Effects of Sr^{2+} on the preparation of *Escherchia coli* DH5α competent cells and plasmid transformation

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ABSTRACT

Bacterial gene transformation used with *Escherichia coli* as a desired microorganism is one of the important techniques in genetic engineering. In this study, the preparation of *E. coli* DH5α competent cells treated with $SrCl_2$ and transformation by heat-shock with pUC19 plasmid was optimized by Response Surface Methodology (RSM). Other five *E. coli* strains including BL21 (DE3), HB-101, JM109, TOP10 and TG1, three different sizes plasmids (pUC19, pET32a, pPIC9k) were used to verify the protocol, respectively. The transformation mechanism was explored by scanning electron microscope combined with energy dispersive spectrometer (SEM-EDS), atomic absorption spectroscopy (AAS) and Fourier-transform infrared spectroscopy (FT-IR). An equation of regression model was obtained, and the ideal parameters were Sr^{2+} ions of 90 mM, heat-shock time of 90 s and 9 ng of plasmid. Under this conditions, the transformation efficiency could almost reach to 10^6 CFU/μg DNA. A small change of the cell surface structure has been observed between *E. coli* DH5α strain and competent cells by abovementioned spectrum technologies, which implied that a strict regulation mechanism involved in the formation of competent cells and transformation of plasmids. An equation of regression model for the competent cells preparation and plasmid transformation could be applied in gene cloning technology

Subjects Biochemistry, Bioengineering, Microbiology
Keywords *Escherchia coli*, Response surface methodology, Competence, Transformation mechanism

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INTRODUCTION

Escherichia coli is extensively applied in genetic engineering as a desired microorganism for various cloning experiments. In 1970, *Mandel & Higa (1970)* firstly found *E. coli* K12 strains treated with calcium chloride solution was easy to be infected by λ phage DNA by a short-time heat shock. Besides, it was proven that the plasmid DNA could also enter the bacteria by the same method by Cohen & Chang (1972). Thereafter, the chemical transformation methods (*Nørgard, Keem & Monahan, 1978*;

Dagert & Ehrlich, 1979; *Zhang, Xu & Xu, 2004*) have continuously been modified with various combinations of chemical solutions including different cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, K^{+}, Na^{+}, Rb^{+}), PEG, DMSO (*Chung & Miller, 1988*; *Chan et al., 2013*) and glycerin *Shanehbandi et al. (2013)*. Considering the practicality and convenience of different artificial methods, more efficient exogenous gene transfer systems also have been developed by physical or chemical methods, including chemical procedures (*Inoue, Nojima & Okayama, 1990*; *Sarkar, Choudhuri & Basu, 2002a*; *Sarkar, Choudhuri & Basu, 2002b*; *Song et al., 2007*), high-voltage electroporation (*Dower, Miller & Ragsdale, 1988*; *Dunny, Lee & LeBlanc, 1991*; *Sheng, Mancino & Birren, 1995*), a biolistic propulsion system (*Shark et al., 1991*), liposome-mediated DNA transfer (*Kawata, Yano & Kojima, 2003*), microwaves and ultrasounds assisted DNA transfer (*Zarnitsyn & Prausnitz, 2004*; *Fregel, Rodriguez & Cabrera, 2008*; *Tripp, Maza & Young, 2013*; *Deeks et al., 2014*) and chemical-physical (Rb^{+}, sepiolite and nanomaterials) induced transformation (*Ren et al., 2017*; *Ren, Na & Yoo, 2018*; *Ren et al., 2019*), etc. The transformation efficiency ($10^{4}\sim10^{8}$ CFU/μg DNA) of different strains can obviously be obtained from these methods described.

Each of the methods mentioned above has their advantages in transformation efficiency, the corresponding experimental complexity and cost-effective limitations (*Chen, Christie & Dubnau, 2005*; *Ren et al., 2017*). However, the higher transformation efficiency in each chemical-mediate method all depended on the function of $CaCl_2$. This implied Ca^{2+} ions played the key role in the preparation and transformation process of the competent cells by plasmid, that can be affected by the growth of the bacterial strain, plasmid DNA concentration, heat shock temperature and duration, cold incubation duration of $CaCl_2$ treated cells (*Singh et al., 2010*). The various strains showed the different transformation efficiency with the same treatment method (*Ren et al., 2017*). In our previous study (*Wang et al., 2013*; *Liu et al., 2014*), the transformation efficiency with pUC19 plasmid DNA was almost equal between the cells prepared by $CaCl_2$ and $SrCl_2$ treatment, respectively. Whereas, how much concentration of Sr^{2+} resulted in maximum transformation efficiency compared with Ca^{2+} has not yet been reported up to now, and the exact mechanism of $SrCl_2$-mediated artificial transformation process is still largely obscure.

Chemical transformation was referred to as one of the most important techniques in genetic manipulation. The transformation efficiency depended upon several factors, including cation type, cation concentration, treatment time, thermal shock, and incubation time (*Huff et al., 1990*; *Cosloy & Oishi, 1973*; *Chan et al., 2002*; *Broetto et al., 2006*; *Singh et al., 2010*). Bacterial transformation is a fundamental technology to deliver engineered plasmids into bacterial cells, which is essential in industrial protein production, chemical production, etc. Hence, a novelty statistical method could be applied in the experimental design for higher transformation efficiency. Response surface methodology (RSM) consists of a group of mathematical and statistical methods, initiates from the design of experiment (DOE) that is generally considered an effective statistical technique for optimizing complex processes, due to its obvious advantages, e.g., the reduced number of experimental trials needed to evaluate multiple parameters and their interactions, less laborious and lesser time-consuming (*Khuri & Mukhopadhyay, 2010*; *Bezerra et al., 2008*). An optimal process with the mathematical equations is obtained by determining the significant factors affecting

an experiment and reducing the number of experimental runs while maximizing output through the data generated. As one of the RSM, Box-Behnken Design (BBD) (*Ferreira et al., 2007*) is introduced to optimize the preparation of *E. coli* DH5 α prepared with $SrCl_2$ andthe transformation with pUC19. The optimal conditions are verified by different *E. coli* strains with different size of plasmid DNA, including pUC19, pET32a and pPIC9k, respectively. In addition, the transformation mechanism is preliminarily explored by SEM, EDS, AAS and FT-IR, respectively.

