Curr Genet (1987) 12:605–610

Current Genetics

Construction of a series of *Pichia stipitis* strains with increased DNA contents

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Summary. Strains of the D-xylose fermenting yeast *Pichia stipitis* containing varying amounts of cellular DNA, were created through polyethylene glycol induced protoplast fusions. The majority of the fusant strains appeared to be very unstable and segregated easily to cells of lower DNA content, as typical of ploidy reduction. However, a few stable strains were obtained and their hybrid nature was confirmed by cell volume estimation, analysis of nuclear condition and the isolation of a variety of mutant phenotypic segregants by mitotic segregation as well as by meiotic segregation in some sporulating hybrids. The effect of gene dosage on ethanolic D-xylose fermentation was also investigated.

Key words: *Pichia stipitis* – Presumptive polyploids D-xylose fermentation

Introduction

Industrial yeast strains have been reported to be polyploid or aneuploid (Spencer and Spencer 1983), suggesting that an increase in chromosome number may be advantageous in processes involving the use of such strains. For example, Scheda (1963) reported a systematic increase in the rate of ethanol production from D-glucose with increasing ploidy in *S. cerevisiae*. Similar trends were also demonstrated with a ploidy series of the yeast *Pachysolen tannophilus* in the fermentation of D-xylose (Maleszka et al. 1983), an aldopentose present in lignocellulosic waste materials (du Preez and van der Walt 1983). However, presumptive polyploid strains of *Candida shehatae*, constructed by protoplast fusion, showed only slight increases in the rate of ethanol production from D-xylose and during prolonged fermentations of 50 to 80 h, the relationship between ploidy level and ethanol production, was not clearly defined (Johannsen et al. 1985).

In this study, protoplast fusion was used to construct a series of *Pichia stipitis* strains with increasing cellular DNA content in order to investigate gene dosage effects on D-xylose fermentation.

Materials and methods

Microorganisms. A rapid xylose-fermenting prototrophic strain of *P. stipitis* CSIR Y633 (du Preez and Prior 1985) served as the parental strain for the breeding programme. The strain was supplied by Dr. J. P. van der Walt of the Council for Scientific and Industrial Research, Pretoria, South Africa. Auxotrophic strains used in this study were derived from the prototroph by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) mutagenesis, as described previously (Gupthar and Garnett 1987). Auxotrophs P2-2 *pro* and P2-2 *cys, trp* were also derived by NTG mutagenesis from the prototrophic fusion product P2-2.

Media. Yeast-malt agar (YMA) or broth (YMB) (Wickerham 1951) were used for the propagation and maintenance of parental and fusant strains. YMA (pH 5.6) was also used as stabilization or salvage medium for fusant strains. Osmotically stabilized basal xylose medium (Gupthar and Garnett 1987) was used for the recovery of prototrophic fusion products. Sporulation of *Pichia* parental and fusant strains was assessed on 5% (w/v) malt extract agar (MEA) (Merck). Medium CA containing 50 g · l^{-1} D-xylose (Merck), vitamin free casamino acids (Difco), added vitamins and mineral salts as described previously (du Preez and van der Walt 1983) was used for fermentation experiments.

Breeding programme. The breeding programme involving the use of auxotrophic derivatives of *P. stipitis* CSIR Y633 (Ps Y633) and strain P2-2, as well as the listing of stable fusion products recovered, is indicated as follows:

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Fusion 1: Ps Y633 (*leu, lys, ade*) x Ps Y633 (*arg*)
Stable Products: P2-1; P2-2; P2-3; P2-4
Fusion 2: P2-2 (*pro*) x Ps Y633 (*leu, lys, ade*)
Stable Product P3-1
Fusion 3: P2-2 (*pro*) x P2-2 (*cys, trp*)
Stable Products: P4-1; P4-2

The experimental protocols involving protoplast preparation, fusion and transfer of products from osmotically stabilized basal xylose recovery medium to YMA (pH 5.6) salvage medium after 10 days of incubation of basal xylose plates at 30 °C, are described in a previous study (Gupthar and Garnett 1987). Prototrophic colony forming units (cfu's) recovered in all fusion experiments, were subjected to 3 passages on YMA medium before further analysis.

