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Optimization of medium composition for two-step fermentation of vitamin C based on artificial neural network – genetic algorithm techniques

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The production of 2-keto-L-gulonic acid (2-KGA) during the conversion from L-sorbose to 2-KGA in the two-step fermentation of vitamin C can be improved by using an efficient companion strain Bacillus subtilis A9 to facilitate the growth of Ketogulonicigenium vulgare and the production of 2-KGA. Two optimization models, namely response surface methodology (RSM) and artificial neural network (ANN), were built to optimize the medium components for mixed-culture fermentation of 2-KGA. The root mean square error, \( R^2 \) and the standard error of prediction given by the ANN model were 0.13%, 0.99% and 0.21%, respectively, while the RSM model gave 1.89%, 0.84% and 2.9%, respectively. This indicated that the fitness and the prediction accuracy of the ANN model were higher than those of the RSM model. Furthermore, using genetic algorithm (GA), the input space of the ANN model was optimized, predicting that the maximum 2-KGA production of 72.54 g L\(^{-1}\) would be obtained at the GA-optimized concentrations of the medium components (L-sorbose, 92.5 g L\(^{-1}\); urea, 10.2 g L\(^{-1}\); corn steep liquor, 16 g L\(^{-1}\); CaCO3, 3.96 g L\(^{-1}\); MgSO4, 0.28 g L\(^{-1}\)). The 2-KGA production experimentally obtained using the ANN–GA-designed medium was 71.21 ± 1.53 g L\(^{-1}\), which was in good agreement with the predicted value. The same optimization process may be used to improve the production during bacterial mixed-cultures fermentation by changing the fermentation parameters.

Keywords: 2-keto-L-gulonic acid; B. subtilis A9; medium optimization; response surface methodology; artificial neural network; genetic algorithm

Introduction

The primary method of industrial vitamin C (Vc) production in China is via mixed-culture fermentation of L-sorbose for producing 2-keto-L-gulonic acid (2-KGA), the precursor of Vc.[1–3] Mixed-culture fermentation is achieved by the cooperation of two microorganisms: Ketogulonicigenium vulgare (the acid-producing strain) and Bacillus megaterium (companion strain, or co-culture helper).[4] Thus, in the light of current systems biology analysis, not only the individual physiological characteristics of the two strains, but also the interactions between them in this ecosystem have been the focus of research.[3,6] Studies have shown that the interactions between B. megaterium and K. vulgare are a synergistic combination of mutualism and antagonism.[7] By reconstructing genome-scale metabolic models of K. vulgare[8] and B. megaterium[9] based on genome annotation and data from the literature and biochemical databases, we found that K. vulgare was deficient in nutrient biosynthetic pathways.[10] To date, many studies on the construction of combinatorial expressions of key enzymes and the related co-factors in K. vulgare[11] and using the proposed high-throughput method to screen target companions from large numbers of random mutants for the co-culture process of 2-KGA biosynthesis[12] have become feasible. However, these studies remain at an exploratory level; they need time for practice, repetition and improvement. The major companion strain currently used in industry is B. megaterium. The conversion rates from L-sorbose to 2-KGA by current mixed-culture strains are difficult to enhance sufficiently. Therefore, scholars from different countries have focused on increasing technical innovations to screen for efficient strains with higher conversion rates to enhance the 2-KGA productivity[13] and reduce industry costs. It is known that bacteria that have the potential to serve as companion strains include Bacillus megaterium, Bacillus thuringiensis, Bacillus cereus, Pseudomonas striata, Sporobolomyces roseus and others.[14] In this study, B. subtilis A9 from fresh milk was studied. It exhibited good companion ability and was able to effectively improve the production of 2-KGA, which is a promising development in terms of application prospects.

