Introduction of flocculation into industrial yeast, *Saccharomyces cerevisiae* saké, by protoplast fusion

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Key words: protoplast fusion, flocculation, *Saccharomyces cerevisiae* saké

Abstract

Protoplast fusion was applied to obtain intraspecific fusion in yeast strains in order to overcome restrictions imposed by the natural mating system. Acriflavine was used to construct *petite* mutants from non-flocculent industrial *Saccharomyces cerevisiae* saké strain. UV radiation was used to construct *ura*$^-$ mutants from a respiratory competent and highly flocculent *S. cerevisiae* NCYC869 strain. Fusion products were selected by complementation on minimal medium. The frequency of appearance of prototrophic hybrids was five per 10^5 protoplasts. The stable hybrids showed not only the *S. cerevisiae* saké characteristics but also flocculation ability, thereby confirming the success of the fusion.

Introduction

Fermentation of sugars to ethanol is one of the oldest applications of biotechnology. The use of ethanol as liquid fuel, blended with gasoline or on its own, has been increasing in many parts of the world (D'Amore *et al.*, 1989). Ethanol is produced by *Saccharomyces cerevisiae* when substrate flow exceeds the respiratory capacity of the yeast cells (supracritical case) and is excreted into the medium (Fiechter and Seghezzi, 1992). Ethanol tolerance is a desirable characteristic for yeasts since ethanol accumulation represents a strong chemical stress to the yeasts with effects on cell multiplication (Troyer, 1953), on growth rate (Aiba *et al.*, 1968), and on cell viability (Thomas *et al.*, 1978).

Any process which results in an increased ethanol concentration in the fermenter effluent is desirable to minimise capital cost and distillation energy requirements. Most batch processes in current commercial use produce *ca* 9% w/v ethanol (Righelato, 1980). The complex saké fermentation results in a beverage of 14% w/v ethanol, and in this process a fraction from the envelope of *Aspergillus oryzae* containing unsaturated fatty-acyl residues, derived from the primary fermentation, is believed to increase the ethanol tolerance of the *S. cerevisiae* saké which effects the secondary fermentation (Hayashida *et al.*, 1974).

On the other hand, flocculation is another important property of some *S. cerevisiae* strains used in obtaining ethanol by fermentation (Teixeira *et al.*, 1990). The use of flocculent yeasts in some industrial fermentations facilitates the efficient separation of yeast cells from the fermentation wort with a considerable reduction in energy costs. Accordingly, Watari *et al.* (1990) constructed new flocculent yeast strains by sexual mating or protoplast fusion

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using a yeast containing the *Flo5* gene. *S. cerevisiae* strains have been improved for biotechnological ethanol production by stabilization of the flocculation character (Kida *et al.*, 1989) and by thermotolerance (Kida *et al.*, 1992) using the protoplast fusion technique.

The present paper reports the utilization of intraspecific protoplast fusion to construct new hybrids of yeasts containing both the fermentation characteristics of industrial *S. cerevisiae* saké and flocculence.

Materials and methods

Strains of yeast

The strains of *Saccharomyces cerevisiae* used in this study, and their relevant genotypes are listed in Table 1.

Media

YEPG complete medium was used and contained 1% Difco yeast extract, 2% Difco peptone and 2% glucose. YEPGly was the same as YEPG except that it contained 2% glycerol instead of glucose. The media for the detection of nutritional requirements of auxotrophic mutants were minimal media (MM) containing 0.67% Difco yeast nitrogen base without amino acids, and 2% glucose supplemented with different amino acids or bases at a final concentration of 50 μg/ml. All stock solutions were sterilized by filtration. MMGly was the same as MM except that it contained 2% glycerol instead of glucose. The sporulation medium (SM) contained 2% $NaH_3C_2O_2.3H_2O$, 0.25% Difco yeast extract, and 0.1% glucose, at pH 7.0. The rich medium (RM) contained 2% glucose, 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% Difco yeast extract, and 0.04% $MgSO_4.7H_2O$ at pH 4.0. Solid media were prepared with 2% Difco agar. All strains were maintained on YEPG or YEPGly slants in a refrigerator at 4°C.

