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Method Development for Electrotransformation of *Acidithiobacillus caldus*

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***Acidithiobacillus caldus* is an acidophilic, chemolithotrophic bacterium that plays an important role in bioleaching. Gene transformation into *A. caldus* is difficult, and only the conjugation method was reported successful, which was a relatively sophisticated method. In this research, electrotransformation of *A. caldus* species was achieved for the first time using *A. caldus* Y-3 and plasmid pJRD215. Transformants were confirmed by colony PCR specific to the *str* gene on pJRD215, and the recovery of the plasmid from the presumptive transformants. Optimizations were made and the transformation efficiency was increased from 0.8 to 3.6×10^4 transformants/μg plasmid DNA. The developed electrotransformation method was convenient in introducing foreign genes into *A. caldus*.**

Keywords: *Acidithiobacillus caldus*, chemolithotrophic, electrotransformation, optimization

A. caldus is an acidophilic (optimal pH 1.5 to 2.5), chemolithotrophic, Gram-negative, aerobic, and moderately thermophilic (40 to 50°C) bacterium [12, 13, 22]. It derives energy by oxidizing reduced or partially reduced sulfur compounds (sulfur, thiosulfate, tetrathionate, *etc.*) and fixes carbon dioxide from the atmosphere [8, 18]. It plays a important role in bioleaching together with iron-oxidizing bacteria [6, 14, 24].

However, *A. caldus* has some drawbacks, for example being sensitive to heavy metals such as As(III), Hg(II), and Ag(I), and growing slowly, which limit its practical applications. Improvement of this organism using recombinant gene technology is needed. This experiment showed that a conjugational gene transfer method using broad-host-range IncP and IncQ exogenous plasmids was successfully developed to transfer the target genes into *A. caldus* MTH-04 from *Escherichia coli* [22]. However, there are some limitations of this method. The apparent gene transfer frequency depends on the incompatibility groups of plasmids, which limits the application of some mobilizable plasmids. Furthermore, the majority of the mobilizable plasmids used in *A. caldus* are large and not suitable for the vectors.

Electrotransformation is highly efficient, and widely used in introducing exogenous DNA into mammalian cells [4], plants cells [9], yeasts [27], and bacteria [3]. *Thiobacillus ferrooxidans*, a chemoautolithotrophic acidophilic bacterium, was electrotransformed in 1992 [19]. However, of 30 strains electrotransformed, only one gave transformants at a low efficiency of 120 to 200 colonies per μg of plasmid DNA. Therefore, it cannot be used as a standard method, for the reasons of low efficiency and reproducibility. Since, then no report has been found on electrotransformation of chemoautolithotrophic acidophilic bacteria.

In this study, an electrotransformation method for *A. caldus* species was developed. Optimizations were made for the electrotransformation protocol for high transformation efficiency and frequency, as well as good accuracy and reproducibility.

Materials and Methods

Bacterial Strains and Cultivation Methods

The bacterial strains and plasmids used are listed in Table 1. *A. caldus* Y-3 was isolated from commercial biooxidation tanks. The media of Luria broth (LB) or agar plate for *E. coli* were described in the reference [25]. Liquid Starkey-S^0 inorganic medium and solid Starkey-$Na_2S_2O_3$ medium for cultivation of *A. caldus* were prepared as described in the reference [17]. Streptomycin (Sm) was added to the final concentrations of 50–100 μg/ml in LB medium, 200 μg/ml in liquid Starkey-S^0 medium, and 100 μg/ml in solid Starkey-$Na_2S_2O_3$ medium, respectively. The cultivation temperatures were 37°C for *E. coli* and 40°C for *A. caldus*. The shake speed for liquid cultivation was 120 rpm if not otherwise specified.

Preparations of Plasmid and Electrocompetent *A. caldus* Cells

Plasmid (pJRD215) was purified using a TaKaRa MiniBEST plasmid purification kit ver.2.0 (TaKaRa Biotechnology Co.) and the concentration was determined using Eppendorf BioPhotometer plus (Eppendorf Co.).

