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Characterization of Alcohol Fermentation and Segregation of Protoplast Fusant of *Saccharomyces cerevisiae* and *Pichia stipitis*

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A study was conducted to investigate the characteristics of segregation and alcohol fermentation of intergeneric fusants. The protoplast fusion of both *Pichia stipitis* CBS 5776 and *Saccharomycess cerevisiae* STV 89 was carried out. The fusion frequency was 5×10^{-8} and among fusants selected, a fusant F5 showed the best results in ethanol production by sucrose and xylose fermentations. The performance of xylose fermentation by this fusant was better than that of *P. stipitis* CBS 5776 and fusant F5 exhibited sucrose fermentation patterns intermediate to the two parent strains. The fusant F5 was segregated into a pair of parental strains during the several culture passages. In the average, 91% of colonies had a similar characteristics of *P. stipitis* while 7% of colonies resembled *S. cerevisiae*. Only 2% of colonies had the characteristics of the original fusants. At the sixth passage, all segregants resembled *P. stipitis*. From these results it is suggested that intergeneric protoplast fusion led to an integration of *S. cerevisiae* genes, rather than whole chromosomes, within the entire genome of *P. stipitis*.

Lignocellulosic materials are abundant in nature and are renewable organic substances composed of 40% cellulose, 30% hemicellulose and 20% lignin (5). Typical hydrolyzates of lignocellulosic materials contain 69% of hexoses and 31% of pentoses. Successful development of processes for ethanol production from lignocellulosic materials requires the efficient conversion of pentoses. Approximately one-third of the total carbohydrate sugars in a lignocellulosic biomass is in the form of pentose, D-xylose. Compared with glucose, the conversion of xylose to ethanol by microorganisms is more difficult. An economic analysis of xylose fermentations conclude that for fixed substrate costs, the yield and final concentration of ethanol are the most important factors in the cost of ethanol production (9).

Previous works showed that *Pichia stipitis* and *Candida shehatae* were the yeast species with the greatest potential for ethanol production from xylose (3, 14). However, the low ethanol tolerance of these pentose-fermenting yeasts would be detrimental to the commercial production of fuel ethanol from xylose (4). While *Saccharomyces cerevisiae* strains generally have a high ethanol tolerance (16), they are unable to convert xylose to ethanol efficiently owing to either the lack of, or the extremely low activities of key enzymes involved in xylose catabolism (1, 22). Hence, research efforts have been directed towards the development of yeast strains which are capable of converting xylose to ethanol with a high degree of efficiency.

Protoplast fusion has been widely used for genetic improvement of industrial yeasts (21). This technique is applicable regardless of ploidy and mating type, facillitating the total or partial exchange of genetic components (7). Consequently it has a great advantage for improving the ethanol productivity of yeast strains (17, 18). Many different traits are required for efficient ethanol production and the wholesale merging of genomes from disparate strains has been attempted as a means to improve performance. However, the precise mechanisms contributing to the ensuing stability or instability of fusant strains isolated from intraspecific, interspecific and intergeneric fusions remain unknown.

Intergeneric protoplast fusion of *S. cerevisiae* and *P. tannophilus* has been reported to result in hybrids that will produce and tolerate up to 10% (v/v) ethanol from

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molasses. However they do not produce as much ethanol as *P. tannophilus* from xylose (23). Heluane *et al.* (8) have also produced hybrids by fusion of *P. tannophilus* and *S. cerevisiae*. The hybrids resembled *S. cerevisiae* morphologically and exhibited sugar assimilation patterns intermediate to the two yeasts. The hybrids assimilated but did not ferment xylose. The resulting prototrophs of protoplast fusions of auxotrophically marked strains of *Pichia stipitis* and *Candida shehatae* appeared to contain DNA from both parents (7). However, the genomes were unequally distributed in all the fusants, despite the close evolutionary relatedness between these yeasts shown in DNA-DNA homology studies and similarities in ribosomal RNA sequence (13). More recently, strains of *C. shehatae* and *P. stipitis* were hybridized with ethanol-tolerant *S. cerevisiae* in an attempt to obtain hybrids that would grow on xylose at high ethanol concentrations (6). Mononucleate fusants were obtained, but these dissociated into segregants resembling the parent strains (6). Although the instability of fusion products is commonly encountered, gene exchange occurring prior to the segregation of parental strains is possible.

