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АНАЛИЗ ЭФФЕКТИВНОСТИ ФАКТОРОВ ЭЛЕКТРОТРАНСФОРМАЦИИ КЛЕТОК *BACILLUS SUBTILIS* ДЛЯ ИНАКТИВАЦИИ ГЕНА *aro*K ПУТЕМ ГОМОЛОГИЧНОЙ РЕКОМБИНАЦИИ

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Оптимизирован метод гиперосмотической электротрансформации для штамма *Bacillus subtilis*. В состав гиперосмотической среды для электропорации и среды для восстановления были введены сорбит и маннит. Эффективность трансформации штамма *B. subtilis* 5434 (не трансформируемого химическими методами) возросла в 430 раз, ее максимальное значение составило $8{,}6 \cdot 10^5$ КОЕ/мкг интегративной плазмидной ДНК. При сохранении параметров 25 мкФ, 23 кВ/см, 200 Ом метод был оптимизирован следующим образом: а) бактериальную культуру при культивировании выращивали до достижения значений оптической плотности OD_{600} около 1,2, что существенно повысило выживаемость бактерий и количество жизнеспособных клеток *B. subtilis* 5434 после электропорации; б) число элюирований промывочным раствором (гиперосмотической средой для электропорации) электрокомпетентных клеток увеличено с 3 до 5 раз, что привело к значительному снижению электропроводимости гиперосмотической электропорационной среды, содержащей компетентные клетки (электрокомпетентной культуры), и эффективному увеличению времени истекания импульса при той же напряженности электрического поля; в) повышено количество интегративной плазмидной ДНК, вносимой в гиперосмотическую электрокомпетентную культуру. Полученные результаты свидетельствуют, что увеличение числа жизнеспособных клеток *B. subtilis* 5434 и уменьшение количества ионов металлов в смеси растворов для электропорации (интегративная плазмидная ДНК, компетентные клетки *B. subtilis* 5434, среда для электропорации) были успешным решением для повышения эффективности трансформации штамма *B. subtilis* 5434.

Ключевые слова: *Bacillus subtilis*; гомологичная рекомбинация; гиперосмотическая электротрансформация; шикимат.

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ANALYSIS OF THE EFFICIENCY FACTORS OF ELECTROTRANSFORMATION OF *BACILLUS SUBTILIS* TO INACTIVATE THE *aro*K GENE BY THE METHOD OF HOMOLOGOUS RECOMBINATION

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A hyper-osmotic electrotransformation method was developed for strain *Bacillus subtilis.* Sorbitol and mannitol are included in the hyper-osmotic electroporation medium and recovery medium. In this study, the hyper-osmotic electroporation method was optimised to increase the transformation efficiency of *B. subtilis* strain 5434 (non-transformable by chemical methods) by 430 fold, with a maximum value of $8.6 \cdot 10^5$ CFU/μg of integrative plasmid DNA. With the electroporation setted 25 μF, 23 kV/cm, 200 Ω, the method was optimised as follows: a) the OD_{600} value of the bacterial culture solution was increased to about 1.2, which significantly enhanced survival of bacteria and quantity of viable *B. subtilis* strain 5434 cells after electroporation; b) the elution frequency of washing solution (hyper-osmotic electroporation medium) for complement cells was increased from 3 to 5 times, resulted in significantly reducing the conductivity of the hyper-osmotic electoporation medium with competent cells (electrocompetent cultue), and effectively extending the pulse time under the same electric field strength; c) quantity of integrative plasmid DNA added to hyper-osmotic electrocompetent culture was optimised. These results indicate that increasing the number of viable *B. subtilis* strain 5434 cells and reducing the number of metal ions in the electroporation solution mix (integrative plasmid DNA, competent cells of *B. subtilis* strain 5434, electroporation medium) are useful approach to improve transfomation efficiency of *B. subtilis* strain 5434. Concentration of shikimic acid in the fermentation medium was quantified by high performance liquid chromatography. Quantification of shikimic acid revealed that *B. subtilis* strain 5434p4SA produced 403.98 ± 9.1 μg/mL of shikimic acid.

Keywords: *Bacillus subtilis*; homologous recombination; hyper-osmotic electrotransformation; shikimate.

Acknowledgements. We thank PhD E. O. Korik for helping us with HPLC analysis.