Petite mutation induction by acriflavine

S. cerevisiae saké grown overnight in 50 ml of YEPG was washed once with water and cells were resuspended in fresh YEPG medium containing acriflavine (50 μg/ml) and cultured for 5 h at 30°C in the dark. After being washed twice, the cells were plated on YEPG and the plates were incubated at 30°C for 5 days. Plates were overlaid with 0.6% agar containing 5 mg/ml of a redox indicator, 2,3,5-triphenyltetrazolium chloride (TTC), which stained respiratory-sufficient (*grande*) colonies red, while respiratory-deficient (*petite*) colonies were not stained after 2 h of incubation at room temperature (Figueroa *et al.*, 1984).

The basis of such differential staining is that *petite* strains produce acid as products of fermentation and prevent reduction of TTC dye. In contrast, *grande* strains have the normal ability to reduce the TTC to its red colour form. The resulting small and white colonies were selected and the *petite* phenotypes were confirmed on YEPGly plates.

Auxotrophic mutation induction by ultraviolet light

In order to quantify the survival of cells exposed to ultraviolet (UV) irradiation, cells from the exponential growth phase were first centrifuged and then washed. Serial dilutions of cells were prepared and aliquots of 0.1 ml were spread with a bent glass rod onto the surfaces of YEPGly agar plates poured 24 h before inoculation. Plates were exposed to UV radiation at various intervals during a total period of 2 min, and before colonies were counted they were incubated at 30°C for 5 days in the dark, to prevent photoreactivation. The UV irradiation was carried out with a CAMAG germicidal lamp of 8 W, 254 nm, pre-warmed for 15 min, located at a distance of 12 cm above the plates.

Auxotrophic mutants were random found by colony screening in which single colonies were transferred one by one from complex medium (YEPGly), where the mutation was not expressed, to minimal medium (MMGly), which revealed the mutational auxotrophic lesion. Mutants not growing on MMGly were further analysed using several minimal agar plates supplemented either with amino acids or bases. Auxotrophs requiring uracil were tested for their resistance to 5-fluoro-orotic acid (5-FOA) according to Boeke *et al.* (1984). For this purpose, MMGly agar plates supplemented with 1 mg/ml 5-FOA and 50 μg/ml uracil were used.

Determination of mating type

The mass mating method was used, mixing pairwise on YEPG plates the haploid strains with the two test strains (IGC 4152 and 4153) listed in Table 1. After 2 days of growth at 30°C cells from each cross were transferred to SM and incubated for 10 days at 300 rpm and 30°C. Whenever sporulation occurred, this was regarded as an indication of succcessful mating. A strain was considered at *Matα* when sporulation occurred after mixing with the *Mata* test strain, and as *Mata* when sporulation took place after mixing with the *Matα* test strain (Figueroa *et al.*, 1984).

Table 1 *Saccharomyces cerevisiae* strains used

Strain	Genotype	Source
869	*Matα Flo1 (Flo4)**	NCYC
Saké	Unknown, no nutritional deficiencies	IAM
4152	*Mata*	IGC
4153	*Matα*	IGC
869-A3	*Matα Flo1 (Flo4) ura$^-$*	This work
Saké-R23	Unknown, no nutritional deficiencies, *petite*	This work

**Flo1* is an allele of *Flo4* mapped on chromosome I (Russell *et al.*, 1980).
NCYC, National Collection of Yeast Cultures, Norwich, England; IAM, Institute of Applied Microbiology, Tokyo, Japan; and IGC, Instituto Gulbenkian de Ciência, Oeiras, Portugal.

Sporulation ability

The sporulation abilities of strains were observed by light microscopy after 10 days of incubation at 300 rpm and 30°C in SM medium.