MATERIAL AND METHODS

Bacterial strains and plasmids

E. coli DH5α, JM109, Top10, TG1, HB101 and BL21 strains were employed for the plasmid DNA acceptor and purchased from Sangon Biotech (Shanghai) Co., Ltd. Plasmids pUC19, pET32a and pPIC9k (with an inserted gene encoding ampicillin resistance) were purchased from Solarbio life science (Beijing) Co., Ltd. The plasmid was dissolved in TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.6), and the concentration was measured by their absorbance at 260 nm.

Chemicals, media, and culture conditions

$MgCl_2$, $CaCl_2$, $SrCl_2$ and other reagents used were all of analytical reagent grade. Reagents were purchased from Sangon (Shanghai, China) Biotech Co., Ltd. Tryptone and yeast extract were purchased from Oxoid (England). *E. coli* strains preserved on LB agar plates at 37 °C. Ampicillin (100 μg/ml) purchased from Sigma was added into the medium for screening the transformants.

Preparation of competent cells

Competent cells were prepared according to *Sambrook & Russell (2006)* with some necessary modifications. In detail, a large size (2–3 mm) *E. coli* colony on LB agar plate was picked into three mL of LB liquid medium and the inoculum was cultured at 37 °C, 200 rpm for 12–16 h. The cultures of *E. coli* were diluted 100-fold with the fresh LB medium until OD_{600} reached at approximate 0.4–0.5. Then, the cultures were immediately cooled down to 0 °C by pouring it into a 50 mL sterile and prechilled centrifuge tube, and then centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was poured off and the cell pellets were resuspended with 30 mL 100 mM of different sterile and prechilled solution (only with $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$ and $MnCl_2$. respectively). The mixture was centrifuged again under the same condition. Subsequently, two mL solution containing 15% (v/v) glycerol was added into the tube to resuspend the cell pellets. The treated cells were gently kept in an ice-water bath for 5 min, then packaged in a 1.5 mL Eppendorf tube in a volume of 100 μL/tube. The competent cells were stored at −80 °C until further use.

Standard procedure for transformation

The competent cells were transformed with pUC19 plasmid DNA according to *Sambrook & Russell (2006)*. An aliquot (0.1 mL) of competent cells mixed with 1.0 μL pUC19 DNA (1 ng/L) dissolved in TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.6) were heated

Table 1 The levels of the factors on transformation efficiency of *E. coli* DH5α competent cells.

Factors	Levels		
	−1	0	1
X_1 $SrCl_2$ concentration (mmol/L)	60	80	100
X_2 Heat shock time (s)	75	90	105
X_3 the amount of pUC19 plasmid DNA (ng)	7	9	11

shock 90 s at 42 °C, and then the mixture was kept on ice for 30 min. After the incubation, 0.9 mL of LB medium were added into the mixture and cultured again with shaking at 37 °C for 1 h. 100 μL aliquots of the culture were spread on LB agar plates with 100 μg/mL of ampicillin, which was incubated at 37 °C for 12–16 h. Transformation efficiency was expressed as the number of ampicillin-resistant transformants obtained and was calculated based on the colony-forming units (CFU) per μg DNA used (*Sambrook & Russell, 2006*).

Optimization of transformation of plasmid by RSM

Based on single-factor experiments, RSM with 3 factors and 3 levels was used to estimate the effect of each factor on the transformation efficiency of *E. coli* DH5α competent cells as outlined in Table 1, in which −1, 0, l represented the high, medium and low level, respectively. $SrCl_2$ concentration (X_1), heat shock time (X_2) and the amount of pUC19 plasmid DNA (X_3) were employed as the independent variables, the transformation efficiency as the response value was performed during the RSM analysis. All the series of trials in the BBD experiment were listed in Table 2, including the encoded and non-coded values of the experimental variables and 17 experimental points. Five replications (13–17) were used to evaluate the pure error. A quadratic polynomial model was used to analyze the relationship between transformation efficiency (Y) and the independent variables, then data were fitted to the following equation.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} x_i x_j$$

where Y represented the measured correlation response combined with each factor level; β_0, β_i, β_{ii} and β_{ij} represented the regression coefficients for intercept, linear, quadratic and interaction terms, respectively; x_i and x_j were independent variables of the coding. Design-Expert Software (Trial Version 8.0.5, Stat-Ease Inc., Minneapolis, MN, USA) was used to estimate the response of each set of experimental design and optimized conditions. The applicability of the quadratic polynomial model was tested by the regression coefficient R^2. The significance of the regression coefficients was checked using the F-value and P-value.

SEM and EDS assay

E. coli DH5α cells and competent cells were immobilized with 2.5% glutaraldehyde, washed three times with PBS buffer (pH 7.2, 0.2 M), and vacuum freezing dried (FD-1A-50, Boyikang, Beijing, China), respectively. These dried cells were coated with a thin layer of gold under reduced pressure and examined using a SEM system (JSM-5900LV, JEOL, Japan) at an acceleration voltage of 10 kV with an image magnification of 10000 ×

Table 2 Box-Behnken design and results for transformation efficiency.

No.	Sr^{2+} concentration (mmol/L)	Heat-shock time (s)	Amount of DNA (ng)	Transformation efficiency (CFU/μg of plasmid DNA)
1	−1	−1	0	0.66×10^5
2	1	−1	0	7.68×10^5
3	−1	1	0	0.52×10^5
4	1	1	0	3.28×10^5
5	−1	0	−1	0.62×10^5
6	1	0	−1	5.16×10^5
7	−1	0	1	5.82×10^5
8	1	0	1	6.87×10^5
9	0	−1	−1	5.47×10^5
10	0	1	−1	3.08×10^5
11	0	−1	1	4.87×10^5
12	0	1	1	3.66×10^5
13	0	0	0	1.56×10^6
14	0	0	0	1.63×10^6
15	0	0	0	1.45×10^6
16	0	0	0	1.57×10^6
17	0	0	0	1.79×10^6

~50000×. A series of SEM images were recorded to analyze the surface difference between *E. coli* DH5α and the competent cells. The content of strontium ions in the cell surface was measured by EDS (JEOL, Japan).