Introduction

Bacillus subtilis has been widely used as a model organism for converting exogenous DNA in the past decades. Genetic transformation with exogenous genes allows the improvement of important industrial microorganisms. Exogenous genes could be integrated into microorganisms by this technology [1; 2]. Generally, *E. coli* as a gram-negative bacterium has a higher transformation efficiency than *B. subtilis*. This is due to the influence of the cell wall of *B. subtilis* as a gram-positive bacterium *B. subtilis* has a strong ability to secrete proteins without endotoxin, thus, carbohydrase and protease enzyme preparations from *B. subtilis* are GRAS (GRAS is an acronym for the phrase «generally recognised as safe») for use as direct food ingredient [3]. Therefore, it is more suitable to choose *B. subtilis* as genetically engineered bacteria. And it is necessary to select the most effective transformation method for *B. subtilis* and optimise it. The low competence exhibited by several strains of *B. subtilis* [2; 4] has led to the development of several strategies, including biochemical transformation [5], phage transduction [6], protoplast fusion [7] and electrotransformation [8]. Compared with other transformation methods, electrotransformation can significantly improve the transformation efficiency of gram-positive bacteria. This is due to the temporary weakening of the cell wall and cytoplasmic membrane that requires electro-transformation, so that DNA can effectively penetrate bacterial cells [1]. Therefore, electroporation a more commonly used method is widely accepted and conducted for the transformation of gram-positive bacteria. However, electroporation can permanently damage the cells, resulting in a low transformation efficiency [9]. The *B. subtilis* strain 5434 is characterised by low physiological competence, thus, the most acceptable method is the introduction of exogenous DNA into the cell by electroporation.

For the chemical and pharmaceutical industries, shikimic acid (SA) is an essential raw material. In specific, SA is an intermediate for the anti-viral influenza synthesis of Oseltamivir (Tamiflu®). In 2009, Tamiflu® sales is estimated at 3.5 bln US dollars, with a manufacturing potential of up to 33 mln treatments per month and 400 mln packages per year [10]. Although current commercial production is mostly accomplished extracted from the Illicium plant, the isolation method is tedious and expensive [11]. Microbial fermentation from inexpensive carbon sources such as glucose [12] or glycerol [13] for the shikimate production as an alternative method has gained more and more interest. Moreover, shikimic acid is one of the products in the metabolic pathway of *B. subtilis*. Therefore, the gene of shikimate kinase *aro*K (SA was metabolised by shikimate kinase) can be inactivated, allowing SA to be accumulated in the fermentation medium from *B. subtilis*.

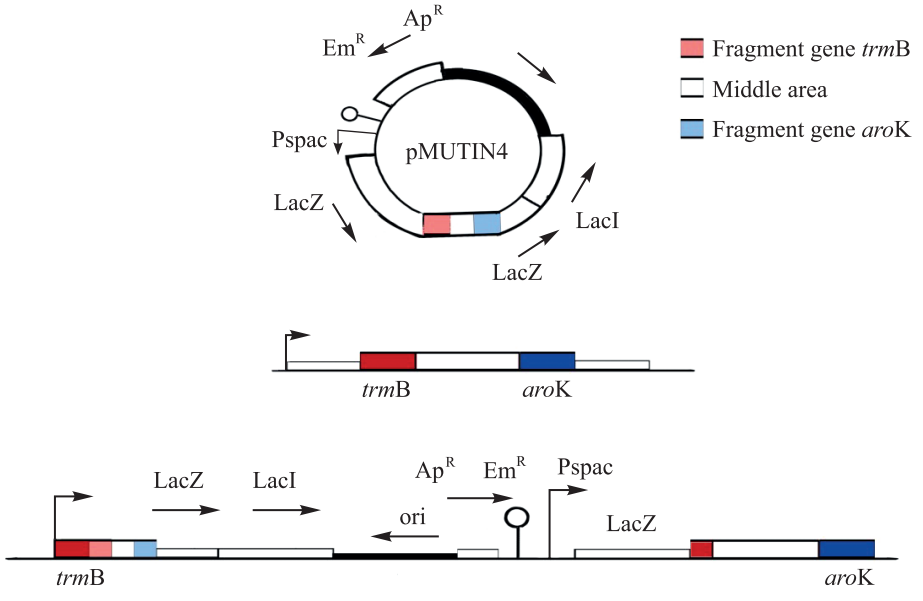

Fig. 1. Design of the integrational vector pMUTIN4 with the homologous sequence (*trm*B-*aro*K) inserted into the bacterial chromosome

In this work, by increasing the number of living competent cells and decreasing the concentration of metal ions in the electroporation solution mix, electroporation efficiency was significantly increased. So optimal method makes it possible to screen viable transformants obtained by homologous recombination. The shikimate kinase gene was inactivated by homologous recombination, which SA was allowed to accumulate and secreted into the medium. The integrational vector pMUTIN4Δ*aro*K containing homologous sequence (*trm*B-*aro*K) was integrated into the strain *B. subtilis* chromosomal DNA by the method electrotransformation and homologous recombination, in order to inactivate the shikimate kinase gene (*aro*K) to accumulate SA in the fermentation medium. Pspac promotor regulated by IPTG was on the integrational vector pMUTIN4Δ*aro*K. The *aro*K was found to be located downstream of the Pspac promoter, which could be blocked in the fermentation medium without IPTG (fig. 1). Thus, those *B. subtilis* cells had a capacity to accumulate SA via modification on this specific metabolic pathway [14].