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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Phenotype or genotype	Source or reference
Strain		
E. coli SM10	Thr leu hsd recA Km^r RP4-2-Tc::Mu integrated	[28]
A. caldus Y-3	Wild type	This study, isolated from biooxidation tanks, identified using physiological and 16s rDNA sequencing methods
Plasmid		
pJRD215	Sm^r Km^r IncQ mob^+, 10.2 kb	[5]

The electrocompetent *A. caldus* Y-3 cells were prepared as follows. Thirty ml of *A. caldus* Y-3 at the late exponential growth phase was inoculated into 300 ml of fresh Starkey-S^0 medium. Cells were harvested at the mid exponential growth phase. The solid sulfur in the cultivation broth was removed by low-speed centrifugation (100 ×*g*) before cell harvest. The harvested cells were washed twice using Starkey salt solution (pH 3.0, without elemental sulfur), once using phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and twice using ice-cold deionized distilled H_2O (ddH_2O). Then, the cells were diluted to a final concentration of about 3.0×10^{10} cells/ml using ddH_2O.

Electrotransformation Protocol and Subsequent Cell Cultivation

Cell suspention of 0.4 ml was mixed with a certain amount (described specifically in later sections) of plasmid DNA and transferred into a precooled cuvette, which had an electrode gap of 2 mm. The pulses were generated by the electrotransformation apparatus (Gene-pulser; Bio-Rad Laboratories). After the pulses were applied, the cells were quickly removed from the electrodes into 10 ml of fresh liquid Starkey-S^0 medium and mixed, incubated for 12 h at 40°C, and shaken at 50 rpm. Then, 2 mg of streptomycin was added, and the shaking speed was increased to 100 rpm to cultivate for another 12 h. After the cultivation, the cells were diluted appropriately, plated on the solid Starkey-$Na_2S_2O_3$ plates (containing Sm of 200 μg/ml or not), and cultivated for 10 days until the colonies appeared. Cell viability was calculated from the number of survived pulsed cells divided by the number of nonpulsed cells, which were counted from the plates. Transformation efficiency was calculated from the number of transformants divided by μg of plasmid DNA added. Transformation frequency was calculated from the number of transformants divided by the number of cells added. All mean values were the average of three replicate measurements.

Verication of Transformation

Colony PCR was used to verify the transformation. A PCR primer set (forward, 5'-TGGCAGGAGGAACAGGA-3'; reverse, 5'-GGAA AGGCACCCATAAGC-3'; synthetized by Invitrogen Biotechnology Co.) was used to amplify the streptomycin resistance gene (*str*) in pJRD215. PCR was carried out in 50 μl of reaction solution containing 25 μl of Premix *Taq* (TaKaRa Biotechnology Co.) and 0.4 μmol of primers. Cells from the colonies grown on selection plates were used as the template DNA sources. PCR reactions were of one cycle at 94°C for 10 min to collapse the cell wall, three 30 cycles at 94°C for 30 sec, at 52°C for 30 sec, and at 72°C for 1.5 min, respectively. Then, one more cycle at 72°C for 10 min for a final extension step was carried out in a Bio-Rad DNA Engine PTC-200 Peltier thermal cycler.

In order to confirm the electrotransformants further, the plasmid pJRD215 was recovered from the *A. caldus* Y-3 transformants using the QIAprep Spin Miniprep Kit (Qigen), digested using HindIII and XhoI (TaKaRa Biotechnology Co.), and analyzed using gel electrophoresis.

