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Development and Validation of a New PCR Optimization Method by Combining Experimental Design and Artificial Neural Network

Ye Li · Xueling Du · Qipeng Yuan · Xinhua Lv

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Abstract Polymerase chain reaction (PCR) is one of the most powerful techniques in a variety of clinical and biological research fields. In this paper, a chemometrics approach, combining experimental design (ED) and artificial neural network (ANN), was proposed for optimization of PCR amplification of lycopene cyclase gene *carRA* in *Blakeslea Trispora*. Five-level star design was carried out to obtain experimental information and provide data source for ANN modeling. Nine variables were used as inputs in ANN, including the added amount of template, primer, dNTP, polymerase and magnesium ion, the temperature of denaturating, annealing and extension, and the number of cycles. The output variable was the efficiency (yield) of the PCR. Based on the developed model, the effects of each parameter on PCR efficiency were predicted and the most suitable operation condition for present system was determined. At last, the validation experiment was performed under the optimized condition, and the expectant results were produced. The results obtained in this paper showed that the combination of ANN and ED provided a satisfactory optimization model with good descriptive and predictive abilities, indicating that the method of combining ANN and ED can be a useful tool in PCR optimization and other biological applications.

Keywords Artificial neural network · Experimental design · Optimization · PCR

Introduction

The polymerase chain reaction (PCR) originates from key research performed by Mullis et al. in the early 1980s [1]. After it is invented, PCR coupled with suitable operation conditions has become one common and often indispensable technique in medical and biological research labs for a variety of applications [2, 3]. In order to reduce sample requirements, save time, and lower experimental cost, more attention has been paid to the

Y. Li · X. Du · Q. Yuan (✉) · X. Lv
Key Laboratory of Bioprocess of Beijing, Beijing University of Chemical Technology, Beijing 100029, China
e-mail: yuanqp@mail.buct.edu.cn

optimization of PCR method [4–8]. Although there are several works that have been done about this subject, most of them are specialized. Moreover, these optimizations are almost traditionally carried out by one-factor-at-a-time method [9]. This method is simple and easy, without the need for statistical analysis, but it involves a relatively large number of experiments, and the interactions among factors are often ignored. Hence, it is not able to completely guarantee the determination of the optimal conditions. Therefore, developing a more general and accurate PCR optimization protocol seems to be necessary.

Artificial neural network (ANN) offers attractive possibilities for nonlinear modeling and optimization with excellent accuracy even though the underlying mechanisms are very complex. Thus, the ANN model has been successfully used by many researchers in the complex systems such as: process control, process prediction, fault diagnosis of processes, and dynamic modeling of process operation [10–15]. Recently, in order to obtain more accurate prediction, some researchers proposed the combination of experimental design (ED) and ANN. Several successful applications of this method in biochemical separation field were reported [16, 17]. Although the ANN has been widely used in a variety of fields, the application of ANN in biological research, particularly in PCR optimization, was seldom reported.

In this paper, the study of ANN conjunction with ED for optimization of PCR amplification of lycopene cyclase gene *carRA* in *Blakeslea Trispora* was reported. The experiments were performed according to a star experimental design to obtain the experimental data. Then, the experimental data were used to develop the ANN model. Subsequently, the effects of each influencing parameters on PCR efficiency were predicted. At last, the proposed method was verified by conducting the corresponding experiments under the optimized conditions. To the best of our knowledge, no chemometrics treatment, which combined ED and ANN, has been reported to provide a more general and accurate PCR optimization strategy.

Theory

ANN is a computer model derived from a simplified concept of the brain. It is a parallel distribution processing system composed of nodes (neurons) and connections (weights), and is based on the principle that a highly interconnected system of simple processing elements that can learn complex interrelationships between independent and dependent variables. Neurons are single processing elements and generally organized into a layered structure, formed by one input layer, one output layer, and at least one hidden layer. The most popular ANN is the back-propagation ANN (BP-ANN), which belongs to the class of supervised learning techniques. Its most important feature is the ability to learn from examples and to generalize, since the learnt information is stored across the network weights. Neural networks do not require the explication of a mathematical model. The final model is built through a continuous and iterative adjustment of weights which, in fact, reflect the relationship of input variables and output variables. Thus, it is not surprising that BP-ANN has gained momentum in numerous industrial and scientific areas. The BP-ANN architecture adopted in the present study consists of nine inputs, one output, and one hidden layer associating the inputs with output (Fig. 1).

