 100 V for 5 - 6 h, stained in ethidium bromide, viewed on a UV transilluminator and photographed.

## 3.3 RESULTS

### 3.3.1 AGAROSE GEL ELECTROPHORESIS

Various methods of isolation of mtDNA were attempted. These include: (a) mechanical disruption (grinding, sonication, french pressure cell) of the yeast cells and the isolation of mtDNA on a cesium-chloride bis-benzimide density gradient; (b) enzymatic hydrolysis of the yeast cell wall and membrane, the isolation of mitochondria by differential centrifugation followed by the lysis of the mitochondrial membrane and the precipitation of mtDNA and (c) enzymatic hydrolysis of the cell wall and membrane followed by the isolation of mitochondria on a sucrose gradient and then lysis of the mitochondrial membrane and precipitation of mtDNA. The first two methods were found to be unsuitable. The first method (a) produced mtDNA with a high level of chromosomal DNA contamination and the second was too tedious because of the many centrifugation steps involved. The third method was finally employed and produced mtDNA with very little chromosomal contamination (Fig. 3.1). In addition to the band corresponding to mtDNA, 2 other bands were present for the *Pichia* parent and all the fusant strains. These were presumed to be mitochondrial plasmid DNA.

### 3.3.2 RFLP ANALYSIS

Mitochondrial DNA was cleaved with several restriction endonucleases: *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Bam*HI and the restriction digests separated by agarose gel electrophoresis.

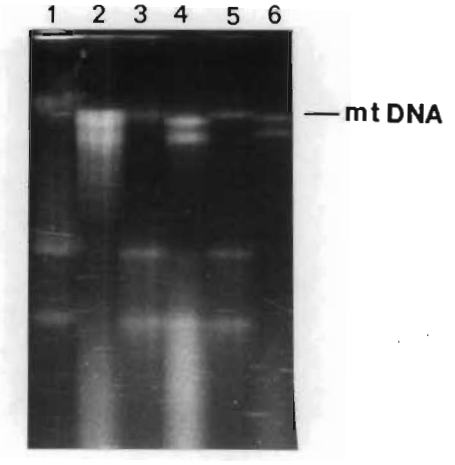



**Fig. 3.1.** Agarose gel electrophoresis of restricted and unrestricted mtDNA of parental and fusant strains. Lane 1: *P. stipitis* 633 *leu lys ala*, unrestricted; lane 2: *P. stipitis* 633 *leu lys ala* restricted with *Pst*I; lane 3: PC1, unrestricted; lane 4: PC1 restricted with *Pst*I; lane 5: PCA1, unrestricted; lane 6: PCA1, restricted with *Pst*I.

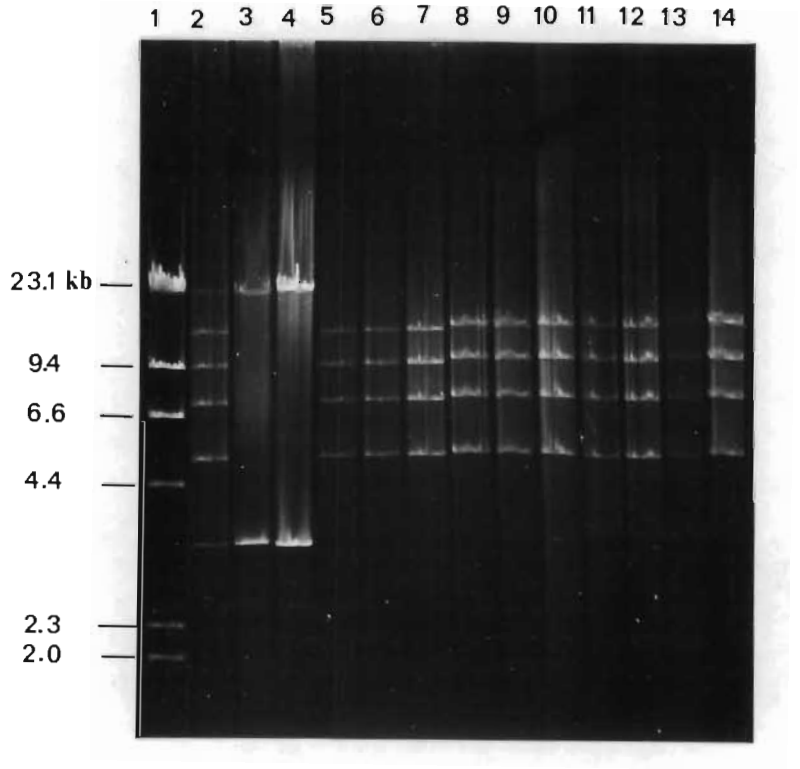



**Fig. 3.2.** RFLP profiles of mtDNA of parental and fusant strains generated by the restriction endonuclease *Eco*RI. Lane 1: Molecular Weight Marker II (Boehringer Mannheim), Lane 2: *P. stipitis* 633 *leu lys ala*, Lane 3: *C. shehatae* 117 A/1 *cys met*, Lane 4: *C. shehatae* 492 *met his*, Lanes 5-9: PC1 - PC5 and Lanes 10-14: PCA1 - PCA5.

Restriction with *Eco*RI produced 4 identical bands for the *P. stipitis* parent and the fusant strains ranging in size from 5 to 13 kb. *Eco*RI cleavage of mtDNA from both *C. shehatae* parents produced a different profile consisting of only 2 bands (Fig 3.2). The sizes of these 2 bands are approximately 23.1 and 3.1 kb. *P. stipitis* apparently has two extra bands not common to the fusants. This is an artefact due to overloading (spilling over) from the well adjacent to it. After *Eco*RI cleavage of the mtDNA, mitochondrial plasmids were no longer observed, indicating that they were cleaved into smaller fragments. Cleavage with *Hin*dIII also produced an identical banding pattern for the *P.*

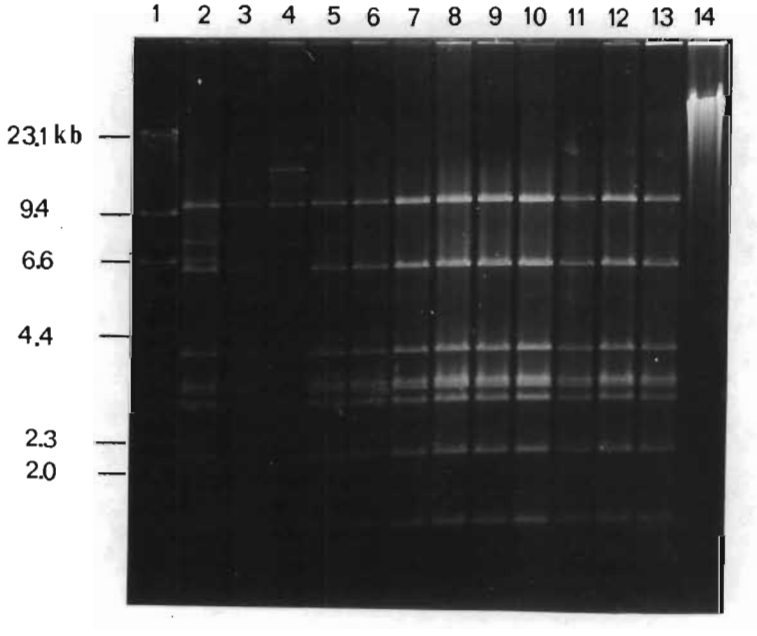



**Fig. 3.3.** RFLP profiles of mtDNA of the parental and fusant strains restricted with *Hin*dIII. Lane 1: Molecular Weight Marker II (Boehringer Mannheim), Lane 2: *P. stipitis* 633 *leu lys ala*, Lane 3: *C. shehatae* 117 A/1 *cys met*, Lane 4: *C. shehatae* 492 *met his*, Lanes 5-9: PC1 - PC5 and Lanes 10-14: PCA1 - PCA5.

*stipitis* parent and the fusants (Fig. 3.3). Eight bands ranging in size from 1 to 10 kb were observed. The mitochondrial plasmid DNA was cleaved by *Hin*dIII. Only 2 bands, both greater than 10 kb were present for the *Candida* parental strains. Double digests with *Eco*RI/*Pst*I (Fig. 3.4) as well as with *Eco*RV/*Bam*HI (not shown) revealed a similar pattern, viz., identical RFLP profiles for the *Pichia* parent and fusant strains. Mitochondrial plasmid DNA is not cleaved by these enzymes. The minor bands in lanes 2, 10, 11 and 12 represent partially cleaved mtDNA.

The *C. shehatae* strains showed differences in restriction profiles for some of the restriction endonucleases and also possessed fewer recognition sites than *P. stipitis* for the same enzymes.

Based on comparisons of the number of fragments obtained with single and double digests we deduced that the mtDNA has a circular form. Single digests of *P. stipitis* mtDNA with *Eco*RV and *Pst*I produced three and two bands, respectively. Double digests with these two enzymes produced five bands. If the mtDNA were linear, *Eco*RV must have two cleavage sites in order to produce three bands and *Pst*I one recognition site in order to produce two bands. The total number of cleavage sites with these two enzymes would therefore be three. This would result in a mtDNA restriction profile containing only four bands for these two enzymes. However, the profile in Fig. 4 shows five bands, suggesting that the mtDNA is not linear. Circular mtDNA on the other hand must have one additional cleavage site in order to produce the same number of bands as linear DNA. The total number of cleavage sites and DNA fragments predicted for a circular molecule i.e., five, corresponds to the profile shown in Fig. 3.4. This suggests that the mtDNA

has a circular form.

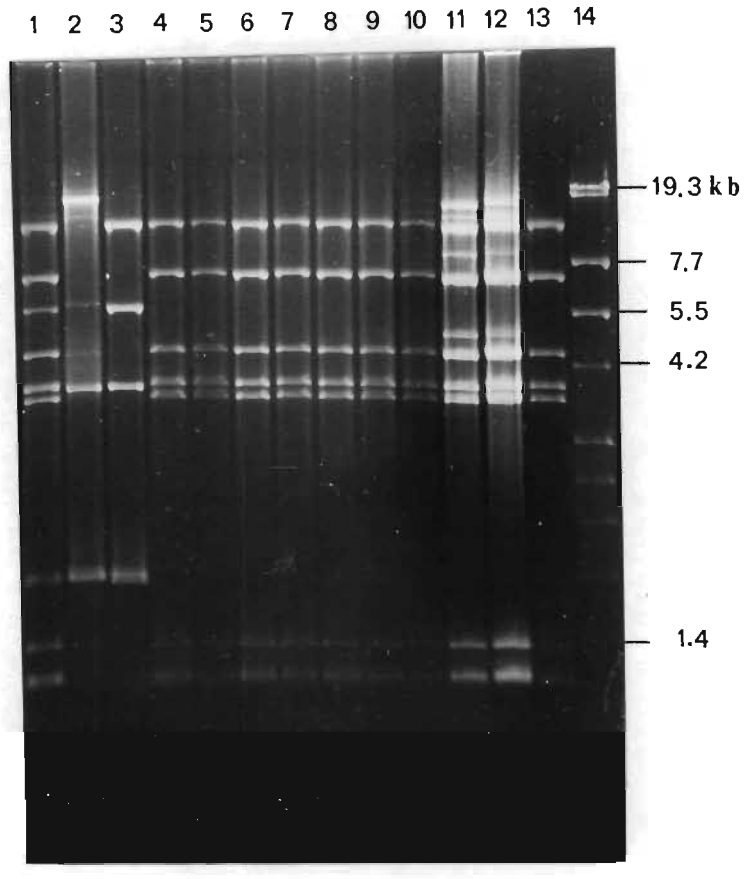



**Fig. 3.4.** RFLP profiles of mtDNA of parental and fusant strains generated by a double digestion with the restriction endonucleases *Eco*RV and *Pst*I. Lane 1: *P. stipitis* 633 *leu lys ala*, lane 2: *C. shehatae* 117 A/1 *cys met*, lane 3: *C. shehatae* 492 *met his*, lanes 4-8: PC1 - PC5, lanes 9-13: PCA1 - PCA5 and lane 14: Molecular Weight Marker IV (Boehringer Mannheim).

## 3.4 DISCUSSION

*P. stipitis* and the fusant strains harbour circular DNA of approximately 35 kb as well as plasmids in their mitochondria. The mtDNA of *C. shehatae* strains is approximately 30 kb. Mitochondrial plasmids are not present in these strains. Compared to other fungi, *P. stipitis* and *C. shehatae* possess relatively small mtDNAs. mtDNA of

most *Pichia* species are circular with a size ranging from 32 to 55 kb, although three species have been reported to have linear mtDNA with sizes ranging from 24 to 50 kb (Fukuhara *et al.*, 1993). *Candida* species have been found to have circular mtDNAs with sizes ranging from 19 kb for *C. glabrata* (O'Connor *et al.*, 1976) to 52 kb for *C. maltosa* (Kunz *et al.*, 1986). *C. tropicalis* has been reported to possess three different forms of mtDNA: linear, open circular and closed circular (Weng *et al.*, 1985 - cited by Su and Meyer, 1991). The mitochondrial plasmids present in the *Pichia* and fusant strains appeared to be small circular molecules. Since they have not been further characterised in the present study, no conclusions can be made regarding the genes they carry or their function. Hybridisation of the plasmid DNA to mtDNA and nuclear DNA may provide information as to whether or not they are of mitochondrial or nuclear origin.

Restriction profiles generated by various restriction endonucleases indicated firstly that *P. stipitis* and *C. shehatae* have unique restriction profiles. Mitochondrial fingerprinting is therefore a suitable technique for differentiating between these two species. This is in accordance with the results reported by Su and Meyer (1991). They studied seven *Candida* species as well as *Lodderomyces elongisporus* and found different patterns for each species. Vezinhet *et al.* (1990) have reported a remarkable polymorphism among 22 enological strains of *S. cerevisiae*. They found 17 different mtDNA profiles for 22 strains using the enzyme *Eco*RV. Querol *et al.* (1992) also reported a large number of mtDNA restriction profiles for enological strains of *S. cerevisiae* (41 and 35 different profiles for 50 strains in 2 different wineries).

Secondly, comparision of the restriction profiles obtained in the present study lead

to the conclusion that all the fusants inherited their mitochondria from the *Pichia* parent. During the protoplast fusion process, the cytoplasms of both cells come into contact with each other and there is generally mixing of the organelles of both cytoplasms. The nuclei may interact with each other to produce hybrids but even in the case where no hybrids are formed and the heterokaryon destabilises to produce 2 cells with one nucleus, these cells possess a mixture of cytoplasmic characters and are known as cybrids (Fig. 1.2a). Collins and Saville (1990) report that during the unstable vegetative fusion in *Neurospora*, although the heterokaryons were unstable, the parents must have remained fused long enough for mixing of the mitochondrial and nuclear populations to occur. Their results show that stable heterokaryons are not a prerequisite for major changes in the mitochondrial genetic system.

The fusants used in this study should contain mitochondria from both parental strains although they inherited their nuclei entirely from one parent with possibly a few gene exchanges. Most of the genes required for mitochondrial biogenesis are coded for by the nucleus. Since the genome of the fusants appears to be almost entirely *Pichia*-inherited, it should therefore code only for *Pichia* mitochondrial proteins. The *Candida* mitochondria that could have been present at the time of the fusion may have been "diluted" out during cell divisions since none of their structural proteins (encoded by the nucleus) would be produced. These mitochondria would eventually disappear in the fusants. This appears to contradict the report by Collins and Saville (1990) who found that even extremely rare and unstable fusions may contribute to the horizontal transfer of mtDNA, plasmids and possibly other mobile elements under laboratory conditions. However, they worked with intrageneric fusions and one would expect a very similar

genetic constituent for the biogenesis of mitochondria for species within the same genus than for different genera as in the case with *P. stipitis* and *C. shehatae*.

The *Candida* mitochondria were therefore lost from the cell lines before any recombination events occurred since the RFLP analyses indicate identical profiles for the *Pichia* parent and fusants. One cannot make any deductions about the mode of inheritance of mitochondria of these fusants because had they possessed a truly hybrid nature, *Candida* mitochondria could have been maintained by the *Candida* genes in the fusants.

# CHAPTER FOUR

# CHARACTERISATION AND COMPARISON OF THE FERMENTATIVE ABILITIES OF *Pichia stipitis*, *Candida shehatae* AND THEIR FUSANTS

## 4.1 INTRODUCTION

Traditionally, glucose-based substrates such as maize and molasses are fermented commercially to ethanol by *S. cerevisiae*. However, this yeast is incapable of fermenting D-xylose to ethanol. Although many yeasts can utilise pentoses oxidatively, it was previously thought that none could ferment them (Barnett, 1978).

Since the discovery of the first yeast capable of D-xylose fermentation by Karczewska in 1959 (Jeffries, 1990), much progress has been made in identifying other yeast strains capable of fermenting D-xylose to ethanol and in establishing conditions for the optimal production of ethanol from this sugar. *P. stipitis* and *C. shehatae* have thus far been identified as the most efficient producers of ethanol from D-xylose. They have several advantages over the other D-xylose fermenting yeasts including: (a) higher volumetric rates of ethanol production; (b) higher ethanol yields; (c) higher ethanol tolerance; and (d) lower xylitol production. *P. stipitis* is superior to *C. shehatae* in that it has a much lower xylitol production and tolerates ethanol somewhat better than *C. shehatae* (Slininger *et al.*, 1985). A number of different conditions influence the production of ethanol from xylose, including inoculum size and age, pH, temperature,

nitrogen source and aeration levels. The age of inocula used in flask experiments had a profound effect on initial volumetric fermentation rate with inocula grown for only 24 h outperforming that grown for 72 h. A critical dissolved oxygen tension has to be maintained in order to maximise ethanol yields in yeasts (du Preez *et al.*, 1984). Under strictly anaerobic conditions, very little ethanol is produced. Sreenath *et al.* (1986) reported 25% more ethanol in one third the reaction time under aerobic fermentation as opposed to anaerobic fermentation for *C. shehatae*.

The maximum ethanol concentration reported from D-xylose is 57 g/l produced by *P. stipitis* Y-7124 in ≈ 13 days with a yield of 0.39 g/g. Although this is a high yield for a D-xylose fermenting yeast, typical industrial D-glucose fermentations by *S. cerevisiae* can achieve almost 80 - 90 g/l ethanol with a yield of 0.5 g/g (Maiorella *et al.*, 1984). A highly fermentative strain of *C. shehatae*, obtained by serial recycling in wood hydrolysates (Parekh *et al.*, 1986), fermented a 70:30 D-glucose/D-xylose mixture to an ethanol concentration of 84 - 100 g/l (Wayman and Parekh, 1985). Jeffries (1990) postulated that these changes may be attributable to altered genome numbers or altered mitochondrial activity. Also, glucose feeding has been found to have a stimulatory effect and significantly reduced the time required for xylose fermentation. Wayman and Parekh (1985) found that *C. shehatae* fermented a mixture of D-glucose/D-xylose at a higher rate than either sugar alone, which suggests that some intermediary metabolite may be limiting the fermentation rate (Jeffries and Sreenath, 1988).

