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Glutathione Fermentation Process Modeling Based on CCTSK Fuzzy Neural Network

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Abstract: As one of the important intelligent modeling methods, BP neural network can be effectively used for GSH fermentation process modeling. However, it usually lacks the nicer interpretation and its performance is often deteriorated by noise existing in the sample data. In order to circumvent theses weaknesses, a robust and interpretable syllogistic-fuzzy-inference based modeling method, called CCTSK (Cascade Centralized TSK) fuzzy neural network, is introduced for GSH fermentation process modeling. Present experimental results demonstrate that the proposed method really has better robustness compared with the traditional BP neural network.

Key words: Glutathione, CCTSK fuzzy neural network, robustness, orthogonal experiment

INTRODUCTION

Glutathione (GSH) is one of the major non-protein thiol compounds, which was first obtained by Hopkins from the extraction of yeast in 1921 (Penninckx and Elskens, 1993) and then its molecular structure was finally confirmed with chemical analysis, acid-base titration, degradation and synthesis (Harington and Mead, 1935). GSH plays many important roles in living cells, such as enhancing the immunity of the body promptly and maintaining the normal redox environment of cells as an antioxidant (Meister and Anderson, 1983; Izawa *et al*., 1995). Moreover, GSH is generally recognized as safe and effective medicine for detoxifcation, knub, cancer and incretion (Meister, 1994). It is also found that GSH can restrain Human Immunodeficiency Virus (HIV) (Jahoor *et al*., 1999). In addition, it is used commercially as food additives. Since GSH is widely used in many fields, such as clinical medicine, food industry and biological research, the demand of GSH has been expanding.

Animalcule fermentative production of GSH is one of the most general ways for GSH production at present. The yeast is usually strains in common use, which can produce and accumulate GSH in broad cultivation conditions. But the product is usually very low. Therefore, in order to achieve the high product of GSH, it is necessary to design the optimal production medium and the process conditions. In the last decade, Artificial Neural Networks (ANNs) have emerged as an attractive tool for predicting and approximating the parameters in fermentation process (Linko and Zhu, 1992) and also achieved the ni-cer effectiveness in the cultivation design (Kennedy *et al*., 1992). The most widely utilized ANN parad-igm is the BP neural network. However, in the practical fermentation processing, the BP neural network has the following major weakness: First, the prediction accuracy of BP is often deteriorated by the noise existing in the experimental data, i.e., the robustness of BP is weak; second, as the BP neural network is usually taken an black-box, it lacks the nicer interpretation for the GSH fermentation process modeling. Therefore, it is very necessary to introduce the robust and interpretable new intelligent modeling method for GSH fermentation process.

In recent years, fuzzy control system applied in industrial bioprocesses has been intensively reviewed (Honda and Kobayashi, 2000). The availability has been demonstrated with a series of concrete examples. For instance, a temperature control of the ginjo sake mashing process based on fuzzy neural networks has been reported (Honda *et al*., 1998). And then fuzzy control of vitamin B2 production has shown that fuzzy control systems can control a large-scale fermentation process and achieve good results (Horiuchi and Hiraga, 1999). In addition, an application of extended recurrent neural network in modeling fed-batch fermentation of Saccharomyces cerevisiae has been maximize the biomass quantities (Chen *et al*., 2004).

In this study, to overcome the weaknesses of BP neural networks mentioned above, we introduce a fuzzy-inference based fuzzy neural network, called the Cascaded Centralized TSK (CCTSK), for GSH fermentation process. Wang *et al*. (2005) presented the CCTSK fuzzy neural network for system modeling. This new modeling method has the following merits: As the CCTSK is based on the fuzzy inference rules, it is not taken as a black box; since

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the CCTSK introduces the syllogism inference and centralized strategy, it has a high robustness to the noise data and a high interpretation for the process modeling compared with traditional fuzzy neural networks.