In addition to the network topology, an important component of the ANN is the learning rule. A learning rule allows the network to adjust its connection weights in order to associate given inputs with corresponding outputs. In this paper, the training of the network is carried out by using a back-propagation algorithm. The complete derivation of the

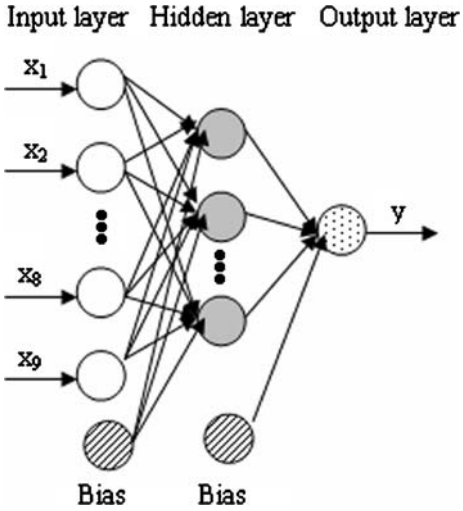


Fig. 1 ANN scheme used in this paper: x_i (I=1, 2, ⋯ 9) represents the nine operation parameters; y represents the PCR efficiency

algorithm can be found elsewhere [18, 19]. By using this algorithm, the network reads inputs and outputs from a proper data set (training set) and iteratively computes weights and biases in order to minimize the sum of the squared error (SSE) of the training data. SSE is an error function composed by squaring the difference between sets of predicted and target values, and by adding these together. SSE computed at the output side is propagated backwards from this layer to the hidden layer and at the end to the input layer. During the training phase, each neuron in the hidden layer sums its input signals x_i after multiplying them by the strengths of the respective connections called weights (w_{ij}), and computes its output y_j as a function of the sum, Eq. 1:

$$y_i = f\left(\sum w_{ij}x_i\right) \tag{1}$$

Where f is the transfer function (Eq. 2) that is necessary to transform the weighted sum of all signals connecting with a neuron. Although the transfer function can take any form and may be linear or nonlinear, the sigmoid function is the most commonly used [20, 21]. Its general form is given as below:

$$f(x) = \frac{1}{1+e^{-x}} \tag{2}$$

The adjustment of the weights can be made immediately after the error is detected when inputting each individual data pair (immediate adjustment) or be made after accumulating the individual errors for all data pairs (deferred adjustment). Herein, the deferred adjustment is used, and the adjustment between the (n)th and the (n+1)th iterative is [22]:

$$\Delta w_{ji}(n+1) = \eta\frac{1}{k}\sum_{k}\delta_{kj}\mathrm{out}_{kj} + \alpha\Delta w_{ji}(n) \tag{3}$$

Where w_{ji} is the weight adjustment between the neuron j in the considering layer and the neuron i in the former layer, $\mathrm{out}_{k,\,j}$ the calculated output of the neuron i for data pair k, the $\delta_{k,\,j}$ the term of the neuron j for data pair k calculated from the calculated and target outputs, η the learning rate, α the momentum factor for preventing oscillations, and k is the number of data pairs. Thus, the unknown parameters (η, α), neuron number in the hidden layer, and

iterative number should be determined in advance. Herein, the trial-and-error method and the split-sample validation procedure were used. The sample data pairs were divided into training set and validation set. The parameters (η, α), hidden neuron number, and iterative number was chosen to minimize the SSE of the validation set.

Experimental

Materials and Methods

Organisms and Cultivation Conditions

Blakeslea Trispora wild-type strain ATCC14271 (−) was maintained on potato dextrose agar (PDA) slants. The strain was grown on Petri dishes at 28 °C for 4 days and then at 20 °C for 2 days to induce the formation of spores. Spores were harvested by rinsing the fully grown agar plates with a solution of 0.9% ($w\ v^{-1}$) NaCl. For submerged culture, 10^7 spores of the strains were inoculated into 50 mL PDA liquid medium in 250-mL Erlenmeyer flasks. The flasks were shaken for 2 days at 180 rpm at 28 °C without light. The moist mycelia were subsequently harvested by filtration and washed with double distilled water.