Measurement of flocculence

A small amount of the yeast strain was inoculated into tubes containing 10 ml of YEPG. After 3 days of growth without shaking at 30°C, the flocculation characteristics of the cultures were distinguished by the nature of the floc. After bringing the sediment back into suspension by shaking the tube with a vortex mixer, the results of this test were expressed on a subjective scale ranging from 0 to 3, *viz* 0, non-flocculent; 1, weakly flocculent; 2, moderately flocculent; and 3, highly flocculent. This method has been routinely used for characterization of the flocculating ability in screening studies (Stewart and Russell, 1977; Holmberg and Kielland-Brandt, 1978).

Protoplast formation

Yeast cells grown overnight in YEPG or YEDGly media were washed twice in water and resuspended in a pretreatment solution consisting of 0.1 M Tris-buffer at pH 7.0 containing 0.01 M EDTA, 1 M KCl and 0.1 M 2-mercapto-ethanol. After 30 min of incubation at 30°C with gentle agitation the cells were centrifuged and washed once with 1 M KCl. Cells were resuspended in 2.5 ml of protoplast solution, 0.05 M phosphate buffer at pH 7.5, containing 1 M KCl and 200 U/ml of lyticase enzyme from *Arthrobacter luteus* (Sigma L-8012, lot 23H6810). The mixture was incubated for 120 min at 30°C with gentle agitation. The rate of protoplast formation was estimated as follows:

$$[(\text{Initial OD}_{620} - \text{Final OD}_{620})/\text{Initial OD}_{620}] \times 100$$

The mixture was diluted with distilled water and the decrease in turbidity caused by rupture of the protoplast was measured at 620 nm (Fukuda and Kimura, 1991). The protoplast regeneration frequency was calculated as the percentage fraction of colonies referred to the plated cells which appeared on osmotically stabilized YEPG.

Protoplast fusion

The protoplasts were centrifuged at 2,500 rpm for 10 min and washed twice in 0.4 M $CaCl_2$. The supernatant was removed and the protoplasts resuspended in 0.4 M $CaCl_2$. Equal amounts of protoplasts (5×10^7 cells/ml) prepared from two different strains were blended. The protoplast mixture was centrifuged as previously described and the supernatant was removed. The protoplasts were resuspended in the residual liquid (about 0.2 ml of 0.4 M $CaCl_2$). An aliquot (2 ml) of 35% polyethylene glycol (PEG), MW 6,000, containing 15% dimethylsulphoxide (DMSO) was added and the protoplasts were gently suspended (Spencer *et al.*, 1989). After 30 min of incubation with gentle shaking at 30°C the protoplasts were diluted in 0.4 M $CaCl_2$.

Aliquots were mixed with osmotically stabilized agar at 43—45°C (1.5% agar in 0.4 M $CaCl_2$) and poured as a thin top layer on pre-warmed osmotically stabilized MMGly plates. The plates were then incubated at 30°C for 5 to 7 days. Hybrids were selected as prototrophic and *grande* colonies in consequence of complementation of nutritional and respiratory deficiencies. The frequency of protoplast fusion was estimated by comparing the number of colonies appearing on selective osmotically stabilized MMGly plates and osmotically stabilized YEPG plates where each original strain grew. As additional control for possible cross feeding a mixture of both types of protoplasts without PEG was inoculated on osmotically stabilized MMGly plates.

Determination of biomass productivity

A small amount of the yeast strain was inoculated into 50 ml Erlenmeyer flasks containing 10 ml of RM medium and incubated at 150 rpm and 30°C in an orbital incubator for 24 h. This culture was used to inoculate 100 ml of the same medium in 250 ml Erlenmeyer flasks containing glucose concentrations ranging from 1 to 20% (w/v), and ethanol concentrations ranging from 0 to 12% (w/v). Each culture was incubated for 8 h in the same conditions. A 5 ml sample was taken at the beginning and at the end of the assay to determine the biomass.

