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# High Frequency Electroporation-transformation of Coryneform Bacteria Grown in the Medium with Penicillin-G

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# Penicillin-G 첨가 배지에서 배양한 코리네형 세균의 전기장 충격법에 의한 고효율 형질전환

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## ABSTRACT

Using the shuttle vector pECCG1 between *Escherichia coli* and *Corynebacterium glutamicum* and *C. glutamicum* strain JS231 grown in the medium supplemented with penicillin-G, which inhibits the formation of cross-links in the peptidoglycan of bacterial cell wall, various parameters involved in electroporation system including resistance, electric field strength, capacitance, DNA concentration, and cell density were investigated independently and optimized for the high efficiency transformation of coryneform bacteria. Using cells grown with 0.3U / ml of penicillin-G and harvested at $A_{600}$ of 0.7–0.8, transformation efficiencies of $10^7$–$10^8$ transformants / $\mu$g of DNA with *Corynebacterium glutamicum* strain JS231 and wild type ATCC13032 were achieved under conditions of 12.5kV / cm of electric field strength, 400 ohms of resistance, 25$\mu$F of capacitance, $3\times10^8$ cells per transformation($1.2\times10^{10}$ cells / ml) and 100ng of plasmid DNA per transformation.

## INTRODUCTION

Various PEG-mediated protoplast transformation methods of coryneform bacteria were reported(1–5). The system met some difficulties in generating protoplast and regeneration to viable cells. Coryneform bacteria are usually more resistant to lysozyme treatment than other gram-positive bacteria and their protoplasts take long time to regenerate to viable cells. So many factors are involved in protoplast transformation system that transformation efficiencies are very variable from experiment to another and from strain to strain.

Recently, there were some reports on the application of the electroporation system to the transformation of both gram-positive and gram-negative bacteria. Electroporation involves the application of a brief, high voltage pulse to

a suspension of cells and DNA. The rationale of the electroporation is making a membrane permeable transiently and the subsequent uptake of DNA possible. Electroporation systems have already been applied successfully to the transformation of some gram-positive bacteria including *Lactobacillus*(6), *Streptococcus*(7), *Bacillus*(8) and *Clostridium* (9). Transformation efficiencies of $10^7$ transformants per $\mu g$ of plasmid DNA have been achieved with intact yeast cells by using the electroporation system(10). The technique was also applied to the transformation of *E. coli* and $10^9$ to $10^{10}$ transformants / $\mu g$ of DNA were obtained with strains LE392 and DH5, and plasmids pUC18 and pBR329(11).

There have been some reports on the application of the electroporation system to the transformation of coryneform bacteria with varying transformation eficiencies of $3.6\times10^3-10^7$ transformants / $\mu g$ of DNA (12-15). Results are summarized in table 1. Dunican and Shivnan (12) reported the highest transformation efficiency of $10^7$ transformants / $\mu g$ of DNA using 1 ng of plasmid DNA pHY416 and *C. glutamicum* strain AS019. Compared with that when 100ng of DNA was used, the transformation efficiency was improved about 20 times. According to the reports of others including ours, there was not such a large improvement in the transformation efficiencies according to the DNA concentration. Haynes and Britz(13) performed the experiment using the same plasmid and host strain, and transformation efficiencies (transformants / $\mu g$ of DNA) were nearly the same with DNA contents between 1 ng and 1000 ng. According to the results of our previous report(15), transformation efficiency with 1 ng of DNA was only 1.3 times as high as tat with 100 ng of DNA. And it seems to be better to compare the results excluding that of Dunican and Shivnan with 1 ng of DNA used. All the experiments proved that optimum electric field strength was 12.5 kV / cm even though culture conditions of host strains and procedure of electroporation were different. Cell concentrations prepared for the electroporation were between $10^{10}$ and $5\times10^{10}$ cells / ml. The reported transformation efficiencies were