Analysis of fusant colonies. The hybrid nature of fusant colonies recovered was analysed by the following methods:

(i) nuclear staining to investigate the condition of the nucleus (Nurse et al. 1976).
(ii) cell volume comparison of stationary phase cells of parental and fusant strains grown in YMB broth (pH 5.6), according to the equation (Morgan et al. 1980) for prolate spheroids ($V = \pi/6 \times W^2 \times l$ were V = cell volume (μm^3), $\pi = 3.14$, W^2 = axial cell breadth, l = axial cell length).
(iii) cellular diphenylamine DNA quantitations in fusant and parental strains (Giles and Myers 1965; Johannsen et al. 1984).
(iv) spontaneous and p-fluoro-phenylalanine induced mitotic segregation of recombinant phenotypes from fusant strains (Morgan et al. 1980; Gupthar and Garnett 1987).
(v) the induction and assessment of sporulation on 5% (w/v) malt extract agar (Merck) (Gupthar and Garnett 1987).
(vi) isolation of spores from some sporulating hybrids and examination of meiotic segregation trends (Gupthar and Garnett 1987).

D-xylose fermentations. The fermentation of D-xylose by fusant and parental strains was carried out in 500 ml Erlenmeyer flasks, each containing 300 ml of CA medium as described by du Preez and Prior (1985). The inocula were prepared sequentially (du Preez and Prior 1985) and fermentation flasks were inoculated with live cell inocula corresponding to a dry cell equivalent of $0.2\ g \cdot l^{-1}$. Ethanol was determined enzymatically using alcohol dehydrogenase test kits and the assay procedure outlined by the supplier, Boehringer-Mannheim.

Residual xylose concentrations at various time intervals during the fermentations were determined by a modification of the DNS reagent technique (Miller 1959). Duplicate 25 µl volumes of cell free fermentation supernatants were diluted 10^{-2} in dH_2O and made alkaline by treatment with 250 µl 2 N NaOH. Samples were then treated with 250 µl of DNS reagent prepared by mixing 250 ml of 2.13 N sodium potassium tartrate, 100 ml of 2 N NaOH containing 0.21 M 3,5 dinitrosalicylic acid (DNS) and 150 ml dH_2O. Samples were heated at 85 °C in a waterbath for 5 min, then treated with 2.5 ml dH_2O and allowed to cool at room temperature. A blank consisting of uninoculated CA medium lacking D-xylose was treated in the same manner and optical densities of the samples were determined spectrophotometrically at 540 nm. Uninoculated CA medium standards containing known concentrations of xylose, were also treated as described. Residual xylose concentrations in fermentation samples were then determined from a linear standard curve.

Three ml samples of fermentation medium were also withdrawn at various time intervals for both ethanol and sugar analysis, as well as the gravimetric determination of dry cell mass.

Results

Recovery of fusion products

A total of 38 prototrophic colony forming units were recovered in all 3 intrastrain fusions. Eighteen, twelve and eight fusion products were initially recovered in Fusions 1, 2 and 3 respectively. The fusion products were screened for increased cellular DNA content after each passage through YMA salvage medium. From the listing

Table 1. Some characteristics of parental and fusant strains of *P. stipitis*

Strain[d]	Cell[a] Volume (μm^3)	Cellular DNA (fg/cell)	DNA[c] IF	Sporulation on MEA
Ps Y633	48.19 ± 5.25 (3)	24.30 ± 0.85 (3)[b]		+
Ps Y633 LLA	49.94 ± 4.99 (3)	24.52 ± 0.68 (4)		+
Ps Y633 A	48.76 ± 4.88 (3)	24.45 ± 0.66 (4)		+
P2-2 P	65.94 ± 4.44 (3)	44.88 ± 1.45 (5)	1.84	+
P2-2 CT	66.05 ± 4.32 (3)	44.98 ± 1.40 (5)	1.84	+
P2-1	68.08 ± 5.23 (3)	43.43 ± 1.68 (5)	1.78	+
P2-2	66.55 ± 4.96 (3)	44.81 ± 1.38 (5)	1.83	+
P2-3	60.67 ± 3.20 (3)	44.54 ± 1.26 (5)	1.82	+
P2-4	62.35 ± 4.66 (3)	45.38 ± 1.62 (5)	1.85	+
P3-1	70.34 ± 6.29 (3)	63.28 ± 2.13 (3)	2.59	−
P4-1	71.30 ± 8.55 (4)	85.22 ± 3.21 (3)	3.49	−
P4-2	70.96 ± 6.88 (3)	85.39 ± 3.30 (4)	3.50	−