Materials and methods

Bacterial strains and plasmids. *E. coli* XL1-Blue was used to amplify all the vectors in this study. *B. subtilis* strain 5434 obtained from the collection of VKMB (Moscow), high-yield tryptophan, Cm^R; *B. subtilis* strain 168wt (wild type).

Plasmid vector pMTL21C was used for cloning the homologous sequences (*trm*B-*aro*K) in *E. coli* XL1-Blue.

Plasmid vector pMUTIN4 is unable to be replicated in *B. subtilis*, containing the Pspac inducible promoter induced by IPTG, which was used as an integrational vector to inactivate the *aro*K gene by the method of homologous recombination [14]. To construct the integrational plasmid pMUTIN4Δ*aro*K, the homologous sequences *trm*B-*aro*K was amplifified by polymerase chain reaction (PCR) and cloned into the pMUTIN4 vector. The resulting plasmid was subsequently linearised using NotI and SacI and then the amplification products with the NotI and SacI cohesive end were ligated into the pMUTIN4 vector.

B. subtilis strain 5434p4SA (Em^R, Cm^R) is a *B. subtilis* strain 5434 derivative containing the *aro*K gene under the control of the Pspac promoter by insertion of integrational vector pMUTIN4Δ*aro*K (with Pspac promoter) in front of *aro*K. *B. subtilis* strain 5434p4SA was constructed by introduction integrational vector pMUTIN4Δ*aro*K into *B. subtilis* strain 5434 cells and resulted in the inactivation of the *aro*K via homologous recombination.

Vector pMTL7-1 [15] is able to be replicated in *B. subtilis*, so when confirming the effectiveness of transformation, pMTL7-1 was used as a positive control of transformation in this study. The vector pMTL7-1 with chloramphenicol resistance could be able to use for screening transformants.

Construction of the vector pMTL21CΔ*aro*K containing homologous sequence (*trm*B-*aro*K) – TA clone. The homologous sequences (target gene sequence *trm*B-*aro*K) was obtained as described in the paper [16]

with modifications. For PCR using primers F1-EcoR1 (G GAA TTC CGG TAT GTA TCT GTC AGA GAA G (5′–3′)) and R1-SacI (C CCG CGG AAC GGT TAT GCA GTA CAG GGC (5′–3′)) with Taq polymerase (*Thermo Scientific*, Lithuania). PCR were carried out with the conditions described by the manufacturer using the following program:

1) denaturation: 5 min at 94 °C;

2) annealing: 10 cycles of 30 s at 94 °C, 10 s at 55 °C, 25 s at 54 °C and then 1.5 min at 68 °C;

3) extension: 25 cycles of 30 s at 94 °C, 30 s at 55 °C, 1.5 min at 68 °C, 5 min at 72 °C.

The PCR product was amplified by Taq polymerase. Therefore, the 3′ end of the PCR product carrying the base adenine A. The vector pMTL21C was digested by restriction enzymes SmaI (*Thermo Scientific*), then a smooth end was formed. Then digestion products with smooth ends was treated with Taq polymerase and dTTP, 3′ ends of the digested product formed a sticky end with a base thymine T. The fragment of vector pMTL21C after digestion by restriction enzymes SmaI with the end base thymine T and the fragment PCR product with the end base adenine A were ligated by the T4 DNA ligase (*Thermo Scientific*). The PCR product (homologous sequence *trm*B-*aro*K) was cloned in the SmaI site of the vector pMTL21C.