**Table 1. Transformation of coryneform bacteria with electroporation system**

| Strains | Plasmids | Plasmid (ng) | Efficiency (Transformants / $\mu g$ of DNA) | Supplements in medium | Ref. |
|---|---|---|---|---|---|
| *C. glutamicum* | | | | | |
| AS019 | pHY416 | 100 | $6.6\times10^5$ | | 12 |
| | | 1 | $1.0\times10^7$ | | |
| ATCC21850 | | 100 | $2.6\times10^3$ | | |
| | | 1 | $6.5\times10^3$ | | |
| *C. glutamicum* | | | | | |
| AS019 | pHY416 | 1000 | $3.9\times10^5$ | 2.5% glycine | 13 |
| | pSCL17 | 1000 | $5.0\times10^5$ | and 4mg / ml | |
| | pUL340 | 1000 | $4.5\times10^5$ | of INH | |
| *B. flavum* | | | | | |
| MJ223 | pCRY3 | 1000 | $6.0\times10^4$ | pen-G(1 U / ml) | 14 |
| *C. glutamicum* | | | | | |
| ATCC31830 | pCG4 | 1000 | $3.6\times10^3$ | | |
| *C. glutamicum* | | | | | |
| JS231 | pECCG1 | 100 | $6.5\times10^5$ | 2% glycine · | 15 |
| ATCC13032 | | 100 | $1.0\times10^6$ | | |
| *C. glutamicum* | | | | | |
| JS231 | pECCG1 | 100 | $1.0\times10^7$ | pen-G | This |
| ATCC13032 | | 100 | $1.0\times10^8$ | (0.3 U / ml) | report |

between $3.6\times10^3$ and $10^6$ transformants / μg of DNA. The transformation efficiencies seem to vary according to the characteristics of plasmids used, host strains and culture conditions of the host strains. Dunican and Shivnan(12) achieved fairly good transformation efficiency of $6.6\times10^5$ transformants / μg of DNA with *C. glutamicum* strain AS019 and plasmid pHY416 without using any cell wall-weakening agents(*e. g.*, glycine or penicillin-G). According to our previous result(15), addition of glycine to culture medium improved the transformation efficiency about 10 to 100 times. And the transformation efficiency ($6.5\times10^5$ transformants / μg of DNA) was similar to that ($6.6\times10^5$ transformants / μg of DNA) of Dunican Shivnan. Considering that they did not use any cell wall-weakening agents, they achieved fairly high transformation efficiencies. Haynes and Britz achieved $5\times10^5$ transformants / μg of DNA using *C. glutamicum* strain AS019 grown with 2.5% of glycine and 4 mg / ml of INH (isonicotinic acid hydrazide). The efficiency was similar to the results of report by Noh *et al.* (15) who also used *C. glutamicum* strain JS231 grown with 2% of glycine. Kurush *et al.* (14) tried to perform the electroporation using *C. glutamicum* and *Brevibacterim flavum* grown with 1U / ml of penicillin G and achieved transformation efficiencies $3.6\times10^3$ and $6\times10^4$ transformants / μg of DNA, respectively. The efficiencies were much lower than that of this reports even though they also used the same cell wall-weakening agent with us, *e. g.*, penicillin-G. The difference seems to result from the differences in medium composition (they used less complex medium for the cultivation of host strains) and electroporation procedure. They used too large volume of cell and DNA mixture in electroporation. We used only 42 μl of mixture and they used as much as 900 μl. The volume of 900 μl seems to be too large for the cells to be transformed efficiently.

By using the electroporation system, we previously reported on the transformation of *C. glutamicum* grown in the medium supplemented with 2% of glycine with transformation efficiencies of $10^6$ transformants / μg of DNA(15). Now, here we report the achievement of 100-fold improvement of transformation efficiencies by some modifications of previous method.

## MATERIALS AND METHODS

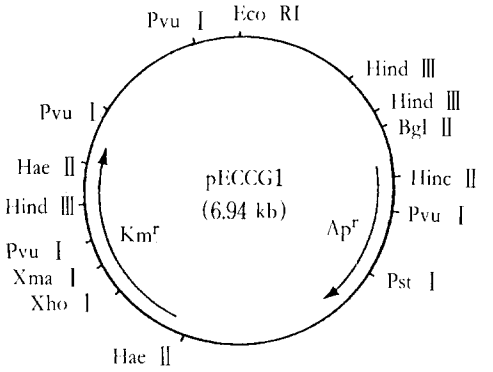


**Fig. 1. Structure of plasmid pECCG1.**

### Bacterial strains and plasmids

Plasmid pECCG1(15), which is 6.94 kb and carries kanamycin resistant marker gene, was used to transform *C. glutamicum* strain ATCC13032 (wild type) and JS231 (leucine$^-$, homoserine$^-$) using electroporation system. At first, we used, *C. glutamicum* strain JS231 as a host strain because JS231 was more sensitive to lysozyme than wild type ATCC 13032 and easier to isolate plasmids from transformants to confirm true transformants. Structure of the plasmid pECCG1 is shown in Fig.1.