In this study, *Candida utilis* WSH 02-08 is selected for GSH production and the essential culture medium is determined to consist of glucose, the sole carbon source, mixed nitrogen source of $(NH_4)_2SO_4$ and urea, KH_2PO_4 and $MgSO_4$. Present experimental data is once used to predict optimal mixture factors with the purpose of maximum products by BP network (Wei *et al.*, 2003). Nevertheless, in this study, based on the results of the L_{16} (4^5) orthogonal experiments, the CCTSK is straight used to predict the product. The experimental results for GSH fermentation process with both CCTSK and BP neural network are reported for comparison study. Present experimental results demonstrate that the introduced CCTSK modeling method really has the better robustness compared with the BP neural network based modeling method.

MATERIALS AND METHODS

The strain: Candida utilis WSH 02-08

Medium

Slant culture (g L^{-1}): glucose 20, peptone 20, yeast extract 10, agar 20 and pH 6.0.

Seed culture (g L^{-1}): glucose 20, peptone 20, yeast extract 10 and pH 6.0.

Fermentative culture (g L^{-1}): glucose 30,$(NH_4)_2SO_4$ 8, KH_2PO_4 2.5, $MgSO_4$ 0.3 and pH 5.5.

Culture methods

Slant culture: The slant is culture at 30°C in constant temperature for three days and then stored in refrigerator. The culture should be transferred each month.

Seed culture: After activated for 3~4 h at 30°C, the slant seed is inoculated by microzyme and carried in a 500 mL shake flask with 50 mL seed culture medium for 20 h at 200 r min^{-1} and 30°C.

Fermentative culture: The seeds are carried in a 500 mL triangle flask with 50 mL seed culture medium for 26 h at 30°C. The speed of HYGIIrotary type constant temperature shake flask closet is 200 r min^{-1}.

Distill of intracellular GSH: The cells are separated by centrifugation (3000 r min^{-1}, 10 min). The fresh wet yeast is washed by distilled water for three times and then carried in shake flask for 2 h by the addition of ethanol (40%). The resulting precipitate is collected by centrifugation (3000 r min^{-1}, 30°C) and re-dissolved in water (Alfafara *et al.*, 1992). The crude GSH solution is dialyzed to estimate the yield.

Analysis

Measurement of Dry Cell Weight (DCW): The fermentation broth (25 mL) are separated by centrifugation (3000 r min^{-1}) and then washed by distilled water for two times. The resulting precipitate is dried for 48 h at 60°C and weighted.

Measurement of glucose concentration: Dinitrosalicylic acid (DNS method).

Measurement of GSH concentration: DTNB (5, 5'-dithio-(2-nitrobenzoic acid)-GSH reductase circulation methods. 100 μL 6 mmol L^{-1} DTNB, 700 μL 0.3 mmol L^{-1} NADPH and 200 μL samples diluted to a suitable level were added to a 2 mL volume quartz cuvette in sequence. 100 μL 5.0 U mL^{-1} GSH reductase is added to start the reaction. The beginning OD value and the OD value after 5 min is detected, according to the relationship of GSH concentration and OD's change rate, GSH concentration can be calculated. Definition of intracellular GSH content:

$$\text{Content}/\% = \frac{\text{GSH}/(\text{mg L}^{-1})}{10\times \text{DCW}/(\text{g L}^{-1})}\times 100\%$$

The cascaded centralized TSK modeling method CCTSK: The idea of CCTSK is first to centralize the classical TSK fuzzy system (Wang, 1998) as the CTSK (centralized TSK) and then by introducing the cascaded structure to organized the CTSK as the CCTSK (Cascaded CTSK). In this section, we first briefly describe the TSK and CTSK and then give the structure and learning rules of CCTSK (Wang *et al.*, 2005; Wang and Korris, 2004).

TSK: The TSK modeling method is based on fuzzy inference mechanism. A TSK system contains the following M fuzzy rules as Eq. 1 and the final input of the system can be written as Eq. 2.

$$\begin{aligned} &R_i\text{: if } x_i \text{ is } A_{i1} \text{ and } x_2 \text{ is } A_{i1}, \ldots \text{ and } x_n \text{ is } A_{in} \\ &\quad\text{then } y_i = p_{i0} + p_{i1}x_1 + \ldots + p_{in}x_n \end{aligned} \tag{1}$$

$$y = \frac{\sum_{i=1}^{M} y_i \prod_{j=1}^{n} A_{ij}(x_j)}{\prod_{j=1}^{n} A_{ij}(x_j)} \tag{2}$$

Where:

- R_i : The ith fuzzy rule (i - 1, 2, ..., M)
- M : The number of fuzzy rules
- x_j : The jth dimensional input variable (j = 1, 2, ..., n)
- n : The number of dimensions;
- A_{ij} : Fuzzy set;
- Y_i : The output of ith fuzzy rule;
- y : Corresponds to the final output of the fuzzy system;
- P_{ij} : The consequence coefficient of the ith fuzzy rule.