Reagents

Solutions and reagents used were as follows: solution I: 100 mM trihydroxymethylamino-methane–HCl (Tris–HCl), 40 mM ethylene diamine tetraacetic acid (EDTA), pH 9.0, benzyl chloride (Sigma Aldrich, Steinheim, Germany), 10% sodium dodecyl sulfate (SDS); extraction solution (solution II): 4 M guanidine thiocyanate (Promega, Madison, USA), 25 mM sodium citrate, 0.5% ($w\ v^{-1}$) sodium lauroyl sarcosine, 0.15 M β-mercaptoethanol (which has to be added into solution II just prior to use), phenol/chloroform/isoamyl alcohol (PCI) 25:24:1; chilled absolute ethanol and 70% ethanol, 2 M sodium acetate, pH 4.0.

Isolation of RNA

Moist mycelia were pretreated with solution I before extraction. First, 5 mL solution I was added into the Erlenmeyer flask containing 1 g moist mycelia and gently vortexed. Then, 1 mL 10% SDS and 3 mL benzyl chloride were added into the flask, which was vortexed vigorously to make the mixture milky and kept stirring at 50 °C for 1 h. Finally, the mixture was filtrated through the paper filters using vacuum pump to harvest the mycelia. The pretreated mycelia were subsequently frozen in the liquid nitrogen for RNA extraction. In extraction process, the frozen samples were first ground with a mortar and a pestle in the presence of liquid nitrogen. The resulting powder was transferred to a 1.5-mL Eppendorf tube containing 500 μL solution II and gently vortexed and inverted two or three times to homogenize the sample. After homogenizing the sample, 0.25 volume of 2 M sodium acetate (pH 4.0) was added into the tube and inverted two or three times. Then, an equal volume of PCI was added into the tube and vortexed the sample until thoroughly suspended (the whole homogenate turns to a turbid solution), and chilled on ice for 5 min. After centrifuging at 16,000×*g*, 4 °C for 5 min, the upper clear aqueous layer was carefully removed and placed into a fresh Eppendorf tube. The obtained solution was retreated by PCI in the same manner. After treating by PCI, the obtained upper phase solution was

transferred into a fresh Eppendorf tube with an equal volume of chilled isopropanol and then incubated at −20 °C for 10 min. At last, the precipitated RNA was collected from the supernatant by centrifugation at 16,000×*g*, 4 °C for 5 min. The collected RNA pellet was washed by 70% ethanol and dried at room temperature and finally dissolved in DEPC-treated autoclaved double-distilled water. The integrity and quality of RNA was estimated by ethidium–bromide staining and UV detector (Bio-Rad GelDoc System 2000).

Reverse Transcription of Total RNA

Reverse transcription was carried out in a separate reaction (two-step RT-PCR); the cDNA from the first step was stored at −20 °C, which could be conveniently used as the template for PCR. The template cDNA was produced using the Easy-Go RT Premix (SBS, Beijing, China). Firstly, a mixture of total RNA (1 μg) and random primer (5 μL, 20 mM) was incubated at 70 °C for 5 min before being placed on ice. Then, the mixture was transferred to an Easy-Go RT Rremix tube, and the final reaction volume was made up to 20 μL with DEPC water. Finally, the mixture was incubated at 40 °C for 60 min and then heated at 94 °C for 5 min to inactivate the reverse transcriptase.

