Electronic Journal of Biotechnology 61 (2023) 54–60

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage: www.elsevier.com/locate/ejbt

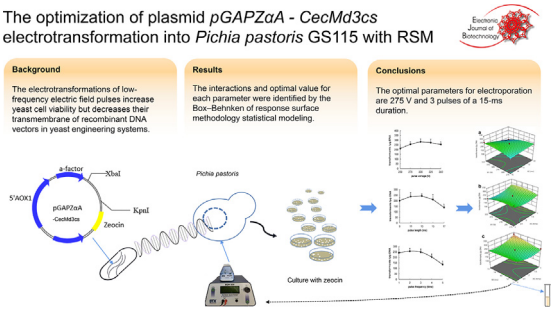
Research Article

Electrotransformation optimization of plasmid *pGAPZαA – CecMd3cs* into *Pichia pastoris* GS115 with response surface methodology

Chenxue Zhang, Jiahua Du, Xueyu Tang, Di Ma, Long Qin, Aizhong Zhang, Ning Jiang *

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, PR China
Key Laboratory of Efficient Utilization of Feed Resources and Nutrition Manipulation in Cold Region of Heilongjiang Province, Daqing, Heilongjiang, PR China
Key Laboratory of Low-Carbon Green Agriculture in Northeastern China, Ministry of Agriculture and Rural Affairs, PR China

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 May 2022
Accepted 21 November 2022
Available online 25 November 2022

Keywords:
Electric field
Electroporation
Electrotransformation
Parameters
pGAPZαA-CecMd3cs
Pichia pastoris GS115
Plasmids
Yeast

ABSTRACT

Background: Industrial production is increasingly reliant upon recombinant microorganisms across sectors. Therefore, it is of great importance for long-term operational viability that care be taken in optimizing the preparation of operationally key organisms. In electrotransformations, low-frequency electric field pulses increase yeast cell viability but decreases their transmembrane of recombinant DNA vectors. Herein, we investigated the optimal parameters for transforming *pGAPZαA-CecMd3cs* into *Pichia pastoris* GS115 and obtaining the efficient production of recombinant cells of interest.

Results: For an initial experiment, we utilized a single-factor experimental design to perform the electroporation and measure the vector transformation efficiency in triplicate, identified three out of five voltages (250–350 V), pulse durations (9–17 ms), and pulse frequencies (1–5 pulses) as candidate values for further analysis. Based on this data, we obtained the optimal value for each parameter by utilizing Box–Behnken of response surface methodology statistical modelling. We found that under a fixed voltage, the primary factors affecting electrotransformation efficiency were pulse duration and frequency. Furthermore, we identified synergistic interactions between pulse length and voltage and between pulse length and frequency.

Conclusions: The optimal parameters for electroporation are 275 V and 3 pulses of a 15-ms duration. We bioinformatically determined the optimal transformation parameters.

Abbreviations: BBD, Box-Behnken design; EF, electric field; EP, electroporation; ET, electrotransformation; RSM, response surface methodology; RT-PCR, reverse transcription-polymerase chain reaction; YPD, Yeast Extract Peptone Dextrose Medium.

* Corresponding author.
E-mail address: byndjn@163.com (N. Jiang).

https://doi.org/10.1016/j.ejbt.2022.11.002

How to cite: Zhang C, Du J, Tang X, et al. Electrotransformation optimization of plasmid *pGAPZαA - CecMd3cs* into *Pichia pastoris* GS115 with response surface methodology. Electron J Biotechnol 2022;61. https://doi.org/10.1016/j.ejbt.2022.11.002

1. Introduction

Pichia pastoris is a crucial yeast species with applications in both industrial and biotechnological sectors [1,2]. Because recombinant *P. Pastoris* can not only synthesize and secrete special proteins at a high level, but also like other yeasts correctly fold, perform post-translational modification, and improve the functioning of expressed proteins [3,4]. While there are several transformation methods available for allowing expression plasmids to enter yeast cells and producing recombinants, a majority suffer from low transformation efficiencies and thus limit the methodological choices available for constructing recombinants. Of these, electrotransformation (ET) is a relatively robust and well-developed technique for constructing *P. Pastoris* recombinants across the life sciences [5,6,7]. It involves a process called electroporation (EP), in which brief electric field (EF) pulses are applied to cells to create transient micropores in the plasma membrane, thereby allowing exogenous nucleic acids to enter cells and be genomically incorporated [7].

The parameters of an applied EF, such as voltage and pulse duration, determine how EFs affect the various membranes of cells and, most saliently, the permeability of the plasma membrane. Additionally, the size of the exogenous DNA, the target cell- or tissue type selected for EP, as well as those cells' environmental conditions are all relevant factors that must be taken into consideration when designing an appropriate ET protocol [7,8,9]. Due to yeast cell walls, the EP-induced permeability of these cells is reduced relative to those of other microbes lacking cell walls [5]. Indeed, cell walls greatly increase the difficulty of yeast ET and remain a major unsolved challenge for effectively introducing nucleic acid vectors into yeasts. Previous research has shown that, for a certain level of *P. Pastoris* transformants to be obtained, the operating parameters of the EP device must first be tested to determine the optimal settings to efficiently and consistently introduce target molecules into the cells [10]. This screening process is typically time intensive; thus, efficient and strategic parameter screening must be employed in discerning optimal ET parameters.