TSK fuzzy system has emergeed as a conventional fuzzy model for nonlinear complex system modeling. Since the exact meaning of the coefficient p_{ij} is not clear, the centralized TSK fuzzy neural network was proposed by Wang *et al*. (2005) to give a more proper interpretation for the coefficient p_{ij}. Centralized TSK (CTSK): In Centralized TSK fuzzy systems, the fuzzy inference rules can be expressed as follows.

$$\begin{aligned}&R_i\text{: if } x_i \text{ is } A_{i1} \text{ and } x_2 \text{ is } A_{i2} \text{ is } Ai_2, \ldots \text{ and } x_n \text{ is } A_{in}\\ &\text{then, } Y_{1i} = p_{i0} + p_{i1}(x_1 - x_{i1}) + \ldots + p_{in}(x_n - m_{in})\end{aligned} \tag{3}$$

Where, n-dimensional vector m = (m_{i1}, m_{i2}, m_{in}) denotes the center of the ith rule and the membership functions of fuzzy set A_{ij} takes Gaussian type, i.e., s $A_{ij}(x_j) = e^{-(x_j - m_{ij})^2/\sigma_{ij}^2}$, the final input of the CTSK can be written as:

$$\begin{aligned}\bar{y} &= \sum_{i=1}^{M} y_i \prod_{j=1}^{n} A_{ij}(x_j) =\\ &\sum_{i=1}^{M}\left\{\begin{matrix}[p_{i0} + p_{i1}(x_1 - m_{i1}) + \cdots +\\ p_{in}(x_n - m_{in})]\prod_{j=1}^{n} e^{-(x_j - m_{ij})^2/\sigma_{ij}^2}\end{matrix}\right\}\end{aligned} \tag{4}$$

From Eq. 3, we can see that the consequence part of each rule in CTSK fuzzy system is similar to the Taylor formula. According to this, p_{ij} (j = 1,2, ..., n) can be viewed as the corresponding first-order derivatives of its output at the rule centers. Therefore, the CTSK fuzzy system is a highly interpretable fuzzy system compared TSK fuzzy neural networks and BP neural networks.

The structure of Cascaded CTSK fuzzy model (CCTSK): The cascaded centralized TSK fuzzy neural network CCTSK is constructed with some CTSK by a cascaded way. For convenience, here we describe a 2-stage CCTSK containing two CTSKs, which are called CCTSK1 and CCTSK2, respectively. x = (x_1, x_2, ...x_n) represents the input variable of the network CCTSK and CCTSK1 simultaneously. z = (z_1, z_2, ..., z_k) is the intermediate variable of the CCTSK, which is the output variable of CCTSK1 and the input variable of CCTSK2 simultaneously. The inference rules of CCTSK1 can be expressed as:

$$\begin{aligned}&R_i^1 \text{ :if } x_1\ A_{i1} \text{ and } x_2 \text{ is } A_{i2}, \ldots \text{and } x_n \text{ is } A_{in}\\ &\text{then, } z_{1i} = p_{i0}^1 + p_{i1}^1(x_1 - m_{i1}) + \cdots + p_{in}^1(x_n - m_{in})\\ &\vdots\\ &\qquad z_{ki} = p_{i0}^k + p_{i1}^k(x_1 - m_{i1}) + \cdots + p_{in}^k(x_n - m_{in})\end{aligned}$$