Most, if not all, strains of *S. cerevisiae* used in commercial fermentations are polyploid or aneuploid, suggesting that for one reason or another, this condition may be

beneficial (James and Zahab, 1983). With this in mind, the construction of strains with increased ploidy was undertaken by intrageneric fusions of *C. shehatae* (Johannsen *et al.*, 1985), *P. tannophilus* (James and Zahab, 1983) and *P. stipitis*, as well as intergeneric fusions between *C. shehatae* and *S. cerevisiae*, *P. stipitis* and *S. cerevisiae* and *P. stipitis* and *C. shehatae* (Gupthar and Garnett, 1987). Very slight increases in ethanol production were reported for the *C. shehatae* and *P. tannophilus* intrageneric fusants. No improvement in ethanol production was apparent for *C. shehatae-S. cerevisiae* or *P. stipitis-S. cerevisiae* fusants. Preliminary analysis of *P. stipitis-C. shehatae* fusants indicated a slight improvement in ethanol production for one of the fusant strains.

The objectives of this study were, therefore, to characterise the fermentative abilities of the *P. stipitis* and *C. shehatae* parental strains and fusant strains resulting from this intergeneric fusion, as well as to compare the fermentative ability of the fusants to that of the parental strains. Fermentation conditions already established by du Preez and Prior (1985) for the wild type parental strains were used since the aim was not to optimise fermentation conditions for these strains but simply to compare their fermentation performance.

## 4.2 MATERIALS AND METHODS

### 4.2.1 SHAKE FLASK FERMENTATIONS

CA Medium : 50 g D-xylose, 5 g casamino acids, 7.5 g $NH_4Cl$, 2.5 g $KH_2PO_4$, 0.5

g $MgSO_4.7H_2O$, 0.05 g $CaCl_2.2H_2O$, 0.5 g citric acid, 35 mg $FeSO_4.7H_2O$, 7 mg $MnSO_4.H_2O$, 11 mg $ZnSO_4.7H_2O$, 1 mg $CuSO_4.5H_2O$, 2 mg $CoCl_2.6H_2O$, 1.3 mg $Na_2MoO_4.2H_2O$, 2 mg $H_3BO_3$, 0.35 mg KI, 0.5 mg $Al_2(SO_4)_3$, 100 mg meso-inositol, 20 mg calcium panthotenate, 5 mg thiamine-HCl, 5 mg pyridoxine-HCl, 5 mg nicotinic acid, 1 mg *p*-amino benzoic acid and 0.1 mg d-biotin

Fermentation conditions established by du Preez and Prior (1985) with two modifications were employed in this study. A preliminary fermentation in a smaller volume (100 ml) of CA medium was carried out using the conditions described below, in order to test the efficiency of the system. Modifications involved the use of a higher inoculum size since several reports suggested that higher inocula improve ethanol yield. A lower agitation speed (which is equivalent to lower aeration) was also used: 90 rpm as opposed to 150 rpm of du Preez and Prior (1985).

Shake flask fermentations were carried out in 1 l Ehrlenmeyer flasks containing 500 ml CA medium. Flasks were gently agitated (90 rpm) on an orbital shaker (Certomat U, Braun) at 30°C to prevent settling out of cells as well as to provide a very low level of aeration. Each flask was inoculated with 50 ml of a cell suspension having an optical density of 30 absorbance units at 600 nm which corresponded to a dry mass of 0.5 to 0.6 g/l. Aliquots of 5 ml were removed every 8 h and immediately centrifuged. Supernatants were stored at -20°C and the pellets used for dry cell mass determinations.

#### 4.2.1.1 Preparation of Inocula

Inocula were prepared in two stages. A loopful of culture was inoculated into 20 ml of CA medium in a 250 ml Ehrlenmeyer flask and incubated for 24 h at 30°C in an orbital shaker at 155 rpm. 5 ml of this culture was transferred to 100 ml of fresh CA medium in a 250 ml Ehrlenmeyer flask and incubated as described above. After 24 h, the cells were pelleted by centrifugation at 8 000 × *g* for 10 min at 4°C. Pellets were resuspended in CA medium. 50 ml of cell suspension with an optical density of 30 absorbance units at 600 nm was used to inoculate 500 ml of CA medium in a 1 l Ehrlenmeyer flask.

#### 4.2.1.2 Fermentation and Sampling

Shake flask fermentations were carried out at 30°C in an orbital shaker at 90 rpm. Aliquots of 5 ml were removed at 8 h intervals and centrifuged as described previously. Supernatants were stored at -20°C and used for quantitative determinations of xylose, xylitol and ethanol. Pellets were used for gravimetric dry cell mass determinations.

#### 4.2.1.3 Dry Cell Mass Determination

Pellets were washed once in sterile distilled water, vacuum filtered onto pre-weighed Millipore type HA filters (0.45 $\mu$m), dried in an oven at 105°C, allowed to cool

in a dessicator and their masses determined.

#### 4.2.1.4 HPLC Analyses

Samples stored at -20°C were allowed to thaw on ice, diluted and filtered through Millipore Millex HV filters (0.45 $\mu$m) and analyzed using a Waters 410 Differential Refractometer (sensitivity = 8×, temperature = 40°C), a Waters autosampler and a Waters Sugar-Pak 1 column maintained at 90°C with a flow rate of 0.5 ml/min. The mobile phase was 50 ppm calcium titriplex dihydrate maintained at 50°C. Injection volumes of 20 $\mu$l were used and the run time was 25 min. Results were recorded by a Waters Maxima 820 Data Chromatography Workstation.

#### 4.2.1.5 Calculation of Fermentation Parameters

The following fermentation parameters were determined: maximum specific growth rate ($\mu_{max}$), volumetric rate of product formation ($Q_p$), specific rate of product formation ($q_p$), volumetric rate of substrate utilisation ($Q_s$), specific rate of substrate utilisation ($q_s$), volumetric rate of biomass production ($Q_x$), biomass yield coefficient with respect to substrate utilisation ($Y_{x/s}$), biomass yield coefficient with respect to product formation ($Y_{x/p}$) and the product-substrate coefficient ($Y_{p/s}$). The units for the volumetric rates were g/l/h, g/g/h were used for specific rates of production and yield coefficients were measured in g/g.

$\mu_{max}$ was determined from the slope of the exponential phase of the natural log of biomass versus time curve. A range of $Q_p$, $Q_s$, and $Q_x$ values were estimated over the entire fermentation period. Time intervals of 8 h were used in calculations. $Q_p$max values were determined from the slope of the exponential phase of ethanol versus time curves. A range of $q_p$ values was also established and calculated by dividing the volumetric rates of ethanol production by the biomass value in the middle of each 8 h time interval. The $q_p$max value was determined by dividing the $Q_p$max value by the biomass value at the middle of the time interval. The $Y_{x/s}$max, $Y_{x/p}$max and $Y_{p/s}$max values were obtained by dividing the $Q_x$max value by the $Q_s$max value, the $Q_x$max value by the $Q_p$max value and the $Q_p$max value by the $Q_s$max value, respectively.

## 4.3 RESULTS

The trial fermentation yielded the following ethanol concentrations for the different strains after 72 h: *P. stipitis* produced 20.2 g/l, *C. shehatae* 117 A/1 *cys met* 18.1 g/l and *C. shehatae* 492 *met his* 14.7 g/l and the fusants had final ethanol concentrations that ranged between 18.0 - 19.6 g/l. Since these results were comparable to those obtained by other authors using similar fermentation conditions (du Preez *et al.*, 1984, 1987, 1989; du Preez and Prior, 1985; du Preez and van der Walt, 1983) and also permitted differentiation between fusant and parental strains with regard to ethanol levels produced, the same conditions were considered suitable for subsequent fermentations.

## 4.3.1 BIOMASS, RESIDUAL XYLOSE, XYLITOL AND ETHANOL CONCENTRATIONS

### 4.3.1.1 Biomass Determination

A lag phase was not observed for any of the strains tested (Fig. 4.3a to 4.15a). This was because actively dividing logarithmic phase cells were used as inocula. The biomass tripled or quadrupled for most of the strains. A final biomass of 4 - 5 g/l was obtained for all the strains, except *C. shehatae* 492 *met his* (2 g/l), *C. shehatae* 117 A/1 *cys met* (3.3 g/l) and PCA1 (3.3 g/l).

### 4.3.1.2 Xylose Utilisation

*C. shehatae* 117 A/1 *cys met* utilised xylose more rapidly when compared to the other strains (Fig 4.4b). After 24 h only 20 g/l xylose remained as opposed to the *P. stipitis* fermentation (Fig. 4.3b) which had 27 g/l xylose after 24 h. Fusant strains PC1 to PC5 (Fig. 4.6b-4.10b) followed the xylose utilisation trend of the *P. stipitis* parent, while PCA1 to PCA5 (Fig. 4.11b-4.15b) possessed characteristics of the *C. shehatae* parent. The exponential phase of xylose utilisation of fusants PC1 to PC5 occurred between 24 and 48 h whereas that of fusants PCA1 to PCA5 was between 8/16 to 48 h. All fusant strains had no residual xylose after 48 h. After 56 h both *P. stipitis* and *C. shehatae* 117 A/1 *cys met* had completely utilised the xylose. *C. shehatae* 492 *met his* utilised xylose very poorly - the residual xylose concentration after 72 h was 6.51 g/l.

### 4.3.1.3 Ethanol Production

Ethanol, the major product of the fermentation was detected in the first sample (8 h) for all the strains. It was accumulated fairly rapidly by all strains. Maximum ethanol concentrations were reached around 48 h by *P. stipitis* (Fig. 4.3b) and the fusants PC1 to PC5 (Fig. 4.6b - 4.10b). The *Pichia* parental strain produced the highest ethanol concentration of 29.9 g/l. Most of the fusant strains produced comparable amounts of ethanol. The *Candida* parental strains produced significantly lower amounts of ethanol. *C. shehatae* 117 A/1 *cys met* (Fig. 4.4b) was the better producer of the two with a maximum ethanol concentration of 25.98 g/l whereas *C. shehatae* 492 *met his* (Fig. 4.5b) produced only 13.92 g/l. The exponential phase of ethanol production occurred at an earlier stage for the fusants PC1 to PC5 as opposed to PCA1 to PCA5, viz., between 24 to 40 h and 40 to 56 h, respectively.

HPLC analysis of the unfermented medium yielded three chromatographic peaks (Fig. 4.1). The first peak corresponded to the inorganic elements in the medium. A major peak was recorded for xylose and a small peak was identified as arabinose. Trace amounts of arabinose were therefore present in the medium at the start of the fermentation. This level increased only marginally during the fermentation for all the strains.

### 4.3.1.4 Xylitol Production

Ethanol was not the sole product of the fermentation. Polyols were also produced,

of which xylitol was the major constituent. As expected, both the *Candida* parental strains produced high levels of xylitol: 3.43 g/l for *C. shehatae* 117 A/1 *cys met* (Fig. 4.4b) and 5.69 g/l for *C. shehatae* 492 *met his* (Fig. 4.5b). *P. stipitis* (Fig. 4.3b) produced 0.2 g/l and the fusant strains (Fig. 4.6b-4.15b) between 0.04 and 0.34 g/l. In addition to ethanol and xylitol, both *Candida* parental strains produced significant levels of glycerol (Figs.4.2a and 4.2b). *C. shehatae* 492 *met his* produced three times the level of *C. shehatae* 117 A/1 *cys met*.

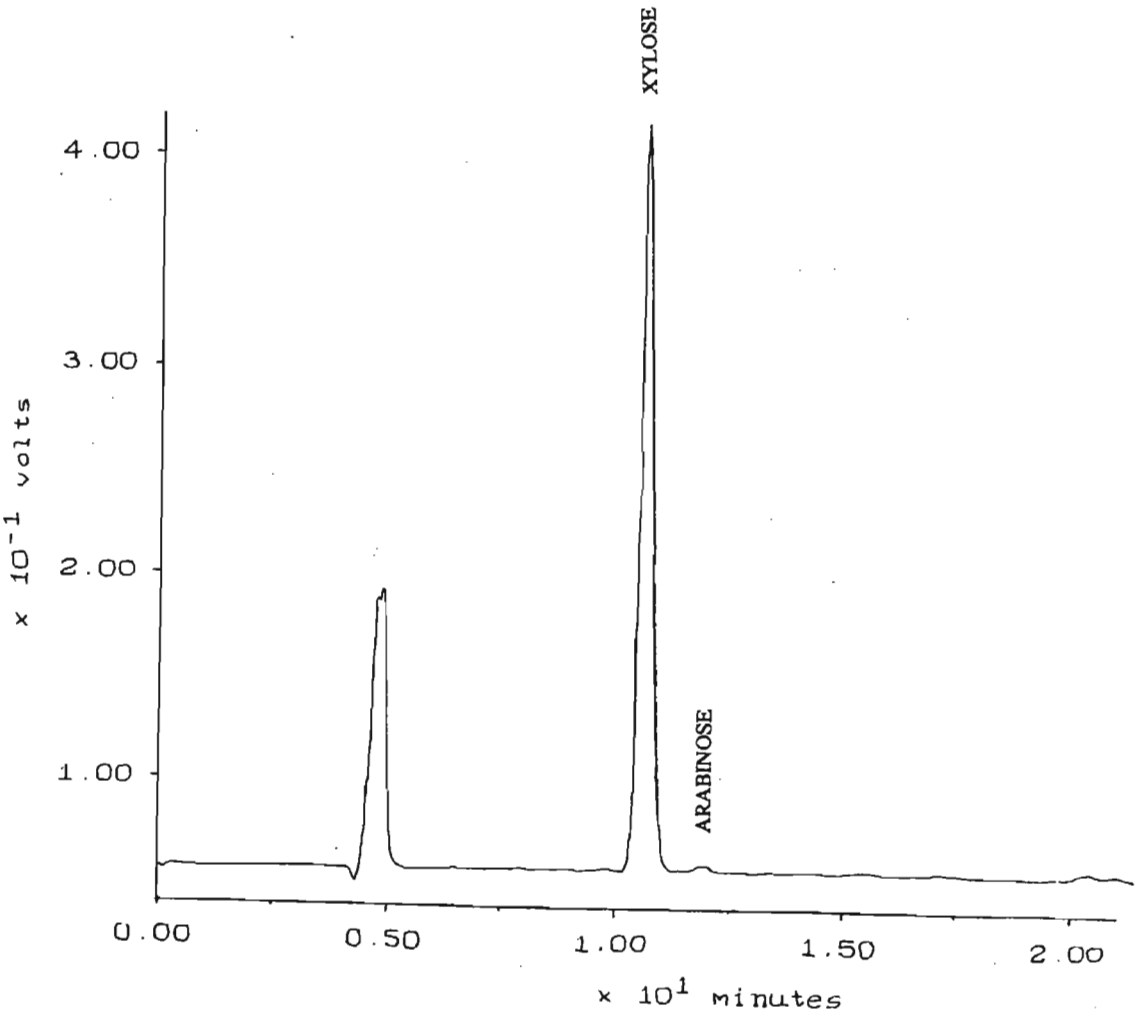
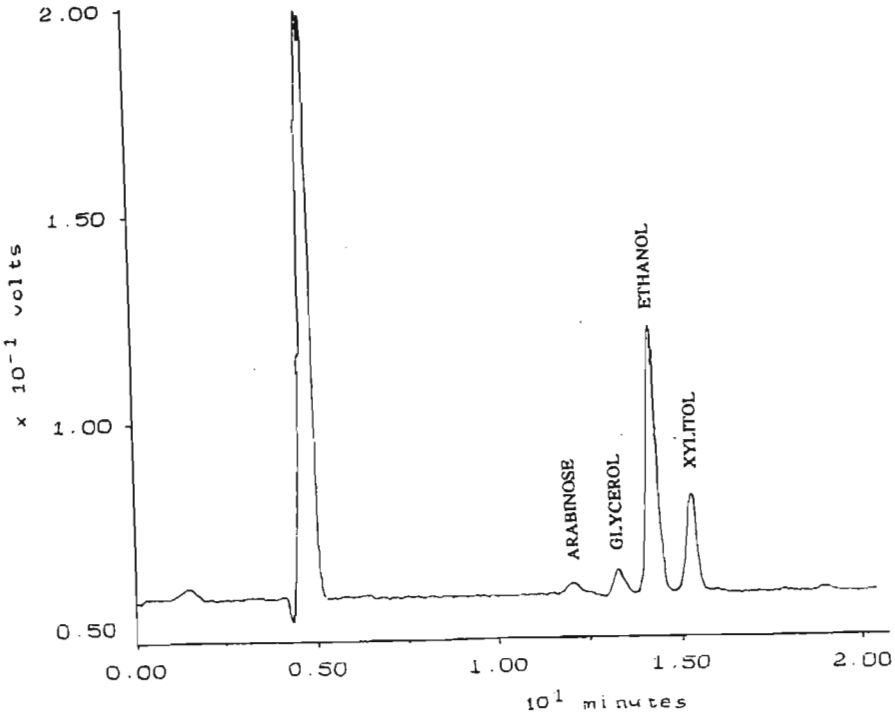



