



Optimization and mechanism exploration for *Escherichia coli* transformed with plasmid pUC19 by the combination with ultrasound treatment and chemical method

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ABSTRACT

As a basic technique of molecular cloning, bio-transformation has been successfully used in the fields of biomedicine and food processing. In this study, we established a transformation system of exogenous DNA into *E. coli* cells mediated by ultrasound. Under the optimal conditions (i.e. 35 °C, 40 W, 25 s, OD600 = 0.4–0.6) optimized by RSM, the transformation efficiency reached at 1.006×10^7 CFU/ μ g DNA. The results of membrane permeability, macromolecular substance and cell structure analysis before and after ultrasound treatment showed that the damage of host cells induced by lower (40 W) ultrasound and shorter ultrasound time (25 s) was reversible, and the transformation efficiency and cell survival rate were not significantly affected under this condition. In brief, proper changes in cell membrane and cell wall were the basic conditions for host cells to uptake exogenous DNA, while, whether exogenous DNA could be replicated and expressed in cells depends on the viability of host cells.

1. Introduction

As a fundamental technique of molecular cloning, bio-transformation was successfully used to produce desirable products such as industrial enzymes, bio-pharmaceuticals, etc. [1]. Efficient means by which to introduce DNA into bacteria is of great practical importance in bio-transformation. Previous studies have demonstrated that *Bacillus subtilis* [2], *Haemophilus influenza* and *Streptococcus pneumonia* [3] could uptake exogenous DNA naturally. However, the vast majority of prokaryotes (>99%) in natural environments are not culturable [4], and therefore could not show a natural transformation ability. Even if prokaryotes can be cultured in vitro, genetic manipulation is frequently impeded because of the lack of efficient, non-invasive and simple methods for DNA delivery. Therefore, artificial transformation such as biological methods (i.e., conjugation, gene transformation and transduction), physical methods (i.e., microinjection, particle bombardment, electroporation, laser irradiation and sonoporation), chemical methods (i.e., calcium chloride, strontium chloride, polyethyleneglycol/dimethyl sulfoxide broth, etc.) is especially

important to solve the above deficiencies.

Chemical method was first demonstrated by Mandel and Higa (1970) [5]. They found that *Escherichia coli* cells soaked in ice-cold calcium chloride solution were susceptible to uptake of bacteriophage DNA. Thereafter, several modifications had been expounded to simplify the process and improve the transformation efficiency. Chung et al. (1989) [1] introduced the method by using TSS, PEG, DMSO to prepare competent cells with only one step, and the efficiency was approximately $10^5 \sim 10^7$ CFU/ μ g DNA. The cetyl trimethyl ammonium bromide (CTAB) was also used to prepare *E. coli* competent cells and routinely yielded $10^5 \sim 10^9$ CFU/ μ g DNA [6]. Although these methods are effective in transferring exogenous DNA into bacterial competent cells, transformation in Gram-positive bacterial strains is a challenge, and little attention has been paid to the development of a universal method for prokaryotes and lower eukaryotes. To overcome these problems, ultrasound treatment was developed.

The cavitation effect produced by ultrasound, which physically generates reversible pores in the cell membrane [7], is the key for exogenous DNA transferring into host cells. Ultrasonic-mediated

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biotransformation was initially applied in eukaryotic cells [8] and gene therapy [9], and even tissue specific gene transformation in human cells can be achieved by artificially designed microvesicles [10]. Song et al., [11] reported that a standard low frequency (40 W) ultrasound clean bath can be used to successfully transferred plasmid pBBR1MCS2 to *Pseudomonas putida* UWC1, *Escherichia coli* DH5 α and *Pseudomonas fluorescens* SBW25, and with a delivery efficiency of $9.8 \pm 2.3 \times 10^{-6}$, $1.16 \pm 0.13 \times 10^{-6}$ and $4.33 \pm 0.78 \times 10^{-6}$ transformants per cell, respectively, which was significantly higher than the results of conjugation and even electroporation. Yang et al., [12] successfully deliver DNA/RNA, proteins (i.e. peptides, antigen, antibody, etc.), lipid, carbohydrate, viruses, small molecules (i.e. inorganic/organic small molecule compounds, molecular probe, gene therapy or medication) into bacillus, streptococcus, vinegar bacteria, clostridium, and successfully converted two or more kinds of Gram-positive bacteria mixed bacteria via ultrasound treatment. In brief, ultrasound is a well-established laboratory technique, in theory and practice, can deliver DNA/RNA to any type of host cells including bacteria [13], fungi, plants [14] and mammalian cells [15]. In addition, low frequency ultrasound can be used at room temperature and in a wide range of media, and allow for the application of ultrasound on large scales.

Response surface methodology (RSM) is a collection of statistical design and numerical optimization techniques used to optimize processes and product designs [16]. Since Box and Wilson developed RSM in 1950 s, it has been widely applied and developed, especially in chemical and biology fields. Guo et al., [17] reported that in the study of sodium alginate-polyethylene for enhancement of enzymatic hydrolysis of microcrystalline cellulose, the reducing sugar yield under the optimal conditions obtained via RSM was 133% of the original yield. In the study of extraction of flavonoids and phenolics from satsuma mandarin reported by Poyraz [18], RSM and particle swarm optimization (PSO) were respectively used to optimize experimental conditions to reduce the number of experiments and achieve optimum yield. The result showed that RSM gave higher satisfactory than PSO, and obtained the highest R-values for total flavonoids content (0.9825) and total phenolics content (0.9947). In addition, in the concrete industry, high-order RSM was used to develop a prediction model to accurately predict the compressive strength of high-strength concrete [19].

In this study, some basic ultrasound-mediated transformation conditions were speculated based on the physiological characteristics of *E. coli* DH5 α , and optimized by response surface methodology (RSM). In addition, a novel mode of AFM based on PeakForce quantitative Nano-mechanics (PF-QNM) measurement was used to evaluate the morphological and mechanical properties changes in *E. coli* induced by ultrasound, which provided more meaningful data for bio-transformation research.

2. Material and methods

2.1. Plasmid and culture conditions

E. Coli DH5 α and pUC19 were provided by laboratory of School Life Science Engineering, Lanzhou University of Technology, Gansu, China. The strain was cultured in Luria-Bertani (LB) medium at 37 °C. When cells carried plasmid pUC19, the growth medium was Super Optimal broth with Catabolite repression (SOC) supplemented and ampicillin (100 μ g/mL).

2.2. Transformation induced by ultrasound

2.2.1. Preparation of competent cells

The *E. coli* cells in logarithmic growth phase were centrifuged at 4500 rpm, 4 °C for 5 min to collect cell precipitation, then washed twice with saline and re-suspend with precooled CaCl₂ solution (0.1 mol/L)

2.2.2. Establishment of ultrasound-mediated transformation process

Plasmid pUC19 (0.8 ng/ μ L) were added to the cell suspension and then treated with ultrasound, thereafter SOC medium was added and the mixture was incubated at 37 °C for 1 h. 100 μ L of mixture was spread onto LB-Amp (100 μ g/mL) agar plate and incubated overnight at 37 °C to score for transformants. Each point was the average of 5 plates and calculated the transformation efficiency, which was defined as the number of transformants per 1 μ g of transforming DNA [11,20].