Generally, there are two EF waveforms generated by modern electroporators utilized in the ET of cells to induce EP: exponential decay waveforms and square waveforms (SWs). In the present study however, we utilized only SW pulses, which offer greater flexibility in setting and controlling pulse strength, duration, and frequency during applications [11,12]. Furthermore, SW devices are considered to more efficiently induce plasmid uptake across various cell types in several applications [6]. They have also been used in clinical research as a delivery system into several fungi of human skin [13], and yeasts are currently under development and need to continue new exploring.

However, importantly, ET transformation efficiency is not uniform across yeast species assayed with an SW device [14,15]. For example, the pulse voltage and duration used for *Zygosaccharomyces rouxii* were 470–1500 V and 6–24 ms, respectively [16]. In contrast, in one study examining *Saccharomyces cerevisiae*, Meilhoc et al. [17] managed to obtain 10^7 transformants using a 2.7 kV/cm pulse with a 15-ms duration. However, Hu et al. [14] used 500 V with a 4-ms duration to obtain 77 transformants. Interestingly, at the relatively higher voltage of 750 V and shorter pulse duration of 400 μs, they obtained fewer successful transformants. In another study examining ET parameters for *P. pastoris*, the pulse voltage and duration were varied between 305–1500 V and from 5–15 ms, respectively; the researchers demonstrated that membrane permeability could both transiently or irreversibly increase under the application of high-voltage EFs [18,19,20]. Thus, the extant literature indicates that both short and long pulse lengths under a certain voltage can, in general, successfully perform ET to yeast strains. However, most existing studies have placed less emphasis on exploring the effect of pulse number upon transformation efficiency and instead investigated the impacts of other factors upon the process. Recently, pulse number has been suggested to also play a role in transformation efficiency by some research [21]. Additionally, there are scarce studies on comprehensive pulse parameters of the electroporation than that in the earlier experiment, especially in *P. pastoris* system.

In our present work, we can obtain less technical information about the process of plasmid *pGAPZαA* transmembrane into *P. pastoris* using an SW device except for being a food-grade, constitutive expression vector, and potentially industrial value. We chose the plasmid *pGAPZαA-CecMd3cs* to *P. pastoris*, in which a gene fragment of the antimicrobial peptide CecMd3cs has been inserted [22]. The optimal ET conditions for this vector, with this novel insertion, have yet to be characterized. Therefore, we sought to identify the optimal combination of ET parameters by first using a single-factor testing paradigm followed by employing response surface methodology (RSM) to provide feasibility data for *P. pastoris* ET.

2. Materials and methods

2.1. Reagents, plasmids, and instruments

The plasmid *p*GAPzαA and yeast *P. pastoris* GS115 were gifts from the Food Science laboratory in Heilongjiang Bayi Agricultural University (Daqing, China). The recombinant plasmid *pGAPzαA/CecMd3cs* were constructed in our Biotechnological laboratory of Animal Science. The Axyprep Plasmid Miniprep and AxyPrep DNA Gel Extraction Kits utilized in the study were purchased from Axygen (New York City, NY, USA). Tryptone, yeast extract, and peptone were purchased from Beijing Lab and Bio-Tech CO. LTD (Beijing, China). Zeocin was obtained from Invitrogen (Waltham, MA, USA). The ECM830 electroporator was purchased from BTX (New York City, NY, USA). The sequence of *CecMd3cs* is:
GGTACCATGGGTTGGTTGAAGAAGATTGGTAAGAAGATTGAAAGAGT
TGGTCAACACACTAGAGATGCTACTATTCAAACTATTGGTGTTGCTCAA
CAAGCTGCCAACGTTGCTGCTACTTTGAAGGGTGATGATGATGATAAG
TGGGAAGTTTCATCGTCGTTGCAACCGTCGAACAACTCGTTGTGGTTA
TCAAACTTATCATCGTAGAGATCACACAACTGGTTGAGAAAGTTAGAA
GAATGGTTAGAAGAAGTTGGTTGGGATGATGATGATAAGGGTTGGTT
GAAGAAGATTGGTAAGAAGATTGAAAGAGTTGGTCAACACACTAGAG
ATGCTACTATTCAAACTATTGGTGTTGCTCAACAAGCTGCCAACGTTG
CTGCTACTTTGAAGGGTTAATCTAGA.

The *CecMd3cs* sequence was designed bioinformatically by our laboratory and was based upon the triploid antimicrobial peptide sequence encoded by *CecMd* (GenBank No. ON152389). It was developed in our laboratory as the parent peptide [23].

2.2. Linearization of recombinant plasmid pGAPzαA-CecMd3cs

First, *p*GAPzαA-CecMd3cs plasmid was synthesized at Sangone Biotech (Shanghai) Co., Ltd. and subsequently extracted from *Escherichia coli* DH5α using an Axyprep Plasmid Miniprep Kit (Axygen). The plasmid was then amplified via -polymerase chain reaction (PCR) and isolated via double enzyme digestion. Finally, linearization of the recombinant plasmid was performed using an *Avr* II enzyme digestion. The digestion system included the addition of the following solutions (in order): 1 μL *Avr*Ⅱ enzyme, 5 μL CutSmart Buffer, and 1 μL recombinant plasmid DNA. The solution volume was brought up to 50 μL with ddH_2O.

