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Characterization of hybrids obtained by protoplast fusion, between *Pachysolen tannophilus* and *Saccharomyces cerevisiae*

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Abstract. The genes for utilization of xylose were transferred from *Pachysolen tannophilus* to *Saccharomyces cerevisiae*. The hybrids resembled the *S. cerevisiae* parent morphologically and in sugar assimilation. Pulsed field gel electrophoresis showed that the chromosome banding pattern was intermediate between the two parental species.

Introduction

Hemicellulose represents a significant fraction of materials that can be said to be under-utilized. Pentosans (xylans) are a potential source of feedstock for fermentation to ethanol, and may become extremely important to the economy of Argentina and numerous other countries. Pentosans constitute 10–40% of the lignocellulosic fraction of plant biomass, and could be an important source of industrial ethanol if an economic fermentation process can be developed.

Pentoses can be converted to ethanol by bacteria, yeasts and filamentous fungi (Skoog and Hahn-Hagerdahl 1988). Some filamentous fungi, mostly *Fusarium* species, and a few yeasts, can convert xylose to ethanol. *Pachysolen tannophilus*, *Pichia stipitis,* and *Candida shehatae* produce significant amounts of ethanol from xylose, and *Pichia farinosa* converts xylose to a potentially useful sugar alcohol, xylitol (Onishi and Perry 1965). There are industrial uses for crude celluloses (bagasse), but not for hemicelluloses and most pectins. Hemicelluloses can be converted into biomass and ethanol or xylitol, but the processes, so far, are not economic. The object of this work is to investigate the characteristics of the yeasts that utilize xylose, and their hybrids with species that ferment sugars more actively but that do not utilize xylose, such as *Saccharomyces cerevisiae* and to attempt to obtain strains that can be used economically to ferment this sugar.

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Materials and methods

Yeast strains. Pachysolen tannophilus met$^-$ was derived from *P. tannophilus* strain NCYC 2460, obtained from the National Collection of Yeast Cultures (Food Research Institute, Norwich, UK).

Media. Yeast cultures were maintained on complete medium (YEPD: 2% glucose, 1% yeast extract, 1% peptone, 1.5% agar), on slants, at 4° C. Selective medium for recovery of fusion products was 0.67% yeast nitrogen base (YNB; Difco), plus 2% xylose. For regeneration of protoplasts, 0.6 M KCl and 3% agar were added.

Protoplast formation, fusion and regeneration. These were carried out according to methods described previously (Spencer et al. 1980). Yeast cells were harvested in the logarithmic growth phase, treated with a solution containing 1% β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA), pH 7.4, at 35° C for 10–15 min. The pretreatment solution was removed, the cells washed once with 0.6 M KCl and treated with Novozyme 234 (5 mg/ml), in phosphate buffer, pH 5.8, at 35° C for 15–30 min. The protoplasts were fused in a solution of 30% polyethylene glycol (PEG), MW 6000, containing 15% dimethylsulphoxide (Becher et al. 1982). The fused protoplasts were regenerated in 1.5% water agar containing 0.4 M $CaCl_2$, on the YNB-xylose regeneration agar previously described. The $CaCl_2$ agar was melted, cooled to about 44° C, the suspension of fused protoplasts, diluted if necessary, was added, quickly mixed and immediately poured over the prewarmed regeneration plates, allowed to spread and then to harden. The plates were incubated at 30° C until colonies appeared and were isolated. Colonies appearing were presumed to be hybrids, since the parental *P. tannophilus* strain was auxotrophic and could not grow on minimal medium, and the *S. cerevisiae* parent could not utilize xylose. A plate of YNB agar without a carbon source was used as a control.

Assimilation tests. These tests on the hybrids were carried out on plates of YNB medium containing the desired sugars, and fermentation tests (Kreger-van Rij 1984) of these sugars in Durham tubes.

Dry weight. Biomass dry weight was determined at intervals during aerobic growth in shaken cultures, and compared with that obtained from *Pichia stipitis* and *C. shehatae.*

F and t tests. These were applied to evaluate the significance of the variance due to differences betwen the strains and due to experimental error (Dixon 1981).