2.2.3. Verification of ultrasound-mediated transformation process

Single-colony from the LB-Amp plate described in step 2.2.2 was randomly selected for colony polymerase chain reaction (PCR) to verify whether the plasmid pUC19 was successfully transfer to *E.coli* DH5 α . The PCR product was detected by 1% agarose gel electrophoresis, and if the product size was consistent with the expectation, it could be proved that the transformation process in this experiment could be used for ultrasonic-mediated transformation of plasmid pUC19 into *E. coli* DH5 α . The PCR condition was shown in Table S1.

2.3. Optimization of response surface methodology (RSM)

The transformation efficiency and host cells survival rates were affected by ultrasonic power (W), ultrasonic time (s), ultrasonic temperature (°C), concentration of recipient cells (OD₆₀₀) [11]. To investigated above factors for this study, the following experiments were carried out.

- 1) The temperature was set as 35 °C, the ultrasonic power was set as 40, 60, 80, 90, 100 W, respectively;
- 2) The temperature was set as 35 °C, the ultrasonic power was set as 40 W, the ultrasonic time was set as 10, 30, 40, 60, 90 s, respectively;
- 3) The temperature was set as 35 °C, the ultrasonic power was set as 40 W, the ultrasonic time was set as 30 s, concentration of recipient cells was 0 ~ 0.2, 0.2 ~ 0.4, 0.4 ~ 0.6, 0.6 ~ 0.8, 0.8 ~ 1.0 respectively;
- 4) The ultrasonic power was set as 40 W, the ultrasonic time was set as 30 s, concentration of recipient cells was 0.4 ~ 0.6, the temperature was set as 25, 35, 45, 55, 65 °C, respectively. Three replicate experiments (samples) were conducted at each parameter.

Taking single factor experiments as index, the optimal ultrasound transformation efficiency of *E. coli* DH5 α with plasmid pUC19 was determined by RSM which designed according to four factors and three levels orthogonal table. Transformation efficiency as response value calculated by $Z_i = (X_i - X_0)/X_i$, studied on four degrees of freedom such as: ultrasonic temperature (X_1), ultrasonic time (X_2), ultrasonic power (X_3), concentration of bacterium (X_4). Z_i and X_i represent the variable of codes and actual values, X_0 represents the center of the coding level, X represents the change of gradient between the actual adjacent levels. Each level sets three gradients -1, 0, 1 which represent the lowest, middle and highest respectively. Each factor and level are presented in Table 1. Five replicate experiments were conducted at the same circumstances and for the error analysis.

The relationship between the transformation efficiency (Y) and independent variables was analyzed by the second-order polynomial equation which can be given as:

Table 1

The factors and level of transformed with *Escherichia coli* DH5 α and pUC19.

| Factors | Levels | | |
|--------------------------------------|--------|------|------|
| | -1 | 0 | 1 |
| X_1 Temperature (°C) | 30 | 35 | 40 |
| X_2 Time (s) | 25 | 30 | 35 |
| X_3 Power (W) | 40 | 50 | 60 |
| X_4 Bacterium (OD ₆₀₀) | 0.35 | 0.45 | 0.55 |

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=1}^3 \beta_{ij} X_i X_j$$

where parameters β_0 , β_i , β_{ii} and β_{ij} are called the regression coefficients. The quality of the fitted model was expressed by the coefficient of determination (R^2), and statistical significance was evaluated based on the p value and F value [21,22].

2.4. Effect of different ultrasonic conditions on *E. coli* DH5 α membrane permeability

2.4.1. The effect of outer membrane permeability

The N-phenyl-1-naphthylamine (NPN) assay [23] was used to analyze the outer membrane (OM) permeation activity of *E. coli* DH5 α under different ultrasound conditions. The *E. coli* DH5 α cells with logarithmic phase were treated with ultrasound under the conditions described in 2.3. Thereafter, adding NPN solution with a final concentration of 10 $\mu\text{mol/L}$ to the ultrasonic treatment solution, and then slight oscillation at 37 $^\circ\text{C}$ for 2 min. The fluorescence value was measured by fluorescence spectrophotometer with excitation and emission wavelengths of 350 nm and 420 nm. Three replicate experiments were used for all statistical analysis.

2.4.2. The effect of inner membrane permeability

The inner membrane (IM) permeation was determined by measuring the activity of the β -galactosidase [24] released by *E. coli* DH5 α . The logarithmic phase *E. coli* DH5 α cells, which had been cultured in LB medium containing 2% lactose, were harvested. Washed twice and re-suspended in 0.85% NaCl solution, then treated with ultrasound under the conditions described in 2.3. Thereafter, adding ONPG solution with a final concentration of 1.5 mmol/L to the ultrasonic treatment solution, and then slight oscillation at 37 $^\circ\text{C}$ for 2 min. The absorption value was measured by Spectrophotometer with 415 nm. Three replicate experiments were used for all statistical analysis.

2.4.3. Flow cytometry experiments

To verify the ultrasonic effect on membrane permeability of *E. coli* DH5 α , flow cytometry (FCM) was used to evaluate cell viability and membrane permeability [25]. The tested *E. coli* cells were cultivated in LB medium to logarithmic-phase previously, then collected, washed twice with distilled water and re-suspended in distilled water. Cell suspensions without ultrasonic treatment were used as controls, the experimental group was treated by ultrasound as the following conditions: 40 W, 35, 25 s, $\text{OD}_{600} = 0.4 \sim 0.6$. Thereafter, the samples were stained with a final concentration of 1 mg/mL of propidium iodide (PI), and left in the dark at room temperature for 10 min. FCM analysis was performed on a FACSAria II flow cytometer (BD, USA), the red fluorescence (PI) was captured through a 670 nm long-pass filter. Samples were acquired in a flow rate of 200 cells/min, with 10,000 events being acquired per sample. The software used for data acquisition and analysis was FACSAria II (BD, USA).

2.4.4. Fluorescence microscope observation

The fluorescein diacetate (FDA) staining method was used to characterize the effect of ultrasound on cell membranes. FDA is a non-fluorescent hydrophilic substance, it will show green fluorescence when it enters the cytoplasm and reacts with water molecules [26]. Logarithmic-phase *E. coli* DH5 α cells were ultrasonically treated under the condition of 40 W, 35, 25 s, $\text{OD}_{600} = 0.4 \sim 0.6$. The cells were washed twice and re-suspended in 0.85% NaCl solution. Thereafter FDA solution with the final concentration was 0.25 mg/mL was added to the cell suspension according to the standard of 1×10^6 bacteria/mL, and then fluorescent imaging after incubated at 20 $^\circ\text{C}$ for 5 min.