[a] The mean of 50 or more cell volumes were considered
[b] The figures in parenthesis indicate the number of determinations
[c] DNA increase factor (IF) calculated on the basis of the mean cell DNA of 24.42 fg for the prototroph Ps Y633 and its immediate auxotrophic derivatives
[d] Auxotrophic strains: Ps Y633 LLA (Ps Y633 *leu, lys, ade*); Ps Y633 A (Ps Y633 *arg*); P2-2P (P2-2 *pro*), P2-2CT (P2-2 *cys, trp*)

Table 2. Random spore analysis in sporulating fusant strains of *P. stipitis*

Fusant strains	P2-1	P2-2	P2-3	P2-4[a]
No. of colonies screened	320	298	311	346
No. of wild colonies[b]	194	166	191	204
% of wild colonies	60.6	55.7	61.4	59.0
No. of mutant colonies	126	132	120	142
% of mutant colonies	39.4	44.3	38.6	41.0
Phenotypes of mutant segregants				
Parental				
leu	66	29	36	54
lys	8	18	0	26
arg	0	7	29	2
leu lys	23	0	12	0
Recombinant				
leu arg	16	48	39	45
lys arg	13	30	4	13

Data represents combinations identifying auxotrophy in all mutant colonies

[a] P2-4: 142 mutants isolated; 140 identified; 2 reverted to the prototrophic state

[b] Prototrophic colonies are referred to as wild

of 7 stable fusion products in the breeding programme, it is evident that many of the initial fusant strains were unstable and easily segregated into cells of lower DNA content. Unstable fusion products of Fusion 1, 2 and 3 constituted 77.77, 91.66 and 75.0 percent respectively of the total numbers of the initial colony forming units isolated. Stable fusion products showing a constant increased quantity of cellular DNA after the first, second and third passages on YMA salvage medium, were characterized further.

Analysis of cell volume and DNA content

All seven fusant strains were uninucleate and showed a general increase in cell volume and cellular DNA content compared to the parental strains (Table 1), both trends typical of plasmogamy and karyogamy, respectively (Morgan et al. 1980; Johannsen et al. 1985). Cells of the fusant strains were spheroidal like the parental strains, although a few large pear-shaped cells of approximately 110 μm^3 were occasionally observed in hybrid strains P4-1 and P4-2. The DNA increase factor (IF) of fusant strains ranged from 1.78 to 3.50, as indicated in Table 1. The staining of nuclei in fusant strains and quantitation of cellular DNA provides evidence that the fusant strains were not stable heterokaryons but nuclear hybrids. However, a progressive loss of cellular DNA content of unstable strains was noted with consecutive passages on YMA medium. Unstable strains which exhibited a 2 to 3-fold increase in total cell DNA compared to parental strains after fusion, segregated easily into cells of lower DNA content with each passage on YMA medium. Finally, all unstable segregants "stabilized" after the third passage on YMA medium and showed constant cell DNA contents resembling parental strains Ps Y633, Ps Y633 (*leu, lys, ade*) and Ps Y633 (*arg*). Interestingly, the cell volume of such unstable strains also showed a decrease with consecutive passages on YMA medium.

Mitotic and meiotic segregation trends

The hybrid constitution of all seven fusant strains was confirmed by spontaneous (s) and induced (I) mitotic segregation, as well as meiotic segregation in some fusants by the isolation of a variety of recombinant diauxotrophic and triauxotrophic segregants. Examples of some of the segregation trends are indicated in Tables 2 and 3. The segregation of auxotrophic markers appeared to be complex and difficult to compare with any known segregational pattern. Interestingly, the frequency of spontaneous segregation increased with strains containing greater quantities of DNA (Table 3). The spontaneous segregation of auxotrophic markers ranged from 0% for strain P2-2 (not shown) to 8.75% for strain P4-1, bearing 85.22 ± 3.21 fg DNA $cell^{-1}$. A similar trend was noted for induced mitotic segregations, as indicated in Table 3.