**Fig. 4.1.** Chromatogram of the unfermented fermentation medium (1:5 dilution).

a




b

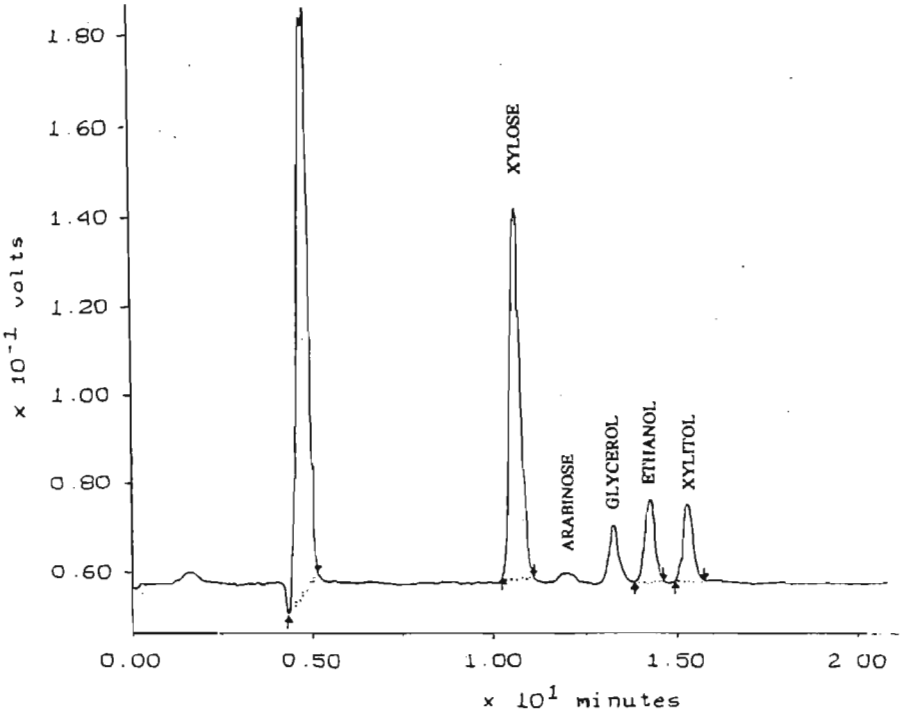
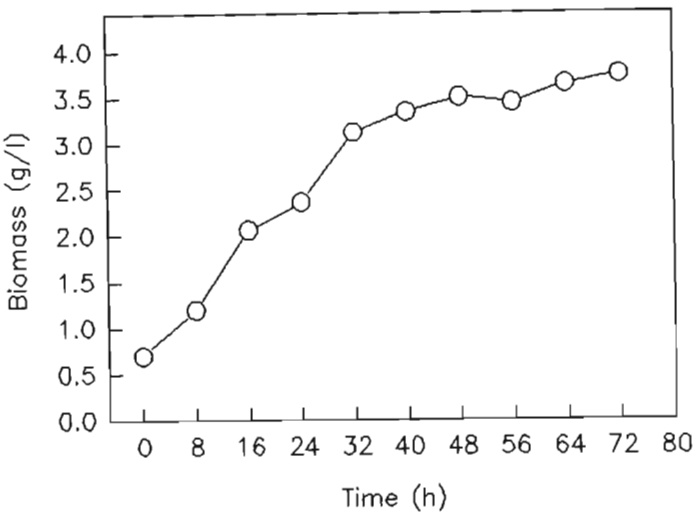



**Fig. 4.2.** Chromatograms of the fermentation products of (a) *C. shehatae* 117 A/1 *cys met* and (b) *C. shehatae* 492 *met his* (1:5 dilution).

a




b

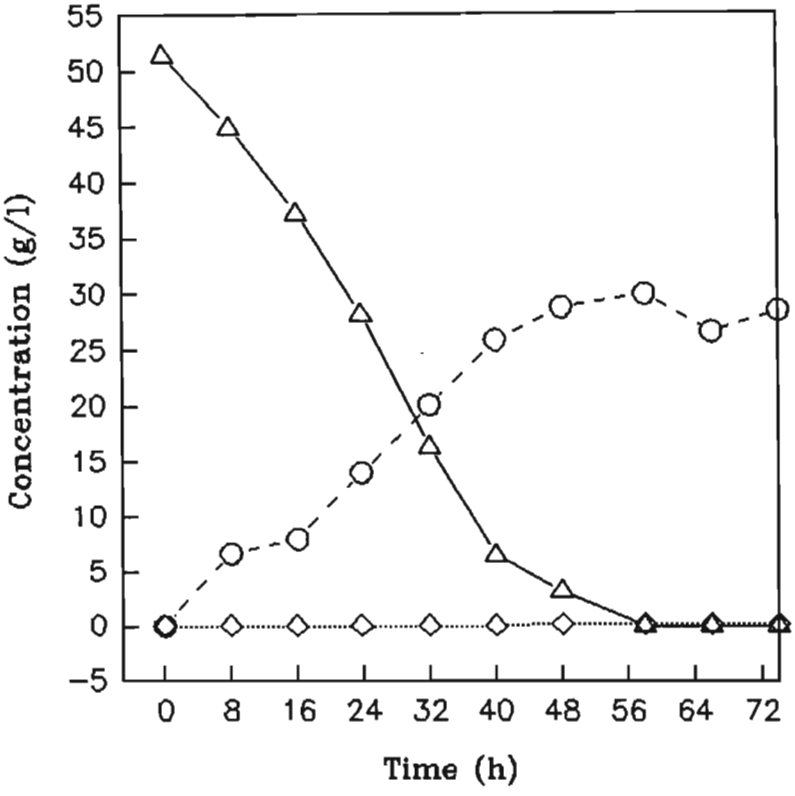
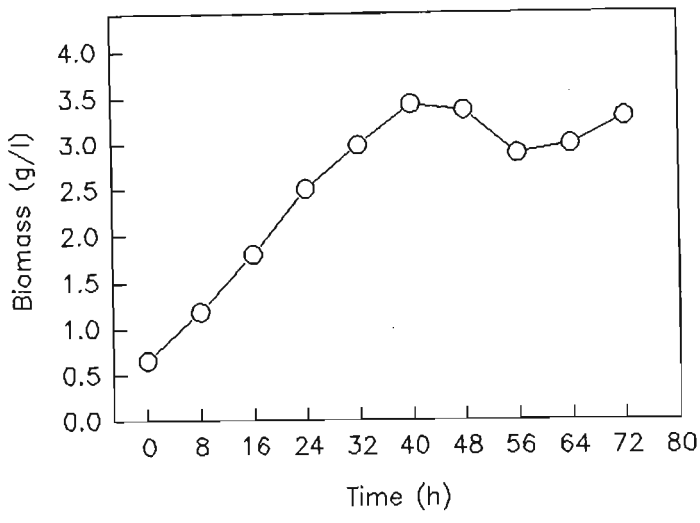



**Fig. 4.3.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by *P. stipitis* 633 *leu lys ala*.

a

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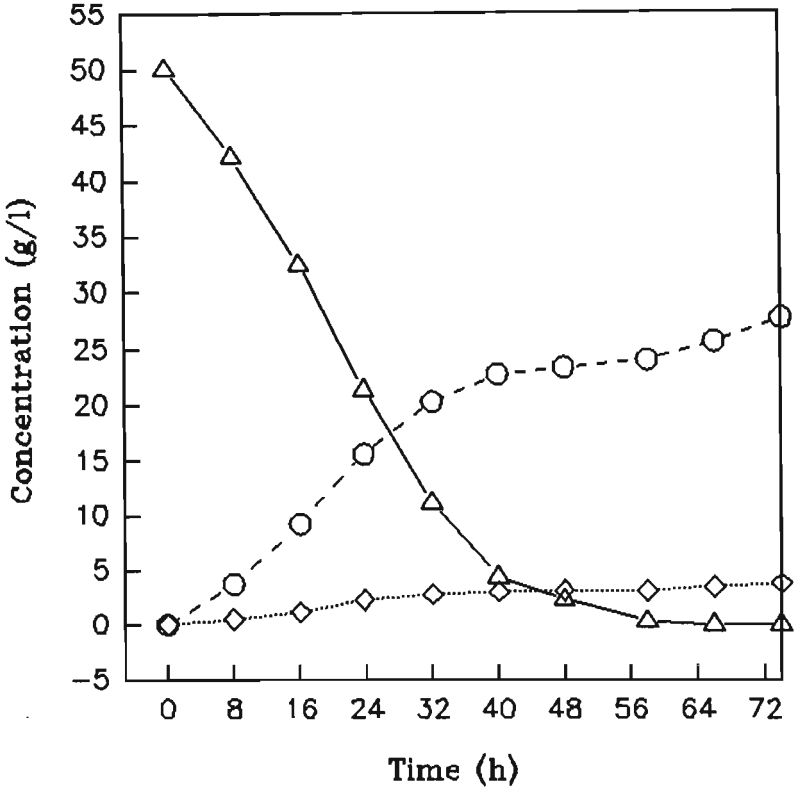
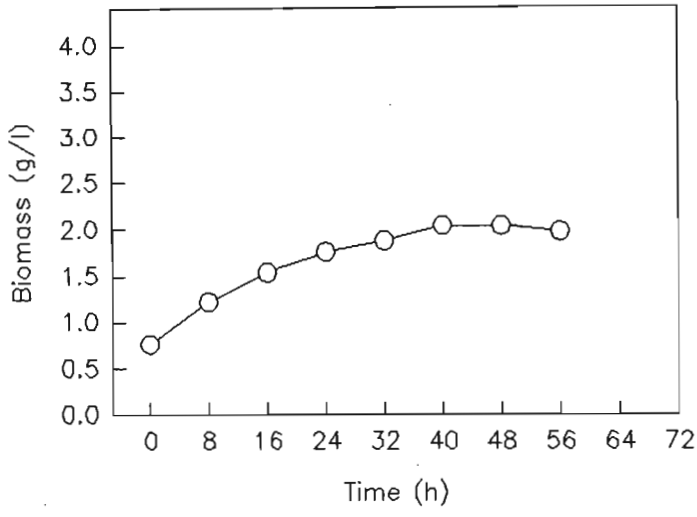



**Fig. 4.4.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by *C. shehatae* 117 A/1 *cys met.*

a

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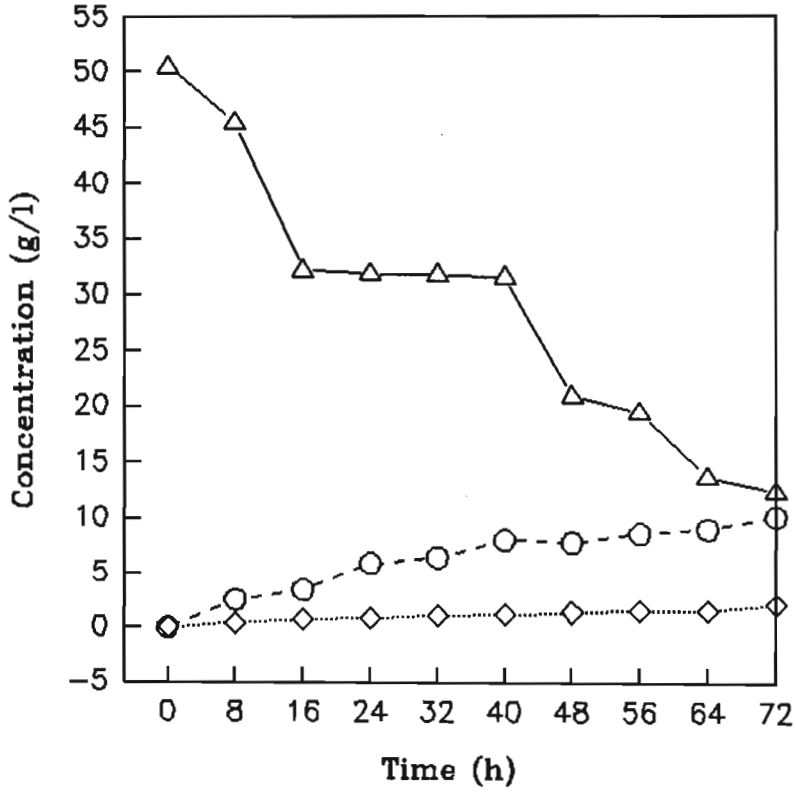
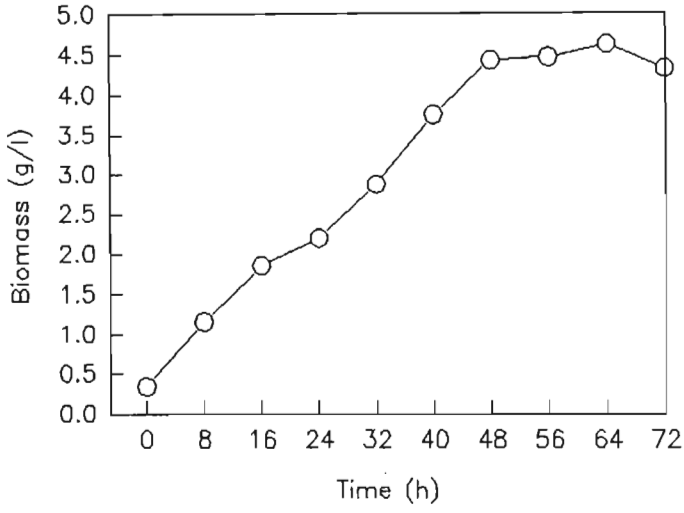



**Fig. 4.5.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by *C. shehatae* 492 *met his*.

a

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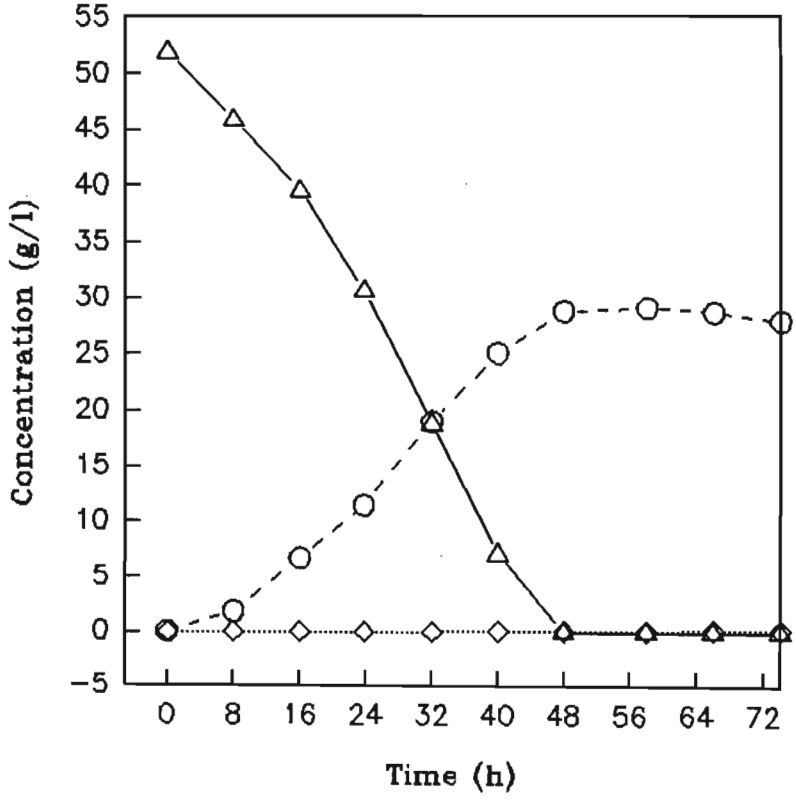
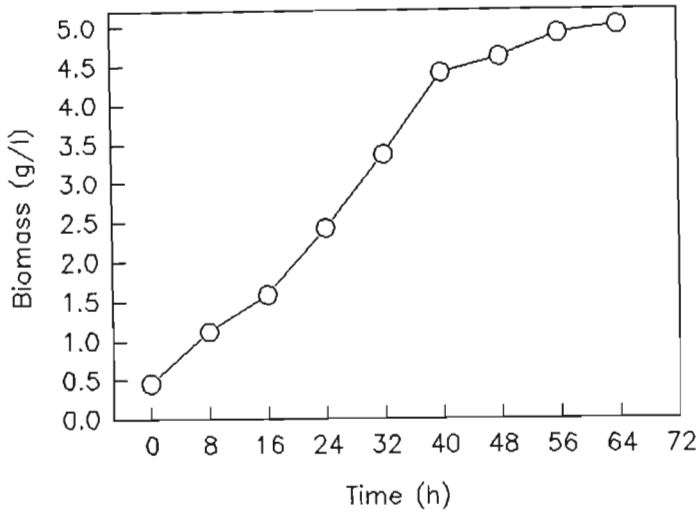



**Fig. 4.6.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, as well as ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PC1.

a




b

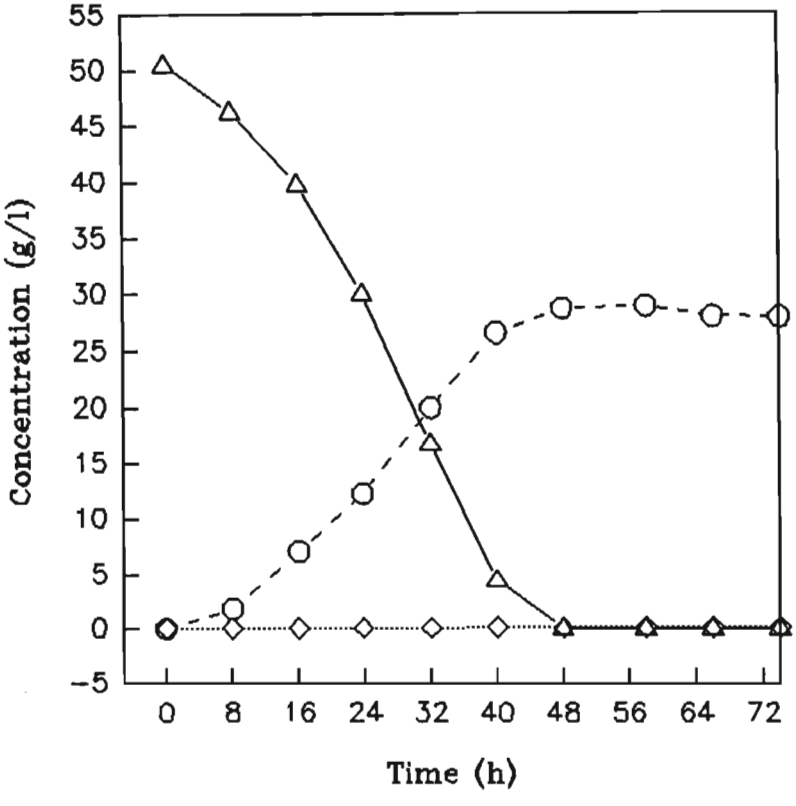
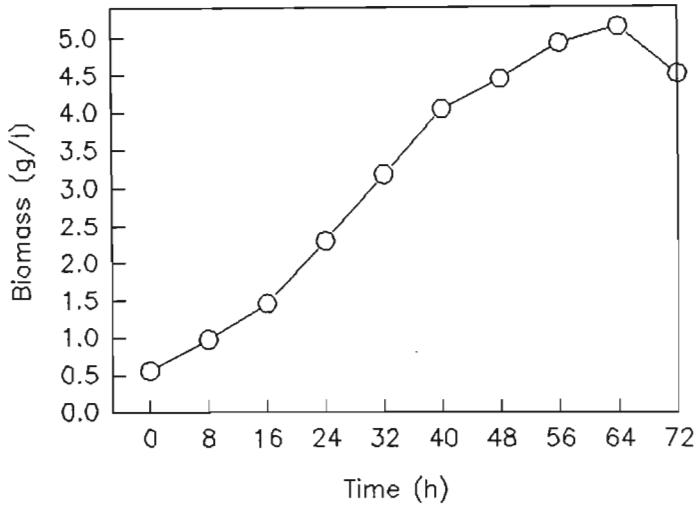