Next, the digested product was identified by performing a 1.5% agarose gel electrophoresis. The resultant isolated and digested plasmid was then recovered using an AxyPrep DNA Gel Extraction Kit (Axygen). The concentration of the linear plasmid was measured using NanoVue Plus and the stock was then stored at −20°C after the condensation of plasmids using ethanol was concluded.

2.3. Preparation of P. pastoris GS115 competent cells

A single *P. pastoris* SMD1168 colony was selected from a yeast extract peptone dextrose (YPD) agar plate, inoculated into 5 mL broth, and incubated for 16 h at 30°C. Next, 1 mL of bacterial solution was added to 50 mL of culture media and incubated until an OD_{600} of 1.3–1.5 was reached using PERSEE T6. The culture was then centrifuged at 4,000 rpm, and the supernatant then discarded. The pellet was resuspended in 20 mL of pre-cooled, sterile ddH_2O and centrifuged once more. Next, the pellet was resuspended in 10 mL of pre-cooled ddH_2O to again wash the cells. This process was repeated twice more with 10 mL and 500 μL, respectively, of a 1 M sorbitol solution to yield *P. pastoris* competent cells, which were stored on ice and used the same day.

2.4. Electrotransformation efficiency determination

To screen through the parameters of the electroporator, first, the values of the target variables are first confirmed by a single-factor design: voltage (250, 275, 300, 325, and 350 V), pulse duration (9, 11, 13, 15, and 17 ms), and pulse frequency (one, two, three, four, and five times). Each condition was performed in triplicate. To proceed with the ET process, second, an 80 μL solution of competent cells and 10 μg of linearized plasmid were added to a pre-cooled cuvette and mixed. Next, EP was performed while keeping two parameters constant and systematically varying the third one. After EP, 1 mL of a sorbitol solution was immediately added to the cuvette and cultivated at 30°C for 4 h. Then, 100 μL of that culture was plated onto YPD agar containing zeocin and incubated for 3 d. Finally, positive clones were counted and used to calculate the transformation rate as **Equation (1)** [16]:

$$\text{Transformation efficiency (number of transformants /μg DNA)} = \frac{\text{colony count} \times \text{dilution ratio} \times \text{total volume of conversion reaction solution}}{\text{volume of coated plate bacteria liquid} \times \text{plasmid DNA addition amount (μg)}} \quad (1)$$

2.5. Response surface test

Based on the results of the initial single-factor test, the ET protocol parameters for the plasmid were designed and performed according to the RSM. The Box-Behnken design (BBD) consisted of 17 randomized runs with five replicates at the central point. In the statistical calculation, voltage (*X1*), pulse length (*X2*), and pulse frequency (*X3*) of extraction independence were coded as −1, 0, and 1, respectively. The variable code count was calculated as **Equation (2)**:

Table 1
Response surface test design and results.

Number	X_1 Voltage (V)	X_2 Pulse length (ms)	X_3 Pulse frequency (time)	Y Electrotransformation efficiency (transformants /μg DNA)
1	0 (300)	0 (13)	0 (2)	257
2	1 (325)	−1 (11)	0 (2)	177
3	0 (300)	−1 (11)	−1 (1)	259
4	−1 (275)	0 (13)	−1 (1)	257
5	−1 (275)	−1 (11)	0 (2)	230
6	0 (300)	0 (13)	0 (2)	239
7	−1 (275)	1 (15)	0 (2)	207
8	0 (300)	1 (15)	−1 (1)	327
9	1 (325)	0 (13)	1 (3)	222
10	1 (325)	0 (13)	−1 (1)	255
11	0 (300)	0 (13)	0 (2)	247
12	0 (300)	0 (13)	0 (2)	227
13	0 (300)	−1 (11)	1 (3)	254
14	−1 (275)	0 (13)	1 (3)	278
15	1 (325)	1 (15)	0 (2)	274
16	0 (300)	1 (15)	1 (3)	233
17	0 (300)	0 (13)	0 (2)	244

$$X_i = \frac{x_i - x_0}{\Delta x_i} \quad (2)$$

where X_i is the code value, x_i is the actual value of the factor, x_0 is the average value of the factor, and Δx_i is the value of the step change.

The design of the test factors and their levels are presented in Table 1. The ET efficiency of the plasmid (*Y*) was calculated using the response value. The model calculates the response, *Y*, for the three independent variables (*X1*, *X2*, and *X3*) using **Equation (3)** [3]:

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_{ii} + \sum b_{ij} X_i X_j \quad (3)$$

where *Y* is the predicted response value, X_i and X_j are independent variables, b_0 is the offset, b_i is the linear coefficient, b_{ii} is the square coefficient, and b_{ij} is the interaction coefficient of the two factors. The calculated optimal parameters of electrotransformation by RSM for *p*GAPzαA/CecMd3cs DNA are shown in Table 1.

2.6. Verification testing

After *in silico* RSM modelling of the optimal ET parameter values for *pGAPzαA/CecMd3cs* uptake into *P. pastoris* GS115 upon ET, we performed three times *in vitro* validation experiments to empirically assess how well our model predicted the actual optimized ET parameter values. The results of our empirical tests were compared with the predicted values obtained from our model's regression equation of the RSM to perform this assessment.