In this study, a protoplast fusion technique was used to develop a genetically constructed yeast strain which is capable of high ethanol tolerance with good xylose fermentation ability for obtaining high ethanol yields and productivity. The segregation of the fusant during the culture passages were also studied.

MATERIALS AND METHODS

Microorganisms and Media

The yeast strains used in this study were *Pichia stipitis* CBS 5776 and *Saccharomyces cerevisiae* STV 89 which were reported to produce high ethanol yields (16). Both yeast strains were grown and maintained on YMPD medium consisting of 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% glucose and 2% agar. The YNB medium containing 0.67% yeast nitrogen base (without amino acids) and 0.5% xylose was used as the minimal medium. The completely synthetic minimal medium (SMM) of yeast nitrogen base without amino acids described in the Difco manual (2) was used to confirm auxotrophic mutants and to select a fusant. Inocula were prepared by growing the strain in YMPD medium for 16 h with agitation at 150 rpm at 30°C. Fermentation medium consisted of 0.5% yeast extract, 0.5% peptone, 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.04% $MgSO_4 \cdot 7H_2O$ and a carbon source. The pH of the medium was adjusted to 5.0 by adding 2 N NaOH or 2 N HCl.

Mutagenesis and Auxotrophic Mutant Selection

The procedure as reported by Ryu *et al.* (17) was used for the formation and the isolation of spores of *P. stipitis*. Spores were washed three times with sterile distilled water, suspended in a solution containing 0.1 M potassium phosphate buffer (pH 8.0) and 0.8% ethylmethane sulfonate (EMS), and incubated at 30°C for 30 min with occasional shaking. After this period, the same volume of 5% sodium thiosulfate was added and the spores were incubated for 15 min to stop mutagenesis. The mutated spores were harvested by centrifugation and washed three times with sterile distilled water. To increase the population of mutants, the enriched culture was subjected to the nystatin procedure as reported by Snow (20). The cells harvested from the enriched culture were washed three times with sterile distilled water and 0.1 ml was spread onto YMPD plates and incubated for 3 days at 30°C. After the colonies appeared, they were replica plated onto YNB medium to screen for auxotrophic mutants. Colonies which grew on YMPD medium but were unable to grow on YNB medium were further transferred to SM medium containing different pools of amino acids in order to elucidate their auxotrophic requirments.

Protoplast Preparation

S. cerevisiae and *P. stipitis* auxotrophic mutant (Arg^-) were incubated aerobically at 30°C to early stationary phase in 250 ml flasks containing 20 ml of YMPD medium. The yeasts (5×10^8 cells) of each culture were collected and centrifuged at $500 \times g$ for 5 min. Cells were then washed three times with sterile distilled water and resuspended in the pretreatment buffer containing 0.1 M sodium phosphate buffer (pH 7.0), 0.1M β-mercaptoethanol and 0.02 M EDTA. After incubating for 30 min at 30°C, cells were washed twice with steriledistilled water and then resuspended in 1 ml of protoplasting solution containing 0.1 M sodium phosphate buffer (pH 7.0), 0.8 M sorbitol, 0.02% lyticase (Sigma) for *S. cerevisiae* and 0.2% Novozyme 234 (Novo) for *P. stipitis*, respectively. The suspension was incubated at 30°C for 60 min with shaking (75 rpm). Protoplasts were collected by centrifugation at $1{,}000 \times g$ for 10 min and washed three times with osmotic stabilizer (0.1 M phosphate buffer, pH 7.0 and 0.8 M sorbitol).

