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Transformation of *Bacillus stearothermophilus* No. 236 by Changing Incubation Temperature after Electroporation

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Abstract *Bacillus stearothermophilus* No. 236 isolated from the soil is a strong xylan degrader producing all the xylanolytic enzymes. However, the strain was discovered to be highly intractable to its transformation. In the present study, we have developed a reliable method for transformation of *B. stearothermophilus* No. 236 by a systematic examination of several factors which might have an influence on the efficiency of electrotransformation. Notably, we found that the most critical factor influencing the transformation efficiency (TE) was the incubation temperature after pulsing, with its optimum incubation of 37°C. At 50°C, the optimum growth temperature of the *B. stearothermophilus* strain, the transformants could not be obtained at a recognizable level. The combination of field strength of 7.5 kV/cm along with pulse duration of 10 msec (resistance of 400 Ω and capacitance of 25 μF) was shown to be the best electrical parameters at the incubation temperature of 37°C. A higher TE was obtained when the cells were harvested at an early-exponential phase. Twenty percent of PEG-8000 in a suspension buffer and an addition of 0.1% glycine in the growth medium resulted in about 4-fold and 3-fold increases in TE, respectively. We also found that the plasmid DNA which had been cycled through the host *B. stearothermophilus* cells enhanced TE by one order of magnitude higher. Under the presently described conditions, 2.5×10^5 transformants per μg DNA was attained.

Key words: *Bacillus stearothermophilus*, electrotransformation, transformation efficiency

Bacillus stearothermophilus No. 236 [21] isolated from the soil is a strong xylan degrader which produces the multiple xylanolytic enzymes including the major xylanolytic enzymes (xylanase and β-xylosidase) and the debranching enzymes (acetylxylan esterase, α-arabinofuranosidase, and α-glucuronidase) [1, 2]. Previously, we have isolated, and cloned all the genes encoding the above xylanolytic enzymes into *E. coli,* and those genes and their protein products were characterized at the molecular level. However, it was discovered that the *B. stearothermophilus* strain was highly intractable to transformation by using a normal method for *Bacillus subtilis.* This characteristic of *B. stearothermophilus* No. 236 interferes with the process for understanding molecular mechanisms by which synthesis of the xylanolytic enzymes are regulated in this bacterium.

Currently, electrotransformations of *E. coli* [3, 8, 13] and *B. subtilis* [9, 12, 16, 17] have been well established, and the techniques developed for the two bacterial species have also been successfully applied to other bacterial species, including gram-negative and gram-positive bacteria [4, 7, 11, 14, 15, 18, 19, 24]. Nevertheless, few reports are available today in regards to the electrotransformation of the thermophilic bacterium, *B. stearothermophilus.*

Therefore, in the present study, we made an attempt to establish an optimum experimental procedure for transformation of *B. stearothermophilus* No. 236, especially, by making use of electroporation techniques.

Bacteria and Plasmid

B. stearothermophilus No. 236 isolated from soil [21] was used as a recipient bacterium in the transformation experiments. *B. stearothermophilus* No. 236 was grown in the basal medium [10] supplemented with 0.75% xylose at 50°C on a rotary shaker (200 rpm). *B. subtilis* DB104 *(his nprR2 nprE18 aprEΔ 3 Cm^s)* was used as a host strain for making plasmid preparations. Plasmid pC194 [5] containing the CAT (chloramphenicol acetyltransferase) gene was used as a donor DNA for electrotransformation of *B. stearothermophilus.*

Electroporation Procedure

Electroporation was carried out by using a Gene-Pluser II system (Bio-RAD Laboratories, Richmond, U.S.A.). The *B. stearothermophilus* cells were grown at 50°C until it

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reached the mid-exponential phase of growth, and then were harvested and chilled on ice for 30 min [20]. The chilled culture was centrifuged at 5,000 rpm for 10 min, and washed three times with SHC buffer (10% sucrose, 16 mM HEPES, 1 mM $CaCl_2$, and 15% glycerol, pH 7.0) [18]. The washed cells were then resuspended in a SHC buffer at a density of about 1.0×10^{10} cells per ml. Two hundred ng of plasmid DNA was added into 100 μl of cell suspension. The mixture was transferred to a pre-cooled cuvette of 0.2 cm electrode gap, followed by pulsing at an electric field strength of 11.5 kV/cm with pulse duration of 5 msec (resistance of 200 Ω and capacitance of 25 μF). After pulsing, 900 μl of the basal medium containing 0.75% xylose was immediately added into the cuvette and gently mixed. The transformation mixture was transferred to a test tube and incubated for 90 min at 50°C while shaking at 150 rpm. After the incubation, the cells were diluted, plated on the selective agar medium supplemented with 5 μg/ml chloramphenicol, and incubated for 48 h at 50°C. The measurement of transformation efficiency (TE) was done by counting the numbers of transformants per μg DNA.