AAS analysis

one mL of the competent *E. coli* DH5α cells was centrifuged at 10,000 rpm for 5 min and 0.5 mL of the supernatant was transferred to a 50 mL volumetric flask and diluted with 0.1% HNO_3 solution. The optical density of this solution was measured by air-acetylene flame atomic spectrum (AAS, Z-5000, Hitachi, Japan) at 460.73 nm with the lamp current of 5 mA and spectral bandwidth of 0.5 nm. The acetylene and the airflow rate were set as 1.7 L/min and 6.0 L/min, respectively The concentration of Sr^{2+} was calculated according to the linear regression equation of the standard curve ($Y = 0.061x$ □0.0061, $R^2 = 0.9998$, linear range of 0.5–6.5 μg/mL), in which, the horizontal and vertical coordinates represented the concentration of Sr^{2+} (g/mL) and $OD_{460.73}$, respectively.

FT-IR spectroscopy analysis

The freezing-dried *E. coli* DH5α and competent cells were prepared via the KBr pressed-disc method (*Xiang, Sai & Yu, 2002*). The functional groups difference on the cell surface was recorded by FT-IR spectrometer (Nexus670, Nicolet, American) with the range of 4,000–400 cm^{-1} at a resolution of four cm^{-1}. All spectra were acquired over 20 scans.

Statistical analysis

All data were shown as mean ± SD values by three replicates. The difference was to be significant when $P < 0.05$. Statistical analysis was performed using the OriginPro software package 8.5 (OriginLab Corp.) and the Design-Expert software version 8.0.5 (Stat-Ease Inc., Minneapolis, MN, USA).

RESULTS

Effects of different divalent metal ions on transformation efficiency

Five strains of *E. coli* were treated by five kinds of divalent metal ions (100 mM) under the same condition and then transformed with 10.0 ng pUC19. The results shown in Fig. 1 displayed that the transformation efficiency of strains treated by Mg^{2+} or Mn^{2+} was lower than those prepared by Ca^{2+}, Sr^{2+} or Ba^{2+}. The transformation efficiencies of *E. coli* BL21, DH5α, HB101 and JM109 induced by $CaCl_2$ were the highest. However, *E. coli* TG1 and TOP10 treatment with $SrCl_2$ obtained the highest transformation efficiencies. Differences between these strains maybe could take account for this phenomenon. Some receptor proteins existed in the surface of *E. coli* cells, such as outer membrane proteins (OmpA, OmpC and so on), responsing the signal of divalent metal ions, and then the receptor signalling pathways were triggered for the absorption of exogenous DNA (*Aich et al., 2012*; *Finkel & Kolter, 2001*). Moreover, the previous reports displayed RNase, DNase, β-galactosidase and alkaline phosphatase could be released on the cell surface or extracellular by osmotic shock (*Nossal & Heppel, 1966*). The different strains also showed different changes (permeability, enzyme activities) with the chemical treatment. The different enzymatic reactions in the cells may be activated or inhibited by the metal ions. For example, nuclease can hydrolyze DNA with the assistant of Mg^{2+} or Mn^{2+} to reduce the transformation efficiencies for different plasmids.

Effects of Sr^{2+} concentration on transformation efficiency

As we all know, divalent metal ions and their concentration play an important influence on the formation of competent cells and transformation efficiency for exogenous DNA. In this experiment, the effects of different Sr^{2+} concentrations on transformation efficiency were examined, while Heat-shock time and the amount of pUC19 plasmid DNA was fixed at 45 s and 10.0 ng. Plots of transformation efficiency against Sr^{2+} concentrations were shown in Fig. 2A, the transformation efficiency was significantly enhanced when the concentration of Sr^{2+} increased from 20 mM to 80 mM, and the value reached at a maximum at the concentration of 80 mM Sr^{2+}. However, transformation efficiency kept stable between the Sr^{2+} treatment of 80 mM and 100 mM, and then decreased gradually when the amount of Sr^{2+} exceeded 100 mM.

Effects of heat-shock time on transformation efficiency

Many heat-shock proteins existed in *E. coli* cell surface, such as GroEL, which has an exclusive role in plasmid transforming into *E. coli* (*Aich et al., 2011*). The effects of different heat-shock time such as 30 s, 45 s, 60 s, 75 s, 90 s and 105 s on transformation efficiency were explored, and Sr^{2+} concentration, the amount of pUC19 plasmid DNA was fixed

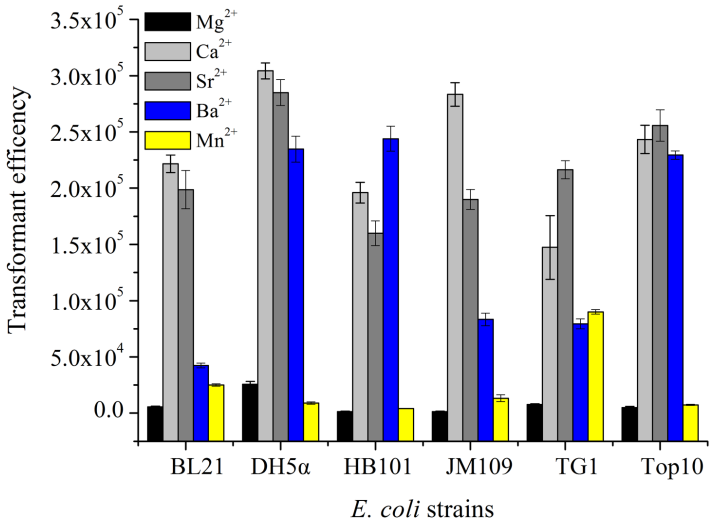

Figure 1 The transformation efficiency of *E. coli* strain treated by different divalent metal ions. Six strains of *E. coli* were used to prepare competent cells treated by the concentration of 100 mM of five kinds of divalent metal ions under the same condition and transformed with 10 ng pUC19 plasmid DNA.
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at 80 mM and 10 ng. As shown in Fig. 2B, the transformation efficiency increased with the increase of the heat-shock in the range of 30–90 s and exhibited the maximum value at 90 s. However, the longer heat shock treatment, the lower transformation efficiency was obtained, which possibly be explained by the cell damage and protection theory. Once the cells were stimulated for a long period of heat-shock, some proteins on the cell surface could reduce cell damage by shutting down some pathways, so the transformation efficiency declined (*Chung, Niemela & Miller, 1989*; *Claverys & Martin, 2003*).

