Electrotransformation of \textit{Saccharomyces cerevisiae} protoplast by a plasmid of \textit{Escherichia coli}

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Abstract

The experiments reported here are aimed at obtaining optimum conditions for gene transformation after electric field pulses. \textit{Saccharomyces cerevisiae} DBY746 is used as the recipient strain for shuttle plasmid (YRp group). From the relationship between the optimum electric field conditions and the transformation efficiency it is discovered that the maximum transformation efficiency appears at a wide pulse length of 400 μs with an electric field strength of 4 kV/cm, yielding up to 273 transformants/μg DNA. The electroporation unit used in the experiment is a home-made set featuring simplicity, readiness and practicality.

INTRODUCTION

The electric penetration technique is an up-to-date bioelectric technology. It allows injection of an external gene into a cell by a dc high voltage electric pulse applied to the biological membrane, i.e. a transient disturbance will appear on the lipid bilayer, resulting in pore formation. Thus, the membrane permeability increases temporarily due to the effect of this short range and high strength electric pulse. This technique is also called “electroporation”. In the past decade, it has been proved that for gene transformation of many cells from animals, plants and microbial organisms, the electroporation technique has some advantages, which cannot be reached by chemical methods. At present, electroporation is widely used for penetration techniques not only for genes but also for drugs. Today there are several models for explaining its mechanism.

This paper is concerned with the measurement of optimum electric field conditions for the transformation of yeast protoplast after transducing plasmid DNA.

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EXPERIMENTAL

Materials

Bacterial strains *Escherichia coli* C600 (F-, thi-1, Leu B6, Lac Y1, ton A21, Sup E44, λ-) was the plasmid donor strain (including Amp resistance), while *E. coli* HB101 (F-, Hsd20, rec A13, ara-14, pro A2, Lac Y1, gal K2, rps L20, xyl-5, mtl-1, Sup E44, λ-) was used for analysis of the plasmid.

Yeast strain

*Saccharomyces cerevisiae* DBY746 (λ, Leu 2-3, Leu 2-112, his 3-1, trp 1-289a, ura 3-52, gal 80) was used. It is a mononutrition defect type strain.

Plasmid

The plasmid employed is a structural plasmid (YRp group) containing the trp-1 gene, with a size of 4.15 kb. This plasmid is a shuttle one consisting of bacterial plasmid and the yeast gene with yeast duplicator and selectable marker, and its features can be revealed in yeast and *E. coli*.

LB (Luria–Bertani) culture medium [1]

This medium was used for the culture of *E. coli* with solid agar powder at a concentration of 1.5%.

Complete yeast culture medium YEPD (*Yeast Extract Peptone Dextrose*)

This medium was used for the culture of yeast cells. The main ingredients are 1% yeast powder, 2% protein gel, 2% dextrose (pH 6.0). The density of solid agar powder was 2% in the culture medium.

Basic yeast culture medium YNB/oAA [2] (*Yeast Nitrogen Base without Amino Acids*)

This medium was used for the selected culture of yeast transformants. The main ingredients are 0.67% Difco YNB, 2% dextrose, 1 M sorbitol (pH 6.5). The concentration of solid and soft agar powder was 2% and 0.9%, respectively, in the culture medium. When YNB/oAA is used for an isotonic culture, different additives [3] should be added in conformity with the nutrition as requirements of the recipient strain and transformant as shown in Table 1.

**TABLE 1**

Additives to YNB/oAA used

<table>
<thead>
<tr>
<th>Additives</th>
<th>Concentration of stored liquid</th>
<th>Concentration of working liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>50 mg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>His</td>
<td>50 mg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Leu</td>
<td>50 mg/µl</td>
<td>50 µg/µl</td>
</tr>
<tr>
<td>Ura</td>
<td>40 mg/ml</td>
<td>40 µg/ml</td>
</tr>
<tr>
<td>Gal</td>
<td>5%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>
Large scale preparation and detection of plasmids

Preparation of the plasmid. The plasmid was prepared from the *E. coli* C600 strain, which was cultured in LB culture medium (containing 50 μg/ml Amp), by the alkaline splitting method [1] and was purified [1] by EB-C,Cl gradient centrifugation. The amount of purified DNA in the sample was measured and analyzed by the action of a cutting enzyme. The limited internal cutting enzyme EcoRI was used to digest an appropriate quantity of the DNA samples [1] and then electrophoresis detection was performed, the results of which read as follows: the size of the plasmid was 4.15 kb and those of the two fragments were 1.45 and 2.7 kb, respectively.