2.7. Data processing

Data from our single-factor experiment were analyzed in Microsoft Excel 2016 and ANOVA of SPSS 23, and the response surface data were analyzed using Design-Expert 12.

3. Results

3.1. Linearization of the recombinant plasmid, pGAPzαA/CecMd3cs

The linearization of *pGAPzαA/CecMd3cs* was performed using the *Avr* II restriction enzyme. The resultant sample was then loaded onto an agarose gel and electrophoretically purified. Upon

visualization, we observed an approximately 3,555 bp band after agarose gel electrophoresis (Fig. 1), which demonstrated that the digested product was linearized.

3.2. Single factor analysis

ET efficiency as determined by our initial single-factor experimental ET parameter screening methodology (Fig. 2) yielded candidate values for each parameter of interest. The ET voltages 275, 300, and 325 V were found to attain a relative high ET rate for vector *pGAPZαA-CecMD3cs*, ET efficiently delivered 255.96, 282.96 and 273.24 μg DNA/mL. We also found that under the voltage 325 V, the pulse duration to be within the range of 11–15 ms, and the pulse frequencies were 1–3 times, the relatively high ET rates have to be determined.

3.3. Response surface test and variance analysis

As a next step, we used Design-Expert 12 software to run multiple quadratic regressions according to our test data (Table 2), and obtained the following multivariate quadratic regression equation [**Equation 4**]:

$$Y = 132.15X_2 + 179.85X_3 - 0.6X_1X_3 - 11.125X_2X_3 - 0.02884X_1^2 + 28.225X_3^2 - 634.956 \quad (4)$$

The results of the regression coefficients are shown in Table 2. The response surface regression model achieved a significance level of p = 0.0207; that is, the multivariate quadratic equation revealed good concordance between the modelled expected and actual optimal ET parameter values. The correlation coefficient R^2 was 0.8694, which indicated that the model fit well with the parameters in the equation, and the error to be sufficiently low in this experiment. The probability was 0.0863 for lacking fit, indicating that non-experimental factors had little effect on the detected results, as the model predicted the optimal voltage of 275 V, a pulse duration of 15-ms, and a pulse frequency of three times. Thus, we may confidently use the regression equation to analyze the experimental results and use the optimized parameters to perform a transformation of the *pGAPzαA* vector in yeast.

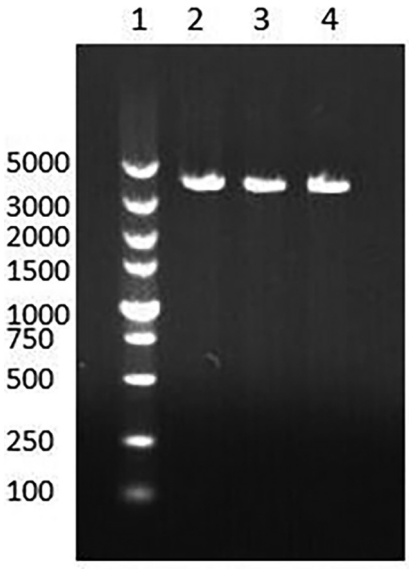


Fig. 1. Electrophoresis of linearized recombinant plasmid *pGAPzαA-CecMd3cs*. lane1: DNA molecular weight marker; 2, 3, and 4: linearized recombinant plasmid *pGAPzαA-CecMd3cs* 3555 bp digested with ArVII.

The product of pulse length to voltage and pulse frequency, the outcome of pulse length, pulse frequency, and quadratic voltage all had significant effects upon the experimental results (p < 0.05), which, interestingly, indicated that the impact of each factor on the response value was not a simple linear relationship. We found that synergistic interactions existed between the pulse duration to both the voltage and pulse frequency, respectively (Table 2).

3.4. Response Surface Interaction Analysis and Optimization

The influence of multiple arguments on the response value was analyzed based on the regression equation (Fig. 3). As shown in