Where, i = 1, 2, ..., M1 and $A_{ij}(x_j) = e^{-(x_j - m_{ij})^2/\sigma_{ij}^2}$

Meanwhile, the inference rules of CCTSK2 can be expressed as:

$$\begin{aligned}z_l &= \sum_{i=1}^{M1} z_{li} \prod_{j=1}^{n} A_{ij}(x_j) =\\ &\sum_{i=1}^{M1}\left\{\begin{matrix}[p_{i0}^1 + p_{i1}^1(x_1 - m_{i1}) + \cdots +\\ p_{in}^1(x_n - m_{in})]\prod_{j=1}^{n} e^{-(x_j - m_{ij})^2/\sigma_{ij}^2}\end{matrix}\right\}\end{aligned} \tag{5}$$

Where, *l* = 1, 2, ..., k.

$$\begin{aligned}&R_i^2 \text{ if } z_1 \text{ is } S_{i1} \text{ and } z_2 \text{ is } B_{i2}, \ldots \text{ and } z_k \text{ is } B_{ik}\\ &\text{then } Y_i = q_{i0} + q_{i1}(z_1 - r_{i1}) + \ldots + q_{ik}(z_k - r_{ik})\end{aligned} \tag{6}$$

Where, i = 1, 2, ..., M2 and $B_{ij}(z_j) = e^{-(z_j - r_{ij})^2/\omega_{ij}^2}$

The final output of the CCTSK fuzzy neural network $\bar{y}$ can be written as:

$$\bar{y} = \sum_{i=1}^{M2} y_i \prod_{j=1}^{k} B_{ij}(z_j) = \sum_{i=1}^{M2}\left\{\begin{matrix}[q_{i0} + q_{i1}(z_1 - r_{i1}) + \cdots +\\ q_{ik}(z_k - r_{ik})]\prod_{j=1}^{k} e^{-(z_j - r_{ij})^2/\omega_{ij}^2}\end{matrix}\right\} \tag{7}$$

Since the CCTSK organize some CTSKs in a cascaded way, it can realize the syllogistic fuzzy inference, which has been proved more robust than the traditional if-then inference (Wang *et al*., 2005; Wang and Korris, 2004).

The learning rules of CCTSK: Given N input-output sample pairs $(x_1^r, x_2^r, \cdots, x_n^r; y^r)$, r = 1, 2, ..., N, we can use the gradient descent algorithm to train the parameters of CCTSK model by minimizing the error objective function $e^r = \frac{1}{2}(\bar{y}^r - y^r)$. The learning rules contain two parts, i.e., the parameter learning rules of CCTSK1 and the parameter learning rules of CCTSK2.

For the CCTSK2, its learning rules are as follows:

$$q_{ij}(t+1) = q_{ij}(t) - \alpha \left.\frac{\partial e^r}{\partial q_{ij}}\right|_t \tag{8}$$

$$r_{ij}(t+1) = r_{ij}(t) - \alpha \left.\frac{\partial e^r}{\partial r_{ij}}\right|_t \tag{9}$$

$$\omega_{ij}(t+1) = \omega_{ij}(t) - \alpha \left.\frac{\partial e^r}{\partial \omega_{ij}}\right|_t \tag{10}$$

Where, t = 0, 1, 2, ... is the iterative learning step index; α is the given learning rate, i = 1, 2, ..., M2, j = 1, 2, ..., k;

$$\frac{\partial e^r}{\partial q_{i0}} = (\overline{y}^r - y^r)\prod_{t=1}^{k} e^{-(z_t^r - r_{it})^2/\omega_{it}^2} \tag{11}$$

$$\frac{\partial e^r}{\partial q_{ij}} = (\overline{y}^r - y^r)(z_j^r - r_{ij})\prod_{t=1}^{k} e^{-(z_t^r - r_{it})^2/\omega_{it}^2} \tag{12}$$

$$\begin{aligned}\frac{\partial e^r}{\partial r_{ij}} &= (\overline{y}^r - y^r)[2(q_{i0} + q_{i1}(z_1^r - r_{i1}) + \cdots + \\ &q_{ik}(z_k^r - r_{ik}))\frac{z_j^r - r_{ij}}{\omega_{ij}^2} - q_{ij}]\prod_{t=1}^{k} e^{-(z_t^r - r_{it})^2/\omega_{it}^2}\end{aligned} \tag{13}$$