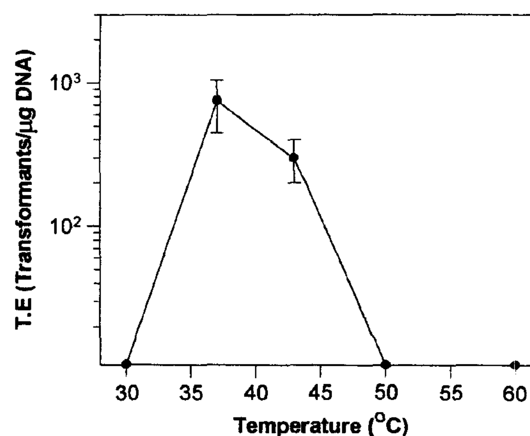

Fig. 1. Effect of incubation temperature on electrotransformation. The cells were cultured at 50°C until reaching the mid-exponential stage, then harvested and washed with SHC buffer. Two-hundred ng of plasmid DNA isolated from *B. subtilis* DB104 were added, followed by electroporation at 11.5 kV, 25 μF, and 200 Ω, and the cells were incubated for 90 min at various temperatures. The cells were diluted, plated, and incubated for 48 h at each temperature. The bar represents the standard deviation of triplicate samples.

Effect of Incubation Temperature and Time

For genetic transformation of *B. stearothermophilus* No. 236, we first tried the usual method to transform *B. subtilis* [23], and next the protoplast method of Imanaka *et al.* [6]. Unfortunately, however, we did not obtain any satisfactory results from both transformation experiments. We also made an attempt to transfer the plasmid DNA into the *B. stearothermophilus* cell by using electroporation as described above. However, we obtained only a few transformants in this experiment even after repeated trials. Hence, we suspected that one of the reasons for this extremely low level of TE might be caused by a strong restriction that was put on the plasmid pC194 at 50°C which was the optimum growth temperature of the recipient strain. The *B. stearothermophilus* cells were then incubated for 90 min at various temperatures for phenotypic expression after pulsing, and thereafter incubated on selective agars for 48 h at the same temperature for cell division. As shown in Fig. 1, the maximum efficiency of 7.5×10^2 transformants per μg DNA was obtained at 37°C. However, at lower or higher temperatures than the optimum temperature, TE was shown to have a sharp decline.

On the other hand, incubation after pulsing is reported to be critical in recovering damaged cells caused by pulse and expression of the antibiotic resistant gene [13, 17]. Thus, we analyzed the effect of incubation time after electroporation on transformation of *B. stearothermophilus* No. 236. TE increased sharply during the first 60 min of its incubation phase and showed nearly the same level until 180 min (data not shown). Thereafter, the level of TE again increased slowly with the incubation time. As expected, the effect of incubation time on cell survival was also shown to be a similar pattern to that for the TE. This increase in TE in a later stage might be explained partly by the increase of the number of transformants through cell division. Thus, we concluded from the above results that the incubation of 90 min at 37°C after electroporation was a reliable time for accomplishing phenotypic expression of the recipient cells without cell division.

Factors Affecting Electrotransformation

The electric field strength and pulse duration (time constant) are known to be primary determinants of electrotransformation [3, 13]. Therefore, we examined the effectiveness of the various combinations of electric field strength and pulse duration on electrotransformation of the *B. stearothermophilus* cell under the optimum incubation condition described above. The highest TE was observed when the transformation was performed with a combination of field strength of 7.5 kV/cm with pulse duration of 10 msec at the incubation temperature of 37°C. Under these conditions, TE of 1.4×10^3 transformants per μg DNA was achieved. Next, in order to prepare competent cells for higher efficiency of electrotransformation, the *B. stearothermophilus* cells were harvested at various stages of its growth, and TE was monitored by electroporation at each stage of the growth. The highest efficiency of electrotransformation was obtained when the cells were harvested at an early-exponential phase (data not shown). The effect of DNA dosage on the total number of transformants was also examined with a fixed number of the recipient cells. The yield of the transformants was linearly proportional to the amount of DNA input over a

wide range, but TE was observed to have nearly the same values over the range of 5 ng to 1,000 ng.

According to a recent report, it was shown that PEG-6000 enhanced TE more than one order of magnitude in the electrotransformation of *B. brevis* [18]. Thus, we performed electroporation in 1 mM HEPES (pH 7.0) containing various concentrations of PEG. In the presence of 20% PEG-8000, the highest TE was observed, and about a 4-fold increment was obtained compared to the level in the absence of PEG-8000 (data not shown). In addition, as a means for overcoming the potential barriers of electrotransformation posed by the cell wall, we pretreated the cells of *B. stearothermophilus* with glycine. About a 3-fold enhancement of TE was attained compared to that observed without glycine when 0.1% glycine was added in the culture medium one hour before the early-exponential phase (data not shown).