Effects of the amount of plasmid on transformation efficiency

When the Sr^{2+} concentration of 80 mM and heat-shock time of 90 s was fixed, the effects of the various amount of plasmid on transformation efficiency were studied. As illustrated in Fig. 2C, maximum transformation efficiency could be obtained with 9.0 ng of pUC19 plasmid, then declined with the increasing of the DNA amount, which may be attributed to that the repulsive forces increased with the more addition of negatively-charged pUC19 plasmid, the reduction of random collisions between DNA molecules and competent cells resulted in transformation efficiency decline (*Broetto et al., 2006*).

Optimization of the parameters on transformation efficiency

According to the single-factor experiment results, three independent variables ($SrCl_2$ concentrations, heat-shock time, the amount of pUC19 plasmid DNA) with three levels (Listed in Table 2) were performed for developing the model. The transformation efficiency was regressed to a second-order polynomial function shown in the following equation:

$$Y = -4.41 \times 10^7 + 3.13 \times 10^5 X_1 + 4.92 \times 10^5 X_2 + 2.41 \times 10^6 X_3 - 355.17 X_1 X_2 + 1091.14 X_1 X_3 + 986.49 X_2 X_3 - 1737.21 X_1^2 - 2661.05 X_2^2 - 1.43 \times 10^5 X_3^2$$

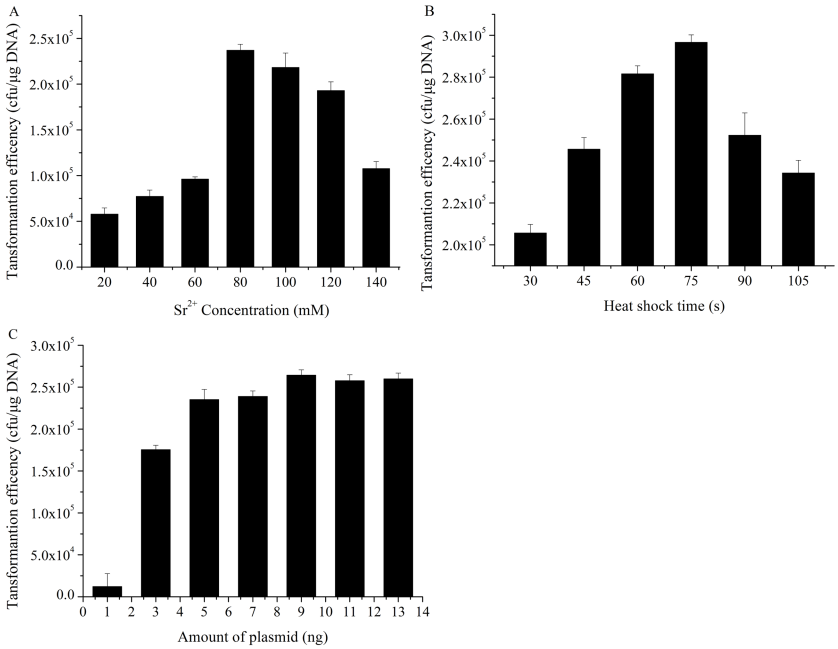

Figure 2 Transformation efficiency affected by the different various factors. (A) Effects of different Sr^{2+} concentrations on transformation efficiency were examined, while heat-shock time and the amount of pUC19 plasmid DNA was fixed at 45 s and 10 ng. (B) Effects of heat-shock time on transformation efficiency was studied, and the other conditions such as Sr^{2+} concentration, the amount of pUC19 plasmid DNA were as well fixed at 80 mM and 10 ng. (C) Effects of the amount of plasmid on transformation efficiency were determined as well. the other conditions such as Sr^{2+} concentration, heat-shock time was fixed at 80 mM and 90 s. All operations were performed three duplicates.

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where Y denoted transformation efficiency, X_1, X_2 and X_3 represented $SrCl_2$ concentrations (mM), heat-shock time (s) and the amount of pUC19 plasmid DNA (ng) in coded values, respectively. Table 3 showed the analysis of variance (ANOVA) for the fitted quadratic polynomial model. Based on this analysis, F-value of 71.59 and P-value < 0.01 indicated that the response surface quadratic model was significant difference. The ANOVA of the quadratic regression model showed that experimental data for transformation efficiency had a correlation coefficient (R^2) of 0.9893 for the calculated model, which implied that the experimental results were agreement with the theoretical values predicted by the polynomial model. The model was suitable for application in the competence cells preparation and plasmid transformation. The Lack of Fit (F-value of 0.13) was not significant compared with pure error, which further indicated this model could accurately predict the preparation of competent cells and plasmid transformation. The data in Table 3 showed that all the linear coefficients (X_1, X_2) and quadratic term coefficients (X_1^2, X_2^2, X_3^2) significantly affected the transformation efficiency (P <0.05). To further understand the relationship between the three independent variables in transformation efficiency, the three-dimensional response surface were plotted in Fig. 3. The interactive effects of $SrCl_2$ concentration and heat-shock time, DNA concentration and heat-shock time, and $SrCl_2$ concentration and DNA concentration on the transformation efficiency

Table 3 Regression coefficients of the predicted quadratic polynomial model.