Protoplast Fusion and Fusant Selection

The protoplasts from both strains of yeast were mixed and carefully suspended in polyethylene glycol solution (40% PEG, 10 mM $CaCl_2$ and 0.8 M sorbitol, pH 7.0) and the suspension was incubated for 30 min at 30°C. The fused cells were washed and resuspended in an osmotic stabilizer. The suspension was then mixed with regeneration medium (SM medium contained 0.5% xylose, 0.8 M sorbitol and 0.5% agar) and poured onto plates containing a thin bottom layer of medium (SM medium contained 0.5% xylose and 2% agar). Plates were sealed and incubated for 7 days at 30°C. The fusants were selected from colonies which appeared on the plates. The colonies were further transferred onto SM medium con-

taining 0.5% raffinose as a sole carbon source and then the fusants were selected by comparing the growth of *S. cerevisiae*.

Analysis of Fusant Stability

The fusant F5 was suspended in 0.9% NaCl solution and spread onto YMPD plates and then incubated for 3 days at 30°C. After the colonies appeared, they were replica plated onto SM medium containing 0.5% raffinose (SMR) and 0.5% xylose (SMX) as the sole carbon source, respectively. Colonies which grew simultaneously on SMR medium and SMX medium were considered as fusants. The best fusant was selected based on the abilities of xylose and sucrose fermentations. The above experimental procedures were repeated five times to test the stability of the fusant.

Alcohol Fermentation

Flask culture experiments were carried out in 500 ml flasks containing 200 ml fermentation medium at 30°C and 150 rpm in a rotary shaker. The fermentation was carried out in 2 liter jar fermentor (NBS, USA) by loading 1 liter of fermentation medium at 30°C and 200 rpm agitation with 0.05 vvm aeration.

Analytical Methods

Cell concentration was determined using a spectrophotometer (Shimadzu) set at an optical density of 620 nm. The concentrations of xylose and sucrose were measured by DNS method (15) and the ethanol concentration was measured by gas chromatography (Hitachi) using a column packed with Parapack Q.

RESULTS AND DISCUSSION

Selection of Auxotrophs and Fusants

Two auxotrophic mutants, A1(Asn$^-$) and A2(Arg$^-$) were isolated and the best mutant for protoplast fusion was selected based on the their high capacity for xylose fermention and rapid cell growth. An arginine auxotroph A2 selected maintained almost the same abilities in cell growth and ethanol production from xylose as *P. stipitis*. The mutant A2 was used in the subsequent protoplast fusion to provide a genetic marker for selecting the fusants.

Primarily the colonies which grew on plates of SM medium containing 0.5% xylose as a sole carbon source were isolated as the fused hybrids of *S. cerevisiae* and *P. stipitis*(Arg$^-$). Complementation must have occurred in these fusants. Since *P. stipitis* was an auxotroph, it is incapable of growing on SM medium. Likewise *S. cerevisiae* is incapable of growing on a xylose carbon source. The hybrid colonies were further transferred onto SM medium containing 0.5% raffinose as the sole carbon source and then finally selected from the fusants when compared with the growth of *S. cerevisiae*. It was necessary to eliminate the reversion prototrophs of the axotrophic mutant, because the wild type of *P. stipitis* can not assimilate raffinose. The fusion frequency was found to be about 5×10^{-8} which was very low compared with those of intraspecific and interspecific fusions (21). To select the best fusant among the 10 fusants isolated, experiments were carried out using fusants and parents in a shaking incubator at 30°C using 100 g/l xylose and 50 g/l sucrose media, respectively. The results are shown in Fig. 1. The ability of alcohol fermentation of xylose was slightly different for each fusant. The highest ethanol concentration from xylose fermentation was obtained by fusant F5. Generally *P. stipitis* can assimilate but does not ferment sucrose (12). In sucrose fermentation, only fusant F5 showed a good fermentation ability which was as effective as *S. cerevisiae*, while other fusants showed almost the same ability in ethanol production from sucrose as *P. stipitis*. These results confirmed that protoplast fusion in yeasts lead to several types of fusion products such as true nuclear hybrids, partial hybids and parental-type revertants (7, 10, 19) by the unequal distribution of genomes in all fusants (13). Therefore, we selected a fusant F5 as the best fusant based on its sucrose and xylose fermentation ability.

