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Full Length Research Paper

Optimization of plasmid electrotransformation into *Escherichia coli* using Taguchi statistical method

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Electroporation is a mechanical method used to introduce polar molecules into a host cell through the cell membrane. In this procedure, a large electric pulse temporarily disturbs the phospholipid bilayer allowing molecules like DNA to pass into the cell. Application of statistical methods to determine the appropriate processes have been suggested for genetic engineering and biotechnology technique such as electroporation. This study explains the use of Taguchi statistical method to optimize the conditions for efficient plasmid transformation into *Escherichia coli* via electroporation. In order to improve electroporation, optical density of bacteria, recovery time and electrical parameter (field strength and capacitance) were optimized using the Taguchi statistical method. ANOVA of obtained data indicated that the optimal conditions of electrotransformation of pET-28a (+) plasmid into *Escherichia coli* BL21(DE3)pLysS was 0.7, 120 min, 12 kV/cm and 50 µF, for optical density of cell culture, recovery time, field strength and capacitance, respectively. The most significant alterations are decrease in field strength and increase in optical density in comparison with common electroporation protocol. The maximum level of plasmid transformation obtained under optimal condition was 8.7×10^8 transformants/µg DNA plasmid, which was 6.7 fold higher than the control condition.

Kay words: Electroporation, Taguchi statistical method, Plasmid.

INTRODUCTION

Many techniques in molecular biology research require a foreign DNA to be inserted into a host cell. Since the phospholipid bilayer of the plasma membrane has a hydrophilic exterior and a hydrophobic interior, any polar molecules, including DNA and protein, are unable to freely pass through the membrane (Cserhati and Szogyi, 1995). Many methods have been developed to pass this barrier and allow the insertion of DNA and other molecules into the cells. One of these methods is electroporation. The concept of electroporation has been capitalized on the relatively weak nature of the phospholipid bilayer's hydrophobic/hydrophilic interacttions and its ability to spontaneously reassemble after disturbance (Cserhati and Szogyi, 1995). Thus, a quick voltage shock may disrupt areas of the membrane temporarily, allowing polar molecules to pass, but sometimes the membrane may reseal quickly and leave the cell intact. Typically, 10,000 to 100,000 V/cm (varying with cell size) in a pulse lasting a few microseconds to a millisecond is necessary for electroporation. This electric pulse disturbs the phospholipid bilayer of the membrane and causes the formation of temporary aqueous pores. The electric potential across the membrane of the cell simultaneously rises by about 0.5 to 1.0 V so that charged molecules (such as DNA) are driven across the membrane through the pores in a manner similar to electrophoresis. Electroporation has some advantages

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Abbreviation: ANOVA, Analysis of variance.

including versatility with nearly all cell and species types, efficiency of cell transformation (Miller and Nickoloff, 1995) and small scale of required DNA (Withers, 1995). Electroporation has also some disadvantages just as cell damage at wrong length or intensity of pulses (Weaver, 1995) and nonspecific transport of material into and out of the cell during the time of electropermeability (Weaver, 1995).

Electroporation is widely used in many areas of molecular biology research and in the medical field. Some applications of electroporation include: DNA transfection (Hoffmann et al., 2000), direct transfer of plasmids into the cells, induced cell fusion (Weber and Berg, 1995), trans-dermal drug delivery (Prausnitz et al., 1993), cancer tumor electrochemotherapy (Mir et al., 2003) and gene therapy. Development of optimized conditions is essential in biology and biotechnology project. Plasmid transformation is one of the most critical step in genetic engineering; the optimization of main factors in DNA plasmid transformation are vital for accessing to better results. Statistical methods are crucial to the improvement of efficiency because they play an important role in experimental design, evaluation and optimization of variables. Several statistical methods are widely used in biological processes (Rao et al., 2008). Among them, the Taguchi method is utilized as a screening filter, which examines the effects of variables and identifies those factors which have major effects on the process. By using this method, optimal conditions are obtained through the use of few experiments (Rao et al., 2008). It is known that compared to super efficient strains like *Escherichia coli* DH5α or Top 10, *E. coli* BL21 (DE3)pLysS, as a expression host, is a strain that gives much lower transformation efficiency. So transformation optimization for this strain is critical for obtaining maximum colony number. In this study, in order to increase the efficiency of plasmid electrotransformation into *E. coli* BL21(DE3)pLysS, Taguchi statistical design was used for the determination and optimization of main variables and how each variable affects conditions for plasmid electrotransformation.