$$\begin{aligned}\frac{\partial e^r}{\partial \omega_{ij}} &= 2(\overline{y}^r - y^r)(q_{i0} + q_{i1}(z_1^r - r_{i1}) + \cdots + \\ &q_{ik}(z_k^r - r_{ik}))(\frac{(z_j^r - r_{ij})^2}{\omega_{ij}^3})\prod_{t=1}^{k} e^{-(z_t^r - r_{it})^2/\omega_{it}^2}\end{aligned} \tag{14}$$

$$\text{Where, } z_1^r = \sum_{i=1}^{M1}\left\{\begin{array}{l}[p_{i0}^l + p_{i1}^l(x_1^r - m_{i1}) + \cdots + \\ p_{in}^l(x_n^r - m_{in})]\prod_{j=1}^{n} e^{-(x_j^r - m_{ij})^2/\sigma_{ij}^2}\end{array}\right\} \tag{15}$$

For the CCTSK1, its parameter learning rules are as follows:

$$p_{ij}^l(t+1) = p_{ij}^l(t) - \beta \left.\frac{\partial e^r}{\partial p_{ij}^1}\right|_t \tag{16}$$

$$m_{ij}(t+1) = m_{ij}(t) - \beta \left.\frac{\partial e^r}{\partial m_{ij}}\right|_t \tag{17}$$

$$\sigma_{ij}(t+1) = \sigma_{ij}(t) - \beta \left.\frac{\partial e^r}{\partial \sigma_{ij}}\right|_t \tag{18}$$

Where, β is the learning rate for CCTSK1; i = 1, 2, ..., M1; l = 1, 2, ..., k; j = 1, 2, ..., n.

$$\frac{\partial e^r}{\partial p_{i0}^l} = (\overline{y}^r - y^r)\frac{\partial e^r}{\partial z_1}\prod_{j=1}^{n} e^{-(x_j^r - m_{ij})^2/\sigma_{ij}^2} \tag{19}$$

$$\frac{\partial e^r}{\partial p_{ij}^l} = (\overline{y}^r - y^r)\frac{\partial e^r}{\partial z_1}(x_j^r - m_{ij})\prod_{t=1}^{n} e^{-(x_t^r - m_{it})^2/\sigma_{it}^2} \tag{20}$$

$$\begin{aligned}\frac{\partial e^r}{\partial m_{ij}} &= (\overline{y}^r - y^r) \\ &\sum_{l=1}^{k}\left\{\begin{array}{l}\frac{\partial e^r}{\partial z_1}\prod_{t=1}^{n} e^{-(x_t^r - m_{it})^2/\sigma_{it}^2}[-p_{ij}^l + 2 \\ (p_{i0}^l + \cdots + p_{ij}^l(x_n^r - m_{in}))\frac{x_j^r - m_{ij}}{\sigma_{ij}^2}]\end{array}\right\}\end{aligned} \tag{21}$$

$$\begin{aligned}\frac{\partial e^r}{\partial \sigma_{ij}} &= 2(\overline{y}^r - y^r) \\ &\sum_{l=1}^{k}\left\{\begin{array}{l}\frac{\partial e^r}{\partial z_l}[p_{i0}^1 + \cdots + \\ p_{in}^l(x_n^r - m_{in})]\prod_{t=1}^{n} e^{-(x_t^r - m_{it})^2/\sigma_{it}^2}\frac{(x_j^r - m_{ij})^2}{\sigma_{ij}^3}\end{array}\right\}\end{aligned} \tag{22}$$

$$\frac{\partial e^r}{\partial z_l} = \sum_{s=1}^{M_2}\prod_{t=1}^{k} e^{-(z_t^r - r_{st})^2/\omega_{st}^2}\left\{\begin{array}{l}q_{sl} - 2[q_{s0} + q_{s1}(z_1^r - r_{s1}) \\ + \cdots + q_{sk}(z_k^r - r_{sk})]\frac{z_1^r - r_{sl}}{\omega_{sl}^2}\end{array}\right\} \tag{23}$$

RESULTS AND DISCUSSION

Orthogonal experiments: According to alimentative needs of *C. utilis* WSH 02-08, the fermentation media for the GSH process comprises following five factors: Glucose, $(NH_4)_2SO_4$ Urea, KH_2PO_4 and $MgSO_4$ and levels of each component are also confirmed (Table 1). In this study, we adopt $L_{16}(4^5)$ orthogonal experiments and the data is shown in Table 2.