Sources	SS[a]	DF[b]	MS[c]	F value	Significance
Model	**6.16×10^{12}**	**9**	**6.85×10^{11}**	**71.58562**	**<0.0001****
X_1	5.31×10^{11}	1	5.31×10^{11}	55.45105	0.0001**
X_2	8.27×10^{10}	1	8.27×10^{10}	8.642465	0.0217*
X_3	3.43×10^{9}	1	3.43×10^{9}	0.358188	0.5684
X_1X_2	4.54×10^{10}	1	4.54×10^{10}	4.746211	0.0658
X_1X_3	7.62×10^{9}	1	7.62×10^{9}	0.796378	0.4018
X_2X_3	3.5×10^{9}	1	3.5×10^{9}	0.366159	0.5642
X_1^2	2.03×10^{12}	1	2.03×10^{12}	212.4936	<0.0001**
X_2^2	1.51×10^{12}	1	1.51×10^{12}	157.757	<0.0001**
X_3^2	1.38×10^{12}	1	1.38×10^{12}	143.8017	<0.0001**
Residue	6.7×10^{10}	7	9.57×10^{9}		
Lack of fit	6.06×10^{9}	3	2.02×10^{9}	0.132688	0.9356
Pure error	6.09×10^{10}	4	1.52×10^{10}		
Cor total	6.23×10^{12}	16			

Notes.

Note: The data with "**" represented extremely significant difference (P <0.01); the data with"*" represented significant difference (0.01 <P <0.05), respectively. SS[a], sum of squares. DF[b], degree of freedom. MS[c], mean square.

of *E. coli* DH5α with pUC19 plasmid were displayed in Figs. 3A–3C, respectively. It was observed that the transformation efficiency enhanced gradually up to a threshold level with the increase of Sr^{2+} concentration, as well as heat-shock time, the amount of pUC19 plasmid. Then, the transformation efficiency slightly decreased beyond this level. The maximum transformation efficiency in *E. coli* DH5α predicted by the model was 1.63 $\times 10^6$, when Sr^{2+} concentration, heat shock time, the amount of pUC19 plasmid DNA were set at 83.87 mM, 88.47 s, and 9.05 ng, respectively. Considering the operability in actual production, the optimal conditions could be modified as follows: Sr^{2+} concentration of 90.0 mM, heat-shock time of 90 s, the amount of pUC19 plasmid DNA of 9 ng. The average transformation efficiency obtained under these conditions was $(1.76 \pm 0.21) \times 10^6$ ($n = 3$). These results confirmed that the response model was sufficient to optimize the preparation of *E. coli* DH5α competent cells and transformation for pUC19 plasmid. Thereafter, other 5 strains of *E. coli* were chosen to verify the optimal conditions with different size of plasmid including pUC19 (2.68 kb), pET32a (5.9 kb) and pPIC9k (9.27 kb). The results were shown in Fig. 4, the strain (*E. coli* DH5α and JM109) with a higher transformation efficiency was obtained, and the transformation efficiency decreased with the increase of molecular weight of the plasmid. A similar result was described by *Chan et al. (2002)*.

Surface observation with SEM and determination of content of Sr^{2+} in the cell surface

SEM is a useful method for checking the higher resolution of cell surfaces (*Li et al., 2004*; *Panja et al., 2008*). The surface structure of *E. coli* DH5α competent cells treated with 90 mM $SrCl_2$ and *E. coli* DH5α cells were observed by SEM at a magnification of 10,000 and 50,000, as shown in Fig. 5. *E. coli* DH5α cells showed the typical rod-shaped bacteria with

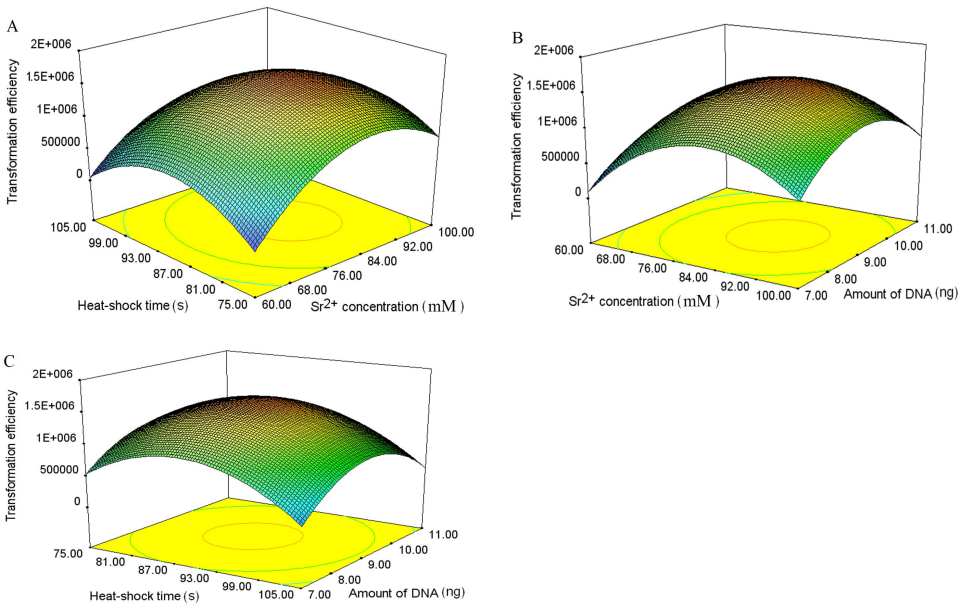

Figure 3 Three-dimensional response surface and contour plots of transformation efficiency against Sr^{2+} concentrations, heat-shock time and the amount of plasmid. Actual factors of were set at 0 in coded levels, respectively. All operations were performed three duplicates. (A). The interaction effect between Sr^{2+} concentrations and heat-shock time on transformation efficiency; (B). The interaction effect between Sr^{2+} concentrations and amount of plasmid on transformation efficiency; (C). The interaction effect between heat-shock time and amount of plasmid on transformation efficiency.