MATERIALS AND METHODS

Bacteria, plasmid, medium and instruments

The *E. coli* BL21(DE3) pLysS, as a host was purchased from Pasteur Institute of Iran, pET-28a(+) as cloning and expression plasmid was purchased from Novagen, Cat No: 69864-3. The pET-28a(+) plasmid genetically engineered for high expression in *E. coli*, contains a *kanamycin resistance* gene for transformant selection. Luria-Bertani medium (LB) and SOC medium were used for cultivation and recovery of bacteria; gene pulser Xcell electroporation system (BioRad) was used as electroporator instrument.

Medium

The Luria-Bertani medium (LB) composed of yeast extract (5 g/L), NaCl (10 g/L) and tryptone (10 g/L), was used for cultivation. *E. coli* in shaking flask, LB agar (LB medium containing 15 g/L agar) was used for bacterial plate cultivations. The pH of the media were adjusted at 7.1 and then sterilized by autoclaving at 121°C for 20 min. SOC medium contains Bacto-tryptone (20 g/L), Bacto-yeast extract (5 g/L), NaCl (0.5 g/L), 1 M KCl (2.5 ml) and ddH_2O to 1000 ml. The media was sterilized, then 10 ml sterile 1 M $MgCl_2$, 10 ml 1 M $MgSO_4$ and 20 ml 1 M glucose was added.

Competent cell preparation

E. coli BL21(DE3)pLysS cells were cultivated in 250 ml flask containing 50 ml of LB medium at 37°C up to optical density at 600 nm of 0.5, 0.7, 0.9 and 1.1. Then cultivation stopped and incubated in ice for 30 min. Thereafter, the bacterial cells were collected by centrifugation at 3000 g for 10 min at 4°C, and the pellet re-suspended in 50 ml sterile ice cold 10% glycerol. The cells were collected again by centrifugation, and re-suspended in 5 ml sterile ice cold 10% glycerol. The cells were collected again and re-suspended in 1 ml sterile ice cold 10% glycerol. Pellets were aliquot in 25 µl and stored at -70°C for subsequent procedure (Kahrizi and Salmanian 2008).

Statistical design

In order to maximize plasmid transformation, the common electroporation method was used and the effect of optical density of bacteria, electrical parameter (field strength and capacitance) and recovery time were selected for screening by using the Taguchi statistical method (Table 1). The L8 orthogonal array was used for examining of the aforementioned factors and interaction between two factors includes: Optical density and field strength at 2 levels (Table 2). After screening, the more effective factors and their interactions were optimized using L4 (Table 3) and L9 (Table 4) orthogonal arrays. L9 was designed for three different levels of optical density and field strength (Table 5). Qualitek-4 software was used for automatic design and standard analysis of variance (ANOVA) of Taguchi experiments. The results from ANOVA showed and identified the effect of each factor and estimated the performance of the optimum condition.

Plasmid transformation (optimum conditions)

Two microliter (2 µl) of pET-28a(+) plasmid (500 pg/µl) was mixed with 25 µl of 10% glyserol-treated competent cell suspension (OD_{600nm} = 0.7), after 10 min incubation in ice, suspension was transferred into the cold cuvette with 0.1 cm inter-electrode gap. Electroporation procedure performed with adjusted 12 kV/cm and 50 µF of field strength and capacitance respectively. Thereafter, 1 ml of SOC medium was added and the mixture was incubated at 37°C for 120 min. Then, the cultures were centrifuged and pellets were suspended in 100 µl of LB medium. 10 µl of each culture was plated on LB agar containing kanamycin (40 µg/ml). The resulting transformants colonies usually appeared after 15 h incubation at 37°C. The transformation efficiency (transformants/µg DNA) was calculated (Roychoudhury et al., 2009; Yari et al., 2010; Kahrizi et al., 2007).

RESULTS

L8 Orthogonal array for evaluation of interaction

In order to optimize the conditions for plasmid DNA

Table 1. Selected variables and their levels for the screening stage by the Taguchi experimental method.