Preparation of the yeast protoplasts [4].

Step 1: *S. cerevisiae* DBY746 was cultured in YEPD medium at 30°C with reciprocal shaking for 12 to 16 h. Fresh logarithmic cells were harvested by centrifugation at 3500 rpm for 5 min.

Step 2: The collected cells were washed with 0.2 M PB buffer solution (0.2 M Na₂HPO₄, 0.1 M citric acid, 1 M sorbitol, pH 5.8) and 0.85% physiological saline; the supernatant was discharged.

Step 3. The cells were suspended in a solution containing 0.1% sulfhydryl alcohol-PB and 0.05 M EDTA by shaking at 28°C for 20 to 30 min. The suspension was then centrifuged for 15 min at 3500 rpm and the supernatant was removed.

Step 4. The cells were washed with PB solution and treated with 1% snail enzyme-PB at 28 to 30°C for 15 to 30 min; the suspension was centrifuged for 10 min at 2000 rpm and the supernatant was removed. The resulting pellet was washed with isotonic solution (10 mM Tris + HCl, 10 mM CaCl₂, 1 M sorbitol, pH 7.5) and finally suspended in this solution.

Conditions for transformation and electroporation. An appropriate quantity of the purified DNA was added to the prepared protoplast suspension (the density was about 7 x 10⁸ cells/ml) and mixed (the final DNA concentration was 5 μg/ml), and after storage at room temperature for 10 min, 0.2 ml of the mixed liquid was put into the small electroporation chamber, which was connected to the power unit. Thus, the electroporation process could be conducted with the needed pulse field strength, pulse width and number of pulses. The resistance value of the mixed liquid in the chamber measured and calculated to be approximately 84 Ω.

After the electric shock, the cell suspension was stored inside the chamber for 20 min, then put into a sterilized Eppendorf tube, and 0.3 ml of YNB isotonic liquid culture medium without trp were added. After cultivation at 30°C for 2 h, the suspension was plated onto a YNB isotonic layer soft agar without trp and cultivated further at 30°C for 2 to 3 days, after which colonies of transformants could be counted.

The unit for electroporation. The complete high-voltage pulse circuit consists of a dc regulated power unit device (model ZGD3A), switch section, capacitor section
and a small electroporation chamber (Fig. 1). The principal wiring of our self-fabricated, simple and practical electroporation unit is shown in Fig. 2.

The voltage in the power unit can be regulated within 0 to 25 kV and the current within 0 to 300 μA. An outstanding feature is its extremely high voltage output. When the switch is turned to “1”, an extra-high voltage will be utilized for charging, i.e. one of the capacitors will be charged within a short period; when the switch is turned to “2”, the capacitor will be discharged for electroporation and electrofusion (compare Table 2).

The small electroporation chamber. The chamber was designed in our laboratory. The shape is cylindrical, the volume is about 0.2 ml and the distance between the two stainless steel electrodes is 2 mm, as shown in Fig. 1. Its design makes it suitable for the electroporation of large quantities of cells and of cell cultures, and resistant to high pressure sterilization.

![Fig. 2. Wiring diagram of electroporation unit. (a) dc power unit; (b) switch section; (c) capacitor section; (d) electroporation chamber.](image-url)
TABLE 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Capacitance/μF</th>
<th>Pulse width/μs</th>
<th>Number of pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5; 40, 4.7; 400, 6.0; 500</td>
<td>2, 4, 6, 8</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.5; 40, 4.7; 400, 6.0; 500</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>0.5; 40, 4.7; 400, 6.0; 500</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

For experiments 1 and 2, separate experiments should be carried out at each pulse width (i.e., changing the pulse strength or the number of pulses).

Detection of transformants. DNA can be extracted from the yeast transformants [5]; however, DNA is thus obtained in very small quantities only and is not directly suitable for electrophoresis. Therefore, it was used for E. coli HB101 transformation, i.e., a retransformation procedure [1].

Detection of the retransformed DNA. This DNA was extracted from E. coli HB101 by the trace plasmid analysis method [6]. This plasmid and the plasmid from E. coli C606 were digested with EcoRI enzyme and examined quickly by electrophoresis with DNA Marker as reference.

Fig. 3. The relation between transformation efficiency and electrical pulse strength: one 400 μs pulse.
RESULTS AND DISCUSSION

Three groups of pulses (see Table 2) were utilized for electroporation of the mixture of protoplasts with E. coli C600 plasmid in order to find the optimum transformation conditions.