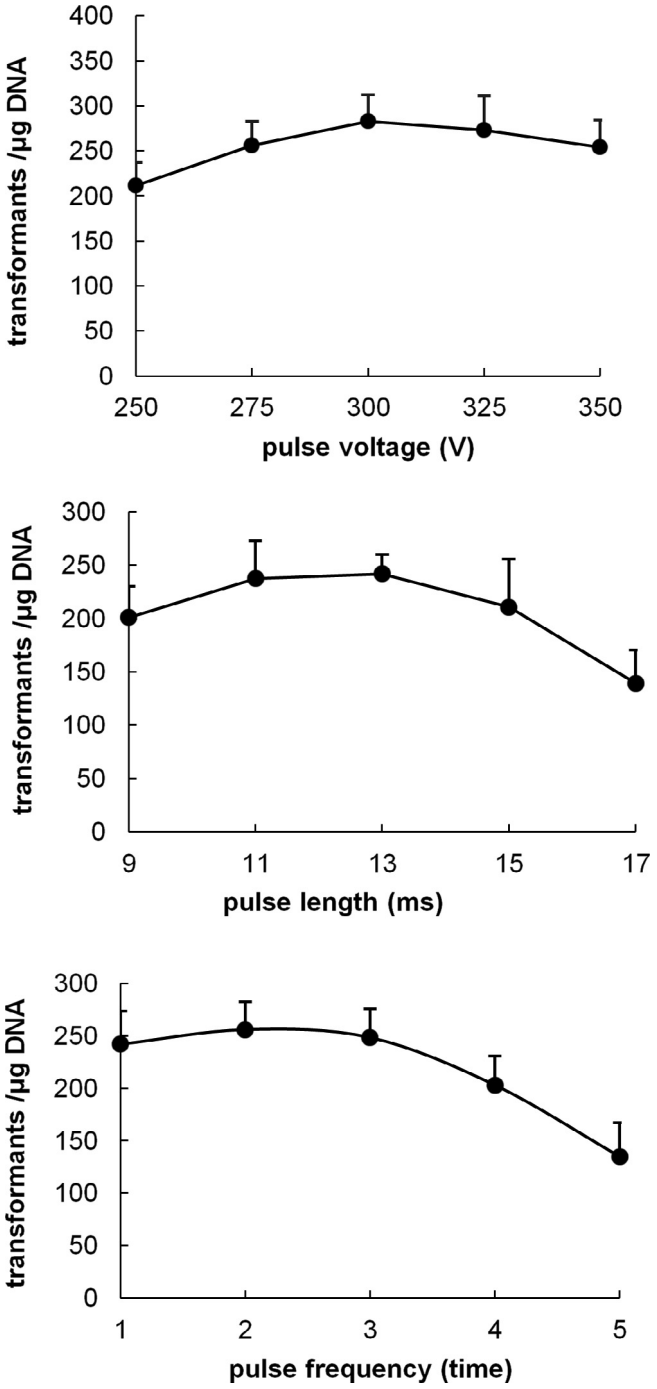


Fig. 2. Effect of pulse voltage, pulse length and pulse frequency on the electrotransformation efficiency of *P. pastoris* GS115.

Table 2
Analysis of variance tables.

Source of Variance	Quadratic Sum	Degree of Freedom	Mean Square	F-value	P-valued
model	14451.48	9	1605.72	5.18	0.0207
X_1	1540.13	1	242	0.78	0.4063
X_2	1830.13	1	1830.13	5.90	0.0454
X_3	242.00	1	1540.13	4.97	0.0611
X_1X_2	1980.25	1	3600.00	11.61	0.0113
X_1X_3	729.00	1	729.00	2.35	0.1690
X_2X_3	3600.00	1	1980.25	6.39	0.0394
X_1^2	3354.32	1	1368.00	4.41	0.0738
X_2^2	32.42	1	32.42	0.1046	0.7558
X_3^2	1368.00	1	3354.32	10.82	0.0133
Residual error	2170.05	7	310.01		
Lack of fit	1685.25	3	561.75	4.63	0.0863
Pure Error	484.80	4	121.20		
total	16621.53	16			

Note: P-values less than 0.0500 indicate model terms are significant. In this case X_1, X_1X_2, X_2X_3, X_3^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.
The **Lack of Fit F-value** of 4.63 implies there is 8.63% chance that a Lack of Fit F-value this large could occur due to noise.