Experimental results: The experimental data in Table 2 is taken to investigate the performance of CCTSK fuzzy

Table 1: Factors and levels in $L_{16}(4^5)$ orthogonal experiments

	Levels			
Factors	1	2	3	4
A: Glucose (g L^{-1})	25.0	30.00	35.0	40.00
B: $(NH_4)_2$ SO_4 (g L^{-1})	2.0	3.00	4.0	5.00
C: Urea (g L^{-1})	3.0	4.00	5.0	6.00
D: KH_2PO_4 (g L^{-1})	1.5	2.00	2.5	3.00
E: $MgSO_4$ (g L^{-1})	0.2	0.25	0.3	0.35

Table 2: Data of the orthogonal experiments

Factors and levels					Results of experiments		
A	B	C	D	E	GSH concentration	DCW	Intracellular GSH content
1	1	1	1	1	166.0	6.9	2.40
1	2	2	2	2	163.5	7.0	2.32
1	3	3	3	3	167.0	6.8	2.44
1	4	4	4	4	160.7	6.8	2.38
2	1	2	3	4	158.2	7.7	2.06
2	2	1	4	3	151.0	7.6	1.98
2	3	4	1	2	156.6	7.6	2.06
2	4	3	2	1	152.6	7.6	2.02
3	1	3	4	2	151.3	7.8	1.95
3	2	4	3	1	142.5	7.5	1.91
3	3	1	2	4	143.5	7.9	1.81
3	4	2	1	3	144.7	8.2	1.77
4	1	4	2	3	143.5	7.4	1.95
4	2	3	1	4	144.7	7.8	1.85
4	3	2	4	1	151.0	8.4	1.80
4	4	1	3	2	144.7	8.2	1.76

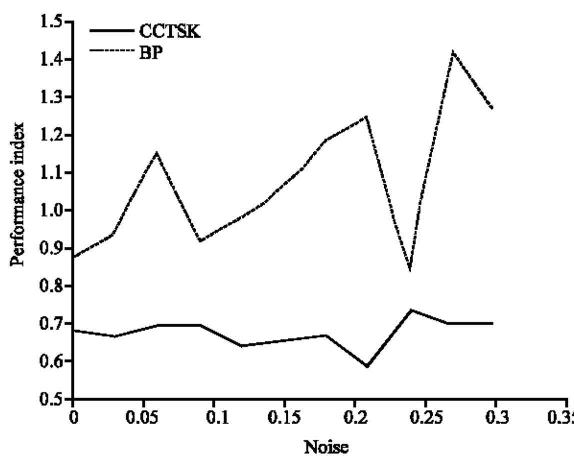

Fig. 1: Comparison of GSH concentration prediction of the orthogonal experiments

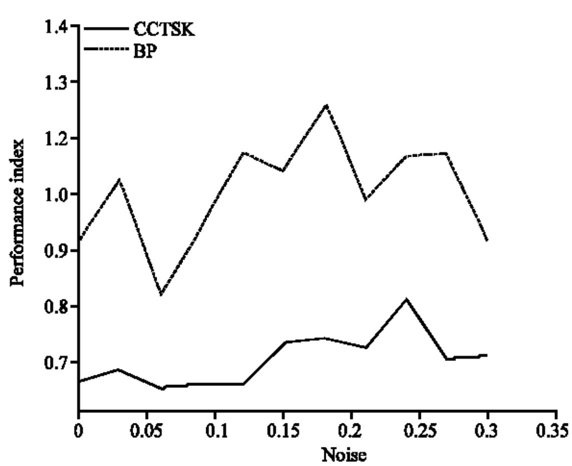

Fig. 2: Comparison of DCW prediction of the orthogonal experiments

neural network for GSH fermentation process modeling and Leave-One-Out cross validation method is used (Kearns and Ron, 1997). In the experiment, the input variables are the factors and levels and GSH, DCW, Intracellular GSH are the outputs of the modeling systems, respectively. In order to evaluate the robustness of CCTSK, the training dataset contains the input and the output variables with different extent noise added, while the test dataset contains the data without noise added.