A major goal in the modelling and optimization of biotechnological processes is to improve the system and increase the process efficiency without increasing the cost.[15] One of the empirical modelling systems that are

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commonly used for development, improvement and optimization of complex processes is response surface methodology (RSM). By RSM, the experimental response(s) are fit to quadratic function(s) and the assessment is made of the effect of the independent variables, alone or in combination, on the process based on their relationships with the response(s). Despite the fact that RSM has many advantages and has been shown to be successful in a wide range of fermentation processes,[16] there are some cases in which it may not prove applicable.[17] Alternative options are approaches based on artificial intelligence, e.g. artificial neural network (ANN) and genetic algorithm (GA). They achieve data modelling based on mimicking different aspects of biological information processing and have been demonstrated to be useful in media optimization.[18,19] ANN offers a powerful solution for studying non-linear problems. In contrast to regression equations, which need to be given as the function, ANN is a mathematical model obtained through finite iterative computation reflecting the connections within the experimental data. GA is a global optimization algorithm based on natural selection and population evolution mechanisms. Through GA, the ANN model can be extended to global training to obtain an optimal algorithm.

Applications of ANN–GA technology in food science, environmental biotechnology and biochemical engineering have been reported,[20] but to the best of our knowledge there are no reports on its application in the field of mixed-culture fermentation for the production of Vc. The aim of this work was to compare two optimization techniques, namely RSM and ANN coupled with GA, in optimizing the concentrations of medium components (L-sorbose, urea, corn steep liquor, CaCO3 and MgSO4) to maximize the production of 2-KGA, using _B. subtilis_ A9 as the companion strain.

**Materials and methods**

**Materials**

The acid-producing strain of _K. vulgare_ 25-B-1 and the companion strain of _B. megaterium_ 2980 were provided by Northeast Pharmaceutical General Factory (Shenyang, China). The companion strain of _B. subtilis_ A9 was screened and maintained in the Shenyang Agriculture University collection (Shenyang, China).

The seed culture medium contained the following: 20 g L⁻¹ L-sorbose; 2 g L⁻¹ glucose; 5 g L⁻¹ corn steep liquor; 1 g L⁻¹ urea and 1 g L⁻¹ CaCO3. The basic fermentation medium contained the following: 80 g L⁻¹ L-sorbose; 12 g L⁻¹ urea; 10 g L⁻¹ corn steep liquor; 5 g L⁻¹ CaCO3; 0.2 g L⁻¹ MgSO4 and 1 g L⁻¹ KH2PO4.

**Methods**

The acid-producing strain colonies and the companion strain colonies were selected, respectively, and put in sterile water. Then, acid-producing strain suspension was added into the companion strain suspension and was shaken to obtain a mixed-culture strain suspension. A sterile inoculation loop was used to streak the mixed-culture strain suspension onto blank slants. This step was repeated 15 to 20 times, until the surface of the slants became wet. Then the mixed-culture strain slants were cultivated upside down at 29 °C for 48–72 h. The seed culture inoculated from a mixed-culture strain slant was cultivated at 29 °C and 180 r min⁻¹ for 24 h.

Fermentation medium (5 mL) was mixed with 10% (v/v) of seed culture in 50 mL flasks. The inoculated mixed culture was incubated at 29 °C for 48 h with orbital shaking at 180 r min⁻¹. The initial culture acidity was adjusted to pH 6.8–7.0.[21] The concentration of 2-KGA was determined using the iodometric method as previously described.[22] For medium optimization, concentrations of L-sorbose, urea, corn steep liquor, CaCO3 and MgSO4 were chosen according to an experimental design matrix (Table 1). The remaining medium components were kept constant.

**Design of CCD**

The preliminary study [23] indicated that L-sorbose, urea, corn steep liquor, CaCO3 and MgSO4 were the significant factors influencing the production of 2-KGA. The symbols and levels of these five variables are shown in Table 1. Using RSM and ANN models, the combined effect of these five factors was evaluated according to the experimental results of the central composite design (CCD).

**RSM model**

Design Expert 8.0 [24,25] was applied to conduct a regression analysis of the experimental data in Table 1. The least square method was used to fit the quadratic polynomial equation and establish a quadratic regression model as follows:

\[ Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \sum_{j=1}^{5} \beta_{ij} X_i X_j + \sum_{j=1}^{5} \beta_{ij} X_j^2, \]

where \( Y \) is the response value (production), \( \beta_0, \beta_i, \beta_{ij} \) and \( \beta_{ij} \) are the coefficients of the equation and \( X_i \) and \( X_j \) are the code values of the variables. The accuracy and general ability of the above polynomial model was evaluated by the regression coefficient (\( R^2 \)).