The biomass concentration was determined by measuring the optical density (OD) of a suspension at 620 nm. A standard curve OD *versus* biomass concentration was previously constructed. Prior to OD determinations, the biomass was deflocculated by the addition of 1.5% NaCl solution at pH 2. The biomass productivity (P_8) was calculated according to Mota (1985) as follows:

$$P_8 = (\text{Final biomass} - \text{Initial biomass})/8 \text{ h}$$

Stability of *petite* and auxotroph mutants or fusants

In order to determine the stability of the mutants a dense suspension of cells of *petite* mutants and auxotrophic mutants was spread onto YEPGly and MMGly plates, respectively. The plates were incubated for 5 days at 30°C and the appearance of colonies indicated the presence of revertants. Input cells were determined by counting the colonies appearing on complete medium plates. The frequencies of back mutations were determined. The genetic stability of fusants was examined by a series of replications on sets of MMGly and YEPG after which the flocculating ability was observed.

Staining procedures

Nuclear staining was performed with 4,6-diamino-2-phenylindole (DAPI) by the method of Williamson and Fennell (1975). The nuclei of the fusion products were examined and counted under a fluorescence microscope (Axioskop, Zeiss) using a 365 nm excitation filter.

Results and discussion

Induction, isolation and characterization of mutants

A series of 23 *petite* mutants was produced from *S. cerevisiae* saké by acriflavine treatment and selected by TTC. The presumptive white *petite* colonies obtained were plated in MMGly and 100% were confirmed as respiratory deficient. The R23 mutant was selected for protoplast fusion because it showed the best back mutation frequency (4.2×10^{-7}).

For the isolation of auxotrophic mutants, *S. cerevisiae* NCYC869 cells were subjected to UV treatment and the survival of the cells as a function of the duration of UV irradiation was determined (Figure 1). The percentage of viable cells was less than 10% after 45 s of UV treatment, which was the time selected to induce auxotrophic mutations. In addition, the NCYC869 strain showed a high spontaneous *petite* mutation (5.4%) and this strain had to be kept under selective pressure using glycerol as the carbon substrate throughout the work.

Auxotrophic mutants were isolated at a frequency of about 0.7% (16 out of 2,250). After looking for the auxotrophic requirements, three uracil auxotrophic mutants, A1, A3 and A94 were found. These mutants were able to grow on MMGly supplemented with uracil and 1 mg/ml 5-FOA, whilst the growth of the 869 wild type strain was totally inhibited by 5-FOA at this

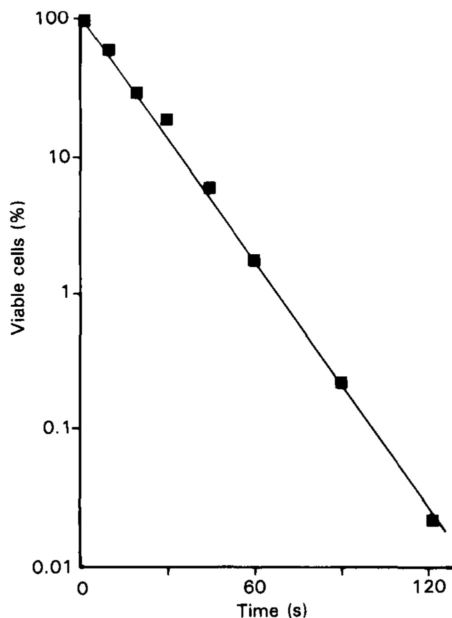

Figure 1 Survival cells of *S. cerevisiae* NCYC869 to UV irradiation.