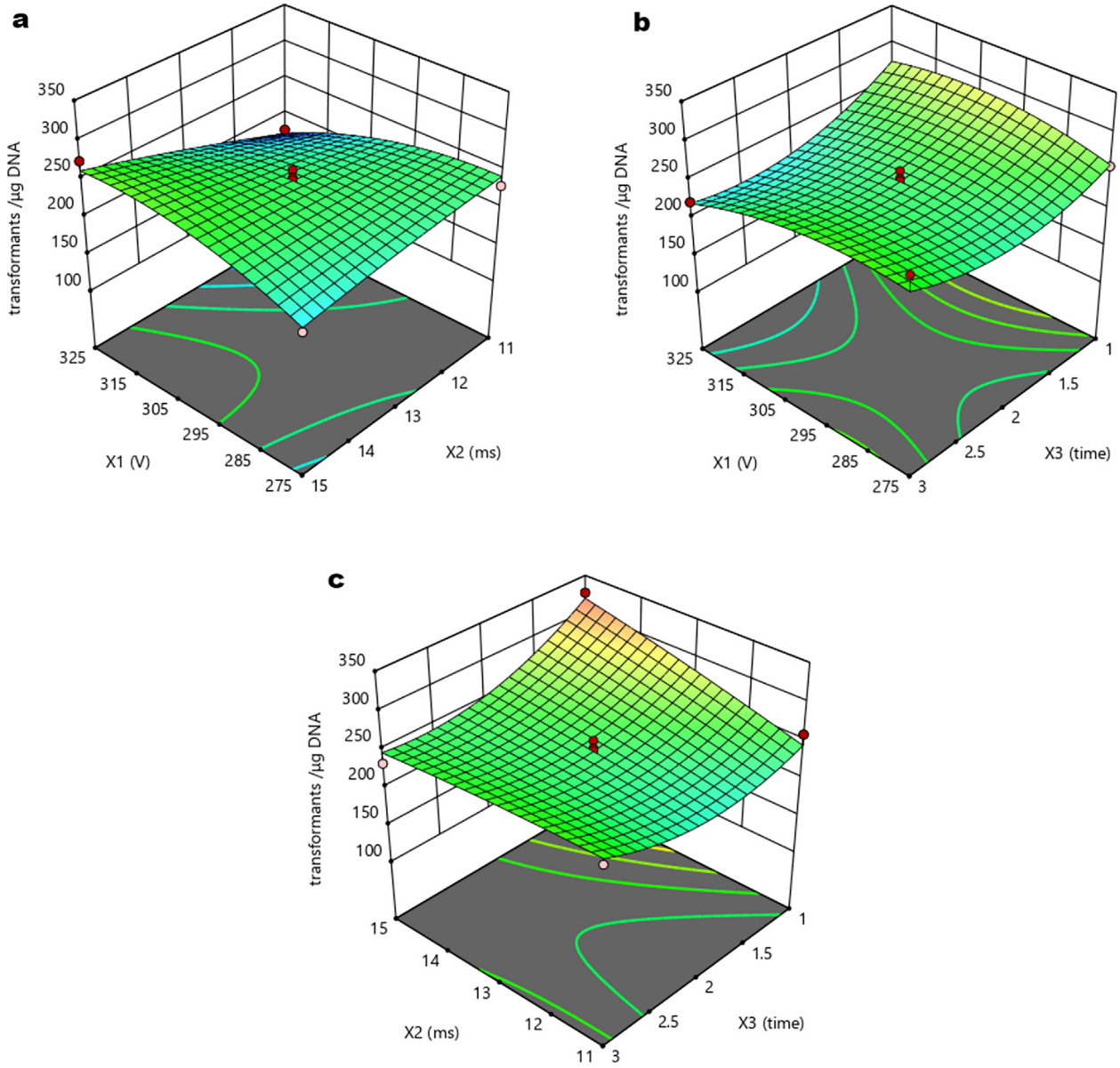


Fig. 3. Three-dimensional response surface plots of interaction between voltage and pulse length (a) = f (X_1, X_2); voltage and pulse frequency (b) = f (X_1, X_3); and pulse length and pulse frequency (c) = f (X_2, X_3). Electrotransformation efficiency (Y), voltage (X_1), pulse length (X_2) and pulse frequency (X_3).

Fig. 3a, the response surface was steeper with a more significant effect of the factor on the response value. Simultaneously, the impact of two interacting arguments on the response value could also be seen on the surface of the response surface (Table 2), while the factors other than the two independent variables are controllable.

We demonstrated the effects of the two variables, voltage, and pulse length, on the response surface (Fig. 3a) on the ET efficiency of plasmids. Although the effect of voltage on the conversion was not significant (p = 0.4063), its interaction with pulse length, however, was p = 0.0113. The curved surface can maintain a constant voltage and the ET efficiency of *P. pastoris* increased gradually with the increased pulse duration, which reached its maximum value at a pulse duration of 15 ms. When the pulse duration was held constant, the ET efficiency of *pGAPzαA-CecMd3cs* in *P. pastoris* increased with voltage before eventually declining and exhibited a longer range.

The curve surface in Fig. 3b shows that voltage and pulse frequency had less robust effects on ET efficiency, with p = 0.4063 and 0.0611, respectively. No significant interactions were observed for the two factors during the transformation of *pGAPzαA-CecMd3cs* into *P. pastoris* GS115 (p = 0.169), as evident on the surface graph. However, the transformation efficiency of the plasmid first increased and then decreased with an increase in the pulse frequency, while voltage was held constant. Furthermore, ET efficiency first decreased and then increased with increasing tension. The pulse frequency did not break, and the variation was in a smaller range.

The interaction between the pulse length and the pulse frequency on the ET efficiency of *P. pastoris* (Fig. 3c) was significant (p = 0.0113). The ET efficiency of *P. pastoris* decreased with an increase in pulse time when the pulse length decreased. However, the ET efficiency was enhanced as the pulse frequency increased when the pulse length increased. In addition, the ET rate decreased when the pulse length increased at a lower pulse frequency. When the pulse frequency was low, the electric conversion rate can also increase as the pulse length increased when the pulse frequency was higher (Fig. 3c).

3.5. Verification experiments

First, optimal solutions of the multivariate quadratic regression equation and the optimal conversion efficiency of *P. pastoris* were both obtained from Design Expert 12 software. The optimal conversion conditions were a voltage of 275 V, pulse duration of 15 ms, and a pulse frequency of 3. Based on these conditions, the theoretical value for optimal *P. pastoris* conversion efficiency was 340 transformants /μg DNA.

Further confidence testing demonstrated that the regression equation was quite reliable, withstanding three validation tests under identical conditions. The ET efficiencies for each verification test were 324, 341, and 348 transformants/μg DNA, respectively, with an average value of 338 transformants/μg DNA and a relative error of 0.6%, which were consistent with theoretical predictions. Therefore, it can be concluded these conditions have strong practical applicability and may be regarded as optimized parameters.