### Media and bacterial cultivation

LG medium (1% Tryptone, 0.5% Yeast extract, 0.5% Glucose, 1% NaCl, pH7.0) and LG agar(1.8%) were used for the routine growth of *C. glutamicum*. The bacteria were grown at 30℃. TYG medium (1% Tryptone, 1% Yeast extract, 0.5% Glucose, 0.5% NaCl, pH 7.0) was used for the cultivation of *C. glutamicum* to isolate plasmid DNA. For electro transformation, *C. glutamicum* strains were grown in LG medium supplemented with 0.3 U / ml of penicillin-G.

### Plasmid DNA preparation and DNA manipulations

Large scale plasmid preparation was done following an alkaline lysis method and further purified with CsCl-ethidium bromide density gradient centrifugation(16). For the rapid confirmation of transformants via plasmid isolation, we followed the method of Kieser(17) with some modifications. Cells were collected from 1ml of overnight culture, resuspended in 0.5ml of TEN buffer(10 mM Tris,

1 mM EDTA, 5 mM NaCl, pH 8.0) containing 5 mg/ml of lysozyme and incubated for 2 hr at 37℃ with vigorous shaking. The cells were collected again, resuspended in 0.2 ml of TEN buffer and 0.1 ml of lysis buffer (0.3M NaOH, 2% SDS) was added. After a vortex-mixing the lysate was incubated at 70℃ for 15 min. After cooling it to room temperature 50 μl of Phenol/Chloroform was added, mixed homogeneously and centrifuged 5 min in an Eppendorf microcentrifuge. The supernatant was taken and 10 μl was applied to the agarose gel(0.9%)-electrophoresis for the confirmation of plasmid DNA. The remaining supernatant was further purified for the later use in other DNA manipulations.

### Electroporation equipment and electroporation procedure.

Electroporation was carried out using a Gene-Pulser system (Bio-Rad Laboratories, Richmond, CA). The system is composed of Gene Pulser apparatus, pulse controller unit and gene pulser cuvettes of 0.2 cm electrode gap.

2 ml of overnight culture of *C. glutamicum* grown aerobically in LG medium at 30℃ was inoculated into 100 ml of fresh LG medium supplemented with 0.3 U/ml of penicillin-G and cultivated at 30℃ with shaking at 260 rpm until an $A_{600}$ reached 0.7−0.8. After chilling on ice for 30 min the cells were harvested, and washed twice with 100 ml of cold 1 mM HEPES (pH 7.0) and once with 20 ml of cold 10% glycerol. The cells were finally resuspended in 3 ml of 10% glycerol. Cuvettes and sliding holder were chilled in ice 5 min prior to use. 40 μl of cell suspension and 2μl of DNA(100 ng, unless otherwise stated) dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) were mixed in cold cuvette and pulsed once. Immediately 1 ml of SOC medium (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) was added to the cuvette and the cells were shaken at 30℃ for 1 hr for the transformed gene to be expressed and followed by spreading on LG agar plate containing 50 μg/ml of kanamycin. Transformants appeared in 12−24 hr after plating.

## RESULTS

### Optimization of conditions for *C. glutamicum* electroporation

There are several reports on the application of electroporation system to the bacterial transformation including both gram-positive and gram-negative bacteria. We tried to apply this system to the transformation of *C. glutamicum* strain JS231 using *C. glutamicum*/*E. coli* shuttle vector pECCG1 is olated from JS231. Several factors which were thought to be most important for the efficient transformation were studied. All the following experiments were performed by using *C. glutamicum* JS231 grown in LG medium supplemented with 0.3 U/ml of penicillin-G because cells grown in LG medium with penicillin-G showed a bout 10 to 100 times higher transformation efficiencies than those grown in the medium without penicillin-G. The penicillin-G inhibits the formation of cross-links in peptidoglycan of growing bacteria and weakens cell wall. The antibiotic is used industrially in the production of glutamic acid which is flavor-enhancing agent, to facilitate the excretion of glutamic acid to the extracellular environment by weakening cell wall. Also penicillin-G is added to the culture medium of some gram-positive bacteria which are somewhat resistant to lysozyme. The cells grown in the presence of penicillin-G are more sensitive to lysozyme and it is easier to isolate DNAs from the cells, or to form protoplasts for PEG-mediated protoplast transformation. We added penicillin G to the culture medium to enhance the transformation efficienies of *C. glutamicum* using electroporation system. In most of experiments, $3\times10^8$ cells of JS231($1.2\times10^{10}$ cells/ml) and 100 ng of plasmid DNA were used per transformation, unless otherwise stated.