PCR Amplification

The PCR experiments were performed with a MyCycler thermal cycler (Bio-Rad Laboratories). Primers were designed based on the sequences of the gene *carRA* (*carRA*-for: CCGTTTCACTCACAGCAAGA; *carRA*-rev: GACAGCCACAACACAAGTAGGA). The PCR amplification mixture consisted of 0.4–2.0 μL of the template cDNA, 0.05–0.25 μL (50 μM) primers, 0.05–0.45 μL (5 U μL^{-1}) Taq DNA polymerase (Takara, Japan), 0.5–4.5 μL dNTPs (2.5 mM), 0.5–4.5 μL $MgCl_2$ (25 μM), and the mixture was finally made up to 20 μL with double distilled water. PCR amplification included 25–45 cycles of denaturating for 30 s at 90–98 °C, annealing for 30 s at 60–66 °C and extension for 60 s at 70–74 °C. The product concentration was measured by a Bio-Rad SmartSpec Plus spectrophotometer (Bio-Rad Laboratories). Then, the product was electrophoretically separated on 1.5% agarose gel. After being stained with ethidium bromide, the gel was visualized and photographed under UV light (Bio-Rad GelDoc System 2000, Bio-Rad Laboratories).

Apparatuses

Bio-Rad MyCycler thermal cycler was purchased from Bio-Rad Laboratories (Beijing). Bio-Rad GelDoc System 2000 was purchased from Bio-Rad Laboratories (Beijing). Bio-Rad SmartSpec Plus spectrophotometer was purchased from Bio-Rad Laboratories (Beijing).

Experimental Design

To locate the optimum condition for PCR amplification of lycopene cyclase gene *carRA* in *Blakeslea Trispora*, an optimization strategy, combining ED and ANN, was adopted. In this strategy, a five-level star design was used to obtain suitable experimental data for ANN modeling [23, 24]. The ranges of each factor considered in this study were based on some preliminary studies. The total number of experiments is 37, and the design matrix with operation conditions and experimental results are shown in Table 1.

Table 1 Experimental design matrix and results.

Run	V_d (μL)	V_p (μL)	V_m (μL)	V_e (μL)	V_{Mg} (μL)	T_d (°C)	T_a (°C)	T_e (°C)	N_c	C (μg mL^{-1})
1	1.2	0.15	2.5	0.25	2.5	94	63	72	35	786.70
2	0.4	0.15	2.5	0.25	2.5	94	63	72	35	585.38
3	0.8	0.15	2.5	0.25	2.5	94	63	72	35	727.40
4	1.6	0.15	2.5	0.25	2.5	94	63	72	35	787.12
5	2	0.15	2.5	0.25	2.5	94	63	72	35	796.20
6	1.2	0.05	2.5	0.25	2.5	94	63	72	35	619.01
7	1.2	0.1	2.5	0.25	2.5	94	63	72	35	777.13
8	1.2	0.2	2.5	0.25	2.5	94	63	72	35	789.98
9	1.2	0.25	2.5	0.25	2.5	94	63	72	35	798.74
10	1.2	0.15	0.5	0.25	2.5	94	63	72	35	283.10
11	1.2	0.15	1.5	0.25	2.5	94	63	72	35	523.26
12	1.2	0.15	3.5	0.25	2.5	94	63	72	35	881.62
13	1.2	0.15	4.5	0.25	2.5	94	63	72	35	890.62
14	1.2	0.15	2.5	0.05	2.5	94	63	72	35	527.27
15	1.2	0.15	2.5	0.15	2.5	94	63	72	35	630.73
16	1.2	0.15	2.5	0.35	2.5	94	63	72	35	812.72
17	1.2	0.15	2.5	0.45	2.5	94	63	72	35	820.42
18	1.2	0.15	2.5	0.25	0.5	94	63	72	35	261.161
19	1.2	0.15	2.5	0.25	1.5	94	63	72	35	662.86
20	1.2	0.15	2.5	0.25	3.5	94	63	72	35	820.10
21	1.2	0.15	2.5	0.25	4.5	94	63	72	35	832.74
22	1.2	0.15	2.5	0.25	2.5	90	63	72	35	122.18
23	1.2	0.15	2.5	0.25	2.5	92	63	72	35	457.71
24	1.2	0.15	2.5	0.25	2.5	96	63	72	35	483.84
25	1.2	0.15	2.5	0.25	2.5	98	63	72	35	218.29
26	1.2	0.15	2.5	0.25	2.5	94	60	72	35	1,281.1
27	1.2	0.15	2.5	0.25	2.5	94	61.5	72	35	1,178.5
28	1.2	0.15	2.5	0.25	2.5	94	64.5	72	35	754.12
29	1.2	0.15	2.5	0.25	2.5	94	66	72	35	685.93
30	1.2	0.15	2.5	0.25	2.5	94	63	70	35	180.44
31	1.2	0.15	2.5	0.25	2.5	94	63	71	35	423.28
32	1.2	0.15	2.5	0.25	2.5	94	63	73	35	383.42
33	1.2	0.15	2.5	0.25	2.5	94	63	74	35	166.86
34	1.2	0.15	2.5	0.25	2.5	94	63	72	25	663.42
35	1.2	0.15	2.5	0.25	2.5	94	63	72	30	967.20
36	1.2	0.15	2.5	0.25	2.5	94	63	72	40	454.74
37	1.2	0.15	2.5	0.25	2.5	94	63	72	45	377.88