4. Discussion

The results of our initial single-factor experiments yielded operationally relevant ranges of ET voltage, pulse duration, and pulse frequency as candidate values for optimizing the ET of *pGAPZαA-CecMd3cs* into *P. pastoris* GS115. SW producing electroporators are widely utilized biotechnological devices that allow the customization of voltage level and pulse duration and produce rapidly repeating pulses [21,22]. Numerous factors, including voltage, pulse duration, frequency, the extracellular environment, and even electroporator characteristics affect ET efficiency [6]. Often, the actual probability of ET producing viable recombinant cells is neither fixed nor readily apparent, perhaps due to differences or heterogeneity of the plasma membrane that affect permeabilization, or from variability in plasmid integration sites into the host genome possibly disrupting indispensable housekeeping functions [23]. Guo et al. [20] reported that using a voltage of 305 V to transform an expression vector into a yeast host can lead to robust heterologous cecropin D secretion. However, in a study conducted by Yamano et al. [24], the authors found that 250 or 150 V allows for robust transformation. It starkly illustrated the important point that optimal biotransformation conditions are not uniform across vectors or cell types. Rehman et al. [25] performed efficient electroporations at relatively lower voltages for the host cell *Verticillium dahliae* at 300, 400, and 500 V, which fall within the optimal range of voltages indicated by our results. Liu et al. [26] indicated that ETs performed in the 600–700 V range could also attain high transformation efficiencies. Transformation efficiency had alternation for not only our studies but also to others [27]. Ji et al. [28] found that the transformation efficiency of electroporated yeast cells increases with voltage but concomitantly reduces survivorship. Liu et al. [26] demonstrated that the electroporation of the high-voltage would easily break down the membrane of *Rhodosporidium toruloides*. Thus, to avoid the drawbacks of high voltage electroporation to the cells, we opted to use low-voltage ET to mediate the uptake of *pGAPZαA - CecMd3cs* in our research into the most feasible and optimal ET conditions.

Finally, we noted that the duration of the pulses exerts a noticeable effect on transformation efficiency apart from either voltage level or pulse frequency. In our assays, we determined the optimal transformation efficiency for our plasmid vector to be 348 transformants/μg DNA at 275 V, with three pulses lasting average 13 ms each time, as determined by our response surface experimental modeling approach. Effective pulse duration fell between 11–15 ms in the two experiments. Our findings are also consistent with those of Hu et al. [14], who presented an inverse relationship between voltage and pulse length upon ET efficiency [29]. In addition, pulse frequency could compensate for a smaller pulse length. Because pulse number affected cell transformation from the perspective of pulse generation [22], but the current result of the research on this topic is limited to Pichia pastoris, even if we further verified our findings on the optimal ET parameter values and obtained a relatively ideal result, which provides merely the basis for yeast engineering with square-wave electroporation in this study. Furthermore, combinations of voltage, pulse length, and pulse frequency for a given ET protocol will still need to be optimized adequately due to environmental conditions and molecular differences thereafter.

In conclusion, we screened through the operating conditions of a SW electroporator, first by using a single-factor experimental paradigm and then followed this up by performing RSM modelling and verification experiments to home in on the true optimal values for ET, which were a voltage of 275 V, pulse length of 15 ms, and frequency of three pulses. We found that the optimum transformation efficiency value for the conversion of *Pichia pastoris* under these conditions was 340 transformants/μg DNA and that the predictive power of our model was quite robust in corresponding with actual observed values. Thus, overall, our findings provide a valuable theoretical reference for optimizing the ET of *P. pastoris*.

Author contributions

- Study conception and design: N Jiang
- Data collection: CX Zhang, JH Du
- Analysis and interpretation of results: CX Zhang, JH Du, D Ma, XY Tang, L Qin
- Draft manuscript preparation: CX Zhang, N Jiang
- Revision of the results and approved the final version of the manuscript: AZ Zhang, N Jiang, CX Zhang

Financial support

This work was supported by the National Natural Science Foundation of China (Grant No. 32072759).

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

We extend our appreciation to our Institutes and Universities.