Effect of the Source of Plasmid DNA

The restriction-modification system often presents a great barrier for the transformation [13, 14]. Therefore it may be advantageous to use the plasmid DNA that had been cycled through the recipient cells which were used in the transformation experiment. As shown in Table 1, about a 10-fold increase in TE was achieved when the plasmid pC194 which had been cycled through the *B. stearothermophilus* cells was used in the electrotransformation. Interestingly, 2.0×10^4 transformants per μg DNA was obtained even at an incubation temperature of 50°C when the plasmid isolated from the *B. stearothermophilus* strain was used in electrotransformation. These results demonstrate that the restriction-modification system actually worked on plasmid pC194 in the *B. stearothermophilus* cells and the restriction might be more effective at an incubation temperature of 50°C than at 37°C.

As described above, the RM system proved to be effective during the first 90 min incubation time, and then it was expected that the incubation temperature for the subsequent cell division would not affect the TE of the bacterium. However, as seen in Table 2, the incubation temperature at the stage of cell division also appeared to influence the efficiency of electrotransformation. At first, we suspected that this temperature effect could possibly result from the relative difference of segregational stability [22] of plasmid pC194 at different incubation temperatures. Contrary to this assumption, the plasmid pC194 in the transformed *B. stearothermophilus* cells was observed to be rather unstable in segregation at 37°C (Fig. 2). At present, we cannot unequivocally explain the reason why TE was higher when the incubation temperature was 37°C for cell division. However, the similar growth rates were observed when the transformants were cultured at 37°C in the presence or absence of chloramphenicol (5 μg/ml), the

Table 1. Effect of the source of plasmid DNA on electrotransformation.

Sources of plasmid pC194	TE (transformants/μg DNA)
B. subtilis DB104	$2.6\pm0.4\times10^4$
B. stearothermophilus No. 236	$2.5\pm0.5\times10^5$

The cells were cultured at 50°C until one hour prior to the early-exponential stage, then 0.1% glycine was added and the culture continued until the early-exponential stage. After the cells were suspended with a 1 mM HEPES-20% PEG concentration solution (pH 7.0), 100 ng of plasmid DNA isolated from *B. subtilis* DB104 or *B. stearothermophilus* No. 236 were added, followed by electroporation at 7.5 kV, 400 Ω, and 25 μF, and the cells were incubated for 90 min at 37°C. The cells were diluted, plated, and incubated for 48 h at 37°C. Values are means±standard deviation of triplicate samples.

Table 2. Effect of incubation temperature on shift electrotransformation.

Pre-incubation temperature[a]	Incubation temperature[b]	Relative TE
37°C	37°C	100%
37°C	50°C	75(±10)%
50°C	37°C	15(±5)%
50°C	50°C	(≒)0%

The cells were cultured at 50°C until one hour prior of reaching the early-exponential stage, then 0.1% glycine was added and the culture continued for one hour further. After the cells were suspended with 1 mM HEPES-20% PEG concentration solution (pH 7.0), 100 ng of plasmid DNA isolated from *B. subtilis* DB104 were added, followed by electroporation at 7.5 kV, 400 Ω, and 25 μF, and the cells were incubated for 90 min at 37°C or 50°C. The cells were diluted, plated, and incubated for 48 h at 37°C or 50°C. Values are means±standard deviation of three independent experiments.

[a]Incubation temperature for phenotypic expression for 90 min.

[b]Incubation temperature for cell division for 48 h.

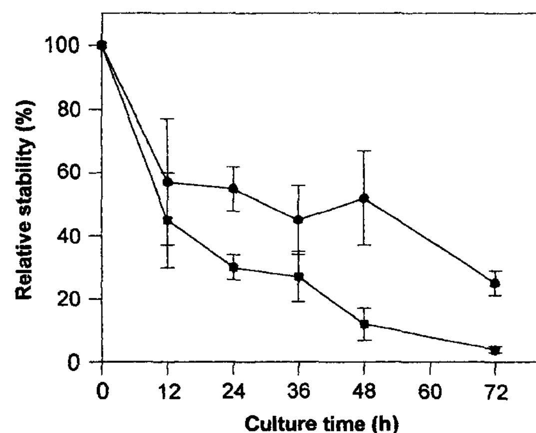

Fig. 2. Effect of temperature on the segregational stability of plasmid pC194.

The transformants were pre-cultured for 12 h in a medium containing chloramphenicol at each temperature, and main-cultured in a medium without chloramphenicol for various times at each temperature. Relative stability was determined by the ratio of cell numbers on plates with or without chloramphenicol. The bar represents the standard deviation of two independent experiments. (●, 50°C; ■, 37°C).

growth rate of the transformants cultured at 50°C in the presence of chloramphenicol was lower than that shown in the absence of chloramphenicol (data not shown). Taken together, thermo-susceptibility of the chloramphenicol resistant gene product of pC194 in the transformed *B. stearothermophilus* could not be excluded as a reason for higher TE at a temperature of 37°C.

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