Results and Discussion

Electrotransformation and Verification of Transformation

Plasmid and electrocompetent cells of *A. caldus* Y-3 were prepared as described in Materials and Methods. Electrotransformation was carried out at 11.25 kV/cm, 200 Ω, and 25 μF, with a plasmid DNA concentration of 1.5 μg/ml. After the recovery incubation, cells were plated on solid Starkey-$Na_2S_2O_3$ plates containing streptomycin as the

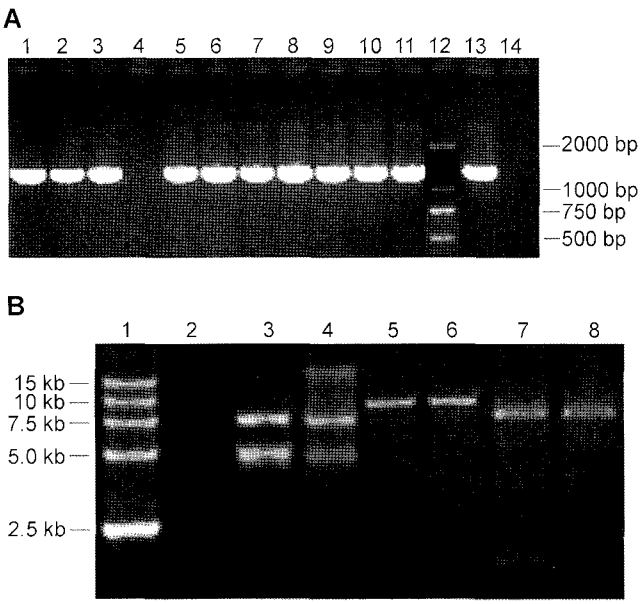

Fig. 1. Confirmation of *A. caldus* Y-3 transformants using (**A**) colony PCR with the primer pair for the *str* gene in pJRD215 and (**B**) electrophoresis analysis of the enzyme-restricted plasmid recovered from the *A. caldus* Y-3 transformant.

A. Lanes 1 to 11, cells of *A. caldus* Y-3 transformants; lane 13, cells of *E. coli* SM10 (pJRD215); lane 14, cells of *A. caldus* Y-3. **B**. Lane 2, plasmid recovered from wild-type *A. caldus* Y-3; lanes 3, 5, and 7, plasmid recovered from *E .coli* SM10 (pJRD215); lanes 4, 6, and 8, plasmid recovered from the *A. caldus* Y-3 transformant; lanes 2, 3, and 4, plasmid without enzyme restriction; lanes 5 and 6, plasmid restricted by HindIII; lanes 7 and 8, plasmid restricted by HindIII and XhoI; lanes 1 and 9.

selection marker. Antibiotic-resistant (Sm^r) colonies were detected at low efficiency (~0.8×10^4 transformants/μg DNA).

Colony PCR was carried out to confirm the transformants using the primers specific to the *str* gene and the cells from Sm^r clones as the DNA template sources (Fig. 1A). As shown in Fig. 1A, fragments with the expected size of 1,343 bp were amplified from 10 Sm^r clones of *A. caldus* Y-3 (lanes 1 to 3, and lanes 5 to 11), as well as from the positive control (lane 13), but not from the negative control (lane 14). Then, plasmid was recovered from one *A. caldus* Y-3 transformant to further confirm the transformation, and the analysis results are shown in Fig. 1B. The plasmid pJRD215 was recovered from *A. caldus* Y-3 transformant (lane 4), as well as from *E. coli* SM10 (pJRD 215) (lane 3), but not from the wild-type *A. caldus* Y-3 (lane 2) (Fig. 1B). Enzyme restriction analysis of the plasmid recovered from *A. caldus* Y-3 transformant and *E. coli* SM10 (pJRD 215) showed that the transformants carried a single plasmid that was the same as the original plasmid (lanes 5 to 8) (Fig. 1B). The above results of colony PCR and plasmid recovery experiments demonstrated that electrotransformation of *A. caldus* Y-3 was successful.

In preparing the electrocompetent cells, no organic components were contained in the culture media or cell washing solutions, as the growth of *A. caldus* was inhibited by organic compounds [1, 30]. The collected cells were washed using Starkey salt solution, PBS buffer, and ddH_2O in series, and then diluted using ddH_2O, which caused the cell suspension to have high resistance and the cell wall to expand.