**Fig. 4.7.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PC2.

a




b

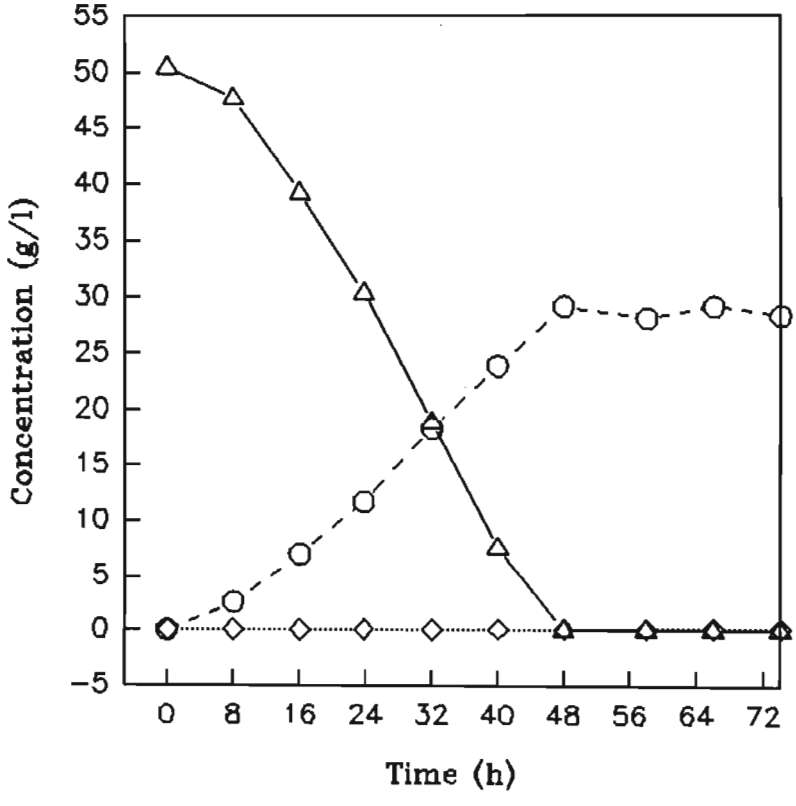
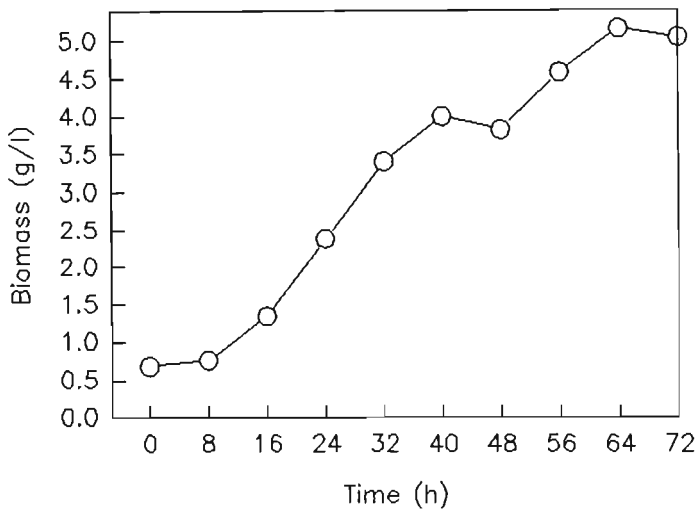



**Fig. 4.8.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PC3.

a




b

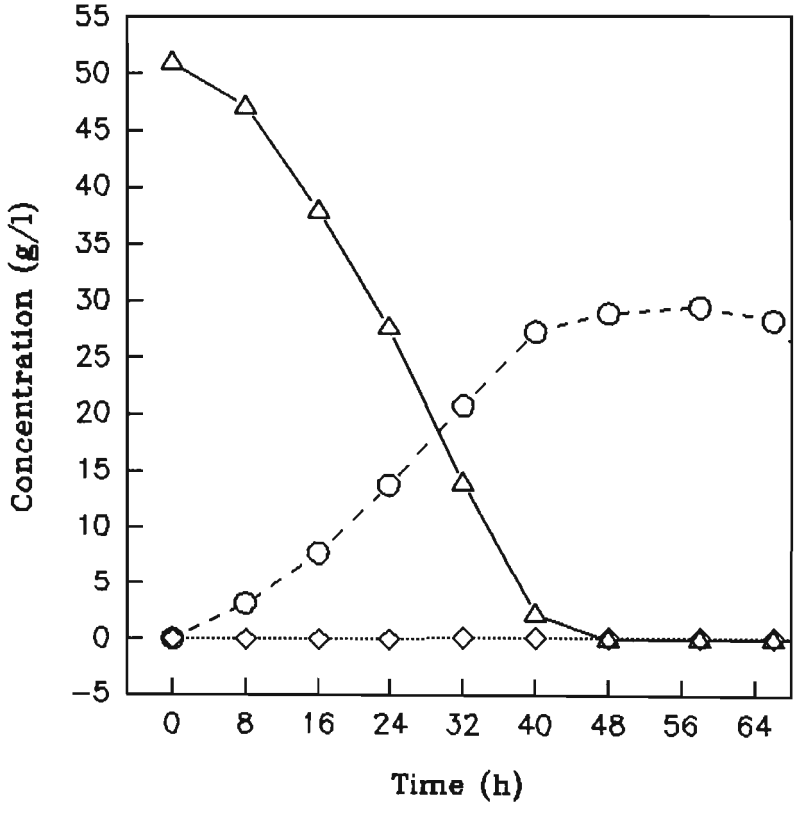
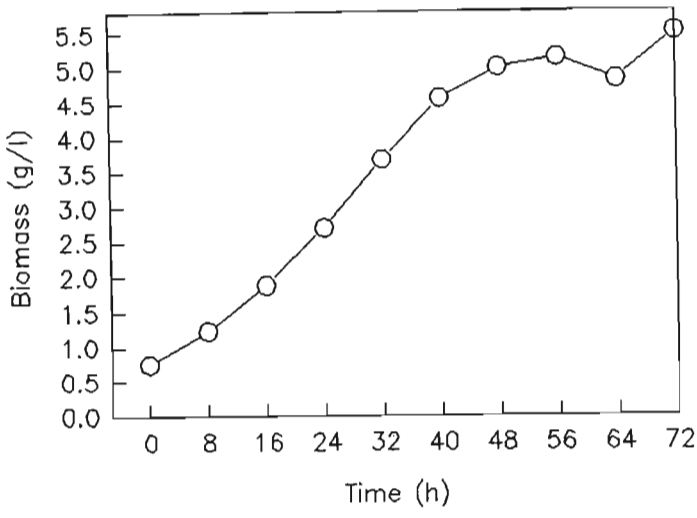



**Fig. 4.9.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PC4.

a




b

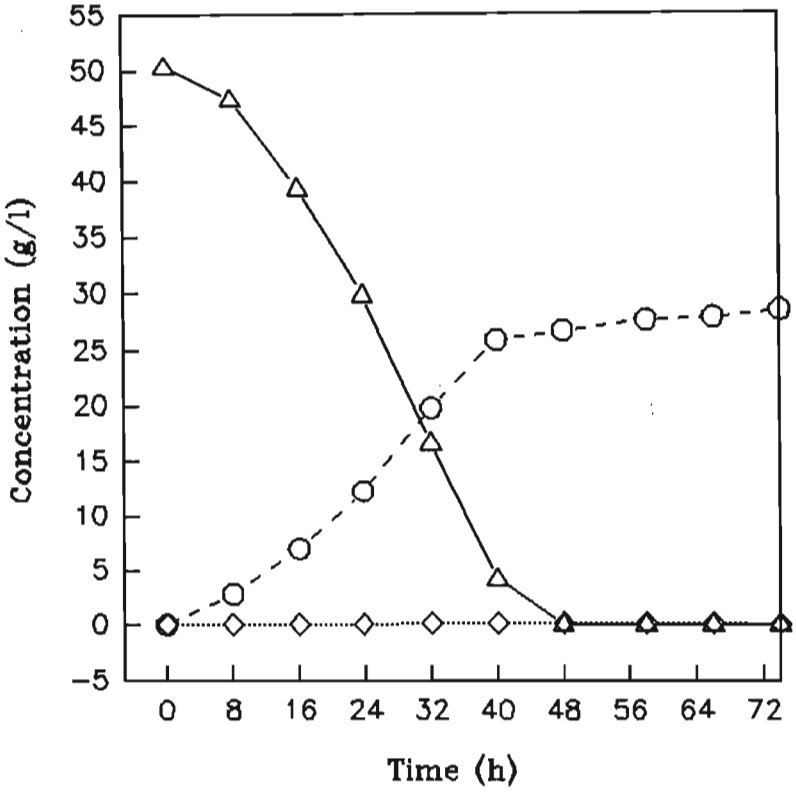
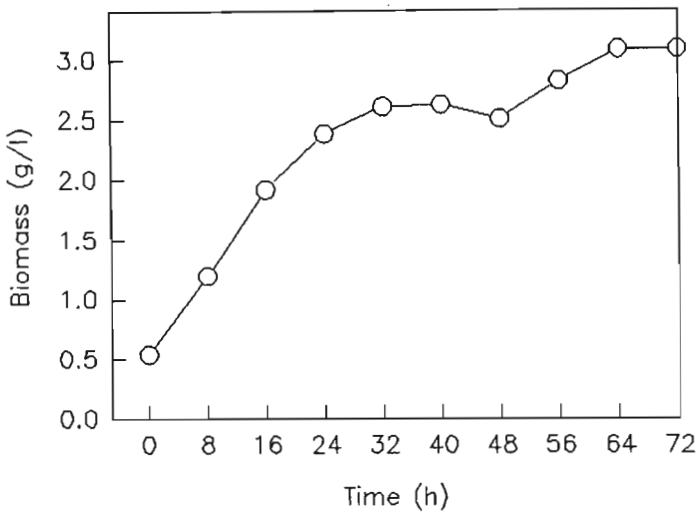



**Fig. 4.10.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PC5.

a

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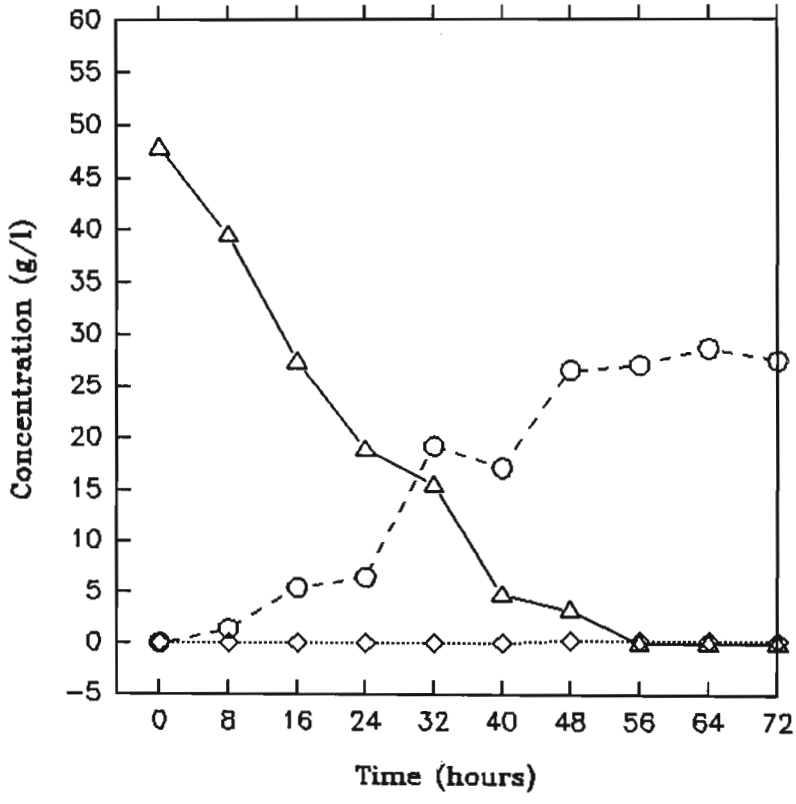
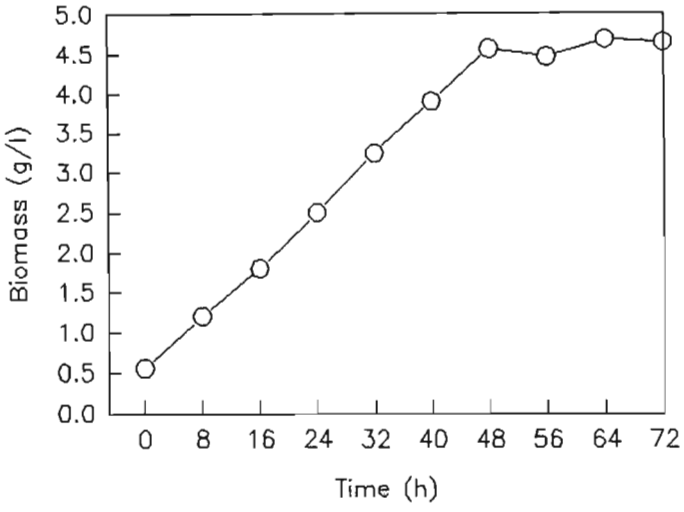



Fig. 4.11. Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PCA1.

a




b

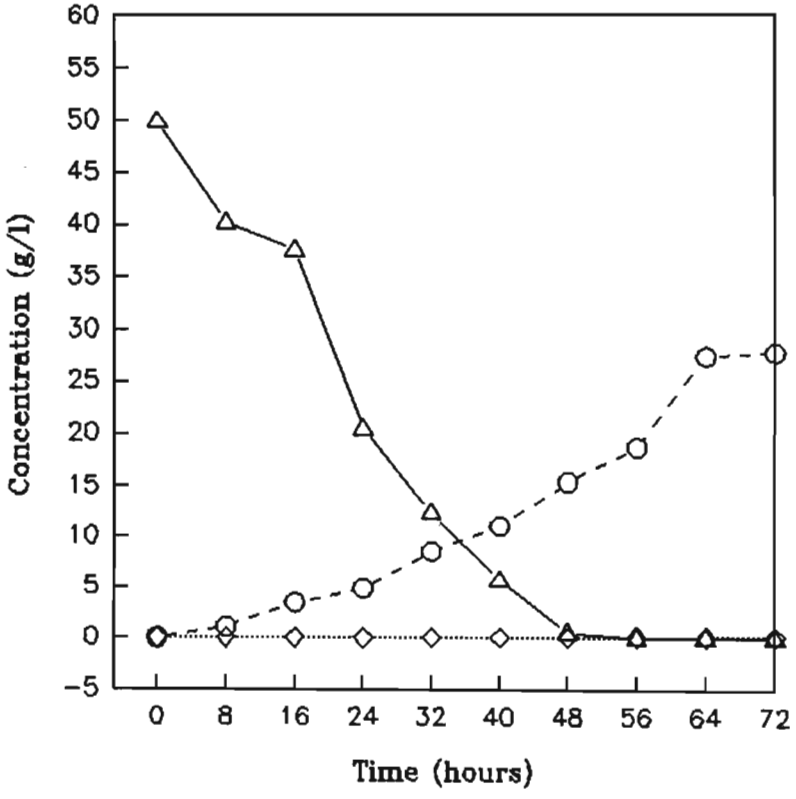
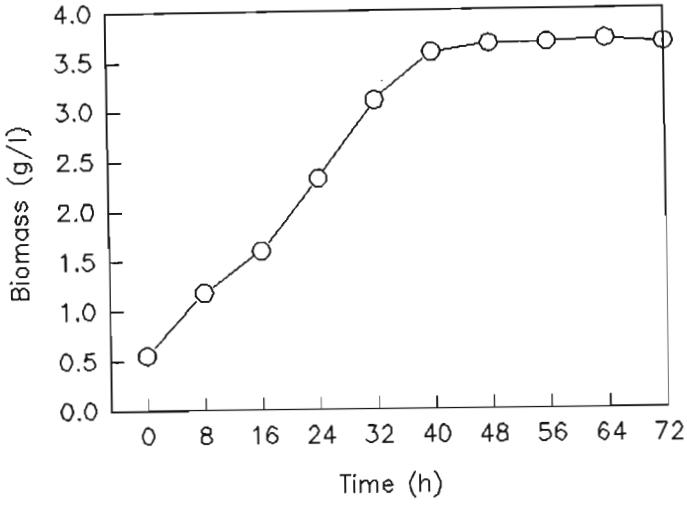



**Fig. 4.12.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PCA2.

a




b

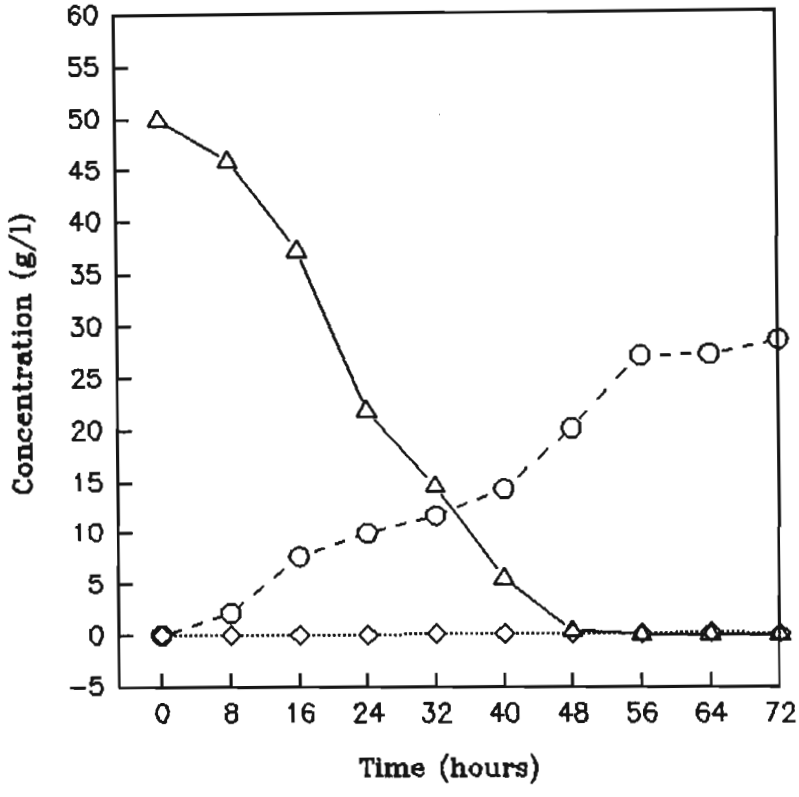
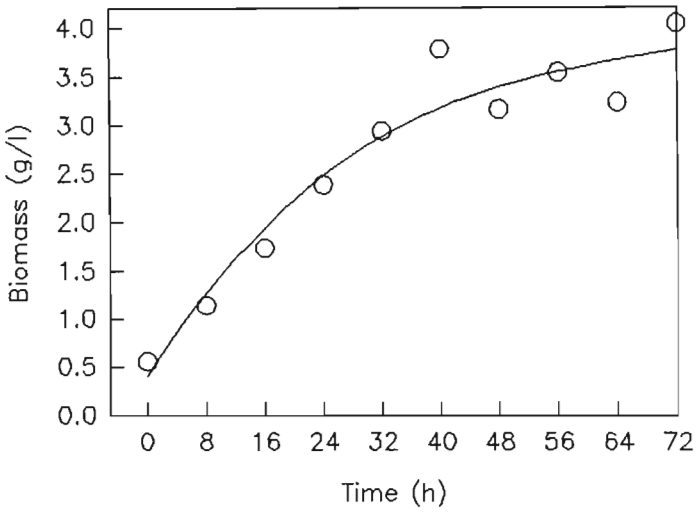