Variable	Low level	High level
Optical density	0.5	0.9
field strength (kV/cm)	10	20
apacitance (µF)	25	50
Recovery time (min)	60	120

Table 2. L8 Orthogonal array of the Taguchi design for screening of selected factors.

Trial number	Optical density	Field strength	Capacitance	Recovery time
1	1	1	1	1
2	1	1	2	2
3	1	2	1	2
4	1	2	2	1
5	2	1	1	2
6	2	1	2	1
7	2	2	1	1
8	2	2	2	2

Table 3. L4 Orthogonal array for studying the interaction between optical density and field strength.

Trial number	Optical density	field strength
1	0.5	10
2	0.5	20
3	0.9	20
4	0.9	10

Table 4. L9 Orthogonal array of the Taguchi design for optimization of significant factors.

Trial number	Optical density	Field strength
1	1	1
2	1	2
3	1	3
4	2	1
5	2	2
6	2	3
7	3	1
8	3	2
9	3	3

transformation into *E. coli*, the Taguchi statistical design was applied. The L8 orthogonal array was used for optimization of four variables including optical density of bacteria, recovery time and electrical parameter (field strength and capacitance). Interaction between optical density of bacteria and field strength was also considered. Results were analyzed by standard ANOVA for the determination of the percentage contribution of each variable and the optimum level. These data show that the DNA plasmid transformation into *E. coli* cells was affected by the optical density of bacteria and field strength. The results show that optimum levels are 0.9,

Table 5. Significant variables and their levels employed for optimization experiments using the L9 Taguchi design.

Variable	Low level	Medium	High level
Optical density	0.7	0.9	1.1
field strength (kV/cm)	8	10	12

Table 6. ANOVA of the effects of assigned variables and interaction on plasmid electrotransformation obtained from L8 orthogonal array.

Factor	DOF (f)	Variance (V)	F – ratio (F)	Contribution (%)	Optimized level
Optical density	1	2312	544	47.705	2
Field strength	1	1860.5	437.764	38.372	1
A×B	1	578	136	11.86	-
Capacitance	1	18	4.235	0.911	2
Recovery time	1	60.5	14.235	1.162	2

50 µF, 10 kV/cm and 120 min for OD, capacitance, voltage and time of the recovery, respectively. The ANOVA results indicate contribution percentages of variables are 47.70, 38.37, 0.911 and 1.162 for $OD_{600\ nm}$ field strength, capacitance and recovery time respectively. However, the interaction between OD and field strength was significant in electrotransformation (Table 6).

L4 Orthogonal array for evaluation of interaction

While keeping the optimized conditions of recovery time and capacitance at the suggested level of the initial screening design, interaction between optical density of bacteria and field strength was studied by the L4 orthogonal array. The data were analyzed by standard ANOVA, and the percentage contribution of each variable and the optimum level were obtained. Hence, the optimum levels obtained for optical density of bacteria and field strength variables were 0.9 and 10 kV/cm, respectively (Data not shown).

L9 Orthogonal array for optimization of significant variables

After screening the significant variables, the effective factors (optical density of bacteria and field strength) were selected for the final optimization stage. The L9 orthogonal array was used to investigate the effects of two main factors, optical density of bacteria and field strength at three levels while the other variables were kept constant at optimum levels. The ANOVA of the obtained result indicated the optimal conditions of field strength and optical density are 12 kV/cm and 0.7, respectively (Table 7). After optimization, the transformants colonies were about 6.7 fold more than the basic conditions (Table 8).

Finally, optimal conditions for great transformation of pET-28a (+) plasmid into *E. coli* BL21(DE3) pLysS are 0.7, 120 min, 12 kV/cm and 50 µF, respectively optical density of cell culture, recovery time, field strength and capacitance.