V_d the added amount of template (μL), V_p the added amount of primer (μL), V_m the added amount of dNTP (μL), V_e the added amount of polymerase (μL), V_{Mg} the added amount of magnesium ion (μL), T_d the denaturating temperature (°C), T_a the annealing temperature (°C), T_e the extension temperature (°C), N_c the number of cycles, C the efficiency (yield) of the PCR

Software

In this study, the BP-ANN was constructed using artificial neural network toolbox in MATLAB simulation software with the version of 6.1.

Results and Discussion

Artificial Neural Network

Optimization of ANN

In this paper, one hidden layer back-propagation artificial neural network with nine inputs and one output was developed. Before fitting the data, input and output variables were normalized to a range from 0 to 1 using the linear normalization method. All the weights and biases were initialized to small random numbers [25], which ensures that the network is not saturated by large values of the weight, and prevents some training pathologies. According to the trial-and-error calculations, the value of momentum factor is set as 0.9 where the best results are obtained. Besides, due to the learning rate, η has apparent impacts on the error, the adaptive learning rule was chosen with the maximum value is set as 1.05η, and the minimum value is set as 0.7η with original η set as 0.02, which decreased the iterative number remarkably. After the learning rate and the momentum factor were determined, the optimal hidden neuron number and iterative number could be easily found. For the present study, four hidden neurons and 3,000 iterative cycles were chosen as the optimal architecture, with which the minimum error of validation set was obtained. In order to test the predictive power and robustness of the model obtained, cross-validation using leave-one-out method was employed [26–28]. In leave-one-out method, a model is constructed after deleting one observation of the data set, and then this observation is predicted by the model based on the remaining data. This procedure is repeated for the entire data set and cross-validated coefficient is calculated. Figure 2 shows the plot of cross-validated predicted values by ANN versus experimental values. High R^2 values for the plot indicated good stability and predictive ability of the developed ANN model. Therefore, the