### Resistance:

In *E. coli* system, highest transformation frequency was reported at the conditions of 12.5 kV/cm, 25μF and 200 ohms. We postulated tha more stringent conditions will work for *C. glutamicum* than for *E. coli* because *C. glutamicum* has a more rigid cell wall than *E. coli*. Electric field strength and capacitance were set at 12.5 kV/ml and 25 μF, respectively and resistances ranging from 100 ohms to 1000 ohms were tested. The results are shown in Fig. 2. The number of transformants was maximum at 400 ohms and decreased abruptly thereafter. DNA uptake frequency, which we define as the transformation frequency of cells both survived and dead after electroporation to distinguish from transformation frequency

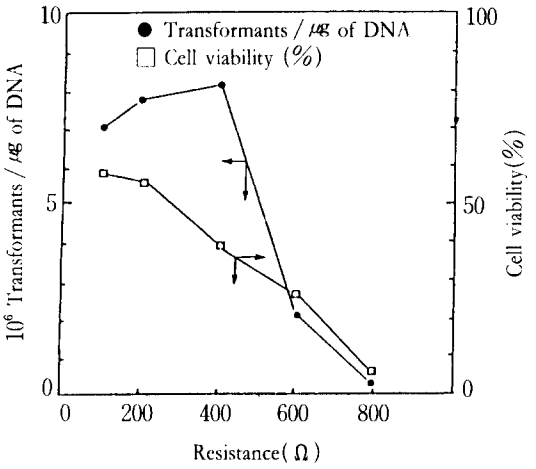


Fig. 2. Effect of resistance on the transformation frequency of *C. glutamicum* JS231.

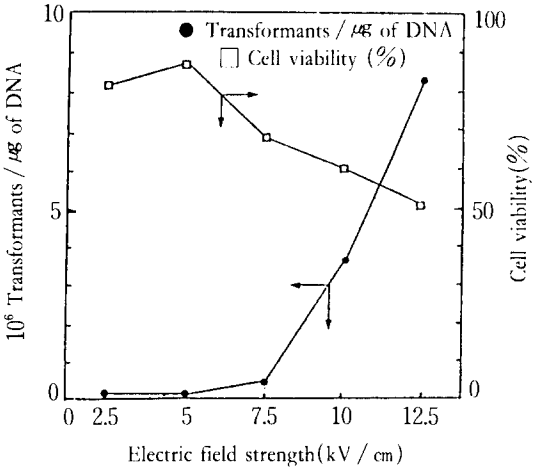


Fig. 3. Effect of electric field strength on the transformation frequency of *C. glutamicum* JS231.

of survived cells, was also highest at 400 ohms. Above 200 ohms, cell viability decreased inversely with increasing resistance. These indicate that the decrease of the transformants at the resistance higher than 400 ohms seemed to result partly from the decrease in cell viability and partly from suboptimal condition for the uptake of DNA into cells.

### Electric field strength:

Now that optimal resistance was determined at the conditions of 12.5 kV / cm and 25 μF, the effect of varying electric field strength was examined with resistance set at 400 ohms. As presented in Fig. 3, transformation efficiencies were very low below 7.5 kV / cm. Transformation efficiency reached maximum at 12.5 kV / cm which was the same value set at the previous experiment. Cell viability was maintained about 90% up to 5 kV / cm and fell down thereafter. As the data illustrate, it seems to be possible that we get higher transformation efficiencies at field strength higher than 12.5 kV / cm. But we could not do the test at field strength higher than 12.5 kV / cm due to instrument limitation. Therefore, it could be concluded that optimal electric field strength is 12.5 kV / cm or higher than 12.5 kV / cm at conditions of 400 ohms and 25 μF.

