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Electrofusion of an Industrial Baker's Yeast Strain with a Sour Dough Yeast

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ABSTRACT

A method for hybridization of yeast protoplasts of *Saccharomyces cerevisiae* and *Candida holmii* by electrofusion was optimized. The hybrids were screened on maltose-acetate agar plates. The average fusion frequency was 1.1×10^{-3}. Two hybrids of 132 collected from selection plates were found to be stable over 15 sequential shake flask cultivations. However, the strains reverted during production of baker's yeast in a laboratory scale process imitating the industrial process.

Index Entries: Electrofusion; baker's yeast; sour dough yeast; acetic acid tolerance; *Saccharomyces cerevisiae; Candida holmii.*

INTRODUCTION

Hybridization of industrially important yeast strains by protoplast fusion has been studied intensively in many laboratories during the 1980s. The main fusogenic agent used has been polyethylene glycol (PEG). Most attention has been paid to hybridization of *Saccharomyces cerevisiae,* either with other *S. cerevisiae* strains (*1–5*), or with *S. diastaticus* (*6–8*), *S. fermentati* (*9*), *S. mellis* (*10*), *S. uvarum* (*11–13*), *Candida tropicalis* (*14*), *C. utilis* (*15*), *Kluyveromyces lactis* (*16*), *K. fragilis* (*17*) and *Zygosaccharomyces fermentati* (*18*).

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In most applications, the aim has been to improve distilling or brewing processes. The properties transferred have mainly been flocculation and utilization of starch (*8,11,19,20*). In order to improve the production of industrial ethanol from cellulolytic wastes, xylose fermenting yeasts such as *Pachysolen tannophilus* (*21*), *Candida shehatae* (*22,23*) and *Pichia stipitis* (*23*) have been hybridized. The properties of baker's yeast have been improved by selective hybridization, (e.g., osmotolerance (*24,25*), acetic acid tolerance (*25*) and freeze resistance (*26,27*)). Pioneer work on protoplast fusion in an electric field was carried out by Zimmermann and his coworkers (*28*). Hitherto, the electrofusion of yeasts (*29–34*) has been studied mainly from the theoretical point of view.

In this paper we describe the optimization of electrofusion as a method for yeast strain improvement. The aim was to improve the acetic acid tolerance of baker's yeast by transferring the tolerance factor(s) from the sour dough yeast *Candida holmii* (ex. *Torulopsis holmii*). A baker's yeast with improved acetic acid tolerance is needed in the baking industry (*35*). Recombinant DNA methods cannot be used because acetic acid tolerance is controlled by several unidentified genes.

MATERIALS AND METHODS

Microorganisms

The yeast strains used were the baker's yeast *Saccharomyces cerevisiae* VTT-B-77044 and the sour dough yeast *Candida holmii* VTT-C-81116 from the VTT Collection of Industrial Microorganisms.

Media and Solutions

Solid media used for regeneration and selection of protoplasts contained, in 1.2 M sorbitol, 0.67% w/v yeast nitrogen base without amino acids, 3% w/v agar and the following ingredients % w/v:

1. SAM/AM: 4% sodium acetate and 0.25% maltose,
2. SA/A: 4% sodium acetate and 2% glucose,
3. SM/M: 0.25% maltose, and
4. SC/C: 2% glucose (control).

The same media without sorbitol were used for testing whole yeast cells (media AM, A, M, and C, respectively). The protoplast incubation medium contained 1.2 *M* sorbitol in 0.02 *M* Tris-HCl buffer, pH 7.5. The electrofusion solution (ES) contained 0.1 m*M* $CaCl_2$, 0.5 m*M* $MgSO_4 \times 7 H_2O$, and 1 mg/mL bovine serum albumin in 1.2*M* sorbitol (*32*). YPD liquid medium (1% w/v Difco yeast extract, 2% w/v Difco peptone, and 2% w/v glucose) was used for shake flask cultivations. When acetic acid was used, it was glacial (Merck, FRG) and added at inoculation.

Acetic Acid Tolerance

The acetic acid tolerance of the parental yeast strains was tested in shake flask cultivations (50 mL of YPD medium, 30°C, 250 rpm, 12 h) at different acetic acid concentrations. Growth was monitored by calculating the maximal specific growth rates, $\mu = (1/dt)\ (\ln X_2/X_1)$, where t = time, h; X = cell dry wt, g/L.

Preparation of Protoplasts

Yeast cells cultured to the early exponential phase of growth were collected by centrifugation and washed once with water and once with 1.2*M* sorbitol (*36*). The procedure for preparation of protoplasts has been described earlier (*36,37*). In the optimization of conditions for protoplast formation, different amounts of Zymolyase 60,000 (Seikaku Kogyo Co Ltd, Japan) and different incubation temperatures and times were used.