Construction of the vector pMUTIN4Δ*aro*K contaning homologous sequence (*trm*B-*aro*K). The vector pMTL21CΔ*aro*K containing homologous sequence (*trm*B-*aro*K) were digested by restriction enzymes NotI and SacI (*Thermo Scientific*), and then cloned into the same digested vector pMUTIN4 by T4 DNA ligase (*Thermo Scientific*) and PEG4000 solution (vector: insert ratio 1 : 5, 10 °C, 18 h). Restriction enzymes, T4 DNA ligase and other DNA manipulating enzymes were used according to their manufacturer's instructions. 5 μL ligation products were used for transformation competent cells *E. coli* XL1-Blue in order to replicate the integrational plasmid pMUTIN4Δ*aro*K. Then the transformed *E. coli* XL1-Blue strains were selected on LB plates containing 100 μg/mL ampicillin. The recombinant plasmids DNA were extracted using the TIANprep MiNi Plasmid Kit for introduction into the *B. subtilis* strain 5434 cells by electrotransformation method. All plasmids constructed in this study (pMTL21CΔ*aro*K and pMUTIN4Δ*aro*K) were verified by DNA sequencing.

Transformation of *B. subtilis* strain 5434. An overnight *B. subtilis* strain 5434 in medium LB (tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L) [17] was diluted in growth medium LBS (LB containing 0.5 mol/L sorbitol) to a final OD_{600} of 0.01 and shaken at 37 °C, 200 r/min with an OD_{600} of 0.5. The cells were cooled for 10 min on ice and then centrifuged for 10 min at 5000g. After four washes in the ice-cold hyper-osmotic electroporation medium SMG (1 mol/L sorbitol, 0.5 mol/L mannitol and 1.5 % v/v glycerol), the cells were suspended in the electroporation medium 1/80 (v/v). If it is necessary, the competent cells were stored at –80 °C for future use. 60 μL of the competent cells were combined with 1 μL vector DNA (60 ng/μL) for electroporation and then moved to an ice-cold electroporation cuvette (1 mm electrode gap). After incubation for 5 min, the cells were exposed to a single electrical pulse using a *GenePulser Xcell*™ (*Bio-Rad*, the USA) set at 23 kV/cm, 25 μF and 200 Ω, 1 mL of recovery medium (LB containing 0.5 mol/L sorbitol and 0.38 mol/L mannitol) was then directly applied to the cells, resulting in a pulse duration of 4.0–5.5 ms. After incubation for 6 h at 37 °C, the cells were placed on an LB plate with 5 μg/mL of the antibiotic erythromycin and preserved overnight at 37 °C. Then positive transformants were chosen [1; 8; 18–20].

Preparation of SA solution for HPLC analysis. Overnight cultures of *B. subtilis* strains 5434 and 5434p4SA were separately inoculated (5 % v/v) into fermentation medium: 4 mL Spizizen minimal medium (0.5 % glucose, 1.4 % K_2HPO_4, 0.6 % KH_2PO_4, 0.072 % anhydrous $MgSO_4$, 0.2 % $(NH_4)_2SO_4$, 0.19 % Na citrate, 0.02 % acid-hydrolysed casein) [5; 21] with 10 μL glutamic acid (5 mg/mL), 10 μL tryptophan (5 mg/mL), 10 μL phenylalanine (5 mg/mL), 100 μL 20 % glucose, and then shaken at 37 °C and 200 r/min for 72 h. At the end of cultivation, the culture was centrifuged at 13 000g for 10 min on *Thermo Scientific® Heraeus Fresco 21 centrifuge* to remove bacterial precipitation. Supernatants contained SA were passed the *CHROMAFIL® Xtra PES-45/25* 0.45 μm filter (*Macherey-Nagel*, Germany). The injection volume was 5 μL, triplicate injections were performed.

Preparation of SA standards solution for HPLC analysis. Sample of SA standards, dissolved in methanol to achieve 2 mg/mL concentrations. Syringe filters with a pore size of 0.45 μm were used to filter the solutions. There have been triplicate injections of 5 μL [22; 23].

Quantitative analysis by HPLC. *LCMS-2020 liquid chromatography* (*Shimadzu*, Japan) was used to detect SA. Absorption spectra were recorded in a flow using a detector based on the *SPD-M20A photodiode array* detector (*Shimadzu*).

The supernatants were analysed by HPLC (C18 Allure column, 4.6 × 150 mm, 5 μm), the column temperature was 40 °C. The HPLC was run with mixture of methanol and formic acid at a flow rate of 0.5 mL/min, the running time was 20 min. SA was detected at 210 nm by the *SPD-M20A photodiode array* detector. The following gradient was described in the table 1 [22–24].