2.5. The effect of ultrasound on the structure of *E. coli* DH5 α

2.5.1. Characterization of *E. coli* DH5 α cells structure before and after ultrasonic treatment by scanning electron microscopy

Logarithmic-phase *E. coli* cells were obtained at 37 $^\circ\text{C}$, washed twice and re-suspended in PBS buffer. The cell suspensions were divided into two groups and treated by ultrasound or not, then collected by centrifugation and fixed in 2.5% glutaraldehyde for 2 h. Cell pellets were re-suspended in sterile water and washed with alcohol (30, 50, 70, 80, 90, 100%), tertiary butanol in turn, then re-suspended in tertiary butanol solution (10^5 CFU). Finally, the sample was dried, placed in the grid-chamber and stored in desiccators before observation by scanning electron microscope (SEM, JSM-6701F) [27].

2.5.2. Characterization of *E. coli* DH5 α cells structure before and after ultrasonic treatment by atomic force microscope

To further evaluate the effects of ultrasound on bacterial cells, high-resolution imaging and mechanical measurement were carried out using atomic force microscope (AFM). Logarithmic-phase *E. coli* cells centrifuged at 4000 rpm for 5 min, washed with 10 mM phosphate buffer solution (PBS) in pH 7.0, and re-suspended in PBS, fixed in 2.5% glutaraldehyde for 1 h at room temperature. The *E. coli* DH5 α were divided into two samples and treated by ultrasound or not, then transferred 20 μL solution to coverslip. Images of the cells were visualized by the AFM (AFM, Agilent 5500 ILM), the scanning areas were chosen as 5 $\mu\text{m} \times 5 \mu\text{m}$. The height and size information was acquired by imaging software from Nova. The images were obtained in the deflection mode for all the samples [28].

2.6. Analysis of macromolecule content of *E. coli* DH5 α cells before and after ultrasonic treatment

Macromolecules such as proteins, phospholipid, alkaline phosphatase are the material basis for maintaining the structural integrity and ensuring the physiological characteristics of cells. In order to research the effect of ultrasonic on cell inclusions, proteins, phospholipid and alkaline phosphatase content were analyzed of *E. coli* DH5 α . The condition of ultrasound treatment was: a:untreated;b:35 $^\circ\text{C}$,30 s,40 W; c:35 $^\circ\text{C}$,30 s,100 W;d:35 $^\circ\text{C}$,120 s,40 W;e:35 $^\circ\text{C}$,120 s,100 W. Three replicate experiments were used for all statistical analysis, respectively.

2.6.1. Analysis of protein content of *E. coli* DH5 α cells before and after ultrasonic treatment

Logarithmic-phase *E. coli* DH5 α cells were washed thrice and re-suspended in sterile water and the suspension was treated according to the condition list as a-e. Protein concentration was determined by the Bradford protein assay which depends upon the binding of Coomassie brilliant blue G-250 dye to protein to produce a complex absorbing at 595 nm, and measured with BSA as standard [29].

2.6.2. Analysis of phospholipid content of *E. coli* DH5 α cells before and after ultrasonic treatment

Samples were prepared as described in 2.7.1. Thereafter, 0.5 mM 1,6-Diphenyl-1,3,5-Hexatriene (DPH) was added into the suspension. The sample was diluted 15 times with 100 mM CaCl_2 , incubated in the dark at room temperature for 40 min, and the baseline fluorescence was measured using excitation and emission wavelengths of 350 nm and 440 nm, respectively [30].

2.6.3. Analysis of alkaline phosphatase content of *E. coli* DH5 α cells before and after ultrasonic treatment

Enzyme activity was investigated using the chromogenic substrate p-nitrophenylphosphate (pNPP) [31]. Logarithmic-phase *E. coli* cells were grown in tris-glucose medium at 37 $^\circ\text{C}$ and centrifuged at $5000 \times g$ for 10 min, washed thrice in non-phosphorus Tris-glucose, and incubated in non-phosphorus tris-glucose medium at 37 $^\circ\text{C}$ for 40 min. The sample

was washed twice and re-suspended in sterile water, and treated as previously described. The reaction mixture containing 1.0 mL of sample and 2.0 mL of substrate (pNPP 200 mM, final concentration 40 mM in 100 μ L) was pipetted in microtitre plates and incubated at 37 °C for 15 min. The formation of the yellow dephosphorylated product was monitored by measuring the absorbance at 420 nm.

3. Results and discussion

3.1. Verification of the presence of the transgenes by colony PCR

Any four single colonies on the LB-Amp plate (Fig. 1A) were identified by colony PCR. The PCR product sizes of the four single colonies were in the range of 1500–2000 bp (Fig. 1B), which were consistent with the expected size of 1760 bp, indicating that the single colony in the plate was the transformant of plasmid pUC19 to *E. coli* DH5 α , which further proved that the ultrasonics-mediated transformation system in this study was effective.

3.2. Effects under different processing conditions

3.2.1. Effect of the ultrasonic power on transformation efficiency and host cells survival rates

Lower ultrasonic power (40 W) seemed to be more conducive to plasmid transformation and host cells survival (Fig. 2A). The effect of ultrasonic power on transformation efficiency and host cell survival rate are consistent, both decreased significantly with the gradual increasing of ultrasonic power. Whether exogenous DNA can enter the host cell depends on the cavitation formed on the cell surface by ultrasound [32]. While the physical wave acts in the host cell, the permeability of the membrane changes, and it is reversible at low ultrasonic power with little or no damage, the host cell can be restored to its original state after brief repair. On the contrary, the damage is permanent while in strong physical wave generated by high ultrasonic power [33]. Meanwhile, the integrity of exogenous DNA could also be affected, which will further affect the transformation efficiency and the host cell survival rate [32,34].

3.2.2. Effect of the ultrasonic temperature on transformation efficiency and host cells survival rates

In accordance with the growth of the strain, the transformation efficiency was the highest when the ultrasonic temperature was 35 °C (Fig. 2B). Obviously, at the high temperature of 65 °C, it would cause cell death due to exceed its own tolerance, and the ability of uptake exogenous DNA was significantly reduced.

3.2.3. Effect of the ultrasonic time on transformation efficiency and host cells survival rates

With the increase of ultrasonic time, the survival rate of host cells decreased obviously, while the transformation efficiency was presented inverted “V” state, and reaching the maximum at 30 s (Fig. 2C). Under the ultrasound was applied for 30 s, the host cell membrane permeability may only allow the exogenous DNA to enter the cystic and ensure the high replication and expression of pUC19 in host cells with little or no damage to the cell. While the ultrasonic time was <30 s, the change of host cell membrane permeability couldn't provide ion channels for exogenous DNA to enter into cystolic, resulting in the decrease of transformation efficiency [34]. Obviously, longer ultrasonic time (>30 s) will lead to the death of host cells and the damage of plasmid DNA, thus reducing the transformation efficiency [7].

3.2.4. Effect of the concentration of bacterium on transformation efficiency and host cells survival rates

As shown in Fig. 2D, both the lower optical density ($OD_{600} = 0-0.2$) and the higher optical density ($OD_{600} = 0.8-1.0$) resulted in a decreased number of transformant, the effect of bacterial concentration on transformation efficiency is presented inverted “V” state with the highest transformation efficiency at $OD_{600} = 0.4-0.6$. Due to optimal metabolism and physiological function of logarithmic-phase ($OD_{600} = 0.4-0.6$) cells, the *E. coli* DH5 α could uptake exogenous DNA with higher efficiency and provide a better environment for exogenous DNA to replicate and express [35]. On the contrary, when the concentration was higher than $OD_{600} = 0.4-0.6$, the cells were in stationary phase and gradually entered the decline phase, their metabolic and physiological functions gradually declined, and their ability to absorb nutrients also decreased, which resulted in the high-quality of exogenous DNA replication and affected the final expression.