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the length of 1.63–2.54 μm, a width of 0.63–0.9 μm (Fig. 5A), and the cells treated by Sr^{2+} kept rod-shaped characteristics with a typical length of 1.63–2.12 μm, a width of 0.43–0.81 μm (Fig. 5B). The cells treated with Sr^{2+} seemed to gather together, which attributed to the cell surface charge is changed. The cell's shape and size almost remained unchanged between the two groups. However, the surface of the treatment cells appeared rough and wrinkled morphology (Fig. 5D) compared with that of normal cells (Fig. 5C). To check whether Sr^{2+} only attached to the cell surface, the contents of Sr^{2+} on the surface between the normal cells and competent cells were determined using an SEM-EDS as shown in Fig. 6. The contents of Sr^{2+} on the surface of competent cells was significantly higher than that of normal cells, which implied that a part of Sr^{2+} remained on the surface of the competent cells. The content of Sr^{2+} in the supernatant of *E. coli* DH5α treated with 90 mM of Sr^{2+} was measured by AAS. The content of Sr^{2+} concentration was measured 82.15 mM according to the calibration curve of $SrCl_2$ solution, which illustrated that a small part of Sr^{2+} may be entered into the cells or adsorbed on the surface of the cells. Based on the analysis results of EDS, we deduced Sr^{2+} indeed was adsorbed on the cell surface, and whether Sr^{2+} could enter into cells needs further research.

FT-IR analysis

Fourier transform infrared spectrum (FT-IR) could not only provide molecular groups characteristics of vibration absorption spectrum band but also detect the change of the

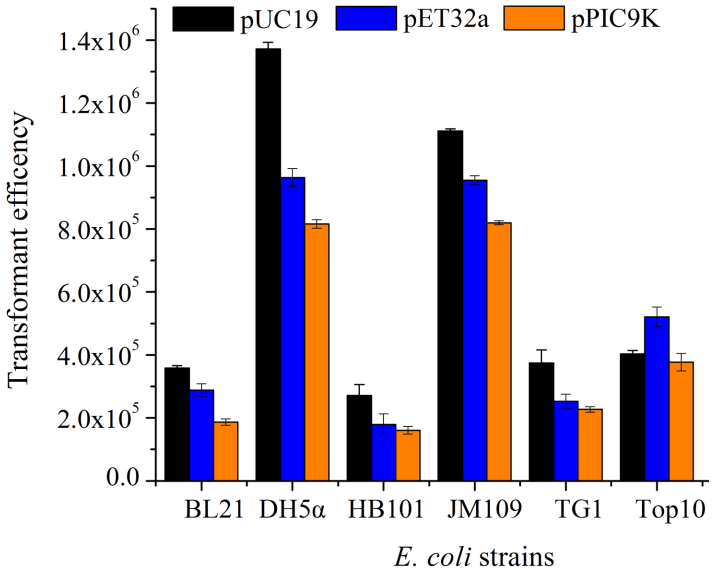

Figure 4 The transformation efficiency of various *E. coli* strains prepared by $SrCl_2$ method for different plasmids. The different *E. coli* strains were treated by sterile and prechilled $SrCl_2$ (90.0 mM), and transformed with 9 ng plasmid at 42 °C for 90 s. Transformation efficiency was calculated in accordance with the colony forming unit (CFU) per μg DNA. All operations were performed three duplicates.
Full-size DOI: 10.7717/peerj.9480/fig-4

molecular groups and the strain identification (*Hellwig, Stolpe & Friedrich, 2004*; *Carlos et al., 2011*). FT-IR of *E. coli* DH5α cells and competent cells were all shown in Fig. 7. A detailed analysis of FT-IR absorbance spectra of the strains was presented as follow: the peak at 1,060 cm^{-1} was attributed to a sugar absorbance, which was mainly caused by the C-OH stretch or the P-O-C stretch form polysaccharide skeleton vibration. A spectral feature between 1,220 cm^{-1} and 1,384 cm^{-1} was assigned to the symmetric stretching vibration ν_s (PO_2^-) and symmetric stretching vibration ν_{as} (PO_2^-) of the phosphodiester group of nucleic acid molecules (*Muntean et al., 2014*). An obvious absorption peak at 1,456.82 cm^{-1} was assigned to a stretching vibration of asymmetric bending vibration δ_s (CH_3) of methyl in a protein molecule. The peaks at 1,537.01 cm^{-1} and 1,614 cm^{-1} were regarded as the stretching vibration of C-N, C=O and the bending vibration of N-H from protein amide (*Goormaghtigh, Cabiaux & Ruysschaert, 1994*). The peaks at 2,919.46 cm^{-1} ν_{as} (CH_2) and 2,937.17 cm^{-1} ν_{as} (CH_3) were the typical stretching vibration of C-H of aliphatic carbon chain, which reflected the essential feature of *E. coli* cell wall including the information of fatty acids, membrane proteins and other amphiphilic molecules (*Muntean et al., 2014*). A broad peak at near 3417 cm^{-1} has been assigned to the stretching vibration of O-H, N-H from polysaccharides, fatty acids and proteins (*Goormaghtigh, Cabiaux & Ruysschaert, 1994*; *Muntean et al., 2014*). According to the above description, the cell displayed slightly different after the Sr^{2+} treatment (Fig. 7), which was consistent with the SEM observation. The differences of the relative intensity and the peak area mainly occurred at 1,537.01 cm^{-1}, 1,384.66 cm^{-1}, 1,220.74 cm^{-1} and 1,060.67 cm^{-1} among three cells, which implied that the cell surface structure including phospholipids and membrane