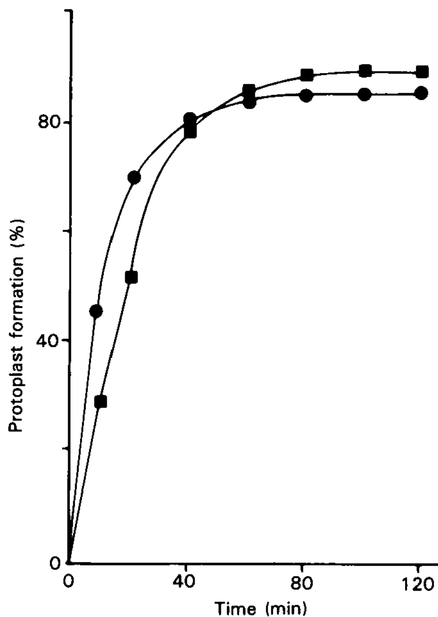

Figure 2 Time course of protoplast formation frequencies of mutant strains: ■, 869-A3; ●, saké-R23.

concentration. Thus, the above three mutants were possibly affected in the genes encoding for orotidine-5′-phosphate decarboxylase or orotidine-5′-phosphate pyrophosphorylase (Boeke *et al.*, 1984; Boeke *et al.*, 1987; Kitamoto *et al.*, 1990). The mutant A3 was selected for protoplast fusion because it showed the best back mutation frequency ($< 6.5 \times 10^{-10}$) and produced the same high degree of flocculation.

Formation, regeneration and fusion of protoplasts

The efficiency of protoplast formation in the R23 and A3 mutant strains was studied (Figure 2). The best result for protoplast formation ($> 85\%$), was obtained after 80 min of incubation and this time was used to form protoplasts for the next stage of fusion. Table 2 shows the results of the regeneration percentage obtained for R23 and A3 mutants. The prototrophic colonies grown in minimal regeneration medium were isolated as the fused hybrids between R23 and A3. Complementation must have occurred in these hybridized fusants, since the parent strains were unable to grow on this medium and the fusion frequency of the protoplasts was two to five orders of magnitude higher than the back mutation frequency of the parental strains (Table 2).

Table 2 Frequency of intraspecific fusion between *S. cerevisiae* (A3) and *S. cerevisiae* saké (R23)

Strain	Regeneration (%)*	Reversion**	Fusion***
R23	27	4.2×10^{-7}	
A3	32	$<6.5 \times 10^{-10}$	
Fusant			5×10^{-5}

* Frequency of protoplasts able to regenerate cells.
** Frequency of back mutation.
*** Frequency of prototrophs against progenies regenerated.

Characterization of fusants

Out of a total of twelve prototrophic and respiratory competent recombinants isolated from MMGly, ten proved to be stable hybrids after weekly transfers to fresh selective MMGly for 2 months. These fusants remained prototrophic not only on selective MMGly, but also on YEPG in the absence of nutritional pressure.

DAPI staining revealed that the prototrophic fusants consisted of uninucleated cells. This finding suggests that karyogamy occurred in the fusion products. The parental and the fusant strains were unable to sporulate. On the other hand, the A3 strain was mated with the *Mata* test strain, and R23 and fusant strains were unable to mate with the test strains. All fusants showed a decrease in flocculation ability when compared with the A3 highly flocculent strain. Fusants F1, F4, F7 and F12 showed degree 2 and the other six fusants manifested degree 1.

In order to confirm the physiological characteristics of the fusants, as a result of genetic recombination of two parental strains, the most promising four fusants with moderate flocculation ability were selected to compare the ability of biomass production in variable glucose and ethanol concentrations with either parental strains (Figure 3). The fusants, as well as the wild type *S. cerevisiae* saké, showed the same profile. In contrast, the wild type flocculent *S. cerevisiae* NCYC869 showed better productivity than the saké and fusant strains. Neither strain grew in 10 or 12% ethanol concentrations. These results suggest that the fusants are physiologically closer to the saké strain than to strain NCYC869, although they acquired the flocculation ability from the other NCYC869 parental strain.