Fermentation of Xylose and Sucrose

P. stipitis is known as one of the most promising

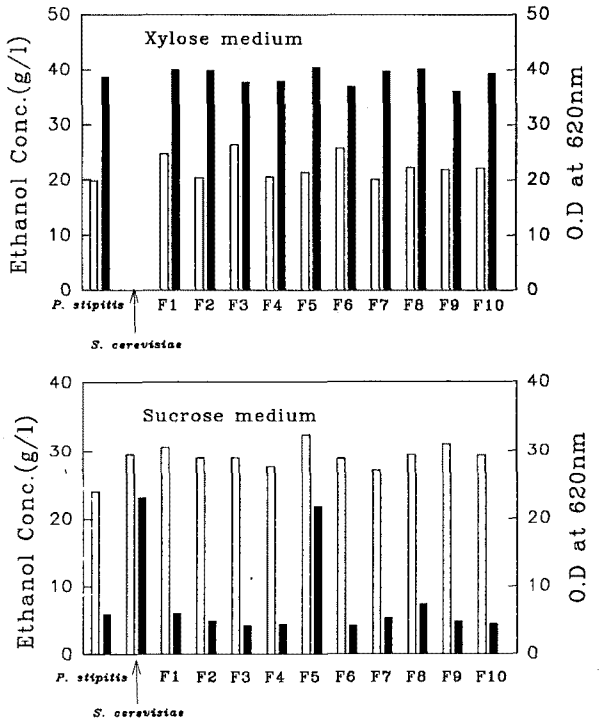

Fig. 1. The cell growth (□) and ethanol production (■) by *S. cerevisiae* STV 89, *P. stipitis* CBS 5776 and fusants from 100 g/l xylose medium after 120 h culture and 50 g/l sucrose medium after 24 h culture.

yeasts for direct fermentation of xylose to ethanol (3, 14) and *S. cerevisiae* showed good ethanol tolerence and fermentation ability (16). To compare the performance of the parent strain (*P. stipitis* CBS 5776) and the fusant F5 alcohol fermetation, experiments were carried out using 100 g/l xylose medium in a jar fermentor. The profiles of cell growth and ethanol production from xylose are presented in Fig. 2 and the various kinetic parameters are shown in Table 1.

The maximum cell and ethanol concentrations of fusant F5 were higher than those of the parent strain, the ethanol production and the cell concentrations obtained from fusant F5 were 13.4% and 22.5% higher as compared to those obtained from the parent strain. The overall ethanol productivity of fusant F5 was slightly lower than that of the parent strain. The ethanol yield of the fusant F5 was 0.44 g-ethanol/g-xylose, which was 86.3% of the theoretical value. This value was slightly higher than that of the parent strain, which is believed to be due to the lower efficiency of fusant F5 for ethanol assimilation and xylitol production than that of parent strain.

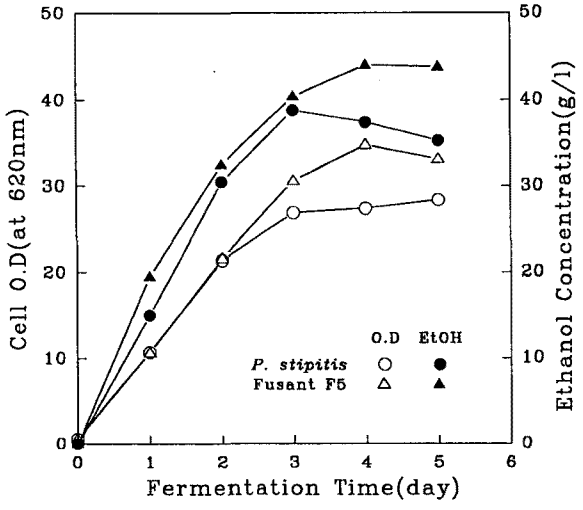

Fig. 2. The profiles of cell growth and ethanol production during the batch fermentation of 100 g/l xylose by *P. stipitis* CBS 5776 and fusant F5.

Table 1. The kinetic parameters in fermentation by *P. stipitis* CBS 5776 and fusant F5 in 100 g/l xylose medium.