**Fig. 4.13.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PCA3.

a




b

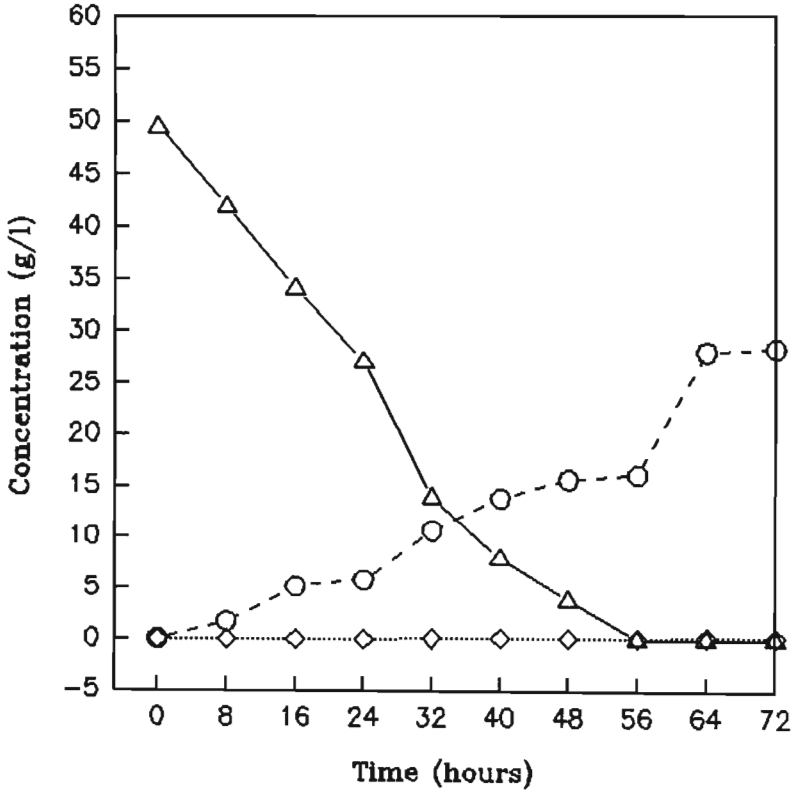
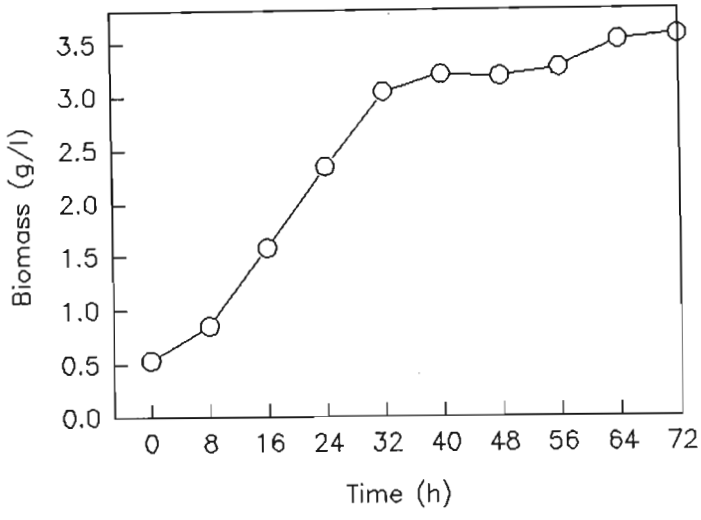



**Fig. 4.14.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PCA4.

a




b

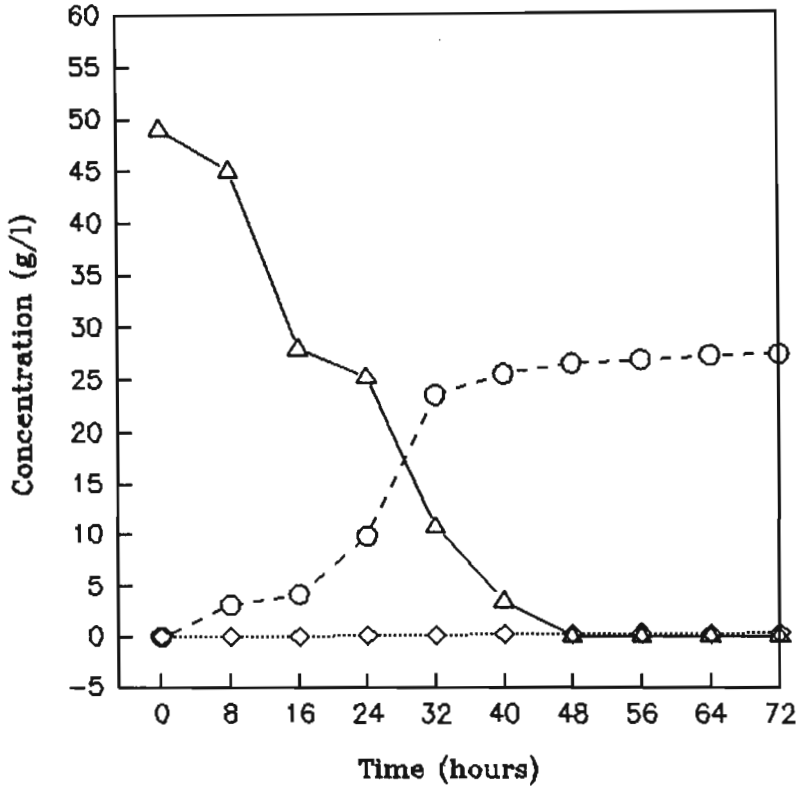



**Fig. 4.15.** Fermentation profiles of (a) biomass production and (b) xylose utilisation {△}, ethanol {○} and xylitol production {◇} for shake-flask D-xylose fermentation by PCA5.

## 4.3.2 CALCULATION OF FERMENTATION PARAMETERS

The values for $Q_p$, $Q_s$ and $Q_x$ were calculated by linear regression analysis of the exponential phase of the curves for ethanol production, substrate utilization and biomass production, respectively (Table 4.1).

**TABLE 4.1.** Quantitative analysis of D-xylose fermentations of *P. stipitis, C. shehatae* and their fusants

| Strain | $Q_p$ | $Q_s$ | $Q_x$ | $q_p$ | $q_s$ | $Y_{x/p}$ | $Y_{x/s}$ | $Y_{p/s}$ |
|---|---|---|---|---|---|---|---|---|
| PsY 633 LLA | 0.75 | 1.34 | 0.08 | 0.24 | 0.43 | 0.06 | 0.11 | 0.56 |
| CsY 117 A/1 CM | 0.75 | 1.31 | 0.07 | 0.42 | 0.62 | 0.05 | 0.09 | 0.57 |
| CsY 492 MH | 0.21 | 1.15 | 0.06 | 0.14 | 0.94 | 0.05 | 0.29 | 0.18 |
| PC1 | 0.84 | 1.45 | 0.10 | 0.28 | 0.48 | 0.07 | 0.12 | 0.58 |
| PC2 | 0.87 | 1.57 | 0.08 | 0.26 | 0.47 | 0.05 | 0.09 | 0.55 |
| PC3 | 0.75 | 1.40 | 0.06 | 0.24 | 0.44 | 0.04 | 0.08 | 0.54 |
| PC4 | 0.83 | 1.56 | 0.07 | 0.24 | 0.46 | 0.04 | 0.08 | 0.53 |
| PC5 | 0.84 | 1.58 | 0.06 | 0.23 | 0.43 | 0.04 | 0.07 | 0.53 |
| PCA1 | 0.84 | 1.22 | 0.10 | 0.31 | 0.81 | 0.08 | 0.12 | 0.69 |
| PCA2 | 0.69 | 1.23 | 0.10 | 0.15 | 0.83 | 0.08 | 0.14 | 0.56 |
| PCA3 | 0.80 | 1.34 | 0.09 | 0.22 | 0.50 | 0.07 | 0.11 | 0.60 |
| PCA4 | 0.69 | 1.09 | 0.09 | 0.15 | 0.41 | 0.08 | 0.13 | 0.63 |
| PCA5 | 0.89 | 1.43 | 0.07 | 0.34 | 0.74 | 0.05 | 0.08 | 0.62 |

The units for $Q_p$, $Q_s$ and $Q_x$ are g/l/h, $q_p$ and q in g/g/h and $Y_{x/p}$, $Y_{x/s}$ and $Y_{p/s}$ in g/g.

*P. stipitis* and *C. shehatae* 117 A/1 *cys met* both had a volumetric rate of ethanol production ($Q_p$) of 0.75 g/l/h. One of the fusant strains (PC3) also produced 0.75 g/l/h. The other fusant strains, with the exception of PCA2 and PCA4, showed higher $Q_p$ values ranging from 0.80 to 0.89 g/l/h. *C. shehatae* 492 *met his* had the lowest $Q_p$ value. In fact, an exponential phase of ethanol production was not observed (Fig. 4.5b). The $Q_p$ value for this strain was therefore calculated from the region of the ethanol versus time curve that showed the greatest increase in ethanol concentration in the smallest time interval.

The volumetric rate of biomass production ($Q_x$) was generally low for all the strains: between 0.6 and 1.0 g/l/h. The three parental strains had $Q_x$ values of 0.08, 0.07 and 0.06 g/l/h for *P. stipitis*, *C. shehatae* 117 A/1 *cys met* and 492 *met his*, respectively. None of the fusant strains possessed lower $Q_x$ values than their parental strains. PC3 and PC5, fusants from the *P. stipitis-C. shehatae* 492 *met his* hybridisation, had the $Q_x$ value of the *C. shehatae* 492 *met his* parent. Fusants of the *P. stipitis-C. shehatae* 117 A/1 *cys met* hybridisation showed slightly higher $Q_x$ values than the other set of fusants. The $Q_s$ values were generally high for all strains. Fusants PC1 to PC5, without exception, possessed higher $Q_s$ values than their parental strains. Fusants PCA1 to PCA5 showed much lower $Q_s$ values, ranging from 1.09 to 1.43 g/l/h, compared to 1.40 to 1.58 g/l/h for the former set of fusants. However, this trend was reversed upon comparison of the specific rates of substrate utilisation. PCA fusants had $q_s$ values ranging from 0.41 to 0.83 g/g/h, whereas, PC fusants showed $q_s$ values of 0.43 to 0.48 g/g/h. *P. stipitis* showed a low $q_s$ value of 0.43 g/g/h compared to 0.62 g/g/h for *C. shehatae* 117 A/1 *cys met*. *P. stipitis* also had a low $q_p$ value compared to that of *C. shehatae* 117 A/1 *cys met* (0.24

and 0.42 g/g/h, respectively). PC fusants had $q_p$ values of aproximately 0.24 g/g/h, although PC1 and PC2 showed slightly higher values of 0.28 and 0.26 g/g/h, respectively. On the other hand, PCA fusants had a wide range of $q_p$ values, from 0.15 to 0.34 g/g/h.

Values for the yield coefficient for biomass, with respect to substrate utilisation ($Y_{x/s}$), was relatively low for all strains. *P. stipitis* had a $Y_{x/s}$ value of 0.06 g/g and *C. shehatae* 117 A/1 *cys met* had a value of 0.05 g/g. Again, a trend emerged where PC fusants showed lower $Y_{x/s}$ values compared to PCA fusants. The $Y_{x/p}$ values also showed a similar trend. *P. stipitis* had a slightly higher value than *C. shehatae* 117 A/1 *cys met*. Similar patterns for the fusants were observed for the product-substrate coefficient ($Y_{p/s}$). A relatively high $Y_{p/s}$ value was observed for all strains. PC fusants displayed values less than or equal to that of the *P. stipitis* parent, whereas, the PCA fusants greater than or equal to either parent.

The general trend for specific ethanol production during the course of the fermentation was similar for the *P. stipitis* (Fig. 4.16a) and *C. shehatae* 117 A/1 *cys met* (Fig. 4.16b) parental strains as well as for most of the fusant strains (Fig. 17a, 17b, 17c, 18a, 18b, 19b). The $q_p$ value was highest at the begining of the fermentation and gradually tapered off to its lowest value towards the end of the fermentation. In the case of *C. shehatae* 492 *met his* (Fig. 4.16c) and a few of the fusant strains (Fig. 19a, 19c, 20), a more ambiguous trend was noticed where the $q_p$ values fluctuated throughout the fermentation.

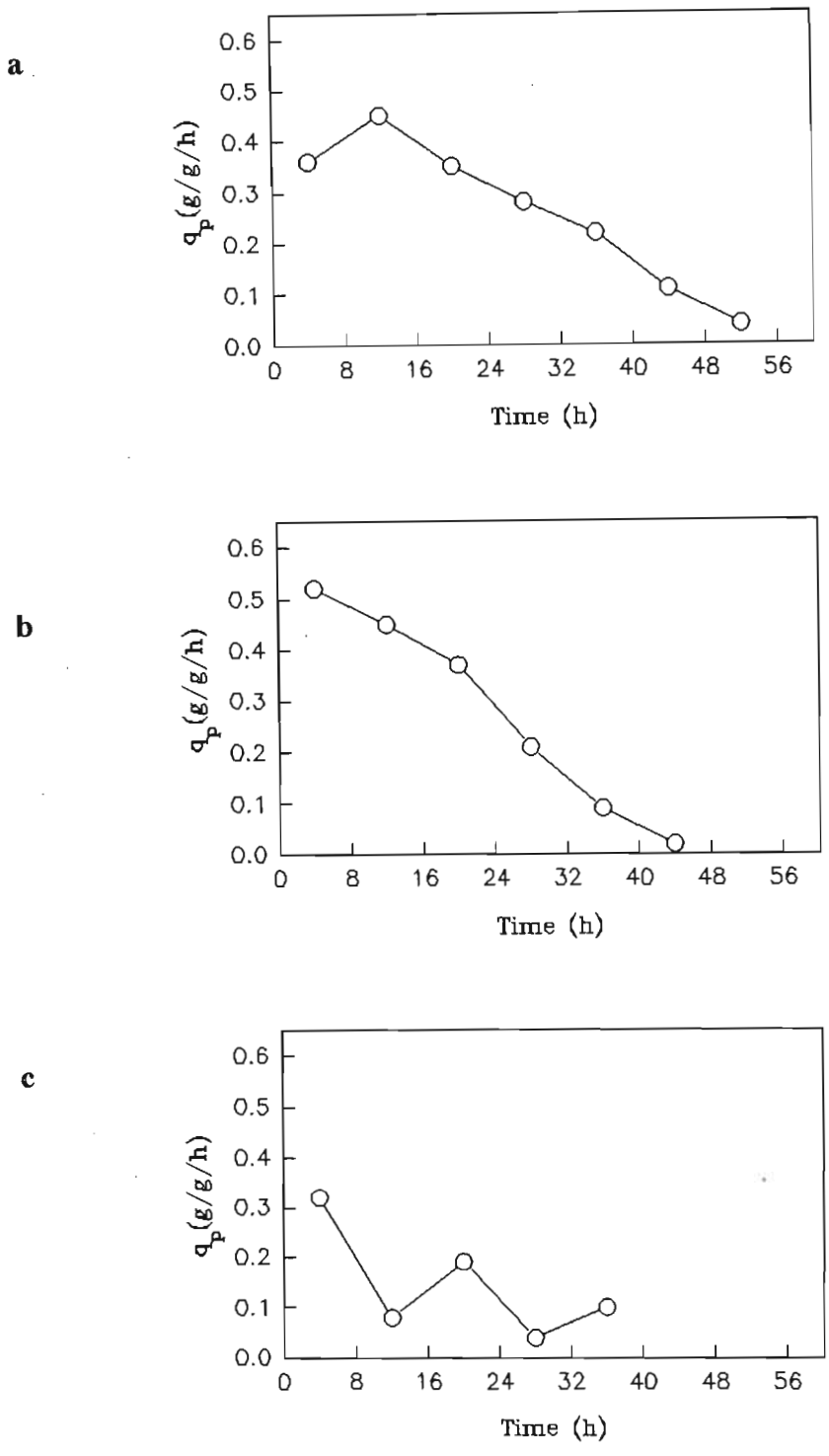
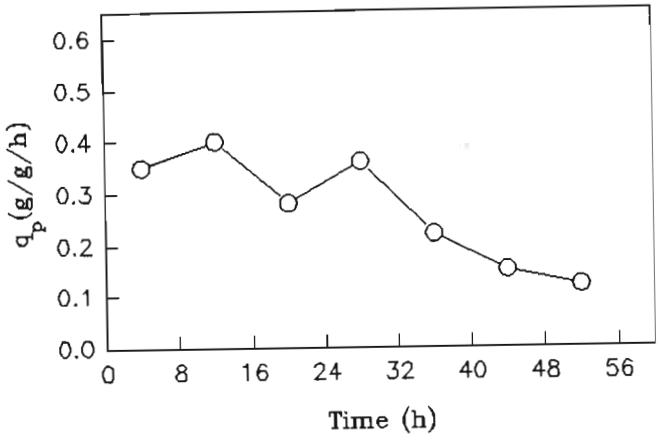



**Fig. 4.16.** Comparative time courses of specific ethanol production during shake-flask fermentations of (a) *P. stipitis* 633 *leu lys ala*; (b) *C. shehatae* 117 A/1 *cys met* and (c) *C. shehatae* 492 *met his*.