Table 1

The condition of following gradient

Time, min	H_2O, %	Methanol, %	1 % formic acid, %
0–12	88–80	2–10	10
12–13	80–0	10–90	10
13–15	0	90	10
15–20	88	2	10

Results and discussion

Optimisation of growth conditions. The optimal growth conditions for preparation of electrocompetent cells of *B. subtilis* were determined by the growth phase (OD_{600} is equal 0.2 (lag-phase), 0.6 (exponential-phase) and 1.6 (stationary-phase)), before washing steps. The cells were grown in LBS medium until $OD_{600} = 0.2–1.6$. Some reports confirmed that an effective electrotransformation protocol also depends on the quantity of viable cells to be transformed [25]. Different phases of culture (lag-, exponential- and stationary-phase) were measured and noticed that cells grown in SLB until $OD_{600} = 1.0–1.6$ produced around $9 \cdot 10^9–9.4 \cdot 10^{10}$ cells/mL strain with the highest number of viable cells.

The highest transformation efficiency was observed at $OD_{600} = 1.2$ (fig. 2). A moderate improvement one was attributable to $OD_{600} = 0.8–1.4$. The hyper-osmotic electroporation medium with glycine supplementation was proved to be the best combination for electrotransformation [1]. The most living cells of *B. subtilis* strain 5434 could achieve $4 \cdot 10^5$ CFU/μg of plasmid DNA. The transformation efficiency at the $OD_{600} = 1.2$ was 50 fold higher than that at $OD_{600} = 0.2$.

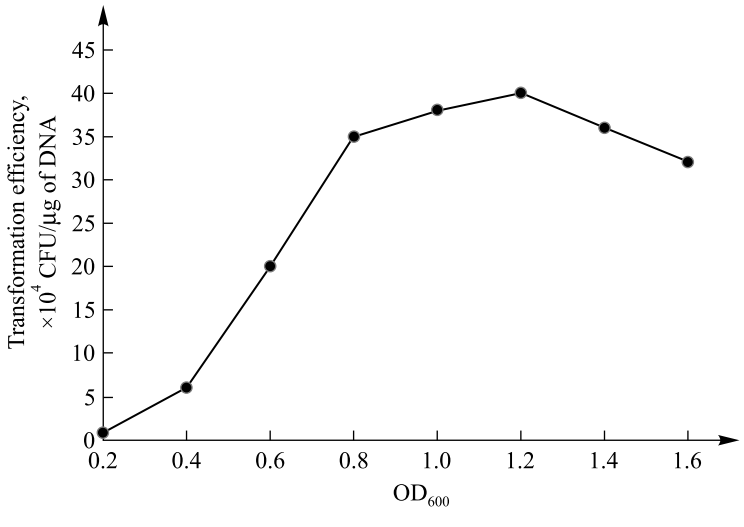

Fig. 2. Effect of growth phase on the electroporation efficiency of *B. subtilis* strain 5434 using the high osmolarity method. The values shown the means of several transformations. The transformation efficiency at different growth phases of *B. subtilis* strain 5434. Cells were grown in LBS medium to various OD_{600} densities for the electrocompetent cell preparation, using SMG solution as the electroporation buffer. 60 ng plasmid pMTL7-1 as a positive control of transformation were used for electroporation with the settings 25 μF, 23 kV/cm, 200 Ω

Improvements in transformation efficiency of *B. subtilis* by increasing the elution frequency. In study [1], mainly by adding trehalose to the electroporation media for improving the survival rate of competent cells, to achieve the purpose of increasing the electrotransformation efficiency. But by the method described in the study [1], couldn't obtain the transformant of *B. subtilis* strain 5434 via homologous recombination (because usually the probability of successfully obtained homologous recombination is generally only one ten thousandth of the transformation efficiency). Therefore, the electrotransformation efficiency by the method described in the study [1] is still lower for obtaining transformants via homologous recombination, and this method is not sufficient to successfully obtain transformants via homologous recombination. So it is necessary to optimise the method described in the study [1]. «An important factor in achieving high transformation

efficiency is to wash competent cells thoroughly to reduce conductivity» was noted in the study [8]. Inspired by this, in our study, the purpose of reducing the conductivity of the electroporation media was achieved by increasing the elution frequency.

Figure 3 indicated that under the same electric field strength, the longer pulse time is due to increase the elution times of competent cells with the electroporation medium for washing. The reason was deduced, because the conductivity was reduced by reducing the number of metal ions in the electrocompetent culture. According to research, have validated that for the electrocompetent culture, the optimum field strength is 23 kV/cm for transformation of *B. subtilis* [1; 8]. Therefore, it is not proper to increase the electric field strength for increasing the pulse time, because higher electric field strength will markedly reduce the cell survivability [26]. To a great extent, exogenous DNA could penetrate through the cell wall when using a longer pulse time, resulting in better transformation and homologous recombination efficiency.