Table 1. Assimilation of sugars by parental strains and hybrids between *Pachysolen tannophilus* and *Saccharomyces cerevisiae*

Strains	Sugars						
	Glucose	Galactose	Sucrose	Trehalose	Cellobiose	Xylose	Succinic acid
P. tannophilus	+ F	−	−	−	+	+ F	+
FP1	+ F	+ F	+ F	+	+	+	+
FP2	+ F	+ F	+ F	+	+	+	+
FP3	+ F	+ F	+ F	+	+	+	+
FP4	+ F	+ F	+ F	+	+	+	+
S. cerevisiae	+ F	+ F	+ F	+	−	−	−

+, assimilation; F, fermentation; FP, fusion products

Results and discussion

Eleven hybrids, morphologically intermediate between the two parental strains, produced by fusion of protoplasts of an auxotrophic strain of *Pachysolen tannophilus* and *S. cerevisiae*, were isolated on selective medium containing xylose as sole carbon source. Of these, four single-cell clones were tested for their ability to assimilate and ferment the sugars glucose, galactose, maltose, sucrose, trehalose, cellobiose, xylose and to assimilate succinic acid. The fusion products assimilated and fermented glucose, galactose, maltose, and sucrose, which is a fermentation pattern like that of *S. cerevisiae*, and assimilated trehalose, cellobiose, xylose and succinic acid. *S. cerevisiae* assimilated only trehalose, in the latter group of compounds, and *P. tannophilus* did not assimilate trehalose, but fermented glucose and xylose (Table 1).

Biomass yields (dry weights and growth constants) for cultures grown with xylose as sole carbon source are given in Table 2. Yields were higher in the hybrids than in any of the xylose-fermenting species tested.

Separation of the chromosomes of the hybrids by field inversion gel electrophoresis, showed that the fusion products had altered genomes (Fig. 1). Gels obtained from *P. tannophilus* showed four chromosomal bands. Preparations from all of the hybrid strains also showed four bands, but the mobility of three of the bands was greater than that of the corresponding bands in the parental strain. Reynolds (1990), however, observed additional chromosomal bands in fusion hybrids between *S. cerevisiae* and *Hansenula wingei* or *Pichia membranaefaciens*.

We have demonstrated that hybrids between *Pachysolen tannophilus* and *S. cerevisiae* can be constructed by protoplast fusion, and that these hybrids are capable of utilizing D-xylose. Further work may yield strains capable of fermenting as well as utilizing xylose, some of which may be superior to strains of the known species of xylose-fermenting yeasts. The occurrence of hybrids, constructed by protoplast fusion and able to ferment xylose, may be a relatively rare event, but their construction should be worthwhile.

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Table 2. Biomass yields from xylose

Strain	μ_{max} (h^{-1})	t_d (h)	Yield[a]
P. tannophilus	0.25	2.8	0.503±0.002
FP1	0.29	2.4	0.578±0.006
FP2	0.31	2.2	0.517±0.004
FP3	0.30	2.3	0.539±0.007
FP4	0.35	2.0	0.626±0.009
P. stipitis	0.36	1.9	0.261±0.002
C. shehatae	0.34	2.1	0.367±0.006

μ_{max}, maximum specific growth rate; t_d, doubling time; yield, biomass yield (g biomass/g consumed xylose)
[a] Mean of four replicates. The differences between strains at the 99% level are significant

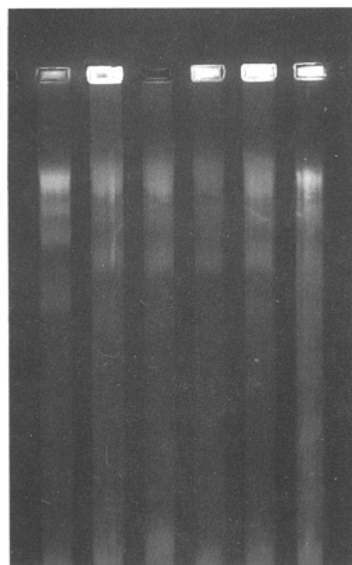

Fig. 1. Chromosomes of hybrids of *Pachysolen tannophilus*, separated by pulsed field gel electrophoresis (FIGE): *lane 1, Saccharomyces cerevisiae; lanes 2–5,* FP1–FP4; *lane 6, P. tannophilus.* Cells were prepared and FIGE gels were run according to methods previously described (Spencer et al. 1989). Field inversion gel electrophoresis was done using a Hybaid (London, UK) control unit, and the gel run at 150 V, one cycle, exponential; forward switch time at the beginning of the cycle, 1 s; forward switch time at the end of the cycle, 60 s; percentage of forward to reverse time phase ratio, 0.33; percentage of cycle time to reach 50%, 40. Running time, 5.5 h, agarose concentration, 1%. After the run, the gels were stained with ethidium bromide (Spencer et al. 1989)

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