a

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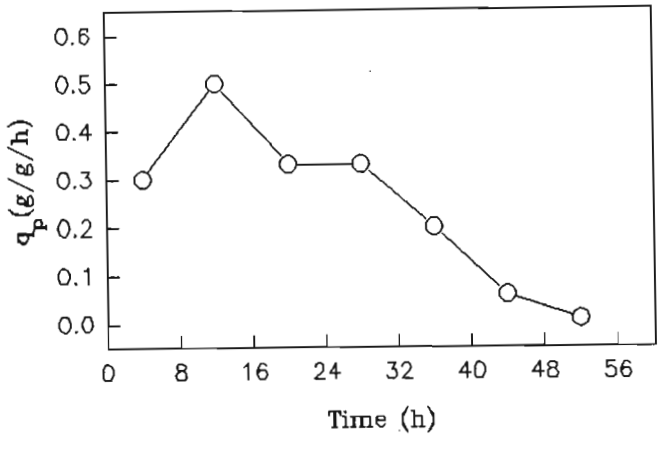


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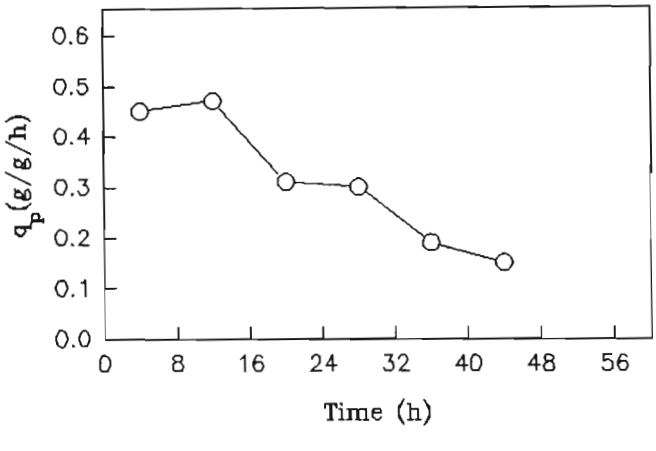
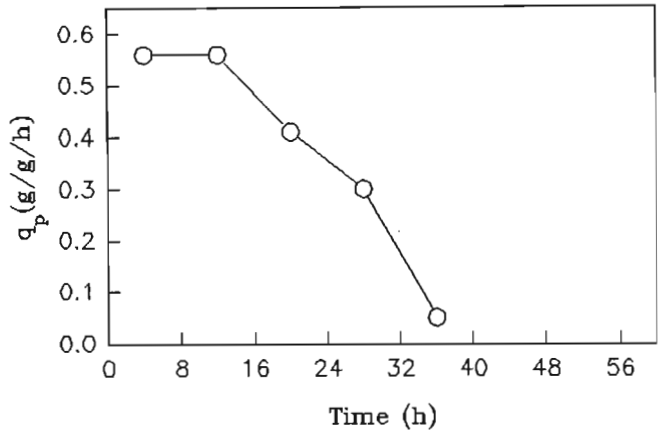



**Fig. 4.17.** Comparative time courses of specific ethanol production during shake-flask fermentations of (a) PC1; (b) PC2 and (c) PC3.

a

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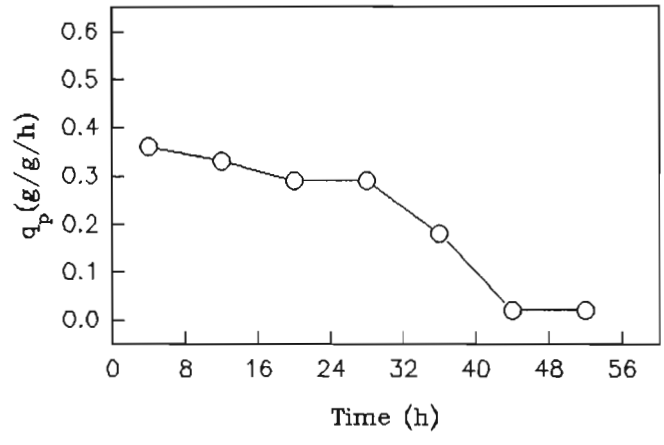


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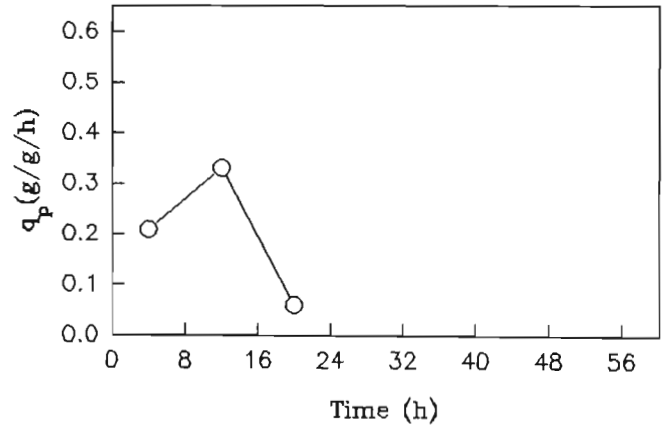
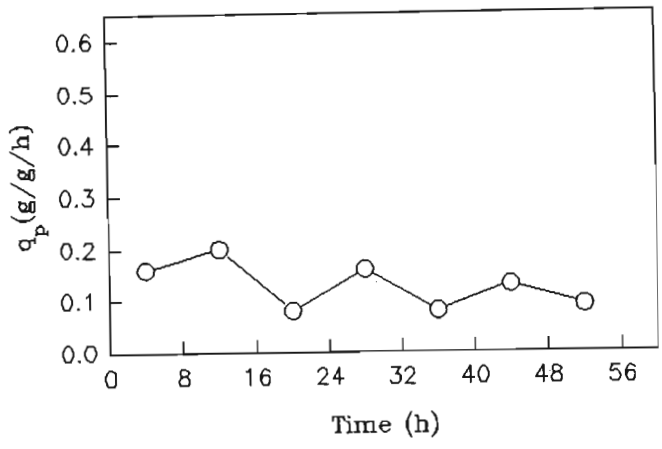



**Fig. 4.18.** Comparative time courses of specific ethanol production during shake-flask fermentations of (a) PC4; (b) PC5 and (c) PCA1.

a

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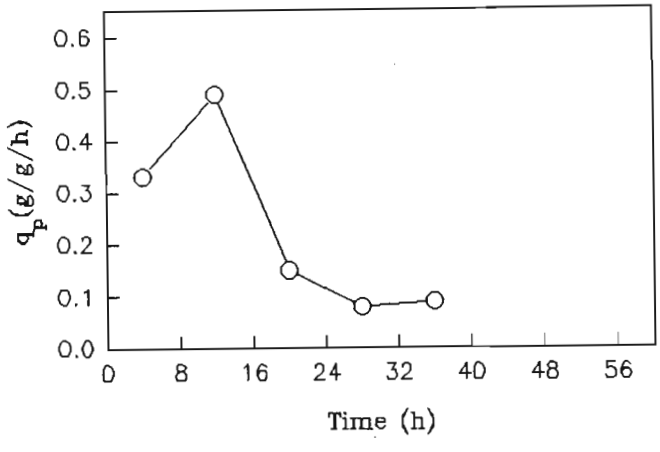


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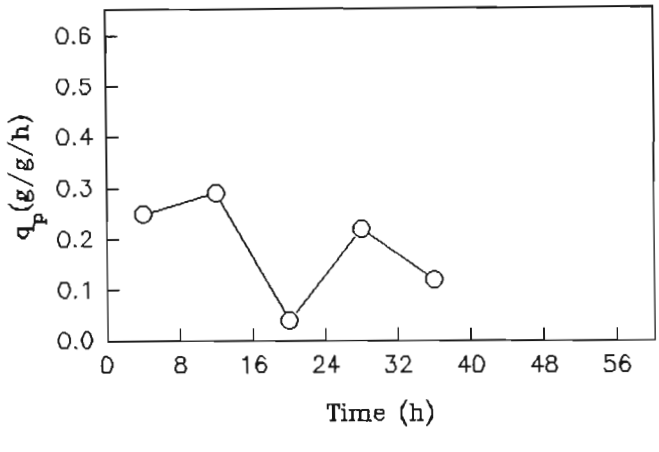


**Fig. 4.19.** Comparative time courses of specific ethanol production during shake-flask fermentations of (a) PCA2; (b) PCA3 and (c) PCA4.

a

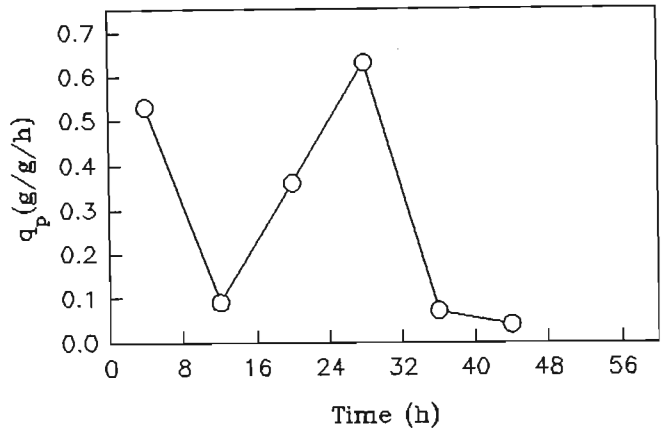


**Fig. 4.18.** Comparative time courses of specific ethanol production during shake-flask fermentations of (a) PCA5.

## 4.4 DISCUSSION

Recent reports by several workers concurred that *P. stipitis* and *C. shehatae* are the most efficient fermentors of D-xylose. They also agreed that fermentation conditions greatly influenced the performance of these strains. In this study, conditions established previously by other workers were used to compare the fermentative abilities of the *P. stipitis* and *C. shehatae* parental strains and their fusants. The wild type strains of all three parental strains have been extensively studied by other workers (du Preez *et al.*, 1984, 1987, 1989; du Preez and Prior, 1985; du Preez and van der Walt 1983; Prior, *et al.*, 1989). Different values for $Q_p$, $q_p$ and $Y_{p/s}$ have been reported by these workers. These variations were due either to differing fermentation conditions such as aeration,

temperature, pH, inoculum size and age, nitrogen source as well as initial sugar concentration used, or because of strain differences.

Maximum ethanol concentrations of 16.5, 26.2 and 18 g/l for *C. shehatae* 492 were reported by du Preez *et al.* (1984), du Preez and van der Walt (1983) and du Preez *et al.* (1989), respectively. In this study, a maximum ethanol concentration of 15 g/l was achieved by the mutant *C. shehatae* 492 *met his*. In accordance with the above-mentioned reports, high levels of xylitol and glycerol were also noted for this strain. In contrast to its very low specific rates of biomass and ethanol production, this strain displayed a relatively high rate of substrate utilisation. Even though substantial amounts of glycerol and xylitol were produced, the total amounts of biomass, ethanol, glycerol and xylitol produced cannot account for the substrate utilised. A probable explanation for this would be that the xylose was metabolised through the oxidative bypass of the Embden-Meyerhoff-Pathway, with the production of $CO_2$ at the expense of ethanol. The *P. stipitis* 633 wild-type strain had a yield coefficient of 0.45 from 5% xylose. The $Y_{p/s}$ value obtained for the mutant *P. stipitis* 633 *leu lys ala* was slightly higher, viz., 0.56. According to the stoichiometry: 3 xylose → 5 ethanol + 5 $CO_2$, which is based on xylose metabolism through the non-oxidative part of the hexose monophosphate pathway (HMP) and which does not take into account the possibility of the production of ethanol through the phosphoketolase pathway (Prior *et al.*, 1989), this $Y_{p/s}$ value would be equivalent to 105% of the theoretical maximum. du Preez and Prior (1985) reported an ethanol yield equivalent to 88% of the theoretical maximum. The very high yield obtained in this study would seem to indicate that the oxidative part of the HMP which is responsible for the recycling of NADPH plays a role in ethanol production. Ligthelm *et al.* (1988) reported

that no recycling of NADPH occurred during anaerobic fermentation whereas Prior *et al.* (1989) suggested that the carbon and nitrogen sources determined the amount of carbon recycyled oxidatively. If this part of the pathway was operational, an additional molecule of ethanol would be produced per three molecules of xylose. Therefore, the yield obtained would represent 90% of the theoretical maximum rather than the unrealistic 105%. du Preez and Prior (1985) also reported a $Y_{p/s}$ of 0.42 for the wild type strain of *C. shehatae* 117 A/1 compared to 0.57 obtained in this study. In contrast, they obtained $\mu_{max}$ values of 0.14, and 0.13 for the wild type *P. stipitis* 633 and *C. shehatae* 117 A/1 respectively, compared to 0.08 and 0.07 obtained for the mutants. These differences in the fermentation parameters can be explained by the lower inocula and higher agitation levels used by du Preez and Prior (1985). The use of a higher inoculum size resulted in a lower specific growth but a higher value for product yield, i.e., a more efficient transformation of xylose to ethanol with a comparatively lower accumulation of biomass. It is well documented that higher aeration levels promote biomass accumulation and xylitol production whereas limited aeration promotes ethanol production. The same growth conditions were used in both the fermentations except that du Preez and Prior (1985) used a rotary shaker speed of 150 rpm compared to the 90 rpm used in this study, which supports the above statement.

The higher ethanol yields and final ethanol concentrations linked with lower specific growth rates obtained for all parental strains in this study can also be explained by the aeration rate - inoculum size differences in the two experiments.

The *P. stipitis* parental strain produced the highest maximum ethanol concentration.

Most of the fusants produced slightly lower levels , although all produced higher levels than both the *C. shehatae* parental strains. In this respect it would therefore appear that they have inherited their fermentation characteristics from the *P. stipitis* parent. All of the fusants, except PCA2, displayed a higher volumetric rate of ethanol production than their parental strains. However, these strains also had a higher specific growth rate and the values for specific ethanol production reduced the differences between parental and fusant strains to $\pm 0.02$ g/g. Three of the fusant strains still had a higher specific rate of ethanol production than the *P. stipitis* parent although none of them displayed a higher $q_p$ value than the *C. shehatae* 117 A/1 *cys met* parent.

From the values for the final ethanol concentration, ethanol yield and specific ethanol production rate, it was evident that the *P. stipitis* parent remained the most efficient fermentor of xylose when compared to both the *C. shehatae* parental strains as well as the fusants. Indeed, these values for most of the fusants are so similar to that of the *P. stipitis* parent, that the question arises as to whether the differences are significant or rather simply due to slight variations in inoculum size, aeration and medium composition. However, examination of the trends in xylose utilisation and ethanol production indicated a character more *C. shehatae* 117 A/1 *cys met*-like for the fusants PCA1 to PCA5.

# CHAPTER FIVE

## GENERAL DISCUSSION

Molecular characterisation of the ethanolic D-xylose fermenting parental and fusant strains used in this study involved electrophoretic karyotyping, RFLP analysis of chromosomal and mitochondrial DNA and electrophoretic analysis of proteins. All the above-mentioned techniques permitted a clear-cut distinction between the *P. stipitis* and *C. shehatae* parental strains. However, all fusants appeared to be identical to one another and the *P. stipitis* parent. Fermentation studies revealed that most of the fusant strains displayed *P. stipitis*-like fermentation parameters except for one set of fusants that resembled the *C. shehatae* 117 A/1 *cys met* parent.

TAFE profiles revealed six and five chromosomes for *P. stipitis* and *C. shehatae*, respectively, and also showed polymorphisms for the *C. shehatae* strains. The polymorphic nature of *C. shehatae* strains has also been reported by Passoth *et al.* (1990). Run conditions were extremely important to obtain optimal separation of the chromosomes. Longer run times coupled with lower voltages, water quality and temperature were crucial. Deionised water with a conductivity of 10 to 2 x 10 mho/cm is ideal (Mary Morgenstern - Beckman Instruments, California, personal communication). If the conductivity of the water exceeds 4 x 10 mho/cm, then distorted, diffuse bands are produced. The preparation of agarose plugs is a relatively simple procedure. Once made, the plugs can be used for up to five months. However, finding a suitable programme for the optimal separation of the chromosomes is a time-consuming procedure. Once this has

been established, generating reproducible and well defined karyotypes becomes routine.

TAFE produced an identical profile for all fusants and the *P. stipitis* parent, indicating that chromosomal inheritance in the fusants was apparently almost entirely from the *P. stipitis* parent. Three possible permutations could result from the protoplast fusion process, viz., a true hybrid possessing both nuclear and cytoplasmic characters of both cells involved in the fusion, cybrids with a mixed cytoplasm but intact nuclei of each cell, or "partial" hybrids having the chromosomes of one cell and a few characters of the other due to partial gene exchange (Provost *et al.*, 1978 - cited by Gupthar and Garnett, 1987).

Protoplast fusion has apparently led to the production of fusants with the chromosomes of *P. stipitis* and a few genes from the *C. shehatae* parental strains integrated in the *P. stipitis* chromosomes. The relatively small increases in chromosome size incurred by gene insertion could not be detected by PFGE. This technique has been successfully applied to characterise hybrids produced by protoplast fusion, e.g., Hoffman *et al.* (1987) performed protoplast fusion with haploid strains of *S. cerevisiae* and *S. diastaticus* and used classical genetic techniques as well as OFAGE to characterise the fusants. Using OFAGE, they successfully identified the following fusants: a fusant having only *S. diastaticus* chromosomes which they identified as a cybrid, a fusant possessing all the chromosomes of both *S. cerevisiae* and *S. diastaticus*, and a fusant with twice the number of chromosomes of *S. cerevisiae* as well as the chromosomes of *S. diastaticus* which resulted from the fusion of two cells of *S. cerevisiae* with one of *S. diastaticus*. Smith *et al.* (1991) were able to detect chromosomal rearrangements in improved

cephalosporin C-producing strains of *Acremonium chrysogenum* using CHEF. Thus, when a more radical/drastic nuclear interaction than a few gene exchanges occurs, it can be detected using PFGE. Although PFGE has not proved successful in distinguishing fusants from the *P. stipitis* parent in this study, it has proved to be useful in the characterisation of fusant and parental strains as well as economically important strains of *S. cerevisiae*.

Where PFGE failed, it was hoped that RFLP analysis would provide information as to which parts of the genome originated from the *C. shehatae* parent. Again, RFLP profiles allowed differentiation between the *P. stipitis* and *C. shehatae* parental strains, with all fusants displaying an identical profile to that of the *P. stipitis* parent. A smear of fragments against which a few high intensity bands were evident was obtained for all the restriction endonucleases. These high intensity bands were generated by the cleavage of ribosomal DNA sequences and probably mitochondrial DNA (Ausubel *et al.*, 1989; Bostock *et al.*, 1993; Meaden, 1990). Insertion of single copy genes would be difficult to detect in the smear of fragments generated by restriction enzymes. The restriction endonucleases employed in this study all recognise six base pair sequences and therefore cleave DNA with lower frequency than those that have four base pair recognition sites. *Dra*I is considered to be a "rare-cutter" and although it produced a higher proportion of larger fragments than the other restriction endonucleases, no discrete bands could be resolved. Since *P. stipitis* has a 43% G+C content, a "rare-cutting" enzyme, with an eight base pair recognition site and which cleaves the DNA preferentially at GC sites may generate fewer fragments and thus produce a more distinct profile rather than a smear.