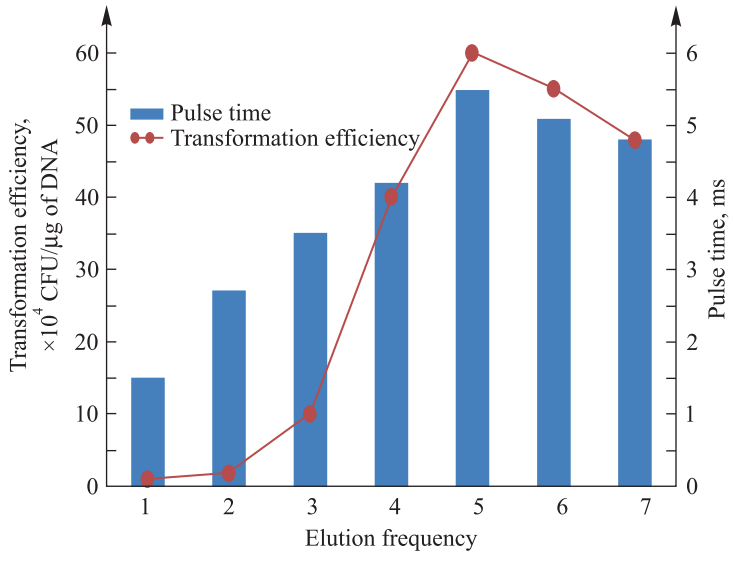

Fig. 3. Effects of elution frequency on the transformation efficiency of *B. subtilis* strain 5434 using the high osmolarity method. The data demonstrated the means of three transformations. The optimised protocol of high osmolarity defined in section «Materials and methods» was followed. Also 60 ng of plasmid pMTL7-1 as a positive control of transformation were used in the transformation with the settings 25 μF, 23 kV/cm, 200 Ω

Although, efficiency transformation could be significantly improved by increasing the elution frequency within a certain range. It is worth noting that the more elution frequency, the fewer living competent cells, resulting in a low transformation efficiency. Under the condition of OD_{600} = 1.2, the highest transformation efficiency was observed at the 5^{th} time elution (see fig. 3). At this moment, a distinct equilibrium between the elution frequency and the number of living competent cells was attained with $6.0 \cdot 10^5$ CFU/μg of plasmid DNA.

This factor was founded that increasing the elution frequency from 1 to 5 times resulted in a 300 fold increase in the transformation efficiency of *B. subtilis* strain 5434. Therefore, this is a useful approach to improve the transformation efficiency of *B. subtilis* by increasing the elution frequency to reduce the conductivity of electrocompetent culture under the condition of ensuring a higher cell survivability and by effectively extending the pulse time under the same electric field strength.

The conclusion could be derived from fig. 3: the pulse time is inversely proportional to the conductivity of electrocompetent culture. Under the same electric field strength, the pulse time is directly proportional to the transformation efficiency. Therefore, the conductivity of electrocompetent culture can be reduced by increasing the elution frequency, thereby achieving the purpose of increasing the transformation efficiency. Increase of elution frequency as a novel applied methodology could significantly improve the efficiency transformation.

Quantity of integrative plasmid DNA. Significant amounts of plasmid DNA are usually used in protocols to acquire transformants of *B. subtilis* and other gram-positive bacteria [25; 27]. To evaluate the optimum concentration of plasmid DNA for *B. subtilis* transformation in the electroporation solution mix, a does-dependency for plasmid DNA ranging from 1 to 140 ng was conducted (fig. 4). Among of them, 65 ng of plasmid DNA was necessary to obtain the most living transformation strain (fig. 5). However, the highest transformation efficiency could be achieved when 20 ng of plasmid DNA applied under OD_{600} = 1.2. Competent cells were washed 5 times

by the electroporation medium, reaching $8.6 \cdot 10^5$ CFU/μg of plasmid DNA. A 2.2 fold reduction in the transformation efficiency of *B. subtilis* strain 5434 was observed in this study by increasing the quantity of plasmid pMTL7-1 from 20 to 140 ng ($3.9 \cdot 10^5$ CFU/μg of DNA at 140 ng integrational plasmid *vs* $8.6 \cdot 10^5$ CFU/μg of DNA at 20 ng of plasmid pMTL7-1). Transformation efficiency was inversely proportional to the quantity of plasmid DNA [26; 28].

The reason can be explained that plasmid pMTL7-1 as a positive control of transformation was extracted from *E. coli* XL1-Blue with metal ions, this situation is unavoidable. Therefore, if the plasmid pMTL7-1 was excessively added to the hyper-osmotic electrocompetent culture, the metal ions in electroporation solution mix and the conductivity of electroporation solution mix were increased, resulting in shorter pulse time and reduced electroporation efficiency. Obviously, the concentration of metal ions introduced to the electroporation solution mix is directly proportional to the concentration of plasmid DNA.