For comparison, we also test the performance of BP neural network with the same dataset. To effectively evaluate the performance, the following index is adopted:

$$J = \sqrt{\frac{\sum_{l=1}^{N}(\widehat{y}_l - y_l^d)^2}{\sum_{l=1}^{N}(y_l^d - \bar{y})^2}}, \text{ with } \bar{y} = \frac{1}{N}\sum_{l=1}^{N} y_l^d \tag{24}$$

Where, N is the total numbers of samples, y_l^d denotes the desired output of the lth sample and $\widehat{y}_l$ represents the real output of the lth sample. Smaller the value of this index is, better of the modeling performance is.

The modeling effects of both CCTSK and BP neural network are showed as Fig. 1-3. In Fig. 1-3, X-axis is the amount of the adding noise and Y-axis is the performance index presented in Eq. 24. From Fig. 1-3, it is easy to observe that with the increases of the noise, the index curve corresponding to BP neural network has a acute change, which ranges over 0.6, i.e., the prediction accuracy is deteriorated greatly with the increasing noise, while the curve corresponding to CCTSK fuzzy neural network is very smooth, whose range is only about 0.15. Thus, the experimental results obviously demonstrate that the CCTSK fuzzy neural network has a better robustness than BP neural networks for GSH fermentation process modeling.

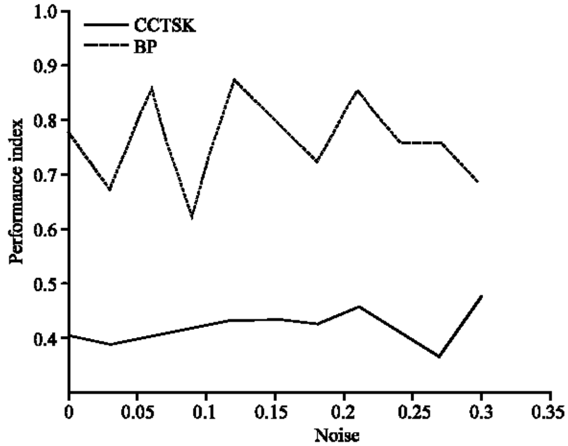

Fig. 3: Comparison of Intracellular GSH content prediction of the orthogonal experiments

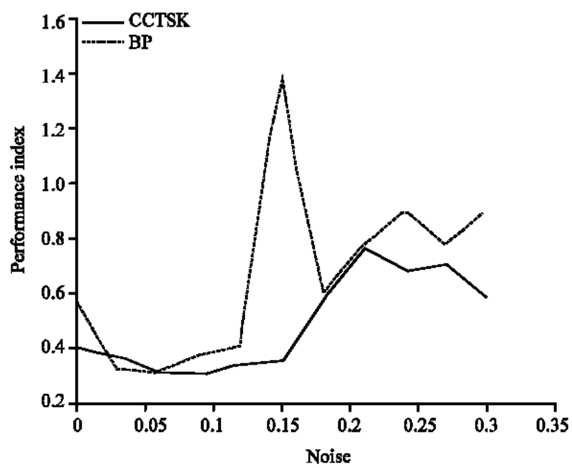

Fig. 4: Comparison of EPS predictions

By a similar way, we also carry out the simialr experiments by using the experimental data of lactobacillus fermented for exopolysaccharide (EPS) in order to further investigate the robustness of CCTSK for fermentation process modeling. The corresponding data is taken from (Desai, 2006), in which 44 of the total 54 data with different extent noised added are taken as training dataset and the other 10 data are taken as the test dataset. The experiment result is as showed in Fig. 4, which also demonstrate that the CCTSK network has better robustness than BP network.

CONCLUSIONS

In the process of GSH fermentation, BP-based method usually lacks the nicer interpretation and its robustness is rather weak to noise data. In this study, the GSH fermentation process modeling based on CCTSK fuzzy neural network is addressed. The adopted new method has a high interpretation in the viewpoint of fuzzy inference and mathematics expression and in theory this method can be proved to have a better robustness to noise compared with some traditional methods. Present experiment results also confirm the robustness of this method. Thus, it is very worthy further studying for fermentation process modeling in depth.

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