In order to determine polmorphisms in single copy genes, fingerprinting using a

number of gene probes is required (Meaden, 1990). In this study, only two gene probes derived from a *P. stipitis* strain were used to probe the products obtained by RFLP analysis and TAFE. These genes are involved in the initial steps of xylose metabolism and were considered important for the determination of gene copy number and linking this to ethanol production. Hybridisation of the *XYL*1 gene probe with the restriction endonuclease-generated profiles indicated that only one copy of the *XYL*1 gene was present in the fusants and that this gene originated from the *P. stipitis* parent. A high degree of homology existed between the probe and the *XYL*1 gene of the *P. stipitis* strain studied (strong signals were obtained under stringent hybridisation and washing conditions). Both *C. shehatae* strains possessed a lower degree of homology, as the signals were only obtained under low stringency conditions (45°C). The *XYL*2 gene probe, on the other hand, only hybridised to *P. stipitis* DNA. No signals were obtained under low stringency conditions, either for the fusants or *C. shehatae* strains, which could indicate that the *XYL*2 gene in the fusants originated from the *C. shehatae* parents. Considering the fact that the gene probe originated from a strain of *P. stipitis*, it would be expected to have a higher degree of homology than that which was evident in this study. Further tests need to be carried out using this probe before conclusions as to the origin of this gene in the fusants can be determined. Also, fingerprinting with other gene probes may reveal additional information on the contribution of *C. shehatae* genes to the fusants.

mtDNA analysis also revealed the predominance of *P. stipitis* DNA in the fusants. Considering the fact that cybrids are formed even if no nuclear interaction occurs during hybridisation, it could be surprising to note the absence of *C. shehatae* mtDNA in the fusants. In retrospect, however, it appears logical that only *P. stipitis* mtDNA should be

predominant in the fusants. If no recombination occurred before dissociation of the heterokaryon, then *P. stipitis* mtDNA should be the sole mitochondrial species in the fusants. The reasoning behind this statement is that the majority (95%) of mitochondrial proteins are encoded by the nucleus. Since the nucleus in the fusants is comprised predominantly of *P. stipitis* chromosomes, it is to be expected that almost entirely only *P. stipitis* mitochondrial proteins are produced. Since the two parental strains belong to two distinct genera and their presumed taxonomic relationship, i.e., perfect-imperfect partners, has been proved otherwise by various workers, it is likely that the *P. stipitis* mitochondrial proteins were not compatible for the biogenesis of *C. shehatae* mitochondria. The existing *C. shehatae* mitochondria present in the fusants immediately after dissociation of the heterokaryon, would have been diluted out in the fusant strains as cell division proceeded, to be eventually lost altogether.

It was interesting to note that *P. stipitis* possessed mitochondrial plasmids, whereas *C. shehatae* did not. The fusants inherited both the circular mtDNA and plasmids from the *P. stipitis* parent. Although the presence of mitochondrial plasmids is widespread among fungi (Collins and Saville, 1990; Meyer, 1991), their role in most cases is unknown. Scazzocchio (1987) speculated that such plasmids represent means by which sequences may transfer from one compartment to another or even between species. In the case of *kalilo* strains of *N. intermedia*, a free nuclear element transposes into the mtDNA and triggers the onset of the senescence phenomenon (Wright *et al.*, 1982). In strains of *Podospora*, the excision of a part of the mtDNA signals the onset of senescence. Since the plasmids in *P. stipitis* and the fusants have not been characterised, their role, if any, is still unknown. It would be of interest to see whether they are derived from nuclear or

mitochondrial sequences and discover their possible role in this yeast.

The collective data from the molecular characterisation studies indicate that the fusants are predominantly *Pichia*-like. Previous experiments also pointed out the resemblance between *P. stipitis* and the fusants. The fusants were found to resemble *P. stipitis* morphologically (Gupthar and Garnett, 1987) and DNA-DNA hybridisation analysis revealed a 74 to 86% homology with the *Pichia* parent (Selebano *et al.*, 1993).

Based on these results, it was anticipated that the fermentative abilities of the fusants would also resemble that of the *P. stipitis* parent. This was found to be true in shake flask fermentations. The *P. stipitis* strain was the most efficient fermenter of xylose and produced the highest maximum ethanol concentration. The fusants produced slightly lower levels of ethanol but the fermentation parameters were similar for *P. stipitis* and most of the fusants. Slight differences noted may be due to differing fermentative abilities of the various strains but was more likely due to inoculum size or slight variations in the concentration of different components of the fermentation medium. Both *C. shehatae* strains produced lower maximum ethanol concentrations than either the fusants or the *P. stipitis* strain. All the fusants, except PCA2, displayed a higher volumetric rate of ethanol production than their parental strains. They also had a higher specific growth rate and the differences between them for the value for specific ethanol production was reduced to 0.02 g/g. Although three of the fusants had a higher specific ethanol productivity than *P. stipitis*, none of them exceeded the $q_s$ value obtained for *C. shehatae* 117 A/1 *cys met*. These three fusants which resulted from the fusion between *P. stipitis* and *C. shehatae* 117 A/1 *cys met*, would seem to have inherited some metabolic properties of the *C. shehatae*

parental strain.

The fusants PC1 to PC5, resulting from the fusion between *P. stipitis* and *C. shehatae* 492 *met his* have without doubt inherited their fermentation characteristics from the *P. stipitis* parent as indicated from the data for final maximum ethanol concentration, ethanol yield, specific ethanol production and substrate utilisation trends. The PCA fusants, on the other hand, exhibited a more ambiguous behaviour. PCA1 and PCA5 displayed specific ethanol production rate values closer to that of the *C. shehatae* 117 A/1 *cys met*, while PCA3 had a value similar to that of the *P. stipitis* parent and PCA2 and PCA4 fusants had values much lower than either parental strain. From the fermentation results it appeared that the PCA fusants possessed a greater hybrid nature than the PC fusants, although this was not supported by molecular evidence. However, the various techniques used for the molecular characterisation of the fusants could not differentiate among the fusants or distinguish them from the *P. stipitis* parent. The differences in the fermentative abilities of the two sets of fusants, therefore remains unexplained. It can be deduced from the results obtained that these fusants are indeed only partial hybrids, most probably resulting from very few gene exchanges in the heterokaryon before its dissociation.

Further molecular characterisation of these fusants could be performed by RAPD fingerprinting. According to Williams *et al.* (1990), RAPDs can be used to identify hybrids that carry deletions and insertions of chromosomal segments, a description which fits the fusants in this study. RAPD fingerprinting of the fusants would, however, be a purely academic excercise because these fusants were constructed with the objective of

obtaining more efficient D-xylose fermenters. Fermentation tests indicated that the *P. stipitis* strain is more efficient than the fusants, therefore, future work should concentrate on finding another means of producing efficient ethanolic D-xylose fermenting strains.

Both desirable and undesirable traits can be conferred on the hybrids by protoplast fusion. A more direct approach of obtaining more efficient xylose fermenters would be to clone genes coding for enzymes involved in D-xylose metabolism into *P. stipitis*, which is one of the most efficient fermenters. Cloning them into *S. cerevisiae* did not produce an efficient D-xylose fermenting *S. cerevisiae* strain. Recently, a system for the transformation of *P. stipitis* was developed (Ho *et al.*, 1991). This technique may prove to be the solution to obtaining a commercially viable strain of ethanolic D- xylose fermenting yeast.

## REFERENCES

**Aguilera, A., and T. Benitez.** 1985. Role of mitochondria in ethanol tolerance of *Saccharomyces cerevisiae. Arch. Microbiol.* **142**:389-392.

**Alexander, M.A., T.W. Chapman, and T.W. Jeffries.** 1988. Continuous xylose fermentation by *Candida shehatae* in a two-stage reactor. *Appl. Biochem. Biotechnol.* **17**:221-229.

**Alexander, N.J.** 1985. Temperature sensitivity of the induction of xylose reductase in *Pachysolen tannophilus. Biotechnol. Bioeng.* **27**:1739-1744.

**Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology, Vol. 2, John Wiley and Sons, New York.

**Baptista-Ferreira, J.L.C., A. Economou, and L.A. Casselton.** 1983. Mitochondrial genetics of *Coprinus*: Recombination of mitochondrial genomes. *Curr. Genet.* **7**:405-407.

**Barnett, J.A.** 1976. The utilization of sugars by yeasts. *Adv. Carbohydr. Chem. Biochem.* **32**:125-234.

**Batt, C.A., S. Carvallo, D.D. Easson Jnr., M. Akedo, and A.J. Sinskey.** 1986. Direct evidence for a xylose metabolic pathway in *Saccharomyces cerevisiae. Biotechnol. Bioeng.* **28**:549-553.

**Beck, M.J.** 1986. Effect of intermittent feeding of cellulose hydrolysate to hemicellulose hydrolysate on ethanol yield by *Pachysolen tannophilus. Biotechnol. Lett.* **8**:513-516.

**Bernardi, G., and G. Bernardi.** 1986. Compositional constraints and genome evolution. *J. Mol. Evol.* **24**:1-11.

**Birnboim, H.C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7**:1513-1523.

**Bolen, P.L., and R.W. Detroy.** 1985. Induction of NADPH-linked D-xylose reductase and NAD-linked xylitol dehydrogenase activities in *Pachysolen tannophilus* by D-xylose, L-arabinose, or D-galactose. *Biotechnol. Bioeng.* **27**:302-307.

**Bolen, P.L., K.A. Roth, and S.N. Freer.** 1986. Affinity purifications of aldose reductase and xylitol dehydrogenase from the xylose-fermenting yeast *Pachysolen tannophilus. Appl. Environ. Microbiol.* **52**:660-664.

**Bolotin-Fukuhara, M., and L.A. Grivell**. 1992. Genetic approaches to the study of mitochondrial biogenesis in yeast. *Antonie van Leeuwenhoek* **62**:131-153.

**Borkhardt, B., and L.W. Olson**. 1983. Paternal inheritance of the mitochondrial DNA in interspecific crosses of the aquatic fungus *Allomyces. Curr. Genet.* **7**:403-405.

**Bostock, A., M.N. Khattak, R. Matthews, and J. Burnie.** 1993. Comparison of PCR fingerprinting, by random amplification of polymorphic DNA, with other molecular typing methods for *Candida albicans. J. Gen. Microbiol.* **139**:2179-2184.

**Bruinenberg, P.M., P.H.M. de Bot, J.P. van Dijken, and W.A. Scheffers.** 1984. NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. *Appl. Microbiol. Biotechnol.* **19**:256-260.

**Bruinenberg, P.M., J.P. van Dijken, and W.A. Scheffers.** 1983. A theoretical analysis of NADPH production and consumption in yeast. *J. Gen. Microbiol.* **129**:953-964.

**Bruinenberg, P.M., J.P. van Dijken, and W.A. Scheffers.** 1986. A radiorespirometric study of the hexose mono-phosphate pathway for glucose metabolism in *Candida utilis* CBS 621 grown in glucose-limited chemostat cultures. *J. Gen. Microbiol.* **132**:221-229.

**Burnette, W.N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulphate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**:195-203.

**Carle, G.F., and M.V. Olson.** 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci.* **82**:3756-3760.

**Clarke-Walker, G.D., and K.S. Sriprakash.** 1981. Sequence rearrangements between mitochondrial DNAs of *Torulopsis glabrata* and *Kloeckera africana* identified by hybridisation with six polypeptide encoding regions from *Saccharomyces cerevisiae* mitochondrial DNA. *J. Mol. Biol.* **151**:367-387.

**Collins, R.A., and B.J. Saville.** 1990. Independent transfer of mitochondrial chromosomes and plasmids during unstable vegetative fusion in *Neurospora. Nature.* **345**:177-179.

**Defontaine, A., F.M. Lecocq, and J.N. Hallet.** 1991. A rapid miniprep method for the preparation of yeast mitochondrial DNA. *Nucl. Acids Res.* **19**:185.

**Dekker, R.F.K.** 1982. Ethanol production from D-xylose and other sugars by the yeast *Pachysolen tannophilus. Biotechnol. Lett.* **4**:411-416.

**Dellweg, H., M. Rissi, H. Methner, and D. Debus.** 1984. Xylose fermentation by yeasts. 3. Comparison of *Pachysolen tannophilus* and *Pichia stipitis. Biotechnol. Lett.* **6**:395-400.

**DeZamaroczy, M., and G. Bernardi.** 1985. Sequence organisation of the mitochondrial genome of yeasts - a review. *Gene* **37**:1-17.

**Ditzelmüller, G., E.M. Kubicek-Pranz, M. Röhr, and C.P. Kubicek.** 1985. NADPH-specific and NADH-specific xylose reductase is catalyzed by two separate enzymes in *P. tannophilus. Appl. Microbiol. Biotechnol.* **22**:297-299.

**Ditzelmüller, G., C.P. Kubicek, W. Wöhrer, and M. Röhr.** 1984a. Xylose metabolism in *P. tannophilus*: purification and properties of xylose reductase. *Can. J. Microbiol.* **30**:1330-1336.

**Ditzelmüller, G., C.P. Kubicek, W. Wöhrer, and M. Röhr.** 1984b. Xylitol dehydrogenase from *P. tannophilus. FEMS Microbiol. Lett.* **25**:195-198.

**D'Souza, T.M., S.B. Dass, A. Rasooly, and C.A. Reddy.** 1993. Electrophoretic karyotyping of the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Mol. Microbiol.* **8**:803-807.

**du Preez, J.C., M. Bosch, and B.A. Prior.** 1986. Xylose fermentation by *Candida shehatae* and *Pichia stipitis*: Effects of pH, temperature and substrate concentration. *Enz. Microb. Technol.* **8**:360-364.

**du Preez, J.C., M. Bosch, and B.A. Prior.** 1987. Temperature profiles of growth and ethanol tolerance of the xylose-fermenting yeasts *Candida shehatae* and *Pichia stipitis. Appl. Microbiol. Biotechnol.* **25**:521-525.

**du Preez, J.C., and B.A. Prior.** 1985. A quantitative screening of some xylose-fermenting yeast isolates. *Biotechnol. Lett.* **7**:241-246.

**du Preez, J.C., B.A. Prior, and M.T. Monteiro.** 1984. The effect of aeration on xylose fermentation by *Candida shehatae* and *Pachysolen tannophilus*, a comparative study. *Appl. Microbiol. Biotechnol.* **19**:261-266.

**du Preez, J.C., and J.P. van der Walt.** 1983. Fermentation of D-xylose to ethanol by a strain of *Candida shehatae. Biotechnol. Lett.* **5**:357-362.

**du Preez, J.C., B. van Driessel, and B.A. Prior.** 1989. Effect of aerobiosis on fermentation and key enzyme levels during growth of *Pichia stipitis, Candida shehatae* and *Candida tenuis* on D-xylose. *Arch. Microbiol.* **152**:143-147.

**Earl, A.J., G. Turner, J.H. Croft, R.B.G. Dales, C.M. Lazarus, H. Lünsdorf, and H. Küntzel.** 1981. High frequency transfer of species specific mitochondrial sequences between members of the Aspergillaceae. *Curr. Genet.* **3**:221-228.

**Ehrlich, S.D., J.P. Thiery, and G. Bernardi.** 1972. The mitochondrial genome of wild-type yeast cells. III. The pyrimidine tracts of mitochondrial DNA. *J. Mol. Biol.* **65**:207-212.

**Evans, C.T., and C. Ratledge.** 1984. Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown on D-xylose: The key to efficient metabolism. *Arch. Microbiol.* **139**:48-52.

**Fukuhara, H., F. Sor, R. Drissi, N. Dinouël, I. Miyakawa, S. Rousset, and A.M. Viola.** 1993. Linear mitochondrial DNAs of yeasts: Frequency of occurrence and general features. *Mol. Cell. Biol.* **13**:2309-2314.

**Gardiner, K., and D. Patterson.** 1988. Transverse alternating electrophoresis. *Nature* **331**:371-372.

**Grootjen, D.R.J., R.G.J.M. van der Lans, and K.C.A.M. Luyben.** 1991. Conversion of glucose/xylose mixtures by *Pichia stipitis* under oxygen-limited conditions. *Enz. Microb. Technol.* **13**:648-654.

**Gupthar, A.S.** 1987. Construction of a series of *Pichia stipitis* strains with increased DNA contents. *Curr. Genet.* **12**:605-610.

**Gupthar, A.S.** 1992. Segregation of altered parental properties in fusions between *Saccharomyces cerevisiae* and the D-xylose fermenting yeasts *Candida shehatae* and *Pichia stipitis*. *Can. J. Microbiol.* **38**:1233-1237.

**Gupthar, A.S., and H.M. Garnett.** 1987. Hybridisation of *Pichia stipitis* with its presumptive imperfect partner *Candida shehatae*. *Curr. Genet.* **12**:199-204.

**Hinze, W.E., M. Mohan, J.B. Anderson, and P.A. Horgen.** 1985. The mitochondrial DNAs of *Agaricus*: Heterogeneity in *A. bitorquis* and homogeneity in *A. brunnescens*. *Curr. Genet.* **9**:127-132.

**Ho, N.W.Y., D. Petros, and X.X. Deng.** 1991. Genetic transformation of xylose-fermenting yeast *Pichia stipitis*. *Appl. Biochem. Biotechnol.* **28/29**:369-375.

**Hoffman, M., M. Zimmerman, and C.-C. Emeis.** 1987. Orthogonal field alternating gel electrophoresis (OFAGE) as a means for the analysis of somatic hybrids obtained by protoplast fusion of different *Saccharomyces* strains. *Curr. Genet.* **11**:599-603.

**Hsiao, H.Y., L.C. Chiang, P.P. Ueng, and G.T. Tsao.** 1982. Sequential utilisation of mixed monosaccharides by yeasts. *Appl. Environ. Microbiol.* **43**:840-845.

**Hyde, J.E.** 1990. Molecular parasitology, pp. 31-35, Open University Press, U.K.

**Iserentant, D.** 1990. Methods for the genetic engineering of yeasts, pp. 35-45. *In* H. Verachtert and R. de Mot (eds.), Yeast: biotechnology and biocatalysis. Bioprocess technology, vol. 5, Marcel Dekker Inc., New York.