**ANN model**

A neural network is a computer program architecture for non-linear computations and it simulates the brain’s learning process by mathematically modelling the network structure of interconnected node cells. The
The architecture of the ANN consisted of a feedforward network with three layers: one input layer with five inputs, which were the content of the L-sorbose, urea, corn steep liquor, CaCO₃ and MgSO₄ in the fermentation medium in Table 1, one hidden layer and an output layer that rendered the predicted production of 2-KGA. The back-propagation method was applied to establish the ANN model. The transfer function of the neurons in the hidden layer was the tansig, and the neurons in the output layer had a linear transfer function. At the same time, the trainbr method was used for training. When the mean squared error (MSE) reached $1 \times 10^{-3}$, the network stopped training.

To evaluate the fitness and prediction accuracy of the RSM and ANN models, the root mean square error (RMSE), $R^2$ and standard error of prediction (SEP) of the model were employed[26]

$$\text{RMSE} = \sqrt{\frac{\sum(Y_{i,e} - Y_{i,p})^2}{n}}$$

$$R^2 = 1 - \frac{\sum(Y_{i,e} - Y_{i,p})^2}{\sum(Y_{i,e} - \bar{Y}_e)^2}$$

$$\text{SEP} = \frac{\text{RMSE}}{\bar{Y}_e} \times 100\%,$$

where $Y_{i,e}$ represents the experimental data, $Y_{i,p}$ is the predictive data and $\bar{Y}_e$ is the mean value of the experimental data.

### Table 1. Design matrix and results obtained in the central composite design (CCD).

| Standard order | $\chi_1$ L-sorbose (g·L⁻¹) | $\chi_2$ Urea (g·L⁻¹) | $\chi_3$ Corn steep liquor (g·L⁻¹) | $\chi_4$ CaCO₃ (g·L⁻¹) | $\chi_5$ MgSO₄ (g·L⁻¹) | 2-KGA production (g·L⁻¹) |
|---------------|--------------------------|----------------------|-----------------------------|----------------------|----------------------|------------------------|
| 1             | 70(-1)                   | 10(-1)               | 14(-1)                      | 3(-1)                | 0.3(1)               | 55.76                  |
| 2             | 90(1)                    | 10(-1)               | 14(-1)                      | 3(-1)                | 0.1(-1)              | 63.46                  |
| 3             | 70(-1)                   | 14(1)                | 14(-1)                      | 3(-1)                | 0.3(1)               | 56.33                  |
| 4             | 90(1)                    | 14(1)                | 14(-1)                      | 3(-1)                | 0.1(-1)              | 63.84                  |
| 5             | 70(-1)                   | 10(-1)               | 16(1)                       | 3(-1)                | 0.3(1)               | 60.22                  |
| 6             | 90(1)                    | 10(-1)               | 16(1)                       | 3(-1)                | 0.3(1)               | 66.12                  |
| 7             | 70(-1)                   | 14(1)                | 16(1)                       | 3(-1)                | 0.3(1)               | 65.42                  |
| 8             | 90(1)                    | 14(1)                | 16(1)                       | 3(-1)                | 0.1(-1)              | 64.22                  |
| 9             | 70(-1)                   | 10(-1)               | 14(-1)                      | 5(-1)                | 0.1(-1)              | 56.92                  |
| 10            | 90(1)                    | 10(-1)               | 14(-1)                      | 5(-1)                | 0.1(-1)              | 56.18                  |
| 11            | 70(-1)                   | 14(1)                | 14(-1)                      | 5(-1)                | 0.1(-1)              | 57.12                  |
| 12            | 90(1)                    | 14(1)                | 14(-1)                      | 5(-1)                | 0.1(-1)              | 57.14                  |
| 13            | 70(-1)                   | 10(-1)               | 16(1)                       | 5(-1)                | 0.1(-1)              | 66.15                  |
| 14            | 90(1)                    | 10(-1)               | 16(1)                       | 5(-1)                | 0.1(-1)              | 63.84                  |
| 15            | 70(-1)                   | 14(1)                | 16(1)                       | 5(-1)                | 0.1(-1)              | 60.38                  |
| 16            | 90(1)                    | 14(1)                | 16(1)                       | 5(-1)                | 0.3(1)               | 65.76                  |
| 17            | 60(-2)                   | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 61.23                  |
| 18            | 100(2)                   | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 71.15                  |
| 19            | 80(0)                    | 8(-2)                | 14(0)                       | 4(0)                 | 0.2(0)               | 61.07                  |
| 20            | 80(0)                    | 16(2)                | 14(0)                       | 4(0)                 | 0.2(0)               | 66.12                  |
| 21            | 80(0)                    | 12(0)                | 10(-2)                      | 4(0)                 | 0.2(0)               | 62.12                  |
| 22            | 80(0)                    | 12(0)                | 18(2)                       | 4(0)                 | 0.2(0)               | 62.69                  |
| 23            | 80(0)                    | 12(0)                | 14(0)                       | 2(-2)                | 0.2(0)               | 66.07                  |
| 24            | 80(0)                    | 12(0)                | 14(0)                       | 6(2)                 | 0.2(0)               | 63.84                  |
| 25            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0(-2)                | 63.26                  |
| 26            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0.4(2)               | 66.19                  |
| 27            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 67.3                   |
| 28            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 69.93                  |
| 29            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 68.46                  |
| 30            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 68.07                  |
| 31            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 69.22                  |
| 32            | 80(0)                    | 12(0)                | 14(0)                       | 4(0)                 | 0.2(0)               | 68.36                  |
Optimization of GA