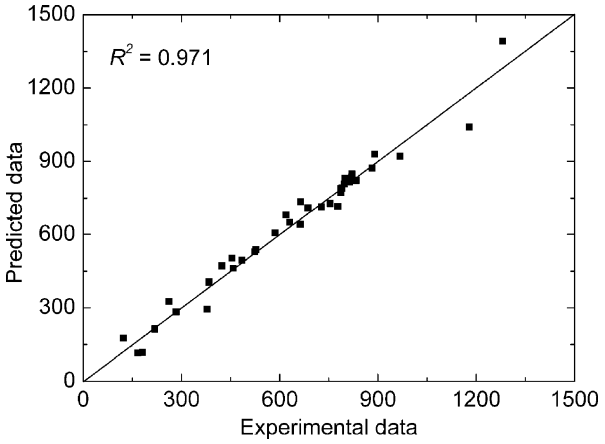
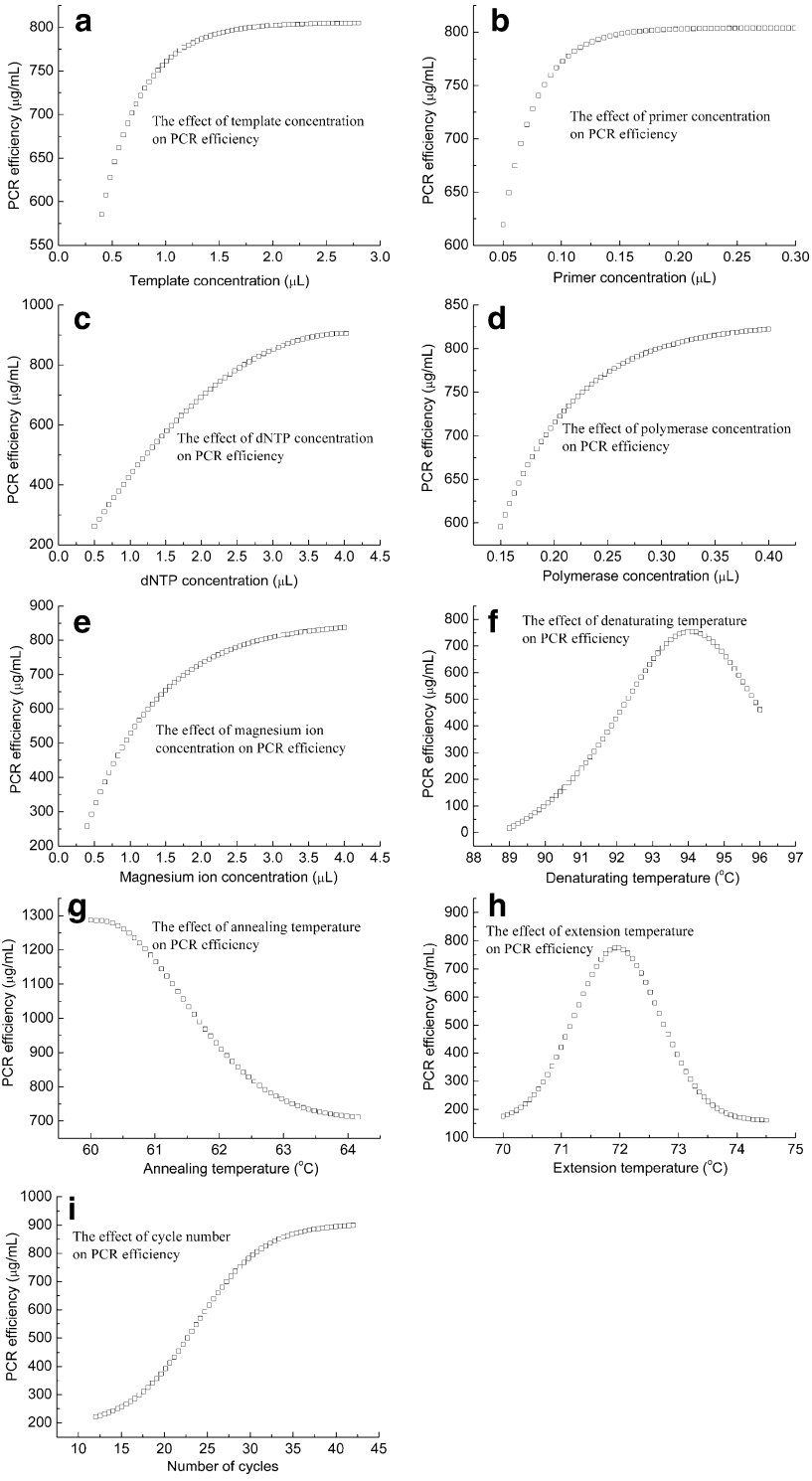


Fig. 2 The plot of cross-validated predicted values by ANN versus experimental values


a
The effect of template concentration on PCR efficiency
PCR efficiency (μg/mL)
Template concentration (μL)
b
The effect of primer concentration on PCR efficiency
PCR efficiency (μg/mL)
Primer concentration (μL)
c
The effect of dNTP concentration on PCR efficiency
PCR efficiency (μg/mL)
dNTP concentration (μL)
d
The effect of polymerase concentration on PCR efficiency
PCR efficiency (μg/mL)
Polymerase concentration (μL)
e
The effect of magnesium ion concentration on PCR efficiency
PCR efficiency (μg/mL)
Magnesium ion concentration (μL)
f
The effect of denaturating temperature on PCR efficiency
PCR efficiency (μg/mL)
Denaturating temperature (°C)
g
The effect of annealing temperature on PCR efficiency
PCR efficiency (μg/mL)
Annealing temperature (°C)
h
The effect of extension temperature on PCR efficiency
PCR efficiency (μg/mL)
Extension temperature (°C)
i
The effect of cycle number on PCR efficiency
PCR efficiency (μg/mL)
Number of cycles

◀ **Fig. 3** The effects of each parameter on PCR efficiency (When investigating one certain parameter other parameters were kept at the mean values)

obtained ANN model can be subsequently used to locate the optimal experimental condition for PCR amplification.