Table 3. Mitotic segregation analysis of fusant strains of *P. stipitis*

Fusant strains Type of segregation[a]	P2-1		P3-1		P4-1	
	S	I	S	I[b]	S	I[b]
Total No. of colonies screened	286	208	203	265	240	236
Total No. of auxotrophic segregants	3	26	8	75	21	72
% segregation	1.05	12.5	3.94	28.3	8.75	30.5
Phenotypes of segregants						
Parental						
leu	2	–	–	–	3	–
lys	–	4	4	–	3	–
arg	–	–	–	–	2	1
pro	n	n	2	2	2	1
trp	n	n	n	–	1	–
leu lys	–	3	–	1	–	–
Recombinant						
lys arg	–	13	–	–	1	–
leu arg	1	6	–	6	–	–
pro arg	n	n	–	12	1	–
pro lys	n	n	2	38	4	–
pro cys	n	n	n	n	–	46
pro leu	n	n	–	–	–	10
cys leu	n	n	n	n	–	6
trp lys	n	n	n	n	3	–
pro leu lys	n	n	–	4	1	–
arg pro cys	n	n	–	n	–	3

Data represents 16 combinations identifying auxotrophy of mutant strains. Twenty eight other combinations tested (not shown) were negative (–)

[a] Types of segregation: spontaneous (S) and induced (I)

[b] Mutants unidentified reverted to the prototrophic state during analysis

n Not determined

In fusant strains P3-1 and P4-1, 12 and 5 mutant segregants reverted to the prototrophic state during the analysis of auxotrophic phenotypes, respectively. As a prerequisite to meiotic segregation analysis, the sporulation ability of all fusant and parental strains was analysed on several stress media (not listed), as well as 5% MEA which showed results identical to those obtained using the other media. Positive sporulation was obtained with fusant strains P2-1, P2-2, P2-3 and P2-4 (Table 1). Fusant strains P3-1, P4-1 and P4-2 with higher cellular DNA contents failed to sporulate on stress media, as well as on 5% MEA (Table 1). Consequently, meiotic segregation analysis was conducted only on sporulating strains and further evidence of the hybrid nature of these strains was provided by the segregation of recombinant diaxotrophic mutants (Table 2). In addition to the above investigations, isolated spores of the various parental and fusant strains were cultured initially on YMA medium and then propagated in YMB broth (pH 5.6) for cellular DNA isolations. The cell DNA content of these cultures was found to be in the range reported (Table 1).

D-xylose fermentations

The results of the fermentation experiments are represented in Table 4. By comparison of the various kinetic parameters between parental and fusant strains, it is evident that the relationship between increased DNA contents and improvement of D-xylose fermentation is not clearly defined. Of the 4 fusant strains showing an approximate 2 fold increase in cellular DNA content compared to parental strains, only strains P2-2 and P2-3 produced higher yields of ethanol over a shorter fermentation period. The highest maximum volumetric rates (Qp) were also recorded with some of these strains. The ethanol yield coefficient (Yp) was improved marginally with strains P2-1, P2-2 and P2-3. Fusant strains showed no systematic trend in the improvement of rates or yields with increased DNA contents. Whilst mutations were clearly necessary in the breeding programme for the selection of prototrophic fusants, this treatment had a detrimental effect on the fermentation of D-xylose by strains such as P2-2 (*cys, trp*) and Ps Y633 (*arg*). To

Table 4. D-xylose fermentation by parental and stable fusant strains of *P. stipitis*

Strains Parental[b]	Cell DNA content[c]	Fermentation parameters[a]							
		μmax	Qp	qp	Yp	Yx	E	ME	FT
Ps Y633	24.35	0.14	0.90	0.46	0.45	0.15	100	21.10	47
Ps Y633 LLA	24.61	0.14	0.66	0.46	0.38	0.10	77.0	16.70	47
Ps Y633 A	24.48	0.17	0.68	0.40	0.40	0.10	68.2	13.24	47
P2-2P	44.92	0.19	0.88	0.40	0.46	0.21	92.0	21.01	47
P2-2 CT	44.80	0.05	0.37	0.66	0.18	0.09	43.9	3.34	47
Fusant									
P2-1	43.66	0.18	0.88	0.44	0.47	0.17	84.6	19.75	47
P2-2	44.10	0.16	1.12	0.48	0.47	0.18	100	21.53	44
P2-3	44.86	0.15	1.00	0.49	0.47	0.18	100	21.30	45
P2-4	44.22	0.11	0.75	0.42	0.41	0.15	76.2	15.25	47
P3-1	63.10	0.18	0.75	0.28	0.43	0.21	79.1	16.93	47
P4-1	86.41	0.18	0.80	0.43	0.46	0.16	84.5	19.50	47
P4-2	85.91	0.16	0.88	0.43	0.46	0.14	100	20.16	46