When comparing the efficiency of transformation of *B. subtilis* strain 5434 on the amount of introduced DNA and the total number of obtained transformants (see fig. 5), the following regularities were revealed.

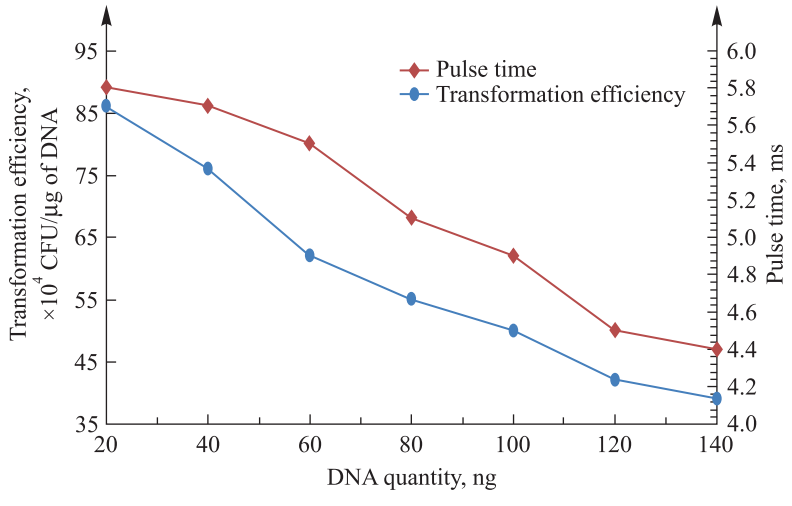

Fig. 4. Effects of plasmid pMTL7-1 quantity on the pulse time and transformation efficiency of *B. subtilis* strain 5434 using the high osmolarity method. The data demonstrated the means of three transformations. The optimised protocol of high osmolarity defined in section «Materials and methods» was followed. Also 20–140 ng of plasmid pMTL7-1 as a positive control of transformation were used in the transformation with the settings 25 μF, 23 kV/cm, 200 Ω

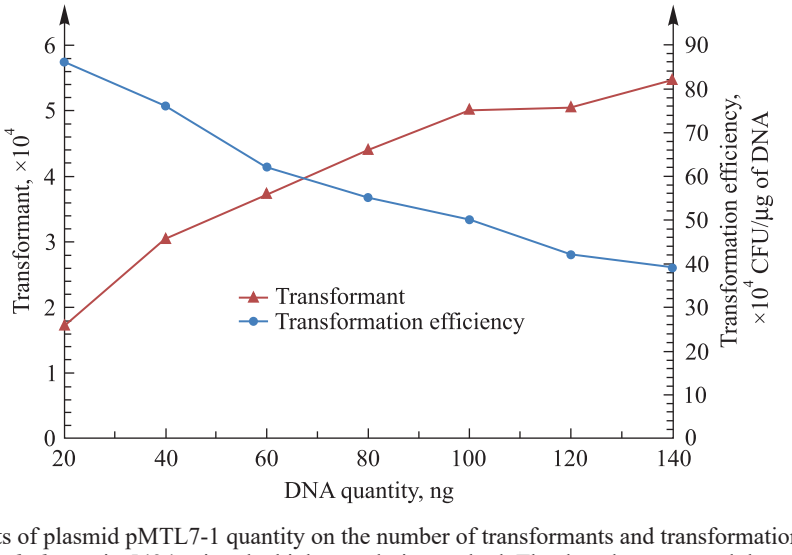

Fig. 5. Effects of plasmid pMTL7-1 quantity on the number of transformants and transformation efficiency of *B. subtilis* strain 5434 using the high osmolarity method. The data demonstrated the means of three transformations. The optimised protocol of high osmolarity defined in section «Materials and methods» was followed. Also 20–140 ng of plasmid pMTL7-1 as a positive control of transformation were used in the transformation with the settings 25 μF, 23 kV/cm, 200 Ω

Low concentrations of DNA (1.083 ng/μL of electrocompetent culture or 65 ng of vector DNA per 60 μL of electrocompetent cells) are optimal for efficient transformation of *B. subtilis* strain 5434. Decreasing concentration of plasmid DNA in the electrocompetent culture leads to a significant increase of the transformation efficiency and a sharp decrease in the total number of transformants (presumably due to the deficiency of introduced DNA). The increase in the amount of introduced DNA (concentration more than 1.083 ng/μL of electrocompetent culture) leads to an increase in the total number of transformed cells and a strong decrease of transformation efficiency. For further work, as the most optimal quantity of plasmid DNA, the concentration of 1.083 ng/μL electrocompetent cells or 65 ng vector DNA per 60 μL electrocompetent cells was chosen as suggested [1]. It should be noted that the key point was not the total amount of plasmid DNA in the electroporation solution mix, but the equivalent ratios between the number of vectors DNA and the number of electrocompetent cells. For different sizes of vectors, this value may be differential. This assumption required a further investigation.