First, the relation between electrical pulse strength and transformation efficiency was determined. The results were as follows:

(1) The highest transformation efficiency will be reached when the capacitance is 4.7 µF ($RC = 400 \mu s$), the electrical field strength is 4 kV/cm: 273 transformants/µg DNA are obtained (see Fig. 3). Similar results are obtained when the capacitance is 6 µF.

(2) The transformation efficiency of a long pulse (400 µs) is about three times higher than of a short one (0.5 µF, 40 µs).

Secondly, when the pulse acts on the mixture of protoplasts and plasmid repeatedly, the number of transformants will be decreased. If the capacitance is 4.7 µF, the field strength is 4 kV/cm and the number of pulses used is 1, 3, 6 or 10 with about 5 s intervals, the results are as follows:

(1) If one pulse is applied, the transformation efficiency is highest (see Fig. 4). If the capacitance is only 0.5 µF, the highest transformation efficiency will be shifted to high electrical field strengths along with a decrease of the number of pulses.

Fig. 4. The relation between transformation efficiency and number of electrical pulses. 4 kV/cm, 400 µs.
Fig. 5. The relation between the viability of protoplasts and the number of electrical pulses. 4 kV/cm, 400 µs.

Fig. 6. DNA electrophoresis pattern obtained from transformants. (A) λDNA marker; (B) original plasmid from C_{600}; (C) transformant's plasmid from HB101. (1) The closed ring form of the plasmid structure from E. coli C_{600}. (2) The opened ring form of the plasmid structure from E. coli C_{600}.

Fig. 7. The enzyme-cutting analysis of transformant's plasmid from E. coli HB101. (a) The result after the original plasmid structure is cut by the EcoRI enzyme; (b) λDNA marker; (c) the result after the transformant's plasmid is cut by the EcoRI enzyme. (1) 1.45 kb fragment after the enzyme cutting. (2) 2.7 kb fragment.
(2) When one pulse is applied, the highest ratio of surviving protoplasts (about comparatively 85–90%) is obtained. With increasing number of pulses, the viable ratio will decrease (see Fig. 5).

Experiment 3 will be based on the description of Table 2 (measured also, i.e. a single pulse). With the field strength at 4 kV/cm, the relation between transformation efficiency and pulse width (or capacitance) was observed. The longer the pulse action lasts, the more serious is the damage to the membrane, and consequently the transformation efficiency decreases.

After the transformation of plasmid DNA into yeast protoplasts is completed, only the selective symbol (trp⁻→trp⁺) can be determined. If the more exact method of electrophoresis determination is used, as mentioned above, the DNA should first be extracted from the retransformed transformants. The result is shown in Fig. 6. It was found that the plasmid extracted from the donor E. coli C₆₀, a shuttle plasmid, has the same position of its electrophoresis-bands as the retransformed plasmid, that is, open and closed rings of the plasmid, respectively, should be in correspondence. According to the electrophoresis pattern obtained with the retransformed plasmid digested by the EcoRI enzyme, the position of the two fragments corresponds to the original structure of C₆₀₀ plasmid digested in the same way. As compared with the DNA marker, the size of the two fragments is correct (see Fig. 7).

While the above-said electrotransformation cells were being cultured, the following comparative experiments were performed:

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Outcome</th>
</tr>
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<tbody>
<tr>
<td>Protoplast + YNB (trp⁻)</td>
<td>no development;</td>
</tr>
<tr>
<td>Protoplast + plasmid from E. coli C₆₀₀ + YNB (trp⁻)</td>
<td>no development;</td>
</tr>
<tr>
<td>Protoplast + electric field + YNB (trp⁻)</td>
<td>no development;</td>
</tr>
<tr>
<td>Plasmid from E. coli C₆₀ + electric field + YNB (trp⁻)</td>
<td>no development;</td>
</tr>
<tr>
<td>Yeast cell + YNB (trp⁻)</td>
<td>no development.</td>
</tr>
</tbody>
</table>

From these results it follows that:

1. If the plasmid DNA is not added to the suspension of protoplasts, trp⁻ will not be transformed to trp⁺ no matter what kind of electrical pulse treatment is applied. Consequently, the (trp⁺) colony is (instead of being induced by pulse treatment) the product of a kind of transformation process during which the plasmid is injected into yeast protoplast.