DISCUSSION

Because plasmid transferring is one of the most important steps in gene cloning, access to suitable conditions for high DNA transformation into bacterial cells is essential. Because the transformation of expression vector into a suitable host is a critical step in recombinant protein production, in this study, the pET28a (+) expression vector and *E. coli* BL21(DE3) pLysS as an expression host were examined. There are several methods such as ultrasound, hydro gel, electroporation and chemical transformation for introducing plasmid into the host. Among them, electroporation is the most effective method for transformation. In recent years, the use of electroporation for transformation of DNA into different host has been improved. Several important factors are involved in electroporation. In this study, the effect of optical density of bacterial culture, field strength, capacitance and recovery time as the important factors in electrotransformation rate were optimized by Taguchi method. Already this technique has been used for the optimization of an electroporation microchip system for gene transfection (Huang et al., 2007). After analysis of L8 orthogonal results by ANOVA for these factors, as shown in Table 6, OD and field strength have significant effects in the electro-transformation of plasmid. Based on Qualitek-4 software and standard ANOVA, 0.9 for OD_{600nm} and 10 kV/cm for field strength were suggested.

Table 7. ANOVA of the effects of assigned variables on plasmid electrotransformation obtained from L9 orthogonal array.

Factor	DOF (f)	Variance (V)	Percent, P(%)	Optimized level
Optical density	2	1011.11	56.9	1
Field strength	2	786.112	43.021	3

Table 8. Verification of the results obtained at control and optimal conditions.

Condition	Maximum rate of plasmid transformation (transformants/µg plasmid)
Basal (control)	1.3×10^8
Optimum	8.7×10^8

Interaction between these factors is important too. After selection of important factors, interaction between them was studied by L4 orthogonal array. By using L9 orthogonal array, the optimum level for the above factors was 0.7 and 12 kV/cm for OD_{600nm} and field strength respectively. In comparison with basic condition, there was 6.7 fold increase in the number of transformant in optimized conditions,

Some researchers have applied Taguchi method for optimization of molecular biology processes. Yari and Mostafaie (2010) reported the effect of various factors for optimization of plasmid transformation by chemical transformation using Taguchi method.

The effect of the number of cell washes prior to electroporation, cell number, DNA amount, and cell growth phase on rate of electroporation was investigated by Wu et al. (2010). They reported that 0.15 is the best $OD_{600\ nm}$ for electrotransformation of pUC19 plasmid into *E. coli* DH10B. Rodríguez et al. (2007) reported that optimized OD of *Pediococcus acidilactici* P60 culture for transformation of pRS4C1 plasmid is 1 to 1.2 in stationary phase. They reported that the optimal field strength is 20 kV/cm. In this study, based on instrument producer instruction (basal conditions: $OD_{600\ nm}$ = 0.6, field strength: 18 kV/cm, capacitance: 50 µF and recovery time: 90 min), two levels of $OD_{600\ nm}$ (0.5 and 0.9) by L8 and three levels (0.7, 0.9 and 1.1) by L9 orthogonal array were examined. Results show that 0.7 is the best level of $OD_{600\ nm}$ in pET28a (+) plasmid transformation into *E. coli* BL21(DE3) pLysS. Our results are different from Wu report (Wu et al., 2010). It may be due to use of different plasmids and hosts. Our study results are more similar to Rodríguez et al. (2007) in the view point of the best stage of culture for electrotransformation of plasmid into bacteria is a stationary phase.

For field strength, four levels, 8, 10, 12 and 20 kV/cm were examined using L8 and L9 orthogonal array and 12 kV/cm was selected as optimized field strength. In this study, the lower OD of bacteria rather than Rodríguez's investigation causes field strength to be less than 20 kV/cm. ANOVA from obtained result indicated that interaction between OD and field strength has the main effect on plasmid uptake by *E. coli* BL21 (DE3) pLysS.

Recovery time after electrical shock is essential for bacterium survival and we showed that 120 min is better than 60 min in the rate of transformation. This time is suitable for host for membrane recovery and expression of the genes that rebound in antibiotic resistance.

Conclusions

In conclusion, our results show that minor alterations in conditions of main variables for plasmid transformation have positive effect in transformation efficiency. Analysis of variance for obtained data indicated that the optimal conditions for plasmid electrotransformation consist of 0.7, 120 min, 12 kV/cm and 50 µF of optical density of bacteria at 600 nm, recovery time, field strength and capacitance, respectively. The study presented here is apparently the first report to use the Taguchi statistical method for the optimization of conditions to introduce pET-28a(+) plasmid DNA molecules into *E. coli* BL21(DE3) pLysS cells by use of electroporation method. This work can thus be used as a basis of future investigations in molecular biology methods.

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