3.3. Optimization of ultrasound-mediated transformation process by RSM

The experimental design matrix and the measurement responses were shown in Table 2, which were consisted of five repetitions at the central points and used in the second-order polynomial model. Through primary tests, the transformation efficiency was achieved 1.006×10^7 CFU/ μ g DNA at 35 °C, 40 W, $OD_{600} = 0.4 \sim 0.6$, 25 s. The purpose of ANOVA was to investigate whether process parameters and the interaction among these parameters had a significant impact on transformation efficiency, and to determine whether the model is meaningful [21].

The ANOVA of the proposed model and the corresponding *p* and *F* values for assessing the significance of the regression coefficients were shown in Table 3. A model is considered the most significant if the *p* (significance probability value) < 0.001, and the model terms are

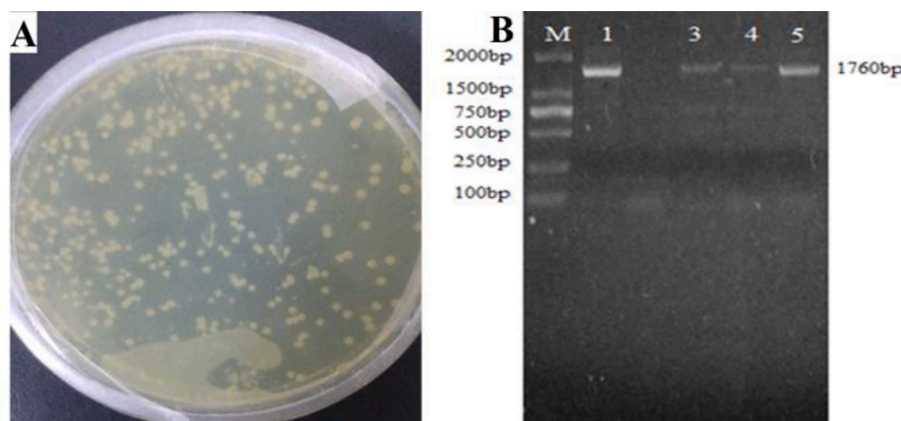


Fig. 1. The transformant on the LB agar medium with ampicillin (A) and verification of the amplified PCR product by agarose gel electrophoresis (B).

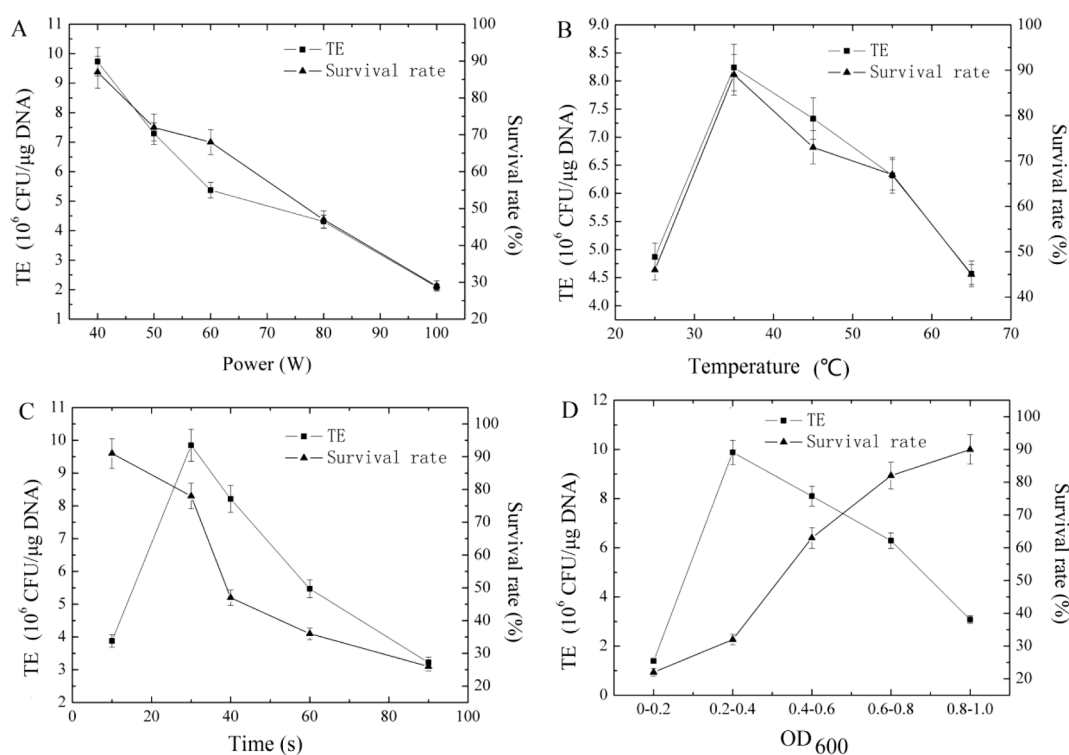


Fig. 2. Effect of different treatment conditions on transformation efficiency and survival rate. Note: Fig. A denotes the effect of ultrasonic power (40 W, 50 W, 60 W, 80 W, 100 W) on transformation efficiency and survival rate; Fig. B denotes the effect of temperature (25, 35, 45, 55, 65 °C) on transformation efficiency and survival rate; Fig. C denotes the effect of time (10 s, 30 s, 40 s, 60 s, 90 s) on transformation efficiency and survival rate; Fig. D denotes the effect of OD_{600} (0 ~ 0.2, 0.2 ~ 0.4, 0.4 ~ 0.6, 0.6 ~ 0.8, 0.8 ~ 1.0) on transformation efficiency and survival rate.

Table 2

Experimental design in terms of coded factors and results of the central composite design.