References

[1] Akishev Z, Kiribayeva A, Mussakhmetov A, et al. Constitutive expression of *Camelus bactrianus* prochymosin B in *Pichia pastoris*. Heliyon 2021;7(5): e07137. https://doi.org/10.1016/j.heliyon.2021.e07137.
[2] Cai P, Duan X, Wu X, et al. Recombination machinery engineering facilitates metabolic engineering of the industrial yeast *Pichia pastoris*. Nucleic Acids Res 2021;49(13):7791–805. https://doi.org/10.1093/nar/gkab535.
[3] Marzban A, Ebrahimipour G, Danesh A. Bioactivity of a novel glycolipid produced by a halophilic *Buttiauxella* sp. and improving submerged fermentation using a response surface method. Molecules 2016;21 (10):1256–66. https://doi.org/10.3390/molecules21101256.
[4] Fischer JE, Glieder A. Current advances in engineering tools for *Pichia pastoris*. Curr Opin Biotechnol 2019;59:175–81. https://doi.org/10.1016/j.copbio.2019.06.002.
[5] Kotnik T, Frey W, Sack M, et al. Electroporation-based applications in biotechnology. Trends Biotechnol 2015;33(8):480–8. https://doi.org/10.1016/j.tibtech.2015.06.002.
[6] Young JL, Dean DA. Electroporation-mediated gene delivery. Adv Genet 2015;89(Chap 3):49–88. https://doi.org/10.1016/bs.adgen.2014.10.003.
[7] Kumar P, Nagarajan A, Uchil PD. Electroporation. Cold Spring Harb Protoc 2019;2019(7):519–26. https://doi.org/10.1101/pdb.top096271.
[8] Swafford AJM, Hussey SP, Fritz-Laylin LK. High-efficiency electroporation of chytrid fungi. Sci Rep 2020;10(1):15145. https://doi.org/10.1038/s41598-020-71618-2.
[9] Potocnik T, Miklavcic D, Macek LA. Effect of electroporation and recovery medium pH on cell membrane permeabilization, cell survival and gene transfer efficiency *in vitro*. Bioelectrochemistry 2019;130:. https://doi.org/10.1016/j.bioelechem.2019.107342107342.
[10] Kumar R. Simplified protocol for faster transformation of (a large number of) *Pichia pastoris* strains. Yeast 2019;36(6):399–410. https://doi.org/10.1002/yea.3383.
[11] Novickij V, Lastauskiene E, Svediene J, et al. Membrane permeabilization of pathogenic yeast in alternating sub-microsecond electromagnetic fields in combination with conventional electroporation. J Membr Biol 2018;251 (2):189–95. https://doi.org/10.1007/s00232-017-9951-4.
[12] Liu Y, Bergan R. Improved intracellular delivery of oligonucleotides by square wave electroporation. Antisense Nucleic Acid Drug Dev 2001;11(1):7–14. https://doi.org/10.1089/108729001750072083.
[13] Novickij V, Grainys A, Svediene J, et al. Controlled inactivation of *Trichophyton rubrum* using shaped electrical pulse bursts: Parametric analysis. Biotechnol Prog 2016;32(4):1056–60. https://doi.org/10.1002/btpr.2276.
[14] Hu M, Bai W, Pan X, et al. Optimization of square wave pulse electrotransformation of Saccharomyces cerevisiae. South China Agriculture 2018;12(34):62–6.
[15] Napotnik BT, Miklavcic D. *In vitro* electroporation detection methods – An overview. Bioelectrochemistry 2018;120:166–82. https://doi.org/10.1016/j.bioelechem.2017.12.005.
[16] Pribylova L, Sychrova H. Efficient transformation of the osmotolerant yeast *Zygosaccharomyces rouxii* by electroporation. J Microbiol Methods 2003;55 (2):481–4. https://doi.org/10.1016/S0167-7012(03)00197-0.
[17] Meilhoc E, Masson JM, Teissie J. High efficiency transformation of intact yeast cells by electric field pulses. Biotechnology (NY) 1990;8(3):223–7. https://doi.org/10.1038/nbt0390-223.
[18] Cregg JM, Tolstorukov I, Kusari A, et al. Expression in the yeast *Pichia pastoris*. Methods in Enzymology 2009;463:169–89. https://doi.org/10.1016/S0076-6879(09)63013-5.
[19] Meng DM, Dai HX, Gao XF, et al. Expression, purification and initial characterization of a novel recombinant antimicrobial peptide Mytichitin-A in *Pichia pastoris*. Protein Expr Purif 2016;127:35–43. https://doi.org/10.1016/j.pep.2016.07.001.
[20] Guo C, Huang Y, Zheng H, et al. Secretion and activity of antimicrobial peptide cecropin D expressed in *Pichia pastoris*. Exp Ther Med 2012;4(6):1063–8. https://doi.org/10.3892/etm.2012.719.
[21] Hyder I, Eghbalsaied S, Kues WA. Systematic optimization of square-wave electroporation conditions for bovine primary fibroblasts. BMC Mol Cell Biol 2020;21(1):9. https://doi.org/10.1186/s12860-020-00254-5.
[22] Murauskas A, Staigvila G, Girkontaite I, et al. Predicting electrotransfer in ultra-high frequency sub-microsecond square wave electric fields. Electromagn Biol Med 2020;39(1):1–8. https://doi.org/10.1080/15368378.2019.1710529.
[23] Eissazadeh S, Moeini H, Dezfouli MG, et al. Production of recombinant human epidermal growth factor in *Pichia pastoris*. Braz J Microbiol 2017;48 (2):286–93. https://doi.org/10.1016/j.bjm.2016.10.017.
[24] Yamano T, Iguchi H, Fukuzawa H. Rapid transformation of *Chlamydomonas reinhardtii* without cell-wall removal. J Biosci Bioeng 2013;115(6):691–4. https://doi.org/10.1016/j.jbiosc.2012.12.020.
[25] Rehman L, Su X, Guo H, et al. Protoplast transformation as a potential platform for exploring gene function in *Verticillium dahliae*. BMC Biotechnol 2016;16 (1):57. https://doi.org/10.1186/s12896-016-0287-4.
[26] Liu H, Jiao X, Wang Y, et al. Fast and efficient genetic transformation of oleaginous yeast *Rhodosporidium toruloides* by using electroporation. FEMS Yeast Res 2017;17(2):fox017. https://doi.org/10.1093/femsyr/fox017.
[27] Wang Y, Okugawa K, Kunitake E, et al. Development of an efficient host-vector system of *Ruminiclostridium josui*. J Basic Microbiol 2018;58(5):448–58. https://doi.org/10.1002/jobm.201700620.
[28] Ji Z-J, Sun B, Chi Y-J, et al. Optimization of square-wave electroporation for increasing the permeability of yeast cells. Food Sci 2010;31 (15):50–4.
[29] Wang L, Yang L, Wen X, et al. Rapid and high efficiency transformation of *Chlamydomonas reinhardtii* by square-wave electroporation BSR20181210. Biosci Rep 2019;39(1). https://doi.org/10.1042/BSR20181210.