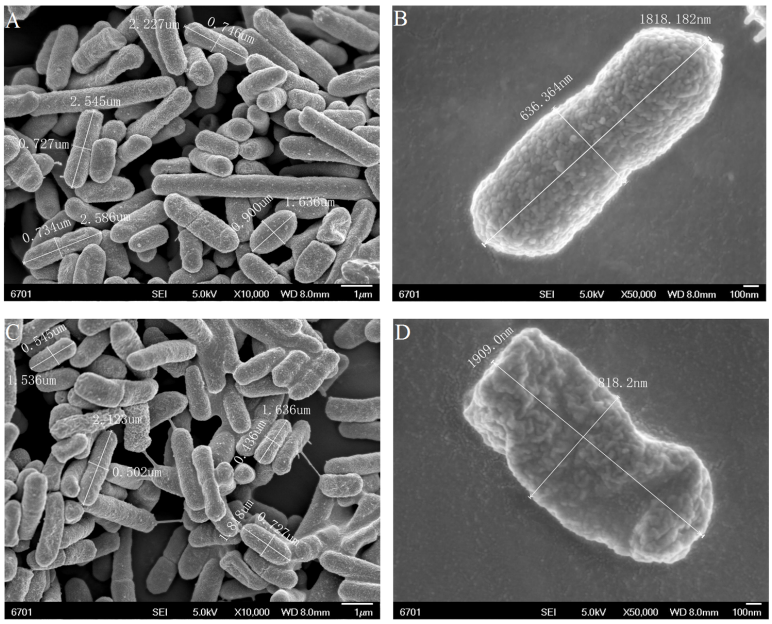

Figure 5 **The surface of the *E. coli* DH5α competent cells and *E. coli* DH5 α cells by SEM analysis.** The iced-drying *E. coli* DH5 α cells and competent cells were coated with a thin layer of gold under reduced pressure. The surface morphology of *E. coli* DH5 α cells at 10000× (A) and 50000× (B), that of competent cells at 10000× (C) and 50000× (D) observed at magnifications of 10000×∼50000× using a SEM system at a 10 kV acceleration voltage.

Full-size DOI: 10.7717/peerj.9480/fig-5

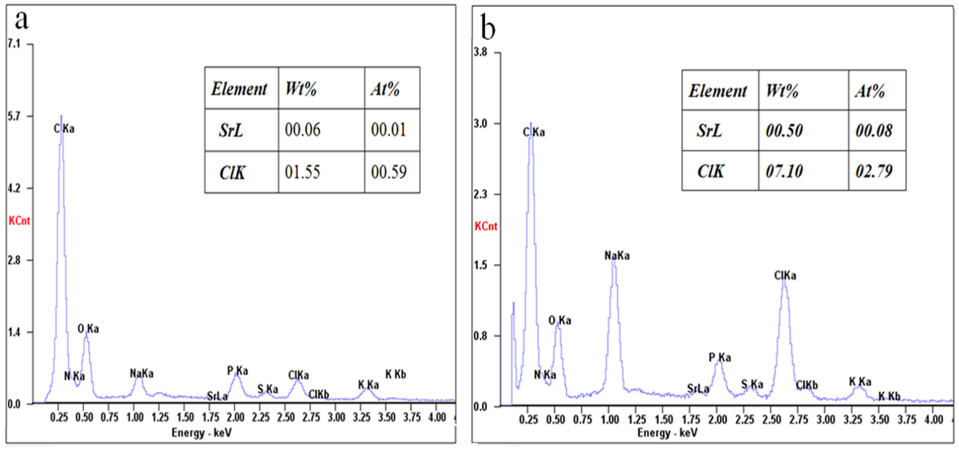

Figure 6 **The content of Sr^{2+} ions on the cell surface between the *E. coli* DH5α competent cells and *E. coli* DH5α cells by SEM-EDS analysis.** The iced-drying *E. coli* DH5α cells and competent cells were coated with a thin layer of gold under reduced pressure. The content of Sr^{2+} ions on the cell surface of the *E. coli* DH5α cells (A) and *E. coli* DH5α competent cells (B) was determined by SEM-EDS analysis.

Full-size DOI: 10.7717/peerj.9480/fig-6

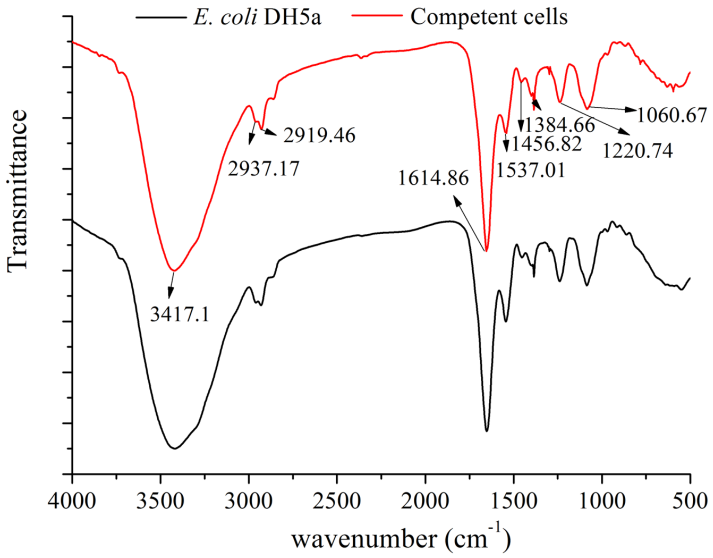

Figure 7 FT-IR of *E. coli* DH5α cells and competent cells. *E. coli* DH5α and competent cells prepared by $SrCl_2$ method was recorded by FT-IR spectrometer (Nexus670, Nicolet, American) with the range of 4,000–400 cm^{-1} at a resolution of four cm^{-1}.

Full-size DOI: 10.7717/peerj.9480/fig-7

proteins. The lipopolysaccharide could generate some changes by the combination with Sr^{2+}. The results were similar to those described in SEM-EDS and AAS analysis.

DISCUSSIONS

The development of competent cells is one of the key techniques for introducing foreign DNA into cells. Many literature have reported the development of chemo-transformation, physical-assisted transformation, electrotransformation and other new nanocarrier-mediated transformation (*Chan et al., 2013*; *Choi et al., 2013*; *Brito et al., 2017*; *Roychoudhury, Basu & Sengupta, 2009*; *Ren et al., 2019*). Among these methods, the chemical artificial transformation was regarded as the simple and efficient bacterial transformation technique. *E. coli* X1776 was optimally transformed by pBR322 DNA and a higher efficiency was obtained under the following conditions: 100 mM C MgCl2aCl_2 in 5 mM Tris buffer (250 mM KCl and 5 mM $MgCl_2$, pH 7.6) (*Nørgard, Keem & Monahan, 1978*). *Ren et al. (2017)*, described a chloride (RbCl)-based chemical-physical method that the best transformation efficiency for *E.coli* DH5α was obtained 4.3 × 10^6 CFU/μg of pUC19 plasmid, in which glycerol (2.6%), $MnCl_2$ (2.5 M), potassium acetate (0.1 M) and $CaCl_2$ (100 mM) was used as transformation solution. *Choi et al. (2013)* also exhibited a new bacterial transformation method by magnesium and calcium aminoclays, and a transformation efficiency of up to 2 ×10^5 CFU/μg pBBR122 in *E. coli* XL-1 and *Streptococcus mutans* (KCTC 3065). According to the abovementioned description, these divalent cations (Mg^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+}), especially Ca^{2+} ions played the most important roles in higher transformation efficiency and displayed different transformation efficiency for various strains. The important factors of competent cells preparation and transformation included

the growth state of bacteria, kinds of the metal ions, the concentration of the metal ions, duration of ice bath, incubation time at 42 °C and concentration of plasmid DNA, which had significant effect on the transformation efficiency.