Electrofusion Experiments

An automatic Cellect-electrofusion instrument (Biofusion AB, Sweden) was used with the following parameters:

1. BUILD: Cell alignment,
2. SINE: The interpulse period,
3. PRE: A short relaxation phase,
4. PULSE: The opening of the membrane,
5. POST: A short relaxation phase,
6. FADE: The reduction of electric field to zero, and
7. LOOP: The number of pulse sequences in a cycle (i.e., sine, pre, pulse, and post phases).

Cells were fused in a CFA frame chamber (Krüss GmbH, FRG). The distance between the electrodes was 50 μm. Washed protoplasts were diluted 1:1000 (baker's yeast) or 1:200 (sour dough yeast) in 1.2*M* sorbitol for dielectrophoresis experiments, whereas in fusion experiments a dilution of 1:50 in ES medium gave approx the same amount of each yeast protoplast. 5 μL of the diluted protoplast solution was pipeted into the space between the electrodes and the behavior of the protoplasts was observed in different electrical fields under a phase contrast microscope (Inverted microscope IMT-2; Olympus Co, Japan). At the end of experiment, the suspension was pipeted from the glass slide to regeneration plates. The glass slide was rinsed once with 200 μL (dielectrophoresis experiments) or 500 μL (electrofusion experiments) of 1.2*M* sorbitol in order to remove adhering protoplasts as completely as possible. At least two replicate experiments were carried out in each set of conditions. In parallel with each experiment, a control was run in duplicate in the beginning and at the end of each series. Controls were not exposed to the electrical field or cultivated on SC plates, but were otherwise subjected to the

same conditions. Protoplasts were regenerated on the SC (dielectrophoresis) or SC and SAM (electrophoresis) plates for 7–10 d.

Primary Selection Tests

Each fusion product growing on SAM plates was collected with a loop, transferred to 1 mL of YPD medium and cultivated for 7 d. The broth was diluted 1:1000 in saline solution and plated on AM plates. After cultivation for 14 d, the growing colonies were suspended in saline solution, plated on AM, A, M, and C plates, and cultivated for 7–14 d. The stabilities of the colonies growing on AM plates were tested.

Stability Tests

100 mL of C and AM liquid medium was inoculated with a fusion product growing on an AM plate. The fusion product was suspended in saline solution before inoculation and cultivated for 48 h (C medium) or for 144 h (AM medium). 1 mL of the culture was transferred to 100 mL of the same fresh medium and cultivated for 24 h (C medium) or for 48 or 72 h (AM medium). Cultivations were repeated 15 times. Stability was monitored by plating a diluted sample on AM, A, M, and C plates after the 1st, 5th, 9th, and 15th cultivation. Plates were incubated for 5–7 d.

Production of Baker's Yeast

Production of baker's yeast in laboratory scale has been described earlier (*35*). In this work, the process was shortened from 3 to 1 aerated stage (*36*).

RESULTS

Acetic Acid Tolerance of the Parental Yeast Strains

Without acetic acid the sour dough yeast and the baker's yeast grew almost equally well (Table 1). The growth of the baker's yeast strain was, however, affected already at a concentration of 0.1% v/v acetic acid and was completely inhibited at concentrations exceeding 0.3% v/v, whereas growth of the sour dough strain was still rather good at a concentration of 0.6% v/v, pH 4.15.

Formation of Protoplasts

Because the presence of whole cells caused disturbance of protoplast fusion, protoplast formation was optimized to give 99–100% yield. Protoplast formation was monitored at 5 min intervals at three different temperatures and with four different amounts of Zymolyase (Figs. 1a and 1b).

Table 1
Maximal Specific Growth Rates (h^{-1})
of the Parental Yeast Strains at Different Acetic
Acid Concentrations and Final pH Values of the Cultures

Yeast	Acetic acid concentration, % v/v						
	0	0.1	0.2	0.3	0.4	0.5	0.6
Specific growth rates							
Baker's	0.42	0.39	0.38	0.31*	0	0	0
Sour dough	0.41	0.40	0.36	0.36	0.35	0.26	0.18*
pH values							
Baker's	5.75	5.75	4.70	4.45	4.35	4.25	4.20
Sour dough	5.65	5.25	4.65	4.45	4.30	4.25	4.15

*Prolonged lag phase.