Effect of Cell Culture Time on Transformation Efficiency

A. caldus Y-3 was cultivated using liquid Starkey-S^0 medium. The exponential growth phase of *A. caldus* Y-3 was between the third and the sixth day of the cultivation. Cells were harvested every 12 h on the fourth, fifth, and sixth days, respectively. The cells were electrotransformed using the same operation steps as described in the Materials and Methods section except for the cell culture time in cell preparation. The transformation efficiency and viability of the cells were calculated and are shown in Fig. 2. The cells cultivated for 4 days had the highest transformation efficiency of about 1.2×10^4 transformants/μg plasmid DNA, with the cell viability at about 54%. The cell viability increased steadily with the extension of culture time. It showed that the culture time of 4 days was optimal for electrotransformation and hence used in the following experiments.

The culture time is important in preparation of *A. caldus* Y-3 electrocompetent cells. There exist extracellular polymeric substances (EPS) outside the cell wall of chemoautolithotrophic acidophilic bacteria [2, 20, 26]. EPS consist of polysaccharides, proteins, and lipids [11], play important roles for cell adherance to the surface of ores in bioleaching [10, 23],

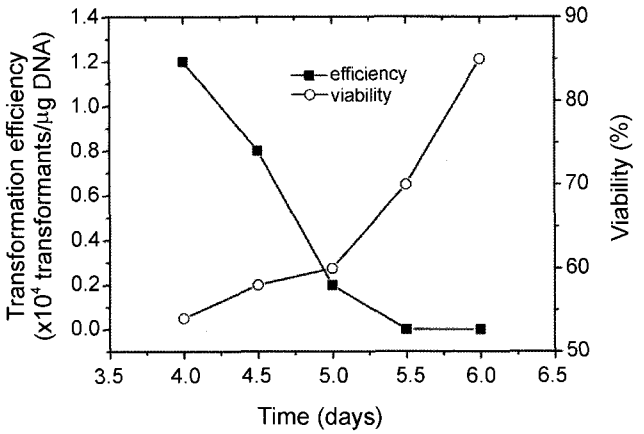

Fig. 2. Changes of transformation efficiency and viability of *A. caldus* Y-3 with culture time.
Field strength of 11.25 kV/cm, pulse time of 4.9 ms (200 Ω), and plasmid DNA concentration of 1.5 μg/ml were used.

and make the cell difficult to be electrotransformed. The negative effect of EPS on electrotransformation was reported for *Xanthobacter autotrophicus* [29]. Our results showed that the cells in the mid exponential growth phase were easier to be electrotransformed. The reasons should be the changes of the amount and compositions of EPS of chemoautolithotrophic acidophilic bacteria with the changes of growth conditions [10, 21].

Effect of Expression Time Length on Transformation Efficiency

Electrotransformation of *A. caldus* Y-3 was carried out using various expression time lengths, and the other operation steps as described in Materials and Methods. After applying the electrical pulses and standing for 12 h for recovery, streptomycin was added and the cells cultivated for 16, 24, 32, and 40 h, to determine the transformation

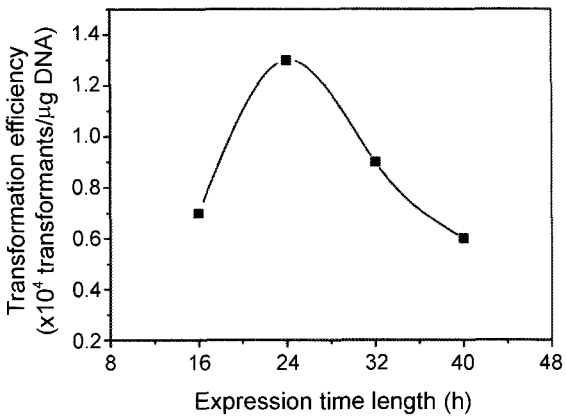

Fig. 3. Changes of transformation efficiency of *A. caldus* Y-3 with expression time length.
Field strength of 11.25 kV/cm, pulse time of 4.9 ms (200 Ω), and plasmid DNA concentration of 1.5 μg/ml were used.

efficiency (Fig. 3). The transformation efficiency was the maximum with the expression time length of 24 h, and then, decreased with the extension of expression time length.