| Run number | Temperature(°C) | Ultrasonic time (s) | Ultrasonic power(W) | Concentration of bacterium (OD_{600}) | Transformation efficiency (CFU/ μ g DNA) |
|------------|-----------------|---------------------|---------------------|---|--|
| 1 | 0 | 0 | 1 | 1 | 0.99×10^6 |
| 2 | 0 | 0 | -1 | -1 | 1.04×10^6 |
| 3 | 0 | 1 | 0 | 1 | 2.17×10^6 |
| 4 | -1 | -1 | 0 | 0 | 3.78×10^6 |
| 5 | 1 | 0 | 1 | 0 | 2.88×10^6 |
| 6 | 1 | 0 | 0 | -1 | 1.06×10^6 |
| 7 | 0 | 1 | -1 | 0 | 7.28×10^6 |
| 8 | 1 | -1 | 0 | 0 | 5.08×10^6 |
| 9 | -1 | 0 | 0 | 1 | 4.47×10^6 |
| 10 | 0 | -1 | 1 | 0 | 3.11×10^6 |
| 11 | 0 | 0 | 1 | -1 | 0.76×10^6 |
| 12 | 0 | -1 | -1 | 0 | 1.006×10^7 |
| 13 | -1 | 0 | -1 | 0 | 7.88×10^6 |
| 14 | -1 | 1 | 0 | 0 | 5.28×10^6 |
| 15 | 0 | -1 | 0 | 1 | 7.2×10^6 |
| 16 | 0 | 1 | 0 | -1 | 1.08×10^6 |
| 17 | 0 | -1 | 0 | -1 | 0.94×10^6 |
| 18 | -1 | 0 | 0 | -1 | 0.84×10^6 |
| 19 | 1 | 0 | 0 | 1 | 4.7×10^6 |
| 20 | 1 | 0 | -1 | 0 | 6.01×10^6 |
| 21 | 0 | 0 | -1 | 1 | 9.02×10^6 |
| 22 | -1 | 0 | 1 | 0 | 2.88×10^6 |
| 23 | 0 | 1 | 1 | 0 | 1.19×10^6 |
| 24 | 1 | 1 | 0 | 0 | 3.11×10^6 |
| 25 | 0 | 0 | 0 | 0 | 7.29×10^6 |
| 26 | 0 | 0 | 0 | 0 | 7.2×10^6 |
| 27 | 0 | 0 | 0 | 0 | 7.17×10^6 |
| 28 | 0 | 0 | 0 | 0 | 7.36×10^6 |
| 29 | 0 | 0 | 0 | 0 | 8.15×10^6 |

statistically significant if the associated $0.001 < p < 0.005$ [21]. The ANOVA study reveals that X_3 (ultrasonic power) ($p < 0.0001$, $SS = 72.42$, $F = 108.88$) and X_4 (concentration of bacterium) ($p < 0.0001$, $SS = 43.43$, $F = 65.30$) had the most significant effect on transformation

efficiency; X_2 (ultrasonic time) ($p = 0.0031$) had significant effect on transformation efficiency; X_1 (temperature) ($p = 0.4312$) had comparatively less significant effect on transformation efficiency. Thus, it is necessary to control the value of X_3 , X_4 to balance the relationship

Table 3
Regression coefficients of the predicated quadratic polynomial model.

| Term | Sum of squares | Degrees of freedom | Mean square | F value | P value Probe > F | Significance |
|-------------------------------|----------------|--------------------|-------------|---------|-------------------|--------------|
| Model | 229.28 | 14 | 16.38 | 24.62 | < 0.0001 | *** |
| X ₁ | 0.44 | 1 | 0.44 | 0.66 | 0.4312 | |
| X ₂ | 8.43 | 1 | 8.43 | 12.68 | 0.0031 | * |
| X ₃ | 72.42 | 1 | 72.42 | 108.88 | < 0.0001 | *** |
| X ₄ | 43.43 | 1 | 43.43 | 65.30 | < 0.0001 | *** |
| X ₁ X ₂ | 3.01 | 1 | 3.01 | 4.53 | 0.0516 | |
| X ₁ X ₃ | 0.87 | 1 | 0.87 | 1.31 | 0.2708 | |
| X ₁ X ₄ | 3.31 | 1 | 3.31 | 7.26 | 0.9952 | |
| X ₂ X ₃ | 0.18 | 1 | 0.18 | 0.28 | 0.6063 | |
| X ₂ X ₄ | 6.68 | 1 | 6.68 | 10.05 | 0.0068 | * |
| X ₃ X ₄ | 15.02 | 1 | 15.02 | 22.57 | 0.0003 | ** |
| X ₁ ² | 16.33 | 1 | 16.33 | 24.56 | 0.0002 | ** |
| X ₂ ² | 10.93 | 1 | 10.93 | 16.43 | 0.0012 | * |
| X ₃ ² | 5.80 | 1 | 5.80 | 8.72 | 0.0015 | * |
| X ₄ ² | 70.61 | 1 | 70.61 | 106.16 | < 0.0001 | *** |
| residual | 9.31 | 14 | 0.67 | | | |
| Lack of fit | 8.65 | 10 | 0.86 | 5.19 | 0.0633 | |
| Pure error | 0.67 | 4 | 0.17 | | | |
| Cor total | 238.59 | 28 | | | | |

between the X₃, X₄.

The interaction effect of temperature, duration of exposure (s), ultrasonic power (W), concentration of bacterium (OD₆₀₀) on the transformation efficiency was shown in Fig. 3. The inclination of the surface reflects the influence of the interaction of two factors, and the shape of the contour can reflect the intensity of the interaction. Oval represents the interaction of the two factors is significant, while circular instead [17,21]. The interaction between the duration of exposure and the concentration of bacterium or with the ultrasonic power, the ultrasonic power and the concentration of bacterium all have a significant effect on the transformation efficiency. In contrast, the interaction between temperature and duration of exposure or with ultrasonic power or with concentration of bacterium has little effect on transformation efficiency.

3.4. Analysis of changes in *E. Coli* DH5 α cell membrane permeability under ultrasonic treatment

3.4.1. The changes of OM permeability of *E. Coli* cell under different ultrasonic treatment

The fluorescence intensity of hydrophobic fluorescence reagent NPN

is very low in aqueous solution. Once it reaches the hydrophobic environment of the outer membrane of cells, its fluorescence value will surge [23,36]. The change of OM permeability and the corresponding transformation efficiency under different ultrasonic treatment conditions were shown in Fig. 4. Obviously, the increased OM permeability of the cells was one of the conditions for exogenous DNA to enter the host cells, but it did not mean that the more exogenous DNA were uptaken, the stronger the replication and expression ability of exogenous DNA. Although a large number of NPN enter into the hydrophobic environment of the OM of the cell will show strong fluorescence under the longer ultrasonic time (> 30 s) and the higher frequency (> 40 W), the structural integrity of the cells has been irreversibly damaged in this case. Even if more exogenous DNA could enter the cells, its replication and expression in the host cell were limited, so it shown a low transformation efficiency (Fig. 4A&4C). There was no significant effect of temperature on the permeability of the OM of cells, but the transformation ability reached the maximum under the optimal growth temperature (Fig. 4B). The higher of OD₆₀₀ value, the higher the number of cells per unit volume, and the more obvious of the change of the permeability of OM of the cell, but the transformation efficiency was