Hence, more efficient methods should be established and employed for multiple microorganisms. In this study, the preparation of *E. coli* DH5α competent cells treated with $SrCl_2$ and the transformation by heat-shock with pUC19 plasmid were optimized by RSM. An equation of the regression model and the optimized value for generation of competent cells and transformation was obtained and was verified by the other five *E. coli* strains with different size of plasmids. The transformants approximately reached to 10^6 CFU/μg DNA, which was sufficient for the gene clone and expression. The ideal parameters to obtain maximum transformation efficiency in *E. coli* DH5α strain with pUC19 plasmid DNA was Sr^{2+} of 90 mM, heat-shock time of 90 s, the amount of pUC19 plasmid DNA of 9 ng. However, the precise details of transformation mechanisms remain vague (*Aich et al., 2012*; *Claverys & Martin, 2003*; *Wang, Sun & Ma, 2016*; *Ren et al., 2019*). Some researchers considered that cell membranes fluidity was weakened by using cations (e.g., Na^+, Mg^{2+}, Ca^{2+}, Ba^{2+}) and low temperature (ice-water bath). The phosphate groups and other negatively charged groups (hydroxyl and the carboxyl group) on the bacterial cell membrane could bind with these cations, that played a bridge to introduce the exogenous DNA to permeabilize the membrane to absorb plasmid DNA. Panja et al. summarized the cations form stable coordination compounds to promote DNA into the cells by eliminating electrostatic repulsion between DNA and lipo-polysaccharide (LPS) molecules on the outer membrane (*Panja et al., 2006*; *Panja et al., 2008*). As an amphiphile, DMSO or glycerin could further improve the efficiency, which might be due to the fact that amphiphiles promotes a better bond between the cation and the outer membrane, thereby facilitate DNA-membrane contact (*Hanahan, 1983*). While some other reports indicated the cations and cold-hot alternation in the process of transformation causes the cell membrane to transform into a more regular packing structure with a less fluid-like behavior. This solidified membrane may increase the permeability to DNA, which may be due to the complex forming of PHB/Ca^{2+}/PPi leading to small pores in the membrane structure (*Addison, Chu & Reusch, 2004*; *Huang & Reusch, 1995*; *Pavlov et al., 2005*). Meanwhile, the pores could be enlarged by adding of other bioorganic compounds, such as 10% ethanol (*Sarkar, Choudhuri & Basu, 2002a*; *Sarkar, Choudhuri & Basu, 2002b*; *Sharma, Singh & Gill, 2007*) and cyclodextrins (*Aachmann & Aune, 2009*). It was recommended to incorporate more unsaturated lipids into the membrane at lower temperatures to maintain membrane fluidity (*Aachmann & Aune, 2009*). Since the membrane is more flexible, this seems to relieve DNA uptake during the shock and recovery phases. In this paper, we preliminarily explored the transformation mechanism by $SrCl_2$ method. The results showed that the surface between *E. coli* DH5α and competent cells treated with 90 mM $SrCl_2$ appeared rough and wrinkled and shrank by SEM analysis. A part of Sr^{2+} was adsorbed on the cell surface, which implied that Sr^{2+} played a role in foreign DNA entering into the competent cells by the interaction between Sr^{2+} andthe cell surface. Also, FT-IR differences of the relative intensity and the area of the peak between *E. coli* DH5α and competent cells mainly occurred at 1,537.01 cm^{-1}, 1,384.66 cm^{-1},

1,220.74 cm^{-1} and 1,060.67 cm^{-1}, respectively. These wavenumbers were related to the phosphate group, hydroxyl and carboxyl group involved in phospholipids, membrane proteins, and lipopolysaccharide (LPS) on the cell surface. The results further were verified by SEM-EDS and AAS, some Sr^{2+} ions were adsorbed on the cell surface, and others may be entered into the cells. However, whether Sr^{2+} could enter into the cell, and why the higher transformation efficiency must be achieved by divalent metal ions such as Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} and Mn^{2+}, rather than K^{+}, Na^{+}, Co^{2+}, Zn^{2+} and Al^{3+}, these questions need to be further explored. Although many studies have shown that membrane proteins and environmental stress factors played important roles in the formation of competent cells and transformation, the mechanisms are still not well known until now. Based on all of these phenomena and conclusions, we also speculate that the transformation is not only a kind of microbial physiological phenomenon but also a physicochemical and biochemical process (*Yoshida & Sato, 2009*; *Aune & Aachmann, 2010*), in which complex regulatory mechanisms maybe existed (*Finkel & Kolter, 2001*; *Claverys & Martin, 2003*; *Aich et al., 2011*).

CONCLUSION

In this study, *E. coli* competent cells were prepared by the $SrCl^{2+}$ treatment, and the method was optimized by Response Surface Methodology (RSM). The differences between the normal cells and competent cells were analyzed by multispectral techniques, that revealed the minor changes occurred and strict regulatory mechanisms existed in *E. coli* cells.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Yonggang Wang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Xinjian Wang performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Linmiao Yu performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Yuan Tian and Shaowei Li analyzed the data, prepared figures and/or tables, and approved the final draft.
- Feifan Leng analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Jianzhong Ma and Jixiang Chen conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

Raw data is available as a Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.9480#supplemental-information.

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