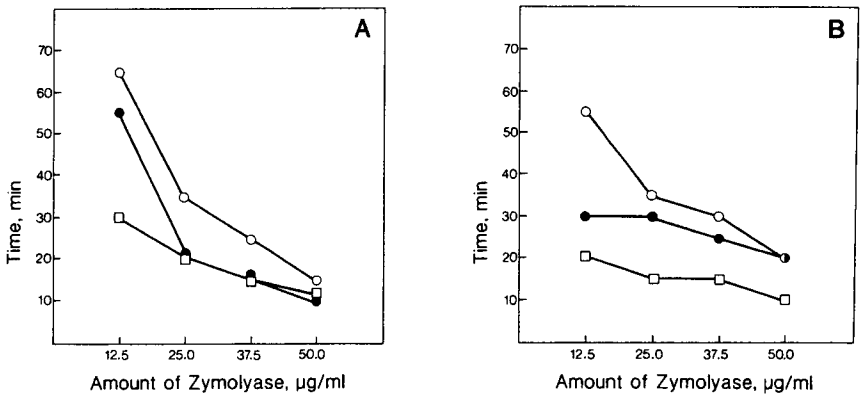

Fig. 1. Times required for 99-100% protoplast yield at different temperatures and amounts of Zymolyase from cells of baker's yeast (A) and sour dough yeast (B). Temperatures: 25°C (○), 30°C (●) and 36°C (□).

When the amount of Zymolyase and the incubation temperature were increased, protoplasts were formed in a shorter time. The number of regenerated baker's yeast protoplasts, however, decreased almost linearly at 30°C and 37°C with increase in the amount of Zymolyase (Fig. 2a). The number of regenerated sour dough yeast protoplasts was lower than that of baker's yeast protoplasts at all the doses of Zymolyase tested. Sour dough yeast protoplasts were, however, less sensitive to temperature differences than baker's yeast protoplasts (Fig. 2b).

In order to facilitate a convenient working routine, the same treatment time and the temperature were used for both yeasts. The conditions chosen for protoplast formation were: temperature 30°C, time 30 min, 25 µg/mL of Zymolyase for baker's yeast and 12.5 µg/mL of Zymolyase for sour dough yeast. Under these conditions, 99-100% of both yeast cells

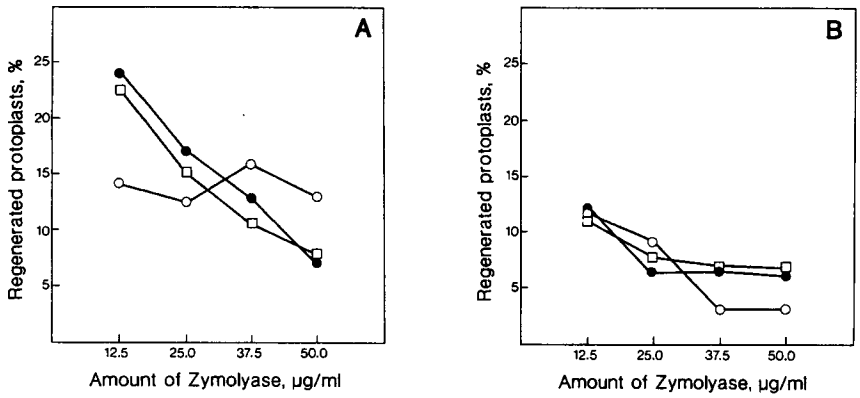

Fig. 2. Percentage of regenerated protoplasts at 99–100% protoplast yield at different temperatures and amounts of Zymolyase from cells of baker's yeast (A) and sour dough yeast (B). Temperatures: 25°C (○), 30°C (●) and 37°C (□).

formed protoplasts, of which approx 10% regenerated. After storage of only a few h at room temperature, the protoplasts lost their viability (e.g., after 2 h at room temperature only 6% of originally regenerating protoplasts were viable), which decreased the efficiency of fusion.

In order to improve the low regeneration and viability of the protoplasts, the effects of sorbitol concentration (0.4*M*, 0.8*M*, 1.2*M*), washing solution (1.2*M* sorbitol, 1.2*M* sorbitol with 10 m*M* $CaCl_2$ or 0.02*M* Tris-HCl buffer, pH 7.5) and temperature (room temperature, 0–5°C) were studied (*36*). The viability of protoplasts remained between 80 and 90% for several hours when they were produced in 1.2*M* sorbitol solution, washed with 1.2*M* sorbitol and handled at low temperature with prechilled solutions and stored in an ice bath.

Dielectrophoresis

The influence of dielectrophoresis (the formation of "pearl chains" in the electrical field) on the viability of protoplasts was studied. The formation of "pearl chains" was studied at different exposure times and voltages at a frequency of 1 mHz. When the time of exposure increased, the viability of protoplasts decreased almost linearly from 98% (1 min) to 43% (5 min). The viability of protoplasts was not affected between the voltages of 1 and 3 V. The conditions chosen for dielectrophoresis were: frequency 1 mHz, time of exposure (BUILD) 90 s and voltage 3.0 V (0.6 kV/cm).