GA is a type of adaptive and global optimization probability search method, which is based on the principle of genetic variation and hybridization in biology. It is a new field which is emerging from the simulation of population evolutionary mechanisms in biological communities through a computer. The solution to a given problem can be considered as an individual coded by chromosome strings. The fitness function values of the individual are regarded as the evaluation index of individual quality. In the process of population evolution, three genetic and evolutionary operators are continuously applied, e.g. selection, crossover and mutation, in order to gradually reach optimal solutions until generating the global optimal solution. GA is especially suitable for complex and non-linear problems which cannot be solved by traditional search methods. The floating-point coding method was adopted for the ANN simulation calculation of L-sorbose, urea, corn steep liquor, CaCO3 and MgSO4 during the fermentation process in their respective ranges. The model fitting data were considered as the fitness function of the GA for selection, variation and exchange. Higher production of 2-KGA corresponded to higher fitness.

The optimization of the neural network was done using the ‘ga’ function of MATLAB 8.0. The input parameters of the ga function were as follows: population type, double vector; population size, 20; crossover fraction, 0.8; elite count, 2; migration direction, forward; migration interval, 20; migration fraction, 0.2; generations, 100; stall generations, 50 and function tolerance, 1e-6.

Results and discussion

Impact of different companion strains on 2-KGA production

The experimental results showed that B. subtilis A9 performed better than B. megaterium 2980 in promoting 2-KGA production as the companion strain (Table 2).

RSM analysis

The regression fitting of the experimental data is shown in Table 1. The ‘lack-of-fit’ value is 0.0030, which means the prediction value cannot give a thorough description of the process of 2-KGA fermentation. The ‘lack-of-fit F-value’ of 18.19 implies that the lack of fit is not significantly relative to the pure error. The value of ‘Model Prob>F’ is less than 0.05, which means that this item is significant at the probability level of 95%. The values $X_1$, $X_3$, $X_4$, $X_5$, and $X_6$ are the significant items in our study. The non-significant terms $X_2$ (urea), $X_4$ (CaCO3), $X_5$ (MgSO4), $X_1X_3$ (L-sorbose × urea), $X_1X_4$ (L-sorbose × CaCO3), $X_1X_5$ (L-sorbose × MgSO4), $X_2X_3$ (urea × corn steep liquor), $X_2X_4$ (urea × CaCO3), $X_2X_5$ (urea × MgSO4), $X_3X_4$ (corn steep liquor × CaCO3), $X_3X_5$ (corn steep liquor × MgSO4), $X_4X_5$ (CaCO3 × MgSO4), $X_4$ (L-sorbose)$^2$ were excluded from the model. The regression equation is as follows:

