Quantification of Physical and Cyto-physiological Conditions for the Electrofusion of Saccharomyces cerevisiae

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Various conditions for obtaining hybrids of the auxotrophic mutants SH1509 and SH1512 of Saccharomyces cerevisiae by electrofusion were investigated. An AC field of 400 V/cm and a DC field of 2 square pulses (7 kV/cm; 60 μsec each) at an interval of 0.5 sec were effective. Treatment with 0.2 (SH1509) or 1.0 mg/ml (SH1512) Zymolyase for 1 or 1.5 hr was essential. As to the molarity of the osmotic stabilizer (sorbitol), the hybrid yield peaked at 0.6 M. The presence of CaCl₂ (up to 0.4 mM) or 0.1 mM CaCl₂ with 0.1 mM MgCl₂ enhanced the yield. The temperature of the spheroplast suspension during pulsations also affected the yield, the most suitable temperature being 28°C.

A number of electrofusion methods have been developed since the first report of plant protoplast fusion done with microelectrodes.¹ Neumann et al.² electrofused Dictyostelium discoideum using exponentially decaying pulses, and Zimmermann et al.³ improved electrofusion by incorporating the dielectrophoresis produced by a non-uniform AC electric field as reported by Pohl.⁴ Electrofusion of many cell species has since been reported: Nicotiana interspecific hybrids,⁵ Daucus carota⁶ for plant protoplasts; mouse fibroblasts in monolayer culture,⁷ antibody-producing hybridomas,⁸ mouse⁹ and bovine¹⁰ embryos for mammalian cells; and yeast,¹¹,¹² basidiomycetes,¹³ and Streptomyces¹⁴ for microorganisms, but few of these studies have shown any quantitative relation between hybrid yield and the electro-physiological conditions used.

Thus we examined the detailed and quantitative effects of electric fields (AC and DC field strengths, pulse duration, and the number and repeated intervals of pulses) produced by parallel electrodes that generate uniform electric fields as reported by Watts et al.¹⁵ We also investigated the effects of cyto-physiological conditions (osmotic pressure, ion composition, the temperature of the spheroplast suspension during electric treatment, and enzyme treatment). We here describe our improved, simple protocol which makes large scale production of hybrids possible.

Materials and Methods

1. Preparation of spheroplasts. S. cerevisiae SH1509 (a, leu2, thr4, trp1, his4, arg4) and S. cerevisiae SH1512 (a, ura2, thr3, trp4, his6, ade1) were gifts from Dr. S. Harashima and Dr. Y. Oshima (Department of Fermentation Technology, Osaka University, Osaka, Japan). Both strains were cultured in 50 ml of YPDA medium (1% yeast extract, 2% polypeptone, 2% glucose, and 0.04% adenine [pH 5.8]) on a rotary shaker (100 rpm) at 27°C. Fresh cells in the logarithmic growth phase (5 x 10⁷-1 x 10⁸ cells/ml) were harvested by centrifugation for 3 min at 1600 × g, then washed with distilled water. After another washing with KT solution (0.45 M KC1, 1 mM EDTA, 10 mM Tris-HCl [pH 7.5]) for 3 min at 1000 × g, then washed with distilled water. After another washing with KT solution (0.45 M KC1, 2 mM EDTA, 57.3 mM 2-mercaptoethanol, and 10 mM Tris-HCl [pH 7.5]) for 30 min on a reciprocating shaker (80 strokes/min) at 35°C. After removing this solution, the cells were shaken (80 strokes/min) for 1 hr at 35°C with enzyme solution (Zymolyase 100T [Kirin Brewery Co., Japan] together with 0.45 M KC1, 1 mM EDTA, 28.7 mM 2-mercaptoethanol, and 10 mM Tris-HCl [pH 7.5]), which had been...
filter-sterilized. The concentration of Zymolyase used was 0.2 (SH1509) or 1.0 mg/ml (SH1512). This suspension was centrifuged for 3 min at 1600 × g. The pellet was washed once and resuspended in fusion buffer (0.7 M sorbitol, 0.1 mM CaCl₂, 0.1 mM MgCl₂, and 0.2 mM Tris–HCl [pH 7.5]) at a density of 5 × 10⁷ spheroplasts/ml. Spheroplast suspensions of both strains were mixed in a 1:1 ratio. The means and standard deviations of the spheroplast diameters were 2.7 ± 0.7 μm (SH1509) and 2.8 ± 0.6 μm (SH1512).