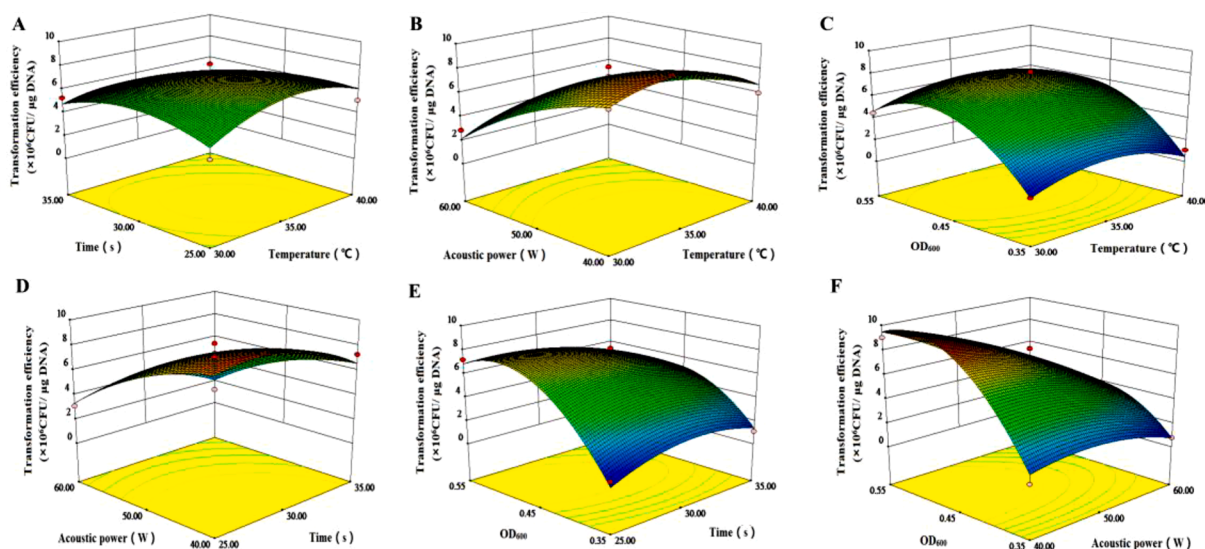


Fig. 3. Interaction effect of temperature (°C), duration of exposure (s), ultrasonic power (W), concentration of bacterium (OD₆₀₀) on the transformation efficiency. Note: A denotes the effect of time and temperature on the transformation efficiency; B denotes the effect of ultrasonic power and temperature on the transformation efficiency; C denotes the OD₆₀₀ and temperature on the transformation efficiency; D denotes the effect of ultrasonic power and time on the transformation efficiency; E denotes the effect of OD₆₀₀ and time on the transformation efficiency; F denotes the effect of OD₆₀₀ and ultrasonic power on the transformation efficiency.

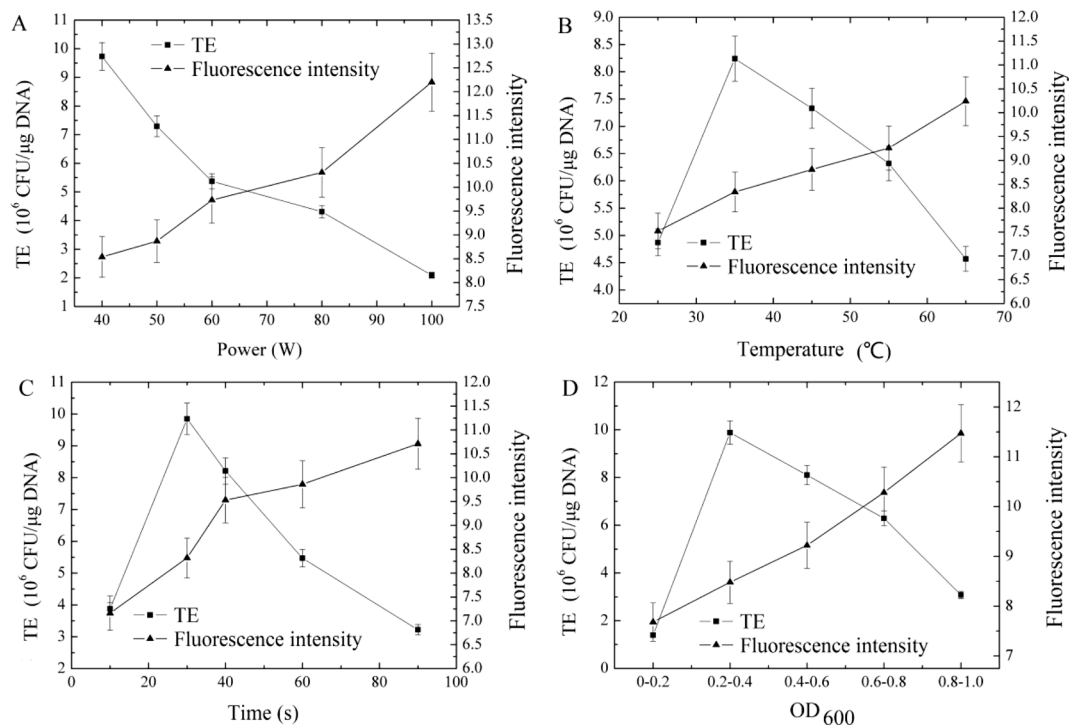


Fig. 4. The Change of outer membrane permeabilization of *E. coli* cell under the different treatments (Note: Fig. A denotes the effect of temperature on the outer membrane permeabilization of *E. coli* cell; Fig. B denotes the effect of ultrasonic power on the outer membrane permeabilization of *E. coli* cell; Fig. C denotes the effect of time on the outer membrane permeabilization of *E. coli* cell; Fig. D denotes the effect of the OD₆₀₀ on the outer membrane permeabilization of *E. coli* cell.)

the highest only in the logarithmic growth phase, which depends on the growth and metabolism process of *E. coli* (Fig. 4D).

3.4.2. The changes of inner membrane (IM) permeability of *E. Coli* cell under different ultrasonic treatment

ONPG as a colorless but chromogenic substrate, when action with β-galactosidase, its product ONP appears yellow and has a strong