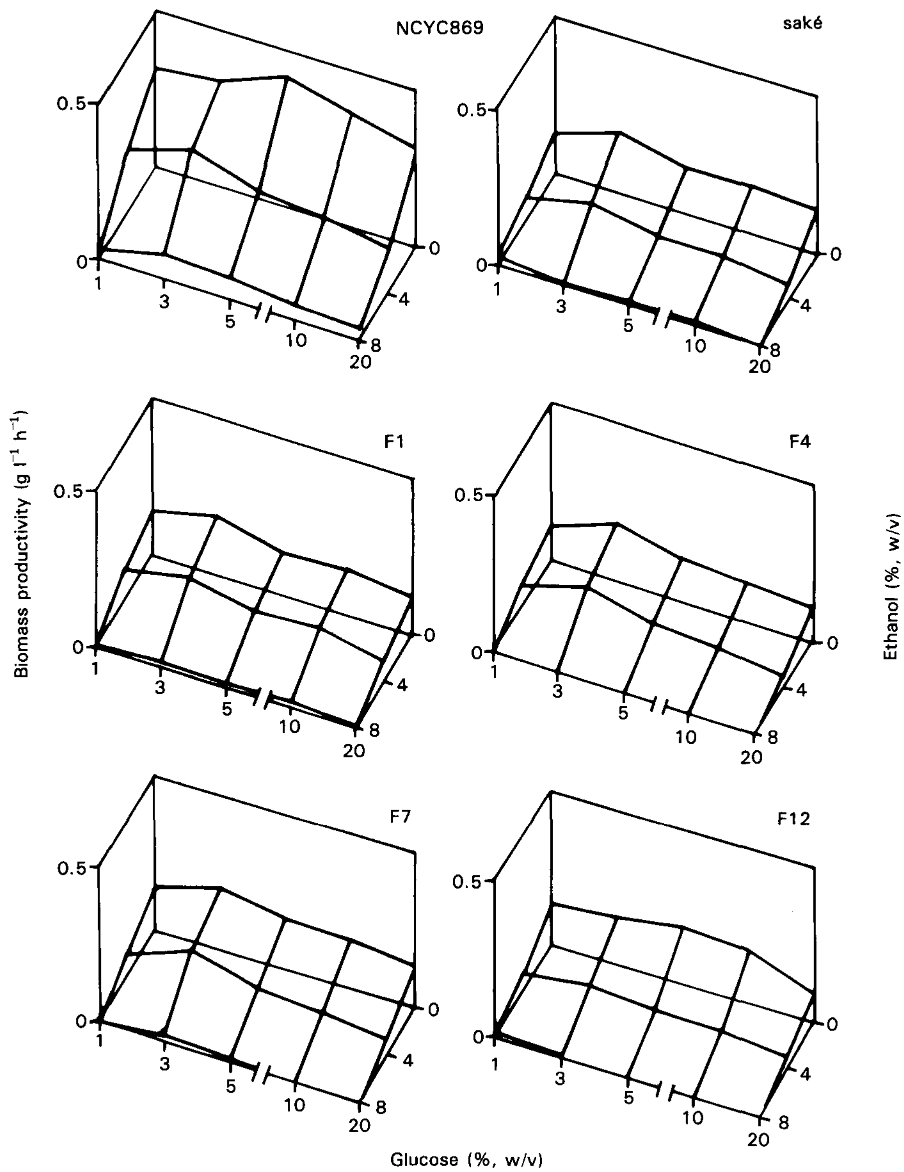

Figure 3 Effect of glucose and ethanol concentrations on the biomass productivity (P_8) for wild type and fusant strains.

The protoplast fusion method proved to be the most effective. Stable new strains were obtained with a set of characters from both *S. cerevisiae* NCYC869 and *S. cerevisiae* saké. The acquired characters of these new strains, namely, tolerance to ethanol, flocculence and non-sporulation are potentially useful for industrial applications. Nevertheless, the evaluation of the industrial applicability of these new strains will require further experimentation lying beyond the scope of the present work.

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Accepted 2 December 1994