Predictions of ANN

Due to the credible predictions, the obtained ANN model was used to predict the effect of each parameter on the process of PCR amplification. When investigating one certain parameter, other parameters were kept at their mean values. Figure 3 shows the influence of each parameter on the PCR efficiency. The results showed that as the added amount of template, primer, dNTP, polymerase and magnesium ion increased the efficiency of PCR, which increased and then reached a plateau; the denaturating temperature markedly affected the efficiency of PCR. Both low and high denaturating temperature decreased the efficiency; as the annealing temperature increased, the efficiency decreased; both low and high extension temperature decreased the efficiency; as the number of cycles increased, the efficiency increased, and too large number of cycles contributed no more to PCR efficiency. According to the results obtained in this study, the most suitable operation conditions for the present system were determined as follows (in 20 μL reaction system): 2.0 μL template, 0.2 μL primer (50 μM), 3.5 μL dNTP (2.5 mM), 0.3 μL polymerase (5 U μL^{-1}), 3.5 μL magnesium ion (25 μM), denaturating temperature 94 °C, annealing temperature 60 °C, extension temperature 72 °C, and number of cycles 35.

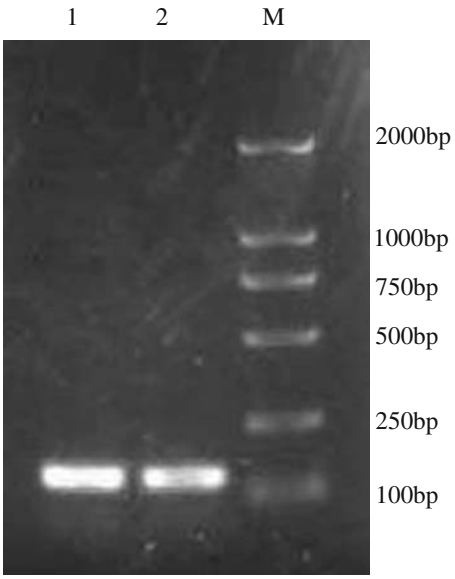


Fig. 4 PCR products detected on agarose gel. *M* DL-2000 Marker (2, 1, 0.75, 0.5, 0.25, 0.1 kb); *lanes 1 and 2*: products under the predicted optimal condition

Experimental Validation

Under the predicted optimal operation conditions obtained in the above section, the corresponding experiments were conducted. The detailed procedure was carried out as described in the section "PCR Amplification." The experimental result (1,296.9 μg mL^{-1}) was well consistent with the predicted result (1,325.7 μg mL^{-1}), which confirmed that the developed method was feasible for present study.

It is well accepted that the specificity is also an important characteristic for PCR amplification. Thus, the specificity of the PCR under the predicted optimal conditions was also investigated by detecting the products on the agarose gel. Figure 4 shows that there was only one band between 100 and 250 bp, confirming the product was correct with high specificity (the size of target product was 135 bp).

Conclusion

The combination of artificial neural network and experimental design has been found to be a feasible tool for optimization of PCR amplification. A five-level star design was performed and confirmed that it was enough to provide data source for ANN modeling. The results showed that the optimized ANN can predict present PCR amplification process with excellent accuracy. With the credible predictions, the influence of each parameter was predicted. The predicted results indicated how each parameter affects the PCR process. Using this information, the PCR process could be optimized. The expectant experimental result was actually produced at the predicted optimal conditions, which confirmed that the proposed method was reliable. Though the ANN model could not provide detailed information about the process like mathematical model, ANN was also a powerful fit and predictive tool as well as a useful compensation to understand the mechanisms of complex systems. To some extended sense, the method of combining the ANN technique and experimental design is probably to take an important role in helping researchers to optimize the PCR process and other processes in biological field.

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