$$Y = 68.36 + 1.75X_1 + 1.93X_3 - 1.5X_5^2 - 1.82X_3^2 - 1.16X_4^2 - 1.22X_5^2.$$
To avoid overfitting, the ANN architecture selected fewer neurons in the hidden layer on the sufficient condition of training accuracy. The network with 10 neurons in the hidden layer gave the lowest error. The performance of the network throughout the training until the final fitness is shown in Figure 1. This figure illustrates the variation of the MSE of the fitness value with the number of generations.

**Comparison of RSM and ANN**

According to the results of the RSM and ANN, the RMSE, $R^2$ and SEP of the ANN model were 0.13%, 0.99% and 0.21%, respectively, while the RMSE, $R^2$ and SEP of the RSM model were 1.89%, 0.84% and 2.9%, respectively. In general, higher RMSE and $R^2$ indicate better fitness between the model and experimental results, while lower SEP indicates better predictability and extrapolation of a model. The comparison results indicated that the fitness and the prediction accuracy of the ANN model were higher compared to those of the RSM model. As the mathematical regression equation which the RSM model established was a quadratic polynomial and had limited fitness ability, it was difficult to reflect the non-linear relationships among all factors of the 2-KGA fermentation process. Figure 2 shows the comparative parity plot for ANN and RSM predictions for the design. The ANN model needs no functions, unlike the regression equation, as it is a mathematical model obtained by using experimental results for limited iterative computations and reflects the inner relationship among experimental results. It has an extremely strong ability to perform non-linear processing. Thus, this experiment ultimately used the ANN model as the fitness function of GA to obtain the best combination of all factors.

**GA optimization**

GA was implemented to optimize the fermentation medium to maximize the 2-KGA production by using the ANN as the fitness function. A population of individuals was randomly generated, and a fitness value was assigned to each individual by the ANN fitness function. The evolution process of GA is shown in Figure 3. After 66 times of optimization calculation through GA, the maximum theoretical production of 2-KGA was 72.54 g·L$^{-1}$. The optimal medium components were L-sorbose, 92.5 g·L$^{-1}$; urea, 10.2 g·L$^{-1}$; corn steep liquor, 16 g·L$^{-1}$; CaCO$_3$, 3.96 g·L$^{-1}$ and MgSO$_4$, 0.28 g·L$^{-1}$. Under such cultivation conditions and after three repeated experiments, the average production of 2-KGA was 71.21 ± 1.53 g·L$^{-1}$. These results indicated that the models of ANN were good for mixed-culture fermentation of Vc. Based on the obtained results, B. subtilis A9 could be recommended as a suitable companion strain in the production of Vc in the pharmaceutical and food industry.
Conclusions

This study demonstrated that *B. subtilis* A9 could serve as a companion strain in the two-step fermentation of VC. ANN was successfully employed to model the complex fermentation process of 2-KGA which could inherently capture almost any form of non-linearity. A maximum 2-KGA production of 72.54 g·L⁻¹ was predicted at the ANN–GA-optimized concentrations of medium.

Figure 2. Comparison of experimental production and predicted production by RSM and ANN.

Figure 3. Evolution of the best and mean fitness in the GA.
components (L-sorbose, 92.5 g·L⁻¹; urea, 10.2 g·L⁻¹; corn steep liquor, 16 g·L⁻¹; CaCO₃, 3.96 g·L⁻¹ and MgSO₄, 0.28 g·L⁻¹). The 2-KGA production experimentally obtained under the above conditions was 71.21 ± 1.53 g·L⁻¹, which was in agreement with the predicted value. ANN–GA can not only effectively improve the 2-KGA production and substantially lower the culture medium cost, but can also dramatically decrease the experimental workload and shorten the study period. Further studies are needed by analyzing complicated mixed-culture fermentation processes of bacteria.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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