As the growth of *A. caldus* was slow, a longer expression time length of 24 h was needed for high transformation efficiency and this value was used in the following experiments. The transformation efficiency decreased obviously when the expression time length was further extended. The reason could be that streptomycin was unstable in low pH for longer time. The relatively higher pH of 4.0 in the recovery and expression steps was tried in order to keep streptomycin stable, but the transformation efficiency decreased significantly owing to the inhibition effect of high pH on cell growth (data not shown).

Effects of Electrical Field Strength and Pulse Time on Transformation Efficiency

Electrical field strength and pulse time have been reported to have significant effects on transformation efficiency [7]. First, electrical field strength was optimized within the range from 10.00 to 15.00 kV/cm, with the other operation steps as described in Materials and Methods. The results are shown in Fig. 4. The transformation efficiency increased with the increase of field strength before reaching the maximum, and then dropped significantly. However, the cell viability decreased from 68 to 30% with the increase of field strength. The highest transformation efficiency of 2.0×10^4 transformants/μg DNA was obtained at the field strength of 12.50 kV/cm with the cell viability of 53%, and this optimized field strength value was used in the following experiments.

Then, electrical pulse time was optimized using the resistor values of 200, 400, 600, 800, and 1,000 Ω for the pulse times of 4.9, 9.1, 12.5, 15.6, and 18.4 ms. The results are shown in Fig. 5. The maximum transformation efficiency

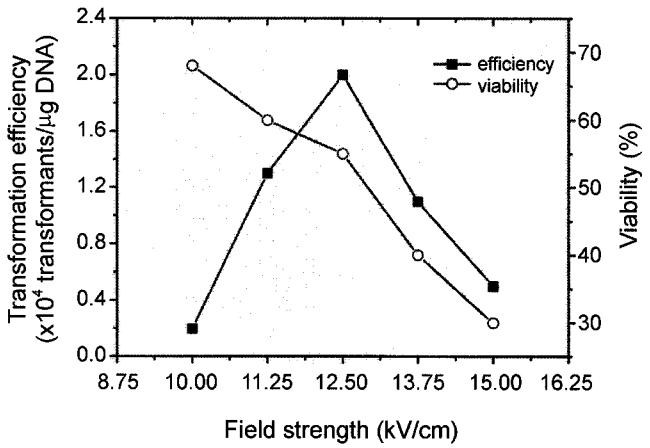

Fig. 4. Effect of electrical field strength on the transformation efficiency of *A. caldus* Y-3.
Pulse time of 4.9 ms (200 Ω) and plasmid DNA concentration of 1.5 μg/ml were used.

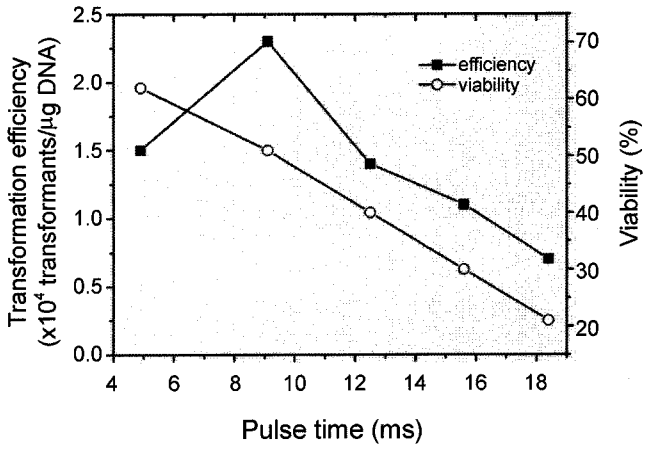

Fig. 5. Effect of electrical pulse time on the transformation efficiency of *A. caldus* Y-3.
Electrical field strength of 12.50 kV/cm and plasmid DNA concentration of 1.5 μg/ml were used. The points were measured at resistor values of 200 (4.9 ms), 400 (9.1 ms), 600 (12.5 ms), 800 (15.6 ms), and 1,000 Ω (18.4 ms).

was reached at the pulse time of 9.1 ms (400 Ω) when about 50% of the cells were killed. Further increase of pulse time led to more cell death and an obvious decline in transformation efficiency. The resistor value of 400 Ω was optimal and was used in the following experiments.