2. Without the electrical pulse, no true trp¹⁺ transformants will be obtained whatever comparative experiment is made.

It was found from the experiments that the preparation of yeast protoplast will affect the transformation result directly. The key to the correct preparation of protoplast lies in obtaining developing cells, i.e. the collection of cells in the logarithmic period. At the same time, better results are obtained, along with the treatment with sulfhydryl alcohol, by the application of EDTA and fast enzymolysis of the cell walls.

Since our experiments were aimed at clarifying the relation between electrical field conditions and gene transformation efficiency, various factors affecting the
transformation efficiency should not be ignored. The main factors include the following.

(1) Since the kind of cells, developing conditions, cell constituents and structures are different, the requirements of electroporation conditions (e.g. pulse strength, width and number), solution composition, pH value and temperature are consequently also different, and should be evaluated experimentally.

(2) The degree of digestion of the walls of the yeast cells will affect the transformation efficiency directly.

(3) Our experiments are characterized by the use of a yeast strain as well as bacteria developed for genetic engineering. However, as a result of this limitation, the yeast strain has several nutritional defects (five kinds altogether). This is one of the factors which affect transformation efficiency and cell development. If the optimum type of bacteria can be found, the gene transformation will be more efficient.

(4) Recently, the electroporation method has been applied to the following recipients [7] with different efficiency (transformed colonies/µg DNA):
- mammalian cells (from mouse, rat, human organs);
- plant cells (tobacco, carrot, rice, maize);
- bacteria [8], e.g.: gram-negative \((E. coli \text{ HB101L} + \text{pKT231}: 1.6 \times 10^5, \ E. coli/\text{WT} + \text{pKT231}: 9 \times 10^2, \ E. coli \text{ LE392/L} + \text{pUC18}: 1 \times 10^{10}, \ S. marcescens/\text{WT} + \text{pKT231}: 4 \times 10^2, \ Enterobacter aerogenes + \text{pKT231: 50}), \) gram positive \((Bacillus cereus 569 + \text{pC194: 5.1} \times 10^3, \ B. thuringiensis \text{ HD-1} + \text{pGK12: 3.4} \times 10^3, \ Enterococcus faecalis \text{ OG1X + pGK12: 61}) ;\)
- yeast cells \((S. cerevisiae [9] \text{ with YEpl3, complementing Leu deficiency: 100, } S. cerevisiae \text{ SE7-6 [10] with pYE shuttle vector from } E. coli: 0.1-4.5 \times 10^3, \ S. cerevisiae \text{ BWG1-7a [11] with Yep351: 2-5 \times 10^3}, \)
- yeast protoplasts \((S. cerevisiae \text{ [12] with } YRp7 \text{ shuttle vector, complementing tryptophan deficiency: } 1 \times 10^3) .\)

From these efficiency (colonies/µg plasmid) data of bacteria a very wide range \(10^1-10^{10}\) is recognizable; however, the results can be modified strongly by changing the conditions [7]. For intact yeast as recipient, values up to \(10^5\) were published [11] depending especially on the kind of plasmid. Our result, \(2.73 \times 10^2\) with yeast protoplasts, belongs to the lower group of data comparable with ref. [12].

(5) Concerning the mechanisms of the fast electroporation and the slow electrotransformation processes there exist at least ten more or less different models only for electroporation [13,14] by Yu. Chizmadzhev (Moscow), D. Dimitrov (Sofia), E. Neumann (Bielefeld), G. Saulis (Vilnius), A. Sowers (Washington), I. Sugar (Budapest), J. Teissié (Toulouse), T. Tsong (Minneapolis), J. Weaver (Cambridge) [15] and U. Zimmermann (Würzburg), taking mostly into account reversible pores \(< 2\) nm in diameter. So, ion and drug incorporation can be increased markedly; however, there are difficulties with the DNA diameter of \(> 2\) nm. Nevertheless the transfection process can be enhanced, sometimes more effectively, than with classical methods. To explain this discrepancy some hypotheses are under discussion, e.g. by a process of electrophoretic percolation [16]. On the other hand, larger pores of 50 nm were
detected very recently by Chang and Reese [17], big enough for the penetration of extended circular DNA. Nowadays the electroporation equipment allows the application of pulses with field strengths of up to about 20 kV/cm in order to avoid protoplast production from bacteria, yeasts, etc. and therefore this method is time-saving and more effective than the classical genetic procedures.

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REFERENCES