### Capacitance:

We also examined the effect of varying capacitance of

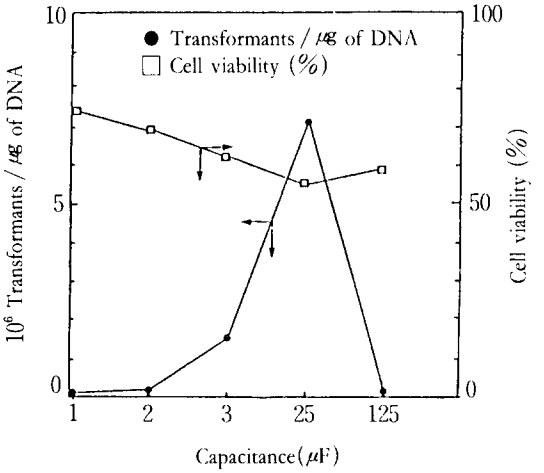


Fig. 4. Effect of capacitance on the transformation frequency of *C. glutamicum* JS231.

the system on the transformation efficiencies. To extend capacitance we used capacitance extender(Bio-Rad Laboratories, Richmond, CA) with which maximum capacitance of the system could be extended from 25 μF to 960 μF. As shwon in Fig. 4, transformation efficiencies were highest at 25 μF and fell down abruptly at capacitances higher

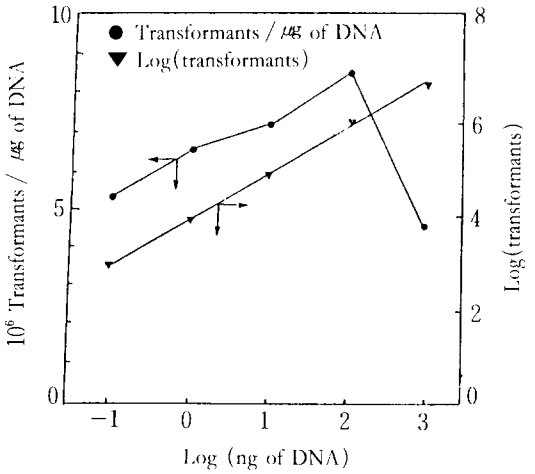


Fig. 5. Effect of increasing amount of DNA on the transformation frequency of *C. glutamicum* JS231.

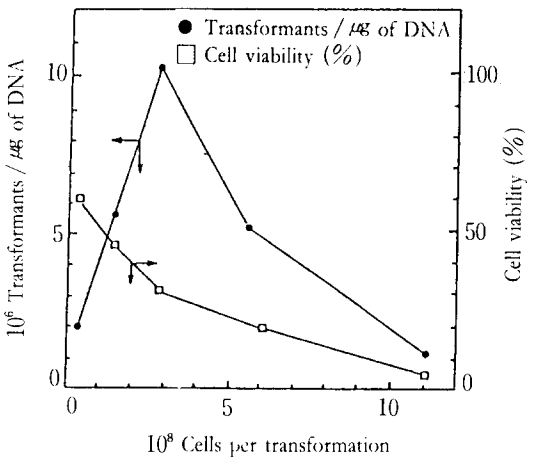


Fig. 6. Effect of cell density on the transformation frequency of *C. glutamicum* JS231.

than 25 μF. Considering that cell viabilities were maintained at nearly the same level, abrupt decrease of transformation efficiency at 125 μF is not caused by cell viability.

### DNA concentration:

$3\times10^8$ cells of *C. glutamicum* JS231 were transformed with increasing concentration of plasmid DNA from 0.1 ng to 1 μg in 42 μl of the mixture volume. As shown in Fig. 5, the number of transformants increased linearly with increasing concentration of plasmid DNA up to 1 μg, when log-log scale was used. The transformation efficiencies, *i. e.*, the number of transformantsper ug of DNA in creased in accordance with increasing DNA concentration upto 100 ng and decreased thereafter. And it was determined that the optimal DNA concentration for the highest transformation efficiency is 100 ng of plasmid DNA in 42μl of volume at conditions of 400 ohms, 25 μF and 12.5 kV / cm. What is interesting is that there exists a definite point of DNA concentration, not a range of DNA concentration, for the highest transformation efficiency. Meanwhile, when PEG-mediated protoplast transformation system was used(5), transformation efficiencies were maintained at constant from 1 to 10 ng of DNA and decreased thereafter up to 10 μg.