Unless otherwise stated, the conditions for the isolation of spheroplasts, the spheroplast density, and the composition of the fusion buffer in the subsequent experiments were the same as above.

2. Electrofusion. A fusion apparatus that is controlled by a microcomputer and generates up to 20 Vp of AC and 700 V of DC electric voltage (Shimadzu SSH-1, Japan) was used. A 0.4-ml portion of the suspension (1 × 10⁷ spheroplasts of each strain) was pipetted into the fusion chamber (Shimadzu FTC-02, electrode distance 1 mm). When DC pulses of more than 8 kV/cm were applied, a 0.2-ml portion was pipetted into a different fusion chamber (Shimadzu FTC-01, electrode distance 0.5 mm). These chambers had been sterilized by autoclaving. They have parallel plate electrodes, as well as transparent bottoms and covers; thus, the fusion process during electric treatment can be followed under an inverted microscope.

DC square pulses were applied during the AC field producing cell-to-cell contact. The AC field was applied before (120 sec), between, and after (2 sec) the DC pulses. "Standard electrical conditions" were 1 MHz, 200 Vp/cm for the AC field and 7 kV/cm, 60 μsec, 2 pulses at an interval of 1 sec for the DC field. After the electric treatment, the suspension was left for 20 min at room temperature (15–20°C).

When we examined the effects of the temperature of the suspension during electric treatment, a temperature control stage (Shimadzu SSH-T1) was used. The chamber was set on it after the suspension had been introduced.

3. Regeneration of spheroplasts. After pulsation, the suspension was pipetted out of the chamber into an Eppendorf tube. The chamber was then washed with fusion buffer (0.4 ml for FTC-02, 0.2 ml for FTC-01), and any spheroplasts remaining between the electrodes were recovered in the same tube. A portion of the suspension in the tube was spread on minimal medium (0.67% Difco yeast nitrogen base w/o amino acids, 2% glucose, 0.5 M KCl, and 2% agar [pH 5.8]) used to select hybrids, then covered with melted minimal medium (43–45°C). After culture at 27°C for 7 to 8 days, the numbers of colonies in media I (a) and II (b) were counted. The isolation frequency was defined as [(b−a)/b] × 100 (%) and the regeneration frequency as [(b−a)/c] × 100 (%) (c: the total number of spheroplasts plated).

Results and Discussion

The ratio of the number of regenerated colonies in minimal medium to the total number of spheroplasts of each parent plated was defined as the hybrid yield. The yield obtained by the standard conditions was of the order of 10⁻⁵. These putative hybrid colonies grew stably in minimal medium for more than 3 weeks. On the other hand, the reversion rates of the control not subjected to electric fields and the electrically treated single strain were less than 10⁻⁹. Thus, we concluded that the colonies selected in minimal medium were hybrids that compensated for the auxotrophy of both strains.

![Fig. 1. Hybrid Yeast Yield as a Function of the Pulse Duration and Electric Field Strength.](image-url)

Two DC pulses of lasting 10 to 480 μsec with field strengths of 2 to 10 kV/cm were applied to the spheroplast suspensions. ×, 10 μsec; ○, 30 μsec; ●, 60 μsec; □, 120 μsec; △, 240 μsec; ▽, 480 μsec. The other experimental conditions are described in the text. The bars indicate standard deviations for three experiments.
1. Effects of electric fields

We first studied the optimization of electrical conditions, including the DC pulse field strength, pulse duration, the number of pulses, the interval of pulses, and AC field strength. Results are shown in Figs. 1 to 4. In these experiments, one or two electrical conditions were changed while the others were fixed to be the same as in the standard conditions (see Materials and Methods). When strength and duration of the pulses were changed (see Fig. 1), three pulse conditions (7 kV/cm; 60 μsec, 7 kV/cm; 30 μsec, and 5 kV/cm; 120 μsec) gave peak yields of similar levels.

Next, strength and the number of pulses were changed (Fig. 2). Two pulses gave the best yield at 7 kV/cm, but 3 to 4 pulses gave a better yield than 2 pulses at a lower (4–5 kV/cm) pulse strength.