Electrofusion

On the bases of visual observations, calculations from SC plates and information from the literature (*29,30,33*), the following conditions were chosen for the electrofusion experiments: polarity +, PRE 10 ms, PULSE 20 µs, amplitude 40 V (8 kV/cm), POST 0 ms, SINE 10 s, LOOPS 4, and

Table 2
Growth of the Parental Yeast Strains and Their Hybrids on Different Media after 15 Sequential Shake Flask Cultivations in Control (C) and Selective (AM) Media

Yeast	Shake flask Media*	Final $Klett_{66}$	Percentage** colony count on different plating media*		
			A	M	AM
Baker's	C	242	0	100	0
	AM	7	-	-	-
Sour dough	C	296	95	0	0
	AM	0	-	-	-
Hybrid 68	C	258	100	100	6
	AM	175	98	97	90
Hybrid 71	C	252	100	100	2
	AM	172	100	100	100

*0.67% Yeast Nitrogen Base with the following incredients: C with 2% glucose, A with 4% sodium acetate and 2% glucose, M with 0.25% maltose, AM with 4% sodium acetate and 0.25% maltose.
**Calculated as a percentage of the colony count on control plates (C).
—Not plated.

FADE 90 s. After electrofusion, protoplasts were regenerated on SAM selection plates for 5–10 d.

Fusion Products

Electrofusion was repeated 59 times and a total of 132 colonies were collected. The number of protoplasts in a 5 mL sample was on average 2000 in each experiment, indicating a rather high average fusion frequency of 1.1×10^{-3}. Published electrofusion frequencies vary between 5×10^{-2} (*29*) and 6×10^{-8} (*32*). 19 hybrids (14%) grew in 1 mL of YPD medium and six of them (4.5%) also grew on AM plates, indicating that the desired properties of the parental strains, utilization of maltose and acetic acid tolerance, were combined in these fusion products. They were tested further on AM, A, M, and C plates. Three hybrids (2.3%) grew on all the plates. The stabilities of these hybrids were tested in shake flask cultivations with (medium AM) and without selection pressure (medium C). One of the hybrids lost its acetic acid tolerance during the first cultivation cycle, whereas the other two fusion products, hybrids 68 and 71, were stable in this test. All cells growing on the control plate were also able to grow on acetate-containing agar (A) even after 15 cultivation cycles under nonselective conditions (Table 2). The number of colonies on AM agar,

however, was low compared with that on the control plate and the hybrids reverted during the strong growth in production of baker's yeast in laboratory scale. The hybrids were uninuclear, when they were microscoped in acridine orange (16.8 mg/L in phosphate buffer, pH 6.3) or DAPI (4,6-diamidino-2-phenylindole 10 μg/mL in water) solution under a fluorescent microscope.

Discussion

The baker's yeast and the sour dough yeast had clearly different conditions for the optimal formation of protoplasts when treated with the same lytic enzyme preparation, indicating different composition of the cell walls. Similar results were earlier obtained by measuring the penetration of labeled acetic acid into cells of baker's yeast and sour dough yeast (*38*). It has been assumed that under continuous acetic acid pressure sour dough yeasts have developed a slower penetration rate of acetic acid than that of industrial baker's yeast.

The technique of protoplast fusion is considered to be of exceptional importance in the construction of industrial yeast strains, because sexual processes are frequently not available or are not effective enough for efficient transfer of genetic information (*19*). PEG is considered to be toxic for protoplasts, whereas the electric field has no adverse side effects. Furthermore, the fusion frequency is much higher in electrofusion than in PEG-fusion and it is possible to follow the fusion under a microscope. Thus, electrofusion is a significant improvement over PEG-fusion for protoplast fusion. As a result of these advantages, it has also been applied for plant (*39–41*) and mammalial (*42*) cells as well as for transformation of intact bacterial (*43–47*) and yeast (*42,48–50*) cells in the process known as electroporation or electroinjection.

The stability of fusion products over several generations has not been tested in any referred electrofusion experiments. Stability was tested in only 8 of over 30 published PEG-induced protoplast fusion experiments. The testing methods used were replica plating (*6–8,51*), shake flask cultivation (*18*), small scale (300 mL) fermenter (*17*), and dough raising test (*52*) and in one study the method of testing was not described (*10*). Hitherto stable polyploid hybrid strains, constructed by protoplast fusion, have not been reported in any pilot scale production process, in which very rapid growth occurs. In the construction of truely stable strains, the fusion of parental protoplasms is evidently not sufficient, but the parental nuclei must also be fused and different DNA's reorganized in the desired way. In order to obtain a permanently stable industrial baker's yeast strain by hybridization, it would be necessary to select from very large numbers of hybrids. The disturbance of ploidy of stable polyploid or aneuploid industrial strains may lead only to varying degrees of instability (*53*).

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