**James, A.P., and D.M. Zahab.** 1982. A genetic system for *Pachysolen tannophilus*, a pentose-fermenting yeast. *J. Gen. Microbiol.* **128**:2297-2301.

**James, A.P., and D.M. Zahab.** 1983. The construction and genetic analysis of polyploids and aneuploids of the pentose-fermenting yeast *Pachysolen tannophilus*. *J. Gen. Microbiol.* **129**:2489-2494.

**Jeffries, T.W.** 1981a. Conversion of xylose to ethanol under aerobic conditions by *Candida tropicalis*. *Biotechnol. Lett.* **3**:213-218.

**Jeffries, T.W.** 1981b. Fermentation of xylulose to ethanol using xylose isomerase and yeasts. *Biotechnol. Bioeng. Symp.* **11**:315-324.

**Jeffries, T.W.** 1982. A comparison of *Candida tropicalis* and *Pachysolen tannophilus* for conversion of xylose to ethanol. *Biotechnol. Bioeng. Symp.* **12**:103-110.

**Jeffries, T.W.** 1983. Effects of nitrate on fermentation of xylose and glucose by *Pachysolen tannophilus. Biotechnology* **1**:503-506.

**Jeffries, T.W.** 1985a. Emerging technology for fermenting D-xylose. *Trends Biotechnol.* **3**:208-212.

**Jeffries, T.W.** 1985b. Effects of culture conditions on the fermentation of xylose to ethanol by *C. shehatae. Biotechnol. Bioeng. Symp.* **15**:149-166.

**Jeffries, T.W.** 1990. Fermentation of D-xylose and cellobiose, pp. 349-394. *In* H. Verachtert and R. de Mot (eds.), Yeast: biotechnology and biocatalysis. Bioprocess technology, vol. 5, Marcel Dekker Inc., New York.

**Jeffries, T.W., J.H. Fady, and E.N. Lightfoot.** 1985. Effect of glucose supplements on fermentation of xylose by *Pachysolen tannophilus. Biotechnol. Bioeng.* **27**:171-176.

**Jeffries, T.W., and H.K. Sreenath.** 1988. Fermentation of hemicellulosic sugars and sugar mixture by *Candida shehatae. Biotechnol. Bioeng.* **31**:502-506.

**Johannsen, E., L. Eagle, and G. Bredenhann.** 1985. Protoplast fusion used for the construction of presumptive polyploids of the D-xylose-fermenting yeast *Candida shehatae. Curr. Genet.* **9**:313-319.

**Jones, R.P., and P.F. Greenfield.** 1985. Replicative inactivation and metabolic inhibition in yeast ethanol fermentations. *Biotechnol. Lett.* **7**:223-228.

**Kilian, S.G., and N. van Uden.** 1988. Transport of xylose and glucose in the xylose fermenting yeast *Pichia stipitis. Appl. Microbiol. Biotechnol.* **27**:545-548.

**Kötter, P., R. Amore, C.P. Hollenberg, and M. Ciriacy.** 1990. Isolation and characterisation of the *Pichia stipitis* xylitol dehydrogenase gene, *XYL2*, and construction of a xylose-utilising *Saccharomyces cerevisiae* transformant. *Curr. Genet.* **18**:493-500.

**Kunz, G., R. Bode, and D. Birnbaum.** 1986. Physical mapping and genome organisation of mitochondrial DNA from *Candida maltosa. Curr. Genet.* **10**:527-530.

**Lachke, A.H., and T.W. Jeffries.** 1986. Levels of enzymes of the pentose phosphate pathway in *Pachysolen tannophilus* Y-2460 and selected mutants. *Enzyme. Microb. Technol.* 8:353-359.

**Laemmli, U.K.** 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

**Laplace, J.M., J.P. Delgenes, R. Moletta, and J.M. Navarro.** 1991. Combined alcoholic fermentation of D-xylose and D-glucose by selected microbial strains: process considerations in relation to ethanol tolerance. *Biotechnol. Lett.* **13**:445-450.

**Laplace, J.M., J.P. Delgenes, R. Moletta, and J.M. Navarro.** 1992. Alcoholic glucose and xylose fermentations by the coculture process: compatibility and typing of associated strains. *Can. J. Microbiol.* **38**:654-658.

**Ligthelm, M.E., B.A. Prior, J.C. du Preez, and V. Brandt.** 1988. An investigation of D-{1-$^{13}$C} xylose metabolism in *Pichia stipitis* under aerobic and anaerobic conditions. *Appl. Microbiol. Biotechnol.* **28**:293-296.

**Linko, Y.Y., H. Kautola, S. Uotila, and P. Linko.** 1986. Alcoholic fermentation of D-xylose by immobilised *Pichia stipitis. Biotechnol. Lett.* 8:47-52.

**Lucas, C., and N. van Uden.** 1985. The temperature profiles of growth, thermal death and ethanol tolerance of the xylose-fermenting yeast, *Candida shehatae. J. Basic. Microbiol.* **25**:547-550.

**Lucas, C., and N. van Uden.** 1986. Transport of hemicellulose monomers in the xylose-fermenting yeast *Candida shehatae. Appl. Microbiol. Biotechnol.* **23**:491-495.

**Maiorella, B.L., H.W. Blanch, and C.R. Wilkie.** 1984. Economic evaluation of alternative ethanol fermentation processes. *Biotechnol. Bioeng.* **26**:1003-1025.

**Maleszka, R., A.P. James, and H. Schneider.** 1983. Ethanol production from various sugars by strains of *Pachysolen tannophilus* bearing different numbers of chromosomes. *J. Gen. Microbiol.* **129**:2495-2500.

**Maleszka, R., and H. Schneider.** 1982. Fermentation of D-xylose, xylitol and D-xylulose by yeasts. *Can. J. Microbiol.* **28**:360-363.

**Maleszka, R., P.Y. Wang, and H. Schneider.** 1982a. Yeasts that ferment D-cellobiose as well as D-xylose. *Biotechnol. Lett.* **4**:133-136.

**Maleszka, R., P.Y. Wang, and H. Schneider.** 1982b. Ethanol production from D-galactose and glycerol by *Pachysolen tannophilus. Enzyme Microb. Technol.* **4**:349-352.

**Mannella, C.A., T.H. Pittenger, and A.M. Lambowitz.** 1979. Transmission of mitochondrial deoxyribonucleic acid in *Neurospora crassa* sexual crosses. *J. Bacteriol.* **137**:1449-1451.

**May, G., and J.W. Taylor.** 1989. Independent transfer of mitochondrial plasmids in *Neurospora crassa. Nature* **339**:320-322.

**Meaden, P.** 1990. DNA fingerprinting of brewers yeast: current perspectives. *J. Inst. Brew.* **96**:195-200.

**Meyer, R.J.** 1991. Mitochondrial DNA's and plasmids as taxonomic characteristics in *Trichoderma viride. Appl. Env. Microbiol.* **57**:2269-2276.

**Miller, M., J.L.F. Kock, G.H.J. Pretorius, and D.J. Coetzee.** 1989. The value of orthogonal-field-alternating gel electrophoresis and other criteria in the taxonomy of the genus *Pichia* Hansen emend. Kurtzman. *Syst. Appl. Microbiol.* **12**:191-202.

**Mitchell, M.B., and H.K. Mitchell.** 1952. A case of "maternal" inheritance in *Neurospora crassa. Proc. Natl. Acad. Sci.* **38**:442-449.

**Morace, G., P. Cattani, S. Manzara, S. Conti, M. Gerloni, M. Sanguinetti, B. Posteraro, and L. Polonelli.** 1992. Genomic studies on killer yeasts belonging to the genus *Pichia. Antonie van Leeuwenhoek* **62**:215-223.

**Morgenstern, M.** - Personal communication. Beckman, Instruments, California, U.S.A.

**Novak, M., P. Strehaiano, M. Moreno, and G. Goma.** 1981. Alcoholic fermentation: on the inhibitory effect of ethanol. *Biotechnol. Bioeng.* **23**:201-211.

**O'Connor, R.M., C.R. McArthur, and G.D. Clark-Walker.** 1976. Respiratory deficient mutants of *Torulopsis glabrata* a yeast with circular mitochondrial deoxyribonucleic acid of 6$\mu$m. *J. Bacteriol.* **126**:959-968.

**O'Farrel, P.Z., H.M. Goodman, and P.H. O'Farrel.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**:133-142.

**Oliver, S.G.** 1991. "Classical" yeast biotechnology, pp. 213-248. *In* M.F. Tuite and S.G. Oliver (eds.), *Saccharomyces* biotechnology handbooks. Plenum Press, New York.

**Orgel, L.E., F.H.C. Crick, and C. Sapienza.** 1980. Selfish DNA. *Nature* **288**:645-646.

**Palnitkar, S., and A. Lachke.** 1992. Effect of nitrogen sources on oxidoreductive enzymes and ethanol production during D-xylose fermentation by *Candida shehatae. Can. J. Microbiol.* **38**:258-260.

**Panchal, C.J., L. Bast, T. Dowhanick, and G.G. Stewart.** 1987. A rapid, simple and reliable method for differentiating brewing yeast strains based on DNA restriction patterns. *J. Inst. Brew.* **93**:325-327.

**Panchal, C.J., L. Bast, I. Russel, and G.G. Stewart.** 1988. Repression of xylose utilisation by glucose in xylose-fermenting yeasts. *Can. J. Microbiol.* **34**:1316-1320.

**Parekh, S.R., S. Yu, and M. Wayman.** 1986. Adaptation of *Candida shehatae* and *Pichia stipitis* to wood hydrolysates for increased ethanol production. *Appl. Microbiol. Biotechnol.* **25**:300-304.

**Passoth, V., M. Hansen, U. Klinner, and C.C. Emeis.** 1992. The electrophoretic banding pattern of the chromosomes of *Pichia stipitis* and *Candida shehatae*. *Curr. Genet.* **22**:429-431.

**Piškur, J.** 1988. Transmission of yeast mitochondrial loci to progeny is reduced when nearby intergenic regions containing *ori/rep* sequences are deleted. *Mol. Gen. Genet.* **214**:425-432.

**Piškur, J.** 1989. Transmission of the yeast mitochondrial genome to progeny: The impact of intergeneric sequences. *Mol. Gen. Genet.* **218**:161-168.

**Prior, B.A., S.G. Kilian, and J.C. du Preez.** 1989. Fermentation of D-xylose by the yeasts *Candida shehatae* and *Pichia stipitis*. Prospects and problems. *Process Biochem.* **24**:21-32.

**Querol, A., and E. Barrio.** 1990. A rapid and simple method for the preparation of yeast mitochondrial DNA. *Nucleic Acids Res.* **18**:1657.

**Querol, A., E. Barrio, T. Huerta, and D. Ramón.** 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl. Environ. Microbiol.* **58**:2948-2953.

**Sarthy, A.V., B.L. McConaughy, Z. Lobo, J.A. Sundstrom, C.E. Furlong, and B.D. Hall.** 1987. Expression of *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **53**:1996-2000.

**Scazzocchio, C.** 1987. Evolutionary biology of the fungi, pp. 52-73. *In* A.D.M. Rayner, C.M. Brasier and D. Moore (eds.), Cambridge University Press, Cambridge.

**Schneider, H., G. Mahmourides, J.L. Mabelle, H. Lee, N. Maki, and H.J. McNeil.** 1985. Correlation between limitation of growth of *Pachysolen tannophilus* on D-xylose with the formation of ethanol and other products. *Biotechnol. Lett.* **7**:361-364.

**Schneider, H., P.Y. Wang, Y.K. Chan, and R. Maleszka.** 1981. Conversion of D-xylose to ethanol by the yeast *Pachysolen tannophilus*. *Biotechnol. Lett.* **3**:89-92.

**Schvester, P., C.W. Robinson, and M. Moo-Young.** 1983. Xylose fermentation to ethanol by *Pachysolen tannophilus*. *Biotechnol. Bioeng. Symp.* **13**:131-152.

**Schwartz, D.C., and C.R. Cantor.** 1984. Separation of chromosomal-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37**:67-75.

**Selebano, E.T., R. Govinden, D. Pillay, B. Pillay, and A.S. Gupthar.** 1993. Genomic comparisons among parental and fusant strains of *Candida shehatae* and *Pichia stipitis*. *Curr. Genet.* **23**:468-471.

**Skoog, K., and B. Hahn-Hagërdal.** 1990. Effect of oxygenation on xylose fermentation by *Pichia stipitis*. *Appl. Environ. Microbiol.* **56**:3389-3394.

**Slininger, P.J., P.L. Bolen, and C.P. Kurtzman.** 1987. *Pachysolen tannophilus*: Properties and process considerations for ethanol production from D-xylose. *Enz. Microb. Technol.* **9**:5-23.

**Slininger, P.J., R.J. Bothast, M.R. Ladisch, and M.R. Okos.** 1990. Optimum pH and temperature conditions for xylose fermentation by *Pichia stipitis. Biotechnol. Bioeng.* **35**:727-731.

**Slininger, P.J., R.J. Bothast, M.R. Okos, and M.R. Ladisch.** 1985. Comparative evaluation of ethanol production by xylose-fermenting yeasts presented high xylose concentrations. *Biotechnol. Lett.* **7**:431-436.

**Slininger, P.J., R.J. Bothast, J.E. van Cauwenberge, and C.P. Kurtzman.** 1982. Conversion of D-xylose to ethanol by the yeast *Pachysolen tannophilus. Biotechnol. Bioeng.* **24**:371-384.

**Slininger, P.J., L.E. Branstrator, R.J. Bothast, M.R. Okos, and M.R. Ladisch.** 1991. Growth, death, and oxygen uptake kinetics of *Pichia stipitis* on xylose. *Biotechnol. Bioeng.* **37**:973-980.

**Smiley, K.L., and P.L. Bolen.** 1982. Demonstration of D-xylose reductase and D-xylitol dehydrogenase in *Pachysolen tannophilus. Biotechnol. Lett.* **4**:607-610.

**Smith, C.L., and C.R. Cantor.** 1989. Purification, fragmentation and separation of large DNA molecules. *Nature* **338**:520-522.

**Smith, A.W., K. Collis, M. Ramsden, H.M. Fox, and J.H. Peberdy.** 1991. Chromosome rearrangements in improved cephalosporin C-producing strains of *Acremonium chrysogenum. Curr. Genet.* **19**:235-237.

**Sreenath, H.K., T.W. Chapman, and T.W. Jeffries.** 1986. Ethanol production from D-xylose in batch fermentations with *Candida shehatae*: process variables. *Appl. Microbiol. Biotechnol.* **24**:294-299.

**Sreenath, H.K., and T.W. Jeffries.** 1987. Batch and membrane-assisted cell recycling in ethanol production by *Candida shehatae. Biotechnol. Lett.* **9**:293-298.

**Su, C.S., and S.A. Meyer.** 1991. Characterisation of mitochondrial DNA in various *Candida* species: Isolation, restriction endonuclease analysis, size and base composition. *Int. J. Syst. Bacteriol.* **41**:6-14.

**Takuma, S., N. Nakashima, M. Tantirungkij, S. Kinoshita, H. Okada, T. Seki, and T. Yoshida.** 1991. Isolation of xylose reductase gene of *Pichia stipitis* and its expression in *Saccharomyces cerevisiae. Appl. Biochem. Biotechnol.* **28/29**:327-340.

**Taylor, J.W.** 1986. Fungal evolutionary biology and mitochondrial DNA. *Exp. Mycol.* **10**:259-269.

**Toivola, A., D. Yarrow, E. van den Bosch, J.P. van Dijken, and W.A. Scheffers.** 1984. Alcoholic fermentation of D-xylose by yeasts. *Appl. Environ. Microbiol.* **47**:1221-1223.

**Tsao, G.T., M.R. Ladisch, M. Voloch, and P. Bienkowski.** 1982. Production of ethanol and chemicals from cellulosic materials. *Process Biochem.* **17**:34-38.

**van der Westhuizen, T.J., and I. S. Pretorius.** 1992. The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Antonie van Leeuwenhoek* **61**:249-257.

**van Vuuren, H.J.J., and L. van der Meer.** 1987. Fingerprinting of yeasts by protein electrophoresis. *Am. J. Enol. Vitic.* **38**:49-53.

**Verduyn, C., J. Frank, Jr., J.P. van Dijken, and W.A. Scheffers.** 1985a. Multiple forms of xylose reductase in *Pachysolen tannophilus. FEMS Microbiol. Lett.* **30**:313-317.

**Verduyn, C., R. van Kleef, J. Frank, Jr., H. Schreuder, J.P. van Dijken, and W.A. Scheffers.** 1985b. Properties of the NAD(P)H-dependant xylose reductase from the xylose fermenting yeast, *P. stipitis. Biochem. J.* **226**:669-677.

**Vezinhet, F, B. Blondin, and J-N. Hallet.** 1990. Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of enological strains of *Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol.* **32**:568-571.

**Viljoen, B.C., J.L.F. Kock, and D.J. Coetzee.** 1988. Orthogonal field alternating gel electrophoretic banding patterns of some asporogenous yeasts and their ascosporogenous states. *System. Appl. Microbiol.* **10**:228-230.

**Wayman, M., and S. Parekh.** 1985. Ethanol and sugar tolerance of *Candida shehatae. Biotechnol. Lett.* **7**:909-912.

**Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**:7213-7218.

**Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**:6531-6535.

**Wilson, J.J., G.G. Khachatourian, and W.M. Ingledew.** 1982. Protoplast fusion in the yeast *Saccharomyces alluvius. Mol. Gen. Genet.* **186**:95-100.

**Wright, R.M., M.A. Horrum, and D.J. Cummings.** 1982. Are mitochondrial structural genes selectively amplified during senescence in *Podospora anserina? Cell* **29**:505-515.

**Yamazaki, M., S. Goto, and K. Komagata.** 1983. An electrophoretic comparison of the enzymes of *Saccharomyces* yeasts. *J. Gen. Appl. Microbiol.* **29**:305-318.

**Yamazaki, M., and K. Komagata.** 1981. Taxonomic significance of electrophoretic comparison of enzymes in the genera *Rhodotorula* and *Rhodosporidium. Int. J. Syst. Bacteriol.* **31**:361-381.

**Yang, V.W., and T.W. Jeffries.** 1990. Purification and properties of xylitol dehydrogenase from the xylose-fermenting yeast *Candida shehatae. Appl. Biochem. Biotechnol.* **19**:197-206.

**Yu, S., M. Wayman, and S.K. Parekh.** 1987. Fermentation to ethanol of pentose containing spent sulphite liquor. *Biotechnol. Bioeng.* **29**:1144-1150.