### Cell density:

$3\times10^8$ cells were used per transformation in all the previous experiments. Optimal cell concentration was examined for higher transformation efficiencies. Number of cells tested per transformation ranged from $0.3\times10^8$ to $1.1\times10^9$. Other parameters were set according to the results of the previous experiments, *i. e.*, 12.5 kV / cm, 400 ohms and 25 μF. As it can be seen in Fig. 6, the number of transformants increased linearly with increasing cell number to $3\times10^8$ cells per transformation and decreased rapidly thereafter. Therefore, optimum cell number per transformation proved to be $3\times10^8$ and it showed $8.5\times10^6$ transformants per μg of plasmid DNA.

## DISCUSSION

All the results presented here suggest that cells of *C. glutamicum* grown in LG medium containing penicillin-G can be efficiently transformed by electroporation. The method proved to have several advantages when compared with PEG-mediated protoplast transformation, *i. e.*, 100 − to 1000−fold higher transformation efficiencies, simple procedure, rapid and reliable results and applicability to frozen cell stocks. The quality of overnight culture to inoculate fresh LG medium with 0.3 U / ml of penicillin-G was very important. Only when the overnight culture was in log stage the culture continued growing in fresh LG medium with penicillin-G, and when the culture in stationary or death phase was inoculated the culture stopped

growing.

Several factors influenced the number of transformants obtained. The addition of penicillin-G to the growth medium showed more than 100-fold enhancement of transformation efficiencies. Cells prepared for electroporation must be suspended completely, otherwise arcing occurs and the number of transformants decreases significantly. When electroporations were performed correctly at the conditions of 25 μF, 12.5 kV / cm and 400 ohms, RC time constants, where R is resistance and C is capacitance, were between 9 to 10 msec. We also made an attempt to pulse the mixture of cells and DNA more than once to see if the number of transformants increases, but the number of transformants did not increase at all.

Even though transformation efficiency with restriction enzyme-digested and religated plasmid DNA was only a quarter level of that with intact plasmid DNA, it was efficient enough to use in gene cloning successfully. Actually, we suceeded in cloning several genes using this transformation system(thrA, thrB, dapA). When a series of manipulations (*e. g.*, digestion and ligation) were carried out with DNAs, it is desirable to remove salts from DNA solution by ethanol precipitation in order to increase the number of transformants by using DNAs of high concentration and with low salt. Also, we tried to reuse the cells prepared for electro transformation and kept at −70℃. There was little difference in transformation efficiencies between cells freshly prepared and cells stored at −70℃ at least for three months, only if cells were frozen in ethanol / dry ice bath and thawed in ice bath. And it is recommendable to prepare cells in aliquots and keep at −70℃, if repeated transformations are required.

After all the conditions were optimized, we finally obtained transformation efficiencies of $10^8$ per μg of DNA by using wild type *C. glutamicum* ATCC13032 as a host strain.

## ABBREVIATIONS

PEG: Polyethylene glycol, $Ap^r$: Ampicillin resistant, $Km^r$: Kanamycin resistant, kb: kilo base pairs, A: Absorbance, INH: isonicotinic acid hydrazide, pen G: penicillin G

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## 요 약

대장균과 코리네형 세균간의 shuttle vector pECCG1과 0.3 U / ml의 penicillin-G가 첨가된 배지에서 배양한 *Corynebacterium glutamicum* JS231을 사용하여 전기상 충격법에 의한 형질전환에 있어서 여러가지 조건들을 조사하였다. 세포 회수시기는 $A_{600}$에서 0.7−0.8로 하고 세포 현탁액 40μl, DNA 2 μl의 혼합액을 사용했을때, 저항 400 ohms, 전기장의 세기 12.5 kV / cm, 정전용량 25 μF, DNA양 100 ng일 때 형질전환시 $3\times10^8$의 세포를 사용했을때 약 $10^7$ transformants / μg of DNA의 형질전환 효율을 보였고, 동일한 조건에서 야생주인 *C. glutamicum* ATCC13032를 사용했을때는 약 $10^8$ transformants / μg of DNA의 형질전환 효율을 보였다.

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