Application of the method for construction of *B. subtilis* strain 5434 to increase the yield of SA from *B. subtilis* strain 5434 and determination of SA concentration by method HPLC. In this study, the *B. subtilis* strain 5434 was successfully transformed with the higher transformation efficiency of $8.6 \cdot 10^5$ CFU/μg of DNA by integrative vector pMUTIN4Δ*aro*K (65 ng) with the method described above. In result, via the inactivation of *aro*K by homologous recombination strain designated as *B. subtilis* strain 5434p4SA was obtained. The fig. 6 shows the elution of SA was detected at 210 nm, resulting in a retention time of 2.9 min.

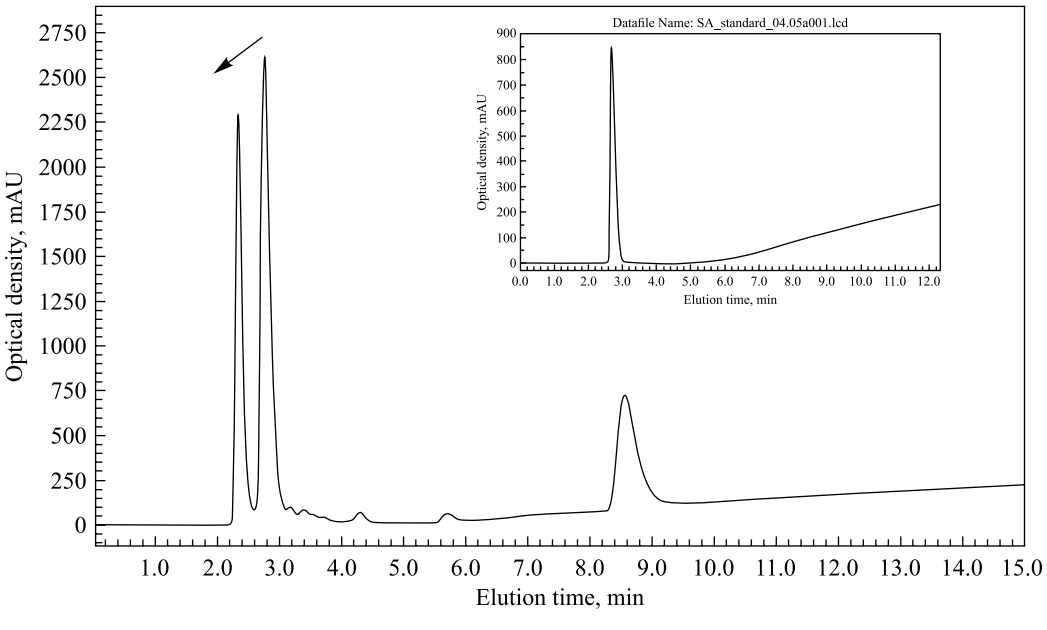

Fig. 6. HPLC chromatograms of the SA production in fermentation liquor.
The peak of SA was indicated by the arrow and the inset is HPLC chromatograms
of the SA standards solution (the concentrations of SA standards solution was 2 mg/mL)

Quantification of SA revealed (table 2) that *B. subtilis* strain 5434p4SA produced 403.98 ± 9.1 μg/mL of SA. This indicates that the method is also appropriate for other *B. subtilis* strains genetic transformation.

The supernatants were assayed and quantified by HPLC, and the values shown for the production of SA are means ± SD of three experiments.

Table 2

Concentration of SA in the fermentation medium was quantified by HPLC

B. subtilis strain	Concentration of SA ± SD, μg/mL
5434	Not detected
5434p4SA	403.98 ± 9.10

Conclusions

We have established a reliable and highly efficient electrotransformation method for *B. subtilis*. On the basis of the approach mentioned in our work, *B. subtilis* strain 5434 was successfully designed to achieve higher levels yield SA. We expect that this approach would be wide used in the transformations of some gram-positive bacterial species which are able to grow in a high osmolarity medium.

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