Strains	μ	X_m	P	P_m	$Y_{P/X}$
P. stipitis CBS 5776	0.12	28.4	0.54	38.8	0.39
Fusant F5	0.12	34.8	0.46	44.0	0.44

μ: Specific growth rate in log phase (h^{-1}).
X_m: Maximum cell concentration (O.D. at 620 nm).
P: Overall ethanol productivity (g/l-h).
P_m: Maximum ethanol concentration (g/l).
$Y_{P/X}$: Ethanol yield (g-ethanol/g-xylose).

Through an economic analysis of xylose fermentations, Hinman *et al.* (9) suggested that the yield and final concentration of ethanol are the most important factors in ethanol production costs. Jeffries (11) also suggested that xylose fermentation processes will be commercially attractive when the "bench mark" performance values of 0.4 g-ethanol/g-xylose of ethanol yield, 50 g/l of final ethanol concentration and 1.4 g/l-h of average ethanol productivity can be achieved. The ethanol yield of fusant F5 was slightly higher than that of the "bench mark" performance value but the final ethanol concentration and ethanol productivity were lower than those of the "bench mark" performance values.

While *S. cerevisiae* strains generally have a good ability for sucrose fermentation, they are unable to convert xylose to ethanol (1, 22). *P. stipitis* has relatively low ethanol tolerance (4) and fermentation of sucrose by *P. stipitis* is not effective due to insufficient invertase activity (12).

In order to compare the characteristics of alcohol fermentation between parents (*S. cerevisiae* STV 89 and *P. stipitis* CBS 5776) and the fusant F5, experiments were carried out using 100 g/l sucrose in a jar fermentor. The profiles of cell growth and ethanol production are shown in Fig. 3 and the various kinetic parameters are shown in Table 2.

S. cerevisiae showed the highest rate of cell growth and ethanol production from sucrose. The maximum ethanol concentration reached 45.5 g/l at 24 h fermentation and overall ethanol productivity obtained was 1.9 g/l-h. The ethanol yield was 0.46 g-ethanol/g-sucrose, which was 90% of the theoretical value. *P. stipitis* showed good cell growth on a sucrose carbon source,

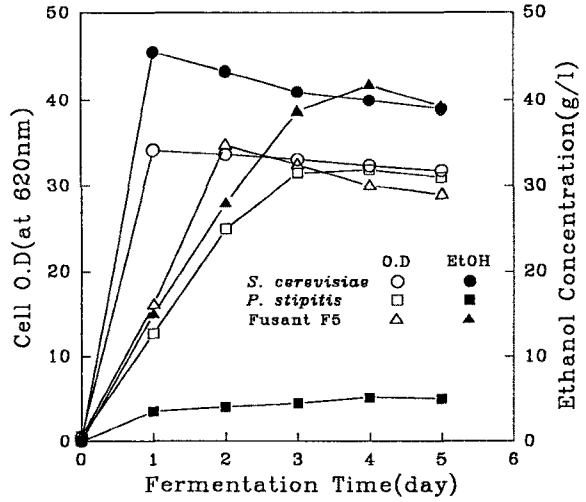

Fig. 3. The profiles of cell growth and ethanol production during the batch fermentation of 100 g/l sucrose by *S. cerevisiae* STV 89, *P. stipitis* CBS 5776 and fusant F5.

Table 2. The kinetic parameters in fermentation by *S. cerevisiae* STV 89, *P. stipitis* CBS 5776 and fusant F5 in 100 g/l sucrose medium.

Strains	μ	X_m	P	P_m	$Y_{P/X}$
S. cerevisiae STV 89	0.18	34.2	1.90	45.5	0.46
P. stipitis CBS 5776	0.13	31.9	0.05	5.2	0.38
Fusant F5	0.15	34.8	0.44	41.8	0.46

but ethanol production was very poor from sucrose. This result suggests that a major factor limiting fermentation of sucrose with *P. stipitis* is insufficient invertase activity as reported by Kurtzman (12). The sucrose fermentation by the fusant F5 showed almost the same properties as *S. cerevisiae*, although the rates of cell growth and ethanol production by fusant F5 were lower than those of *S. cerevisiae*. Fusant F5 was able to produce 41.8 g/l ethanol utilizing 91% of 100 g/l sucrose at 4 days fermentation and the ethanol yield was 0.46 g-ethanol/ g-sucrose consumed, which was 90% of the theoretical value. But the overall ethanol productivity by fusant F5 was much lower than that of *S. cerevisiae*. Fusant F5 exhibited sucrose fermentation patterns intermediate to the two parent strains. These results confirmed that fusant F5 was a hybrid strain of *S. cerevisiae* and *P. stipitis*.