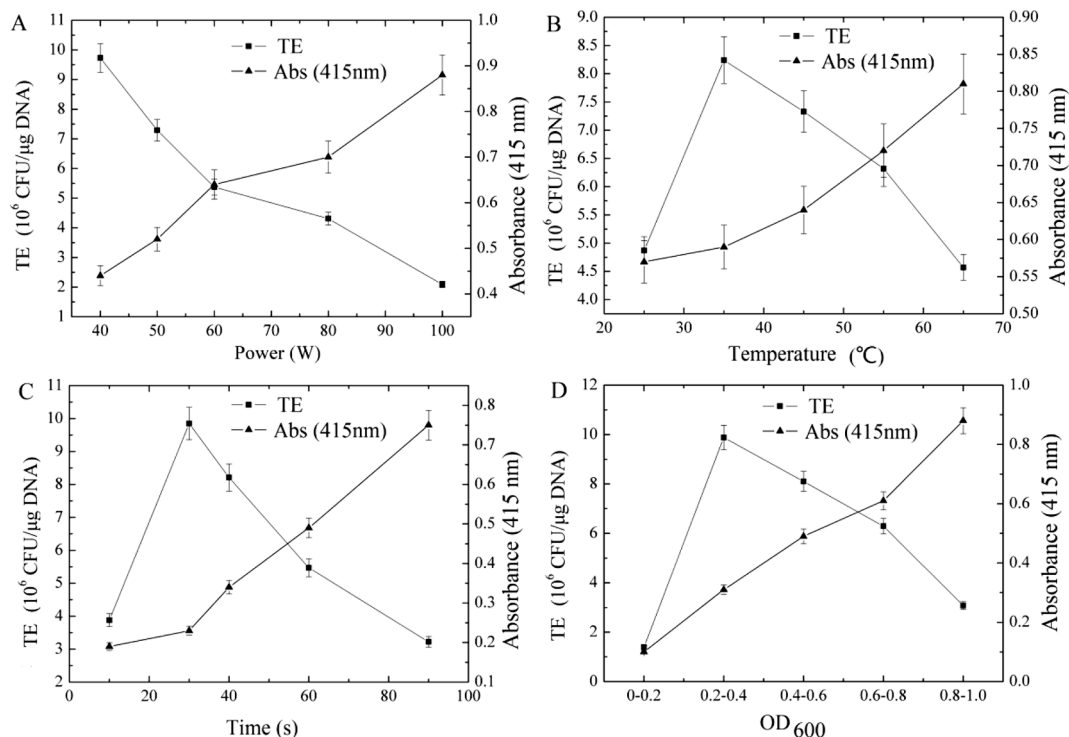


Fig. 5. Inner membrane permeabilization change of *E. coli* cell under the various treatments. (Note: Fig. A denotes the effect of temperature on IM permeabilization change of *E. coli* cell; Fig. B denotes the effect of ultrasonic power on IM permeabilization change of *E. coli* cell; Fig. C denotes the effect of time on IM permeabilization change of *E. coli* cell; Fig. D denotes the effect of the OD₆₀₀ on IM permeabilization change of *E. coli* cell.)

absorption peak at 415 nm [24]. β -galactosidase exists inside the cells of *E. coli* with intact IM. Once the IM structure is broken, β -galactosidase will leak into the surrounding media [37]. Similar to the change trend of OM, both the time and frequency of ultrasound had significant effects on the integrity of cell IM (Fig. 5A and C). The reason why temperature and cells concentration had no significant effect is that they are the necessary factors for cell growth and the embodiment of metabolic capacity (Fig. 5B and D). Furthermore, there was no correlation between the change of membrane permeability and transformation efficiency, which may be because the effect of ultrasound on host cells to mediate the entry of exogenous DNA into the body is not only the result of the change of membrane permeability, but also the change of membrane permeability to remove the obstacle and barrier for the entry of exogenous DNA into host cells [11].

3.4.3. The membrane integrity detection by FCM

The PI single staining is mainly based on the characteristic changes that occur at the cellular, subcellular and molecular levels during apoptosis [25]. These changes included changes in the nucleus, organelles, cell membrane composition and cell morphology, among which the changes in the nucleus were the most characteristic [38]. During cell apoptosis, due to the degradation of chromosomes and the formation of nuclei during apoptosis, intracellular particles tend to increase, the side scattered light of apoptotic cells often increased [25]. Obviously, the intensity of side scattered light of cells treated with ultrasound is significantly higher than that of cells without ultrasonic treatment (Fig. 6A&6D; Fig. 6C & 6F), which showed by green fluorescence. In addition, small fragments of DNA will be lost through the membrane when the cell membrane permeability increased, which lead to the intracellular DNA content decreased, and resulted in a decrease of fluorescence intensity. The peak fluorescence intensity of cells ($\times 600$) (Fig. 6E) treated with ultrasound was significantly lower than that of untreated cells ($\times 800$) (Fig. 6B), which was fully proved that the permeability of the cell membrane was significantly increased after

ultrasonic treatment.

3.4.4. Analysis of membrane permeability with fluorescence microscope

Fluorescein diacetate (FDA) is a polar substance with fluorescence due to the decomposition of lipase after entering the protoplast, which can be detected by fluorescence microscopy [29]. Obviously, FDA successfully enter the cytoplasm and decompose into a polar fluorescent after ultrasonic treatment of cells (Fig. 7B), which was opposite with the control (Fig. 7A).

3.5. *E. Coli* cell structure observation by SEM and AFM

The structure of untreated cells were intact and well-defined, showing a smooth and dense surface, few notable ruptures or pores on the cell surface, without releasing intracellular components (Fig. 7C). While, the ultrasonic treated cells exhibits the significant abnormalities, the surface of the cells was deeply rough and collapsed, the cell brightness dimmed (Fig. 7D). This might due to the destruction of the cell wall structure by ultrasound, which causing wrinkles and bumps on the cell surface, changes of the permeability, and overflow of intracellular substances such as water and macromolecular substances [36,39]. The results of AFM observation were shown in Fig. 7E-H. The 3D images of AFM were described in Fig. 7E&G, and the height mode images were described in Fig. 7F&H, respectively. The results showed that untreated *E. coli* DH5 α cells had a smooth and featureless surface morphology (Fig. 7E), and the corresponding refraction height was low (Fig. 7F). After the ultrasonic treatment, because of the change in the membrane permeability, a large amount of intracellular substances such as proteins, phospholipids, AP were released, the surface morphology changed seriously (Fig. 7F,H), it is sparse and uneven, with corresponding low height protrusion.

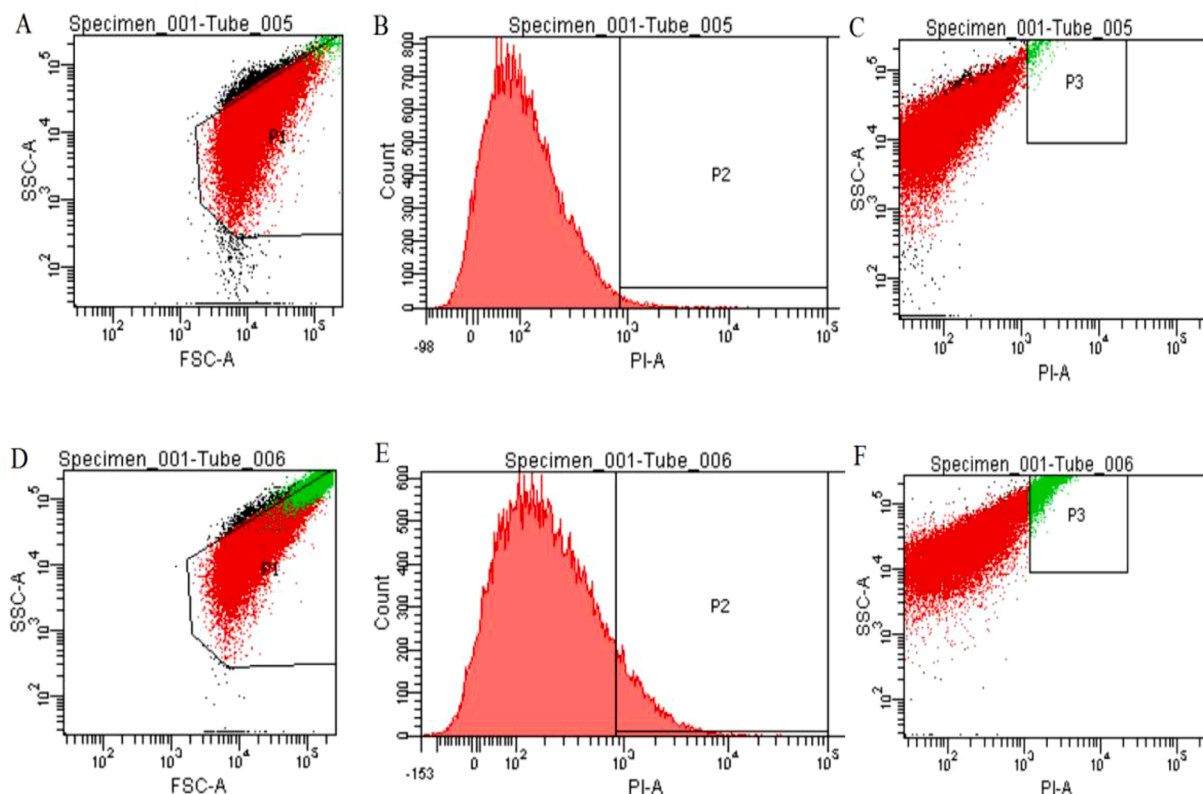


Fig. 6. The result of the reaction between PI and nucleic acid (Note: Fig. A-C denote cell without ultrasonic treatment; Fig. D-F denote cell with ultrasonic treatment).