The above results showed that a relatively high field strength and long pulse time are necessary for the transformation of *A. caldus* Y-3. No transformants were found at an electrical field strength less than 10.00 kV/cm (data not show). Severe eletrotransformation conditions (high field strength and long pulse time) with the cell viability between 40% and 60% were necessary for high transformation efficiency. No transformants was obtained when the cell viability exceeded 70%.

The optimal field strength and pulse time of this research are higher and longer than the published results for Gram-negative bacteria [7, 15, 16]. The special EPS of this strain could be the reason.

Effect of Plasmid DNA Concentration on Transformation Efficiency

The plasmid DNA concentrations ranging from 0.5 to 4.0 μg/ml were optimized for the electrotransformation of *A. caldus* Y-3, with the other operation steps as described in Materials and Methods. The results are shown in Fig. 6. With the increase of plasmid DNA concentration, the transformation efficiency reached the maximum with a plasmid DNA concentration of 1.0 μg/ml, and then decreased from 3.6×10^4 to 0.6×10^4 transformants/μg DNA, and the cell viability decreased from about 55 to 30%. The transformation frequency increased with the plasmid DNA concentration until 2.0 μg/ml and reached the maximum value of 4.7×10^{-6} transformants/cells, and then decreased with the further increase of plasmid DNA concentration.

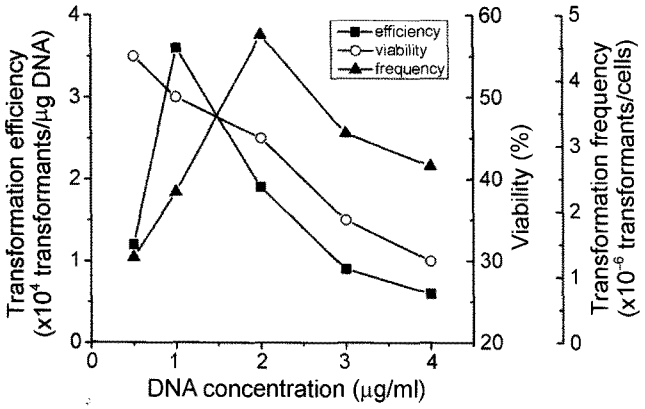

Fig. 6. Changes of transformation efficiency, transformation frequency, and viability of *A. caldus* Y-3 with plasmid DNA concentration.
Field strength of 12.50 kV/cm and resistor of 400 Ω were used.

The ratios of false-positive clones were about 75% and 40% at the plasmid DNA concentrations of 0.5 and 1.0 μg/ml, respectively, whereas no false-positive clones were found at the plasmid DNA concentrations of 2.0, 3.0, or 4.0 μg/ml detected by colony PCR.

As discussed above, the difficulties in electrotransformation of *A. caldus* Y-3 are possibly resulted from the EPS surrounding the cell wall, which hinder the plasmid from entering the cell and need a relatively high plasmid concentration for the transformation. With the further increase of plasmid concentration, the solution conductivity increases, which results in the increase of cell fatality, decrease of pulse time, and decreases of transformation frequency and efficiency.

In summary, *A. caldus* is important in bioleaching. A convenient recombinant gene transfer method is needed for the genetic breeding of this bacterium. Although the conjugational gene transfer method has been developed for *A. caldus* in this experiment [22], it is relatively sophisticated and of relatively low transformation efficiency. In this research, a more convenient electrotransformation method was firstly reported and the key parameters were optimized. The maximum transformation efficiency of 3.6×10^4 transformants/μg DNA and the maximum transformation frequency of 4.7×10^{-6} transformants/cells were obtained in transformation of *A. caldus* Y-3 using the plasmid pJRD215. The method developed in this research can be used as a standard gene transfer method and a powerful and convenient tool for the recombinant gene transformation, gene knockout, and metabolic pathway modification of *A. caldus*.

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