Stability of Fusant

It is desirable that the methods employed in the genetic manipulation with microorganisms should result in the formation of genetically stable products with combined, modified, or intermediate properties capable of subsequent proliferation. The ensuing instability is usually characterized by transient karyogamy, leading to the formation of unstable heterokaryons, gene or chromosomal losses and dissociation of fusion products to parental-type segregants (6, 7, 19, 24). Also the yeast protoplast fusants can be unstable as reported by Gupthar and Garnett (7), requiring an analysis of the stability of fusant F5 during the culture passage.

The stability of fusant F5 during the culture passages is presented in Table 3. The fusant F5 was dissociated into segregants resembling the parent strains as reported by Gupthar (6). On the average, 91% of segregants had similar characteristics to *P. stipitis* while 7% of segregants resembled the characteristics of *S. cerevisiae*. Only 2% of segregants had the properties of an original fusant. To select the best fusant at each culture passage, alcohol fermentations were carried out using 50 g/l xylose and sucrose media in a shaking incubator. The fermentation abilities on xylose and sucrose of the best fusants selected at each culture passage are shown in Fig. 4. The results showed that the best fusant selected at each culture passage maintained the abilities of xylose and sucrose fermentation up to the fifth passage as compared to the parent strains. At the sixth passage, all segregants resembled *P. stipitis* and they were not capable of the assimilation of raffinose. These results confirmed that intergeneric fusants were dissociated into segregants resembling the parent strains by the unequal distribution of genomes during mitosis resulting in the incompatibility of chromosomes of the two parental strains (6, 13). From these experimental observations it is suggested that the fusion led to the integration of *S. cerevisiae* genes, with the entire genome of *P. stipitis*, rather than whole chromosomes as reported by Selebano *et al.* (19).

Table 3. The segregation of fusant F5 into parent strains during culture passage.

Culture passages	Colony No. of segregants resembling *S. cerevisiae*	Colony No. of segregants resembling *P. stipitis*	Colony No. of fusants
1	5	97	3
2	6	120	1
3	18	108	4
4	8	95	3
5	3	99	2
Total	40	519	13

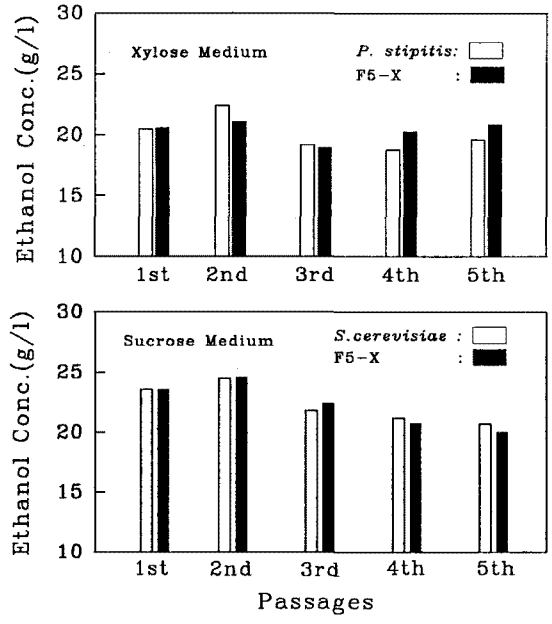

Fig. 4. The ethanol production by *P. stipitis* CBS 5776, *S. cerevisiae* STV 89 and fusant F5-X in 50 g/l xylose medium after 60 h culture and in 50 g/l sucrose medium after 24 h culture. Fusant F5-X is the best fusant selected at each culture passage, which is able to ferment both xylose and sucrose.

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