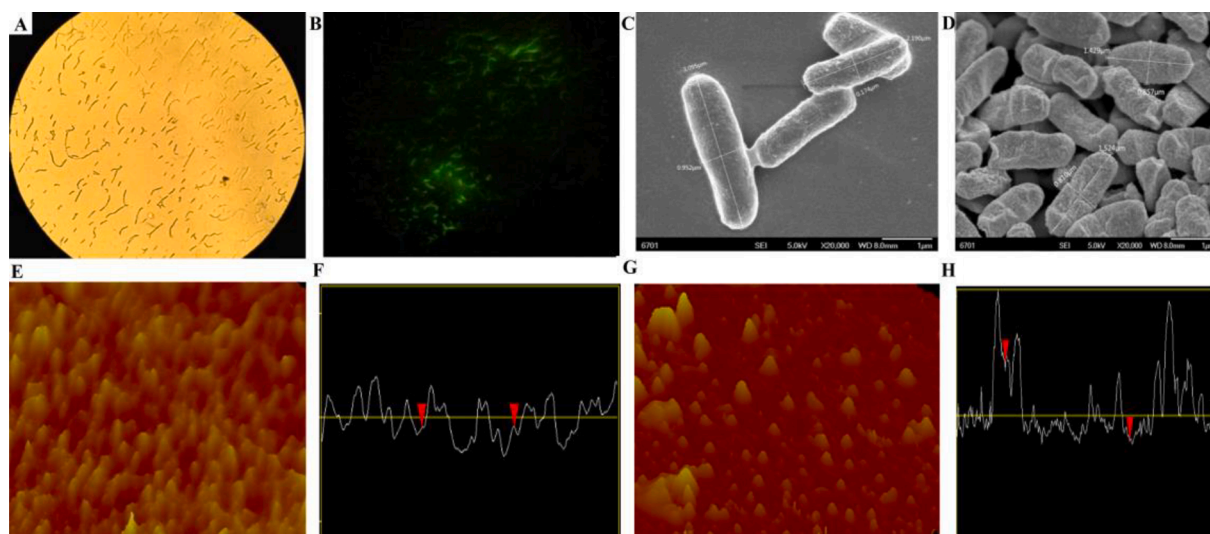


Fig. 7. The result of the reaction between FDA and *E. coli* DH5 α (A&B, 40 \times), Scanning Electron Microscope images (C&D) and Atomic Force Microscope images of cell with different treatments (E&F&G&H) (A denotes cell without ultrasonic treatment; B denotes cell with ultrasonic treatment; C denotes cell without ultrasonic treatment; D denotes cell with ultrasonic treatment; E, F denotes cell without ultrasonic treatment; G, H denotes cell with ultrasonic treatment).

3.6. Effect of ultrasound on the content of bioactive macromolecules in *E. coli* cells

The effects of different ultrasonic treatments on protein, phospholipid and alkaline phosphatase (AP) of *E. coli* DH5 α were shown in Fig. 8. The protein, phospholipid and AP were almost undetectable in culture medium without ultrasonic treatment, while when the cells were stimulated by ultrasound, three macromolecules released rapidly from the inside to the outside of the cells (Fig. 8). Similar to the changes of the permeability of the inner and outer membrane of cells (Fig. 4&Fig. 5), ultrasound frequency and time had significant effects on cell membrane and cell wall, resulting in the release of protein, phospholipid and AP. But this was contrary to the efficiency of exogenous DNA transformation (Fig. 4&Fig. 5), because lower degree of ultrasonic treatment (i.e. 30 s, 40 W) could not only cause less damage to host cells and exogenous DNA, but also make the damaged host cells easier to repair themselves [30,39,40].

4. Conclusion

In this study, we optimized a universal and efficient method of ultrasound-mediated DNA transfer using *E. coli* DH5 α as receptor and pUC19 as exogenous DNA. Under the optimum conditions (35 $^{\circ}$ C, 40 W, OD₆₀₀ = 0.4 ~ 0.6, 30 s), the transformation efficiency can reach 1.006×10^7 CFU/ μ g DNA. The effect of ultrasound on cell membrane and cell wall provides nano-channels for exogenous DNA to enter host cells successfully. However, only under mild ultrasound conditions (40w, 25 s), exogenous DNA could be highly expressed in host cells, because harsh ultrasound conditions will cause permanent damage to host cells and destroy exogenous DNA, which was consistent with the results of Song [11]. The results of this study have important theoretical and practical significance for the biological effects of sonic, and provide evidence for the wide application of sonic.

5. CRediT author statement

YG Wang, FF Leng and JZ Ma planned and designed the experiments. SC Sun and LM Yu performed the experiments. YG Wang, SC Sun, LM Yu, S Hu and WG Fan analyzed the data. YG Wang, SC Sun, and LM Yu wrote the manuscript and passed the revision of FF Leng and JZ Ma. All authors have carefully read and approved the final manuscript.

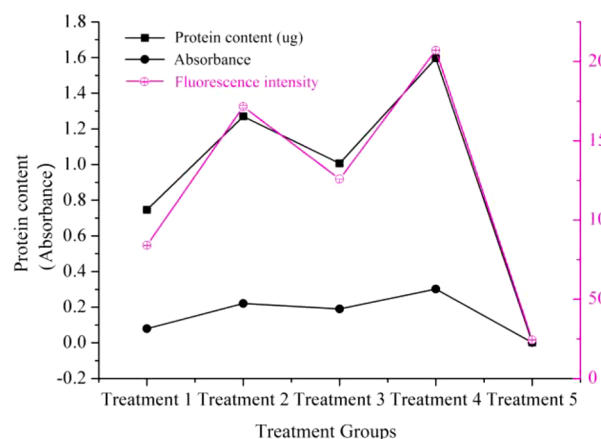


Fig. 8. Protein content, Phospholipid, Alkaline Phosphatase of *E. coli* DH5 α under different ultrasonic conditions. (Note: 1. without treatment; 2. 35 $^{\circ}$ C, 30 s, 40 W; 3. 35 $^{\circ}$ C, 30 s, 100 W; 4. 35 $^{\circ}$ C, 120 s, 40 W; 5. 35, 120 s, 100 W).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ultsonch.2021.105552>.

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