The effects of the interval between 2 pulses are shown in Fig. 3. An interval of 0.5 sec gave the best yield. The yield decreased markedly at intervals of more than 10 sec, probably because the membrane, which fluctuated during or after the first pulse, may have returned to its original state during the 10 sec before the onset of the second pulse.

As to permeability change, Sale et al.17)
reported that it was induced by a transmembrane potential of about 1 V and Dimitrov\textsuperscript{18} reported that it depended on the pulse duration. Our experimental evidence, however, shows that adequate membrane fluctuation for the increase of hybrid yield depended on a combination of the pulse field strength, its duration, the number of pulses, and the intervals between pulses.

The percent of survival (the ratio of pulsed to not pulsed) for both strains was approximately 80\% with 2 DC pulses (7 kV/cm, 60 \mu s each) at 0.5 sec interval.

The effects of the AC field strength are shown in Fig. 4. The yield increased with an increase in the strength up to 400 Vp/cm. As the yield also increased in proportion to the length of the period (200 Vp/cm), the number of chain-formed spheroplasts may have increased (data not shown).

These findings suggested that improved electrical conditions would comprise a 400 Vp/cm AC field and 2 DC pulses (7 kV/cm, 60 \mu s each) at 0.5 sec interval.

2. Effects of isolation conditions

The effects of the concentration of Zymolyase and the incubation period used to isolate the spheroplasts are shown in Table I. The concentration used markedly affected the yield of hybrids. Low concentrations such as 0.02 (SH1509) and 0.1 mg/ml (SH1512) greatly decreased the yield. We speculate that the cells may not have fused because their cell walls were not sufficiently digested. High concentrations such as 1.0 (SH1509) and 5.0 mg/ml (SH1512) also markedly decreased the yield. This may be because most spheroplasts were not able to regenerate cell walls because the severity of the isolation procedure decreased cell viability. When treated with 0.2 (SH1509) or 1.0 mg/ml (SH1512) Zymolyase for 1 hr, the isolation frequency for both strains was almost 100\%, and the regeneration frequencies were 0.6\% (SH1509) and 0.3\% (SH1512).

3. Effects of spheroplast density

The yields at a density of 5 \times 10^6, 5 \times 10^7, and 5 \times 10^8 spheroplasts/ml were 76 \times 10^{-7}, 174 \times 10^{-7}, and 182 \times 10^{-7}, respectively. The yield may not depend on the density if it is sufficient to produce cell-to-cell contact. The ratio of chain-formed to total spheroplasts may have been saturated when a density of more than 5 \times 10^7 spheroplasts/ml was used and an AC field was applied for a sufficient period.

4. Effects of the composition of the fusion buffer

The effects of the osmotic pressure of the fusion buffer are shown in Fig. 5. Sorbitol was used as the osmotic stabilizer. As the approx-
Quantification of Electrofusion Conditions for *S. cerevisiae*

Table II. Effects of the Salt Composition of the Fusion Buffer

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<tr>
<th>Composition of fusion buffer</th>
<th>Hybrid yield ($\times 10^{-7}$)</th>
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<tr>
<td>Sorbitol (m)</td>
<td>CaCl$_2$ (mm)</td>
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<td>27</td>
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<td>1.0</td>
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Appropriate molarity of the stabilizer for culture is 1.0–1.2 m, we speculate that a slightly hypotonic condition induces the appropriate membrane tension for fusion, but that at too low an osmotic pressure, DC pulses cause the spheroplasts to burst because of swelling and increased water permeability. We also speculate that fusion is affected by various factors that have a complex relationship with one another.

Studies in using different strains in yeast reported that the fusion efficiency (number of colonies on minimal medium/number of colonies on complete medium) by electric fields was the order of $10^{-3}$–$10^{-2}$, which was several tens-fold higher than the PEG method. Taking the highest yield (8 $\times 10^{-5}$) and regeneration frequencies (0.6 and 0.3%) into account, the efficiency by the same definition as above in our studies could be approximately $10^{-2}$. Thus we believe that electrofusion can be a useful method for the fusion of yeasts. Though we have no experimental data on other yeast species, we suggest that an especially useful approach for electrofusion is to examine the suitable pulse field strength and duration, and to optimize the enzyme and buffer conditions as done in our findings.

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References