[a] Parameters indicate only the maximum values obtained in two or more determinations. Abbreviations: μ_{max} (h^{-1}) max. specific growth rate; *Qp* max volumetric rate of ethanol production (g/l · h); *qp* (h^{-1}), max. specific rate of ethanol production (g ethanol produced per g dry biomass per h); *Yp* ethanol yield coefficient (g ethanol produced per g xylose utilised); *Yx* cell yield coefficients (g dry biomass produced per g xylose utilised); *E* efficiency of substrate utilization (g xylose utilized/g initial xylose x 100%); *ME* maximum ethanol yield (g/l); *FT (h)* time required for maximum ethanol production, or until termination of the fermentation process

[b] Auxotrophic strains: Ps Y633 LLA (Ps Y633 *leu, lys, ade*); Ps Y633 A (Ps Y633 *arg*); P2-2P (P2-2 *pro*); P2-2CT (P2-2 *cys, trp*)

[c] Final measurement of cell DNA content (fg/cell) of parental and stable fusant strains subjected to propagation after fermentation experiments

test for the stability of fusion products during the fermentation period, cells were removed at the end of the fermentation from fermentation flasks, cultured on YMA medium and subsequently transferred to YMB broth (pH 5.6) for DNA isolations and quantitations. The final measurement of cellular DNA levels of all parental and fusant strains propagated in YMB broth after fermentation experiments, is indicated in Table 4. The DNA levels of the various fusant strains were reproduced in the range indicated (Table 1), thus confirming the stability of these strains during fermentation.

Discussion

This study has demonstrated that protoplast fusion can be applied successfully in the construction of *P. stipitis* strains with increased DNA content. The various fusant strains had a uninucleate condition, increases in cell volume and increased levels of total cellular DNA. The hybrid nature of the prototrophic fusant strains was confirmed by the isolation of recombinant diauxotrophic and triauxotrophic segregants by mitotic segregation analysis (Table 3), as well as meiotic segregation in sporulating fusants (Table 2). The DNA increase factor (IF) ranged from 1.78 to 3.5 in fusant strains. From total cellular DNA quantitations (Table 1), the levels of DNA present in fusant strains was not simply the sum of the DNA contents of the respective parental strains. This suggests that the fusant strains could be aneuploid. Aneuploidy in most of the prototrophic fusants isolated might have contributed to the loss of chromosomes and final stabilization of these strains at a lower ploidy level, leading to the lower DNA content determined after consecutive passages on YMA medium.

The inability of fusant strains P3-1, P4-1 and P4-2 to sporulate on several stress media, as well as 5% MEA, is an unusual event but not uncommon amongst industrial yeast strains bearing higher gene content (Spencer and Spencer 1983). The precise reasons explaining this phenomenon can only be established with further research in the future. However, all parental and sporulating fusant strains contained a maximum of 2 hat-shaped ascospores per ascus. The sporulating ability of the parental strains suggests that they may be at least diploid. The fact that they yield only 2 ascospores per ascus, may be due to a mechanism associated with meiotic or sporulation failure. However, the unknown mechanism seems to result in the restoration of ploidy levels in sporulating parental and fusant strains, as cellular DNA contents of these strains were reproduced from cultured spores. This also implies that reduction division may not be associated with the sporulation procedure. The ploidy of the ascospores of the fusant strains which showed a high viability

ranging from 95 to 98% (data not shown), can only be established by comparing chromosome counts with spores of the parental strains.

Maleszka et al. (1983) reported a trend of systematic increases in the rates and yields of ethanol production from D-xylose with increasing chromosome number in *Pachysolen tannophilus*. However, isogenic presumptive polyploids of *Candida shehatae* constructed by protoplast fusion, showed no relationship between ploidy and improvements in D-xylose fermentation as described above (Johannsen et al. 1985). The current study reports a similar finding (Table 4) with isogenic fusant strains of *P. stipitis* bearing increased cell DNA contents (Table 4). The improvements of D-xylose fermentation by the *Pachysolen* ploidy series may be due largely to heterosis, since the ploidy series was derived from hybridizations between different strains (James and Zahab 1982, 1983; Maleszka et al. 1983).

Acknowledgement. I thank Professor Helen M. Garnett of the Univeristy of Wollongong, Australia, and Dr. Albin A. W. Baecker of the Department of Microbiology, University of the Witwatersrand, for their interest in my investigations.

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Communicated by B. S. Cox

Received July 1/August 17, 1987