

## ARTICLE; FOOD BIOTECHNOLOGY

### Cyclodextrin glycosyltransferase biosynthesis improvement by recombinant *Lactococcus lactis* NZ:NSP:CGT: medium formulation and culture condition optimization

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Cyclodextrin glycosyltransferase (CGTase) is a bacterial glycosyltransferase which catalyses the conversion of starch to cyclodextrin. It can be produced industrially through a fermentation process. The optimal design of the fermentation medium and conditions is critical for metabolic production and microbial growth. Optimization of CGTase biosynthesis by recombinant *Lactococcus lactis* NZ:NSP:CGT was performed using an artificial neural network (ANN) as the main tool in order to improve the CGTase production in the fermentation process. Three parameters, including temperature, carbon source concentration and nitrogen source concentration, were determined as significant factors for improvement of CGTase production by *L. lactis* NZ:NSP:CGT in the fermentation process. Soluble potato starch and yeast extract were chosen as the best carbon and nitrogen sources for higher production of CGTase by *L. lactis* NZ:NSP:CGT. The optimum concentration of soluble starch and yeast extract were 3.82% and 5.67% (w/v), respectively, and the optimum temperature was 20 °C. The final CGTase activity under optimized conditions was 22.09 U/mL, which was close enough to the predicted value of 24.17 U/mL. The obtained results are of particular interest, as CGTase is an industrially important enzyme. In this study, its production through batch fermentation was optimized in order to maximize its production. In future studies, more investigations in various modes of fermentation should be conducted in order to increase the CGTase biosynthesis.

**Keywords:** cyclodextrin glycosyltransferase (CGTase); artificial neural network (ANN); Plackett–Burman design; steepest ascent/descent; optimization

AA: absolute average deviation  
ANN: artificial neural network  
CCD: central composite design  
CD: cyclodextrin  
CGTase: cyclodextrin glycosyltransferase  
IBP: incremental back propagation  
NICE: Nisin controlled expression  
PBD: Plackett–Burman design  
RMSE: root minimum square error

intramolecular cyclization to intermolecular reactions such as coupling and disproportionation as well as weak hydrolytic activity.[3] Microorganisms synthesize CGTase in order to catalyse the conversion of starch present in their environment to cyclodextrin for the purpose of growth and survival.[4] Several CGTase producers are *Bacillus lehensis* S8,[5] *Bacillus* sp. TS1-1,[6] *B.megaterium*,[7] *Paenibacillus pabuli* US132 [8] and *Klebsiella pneumonia* AS-22 .[9] *Bacillus* species are well-known natural producers of CGTase.[10]

CGTase converts starch and related compounds to cyclodextrin through cyclization activity.[11] Cyclodextrin (CD), as the main and most important product of CGTase, is a non-reducing closed-ring maltooligosaccharide consisting of glucose monomers linked to each other by  $\alpha 1 \rightarrow 4$  glycosidic bonds.[12,13] The most common forms of CDs are comprised of six, seven or eight glucose residues and are known as  $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrin, respectively.[4]

#### Introduction

Cyclodextrin glycosyltransferase (CGTase, 1,4- $\alpha$ -D-glucan:1,4- $\alpha$ -D-glucopyranosyltransferase) is a key microbial amylolytic enzyme and a bacterial glycosyl transferase.[1] This enzyme is classified as a member of the glycoside hydrolase family 13, which is recognized as  $\alpha$ -amylase and is the largest family among glycoside hydrolases.[2] CGTase catalytic activities range from

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CD consists of a hydrophilic surface and an apolar cavity. [12] The unique torus-shaped structure of CD with its non-polar interior leads to inclusion complex formation with other molecules. [10] This capability of CDs leads to their wide use in different industries such as foods, cosmetics and toiletries, textiles, agrochemistry. [14,15]

Fermentation is employed for industrial enzyme production by microorganisms such as bacteria and fungi, which is performed under controlled conditions. [16] In a fermentation process, the enhancement of enzyme yields is greatly influenced by physical and biological parameters. [17] The optimal design of the culture medium and the environmental conditions are very critical aspects in the metabolic production and growth of microbial population and development of fermentation processes. [18,19] There are many studies by a number of researchers, e.g. Ravinder et al. [19], Ahmed and El-Refai [7], Kuo et al. [20], Menocci and Goulart [21] and Illias et al. [10], conducted for the purpose of fermentation optimization, which reflects the importance of this issue. According to Khurana et al. [22], the univariate technique is not suitable for use in all cases of optimized factor level determination in the enzyme production process. It is a time-consuming and difficult technique; it neglects the combinatorial effects and there is no guarantee that optimum results will be obtained. [23]

Statistical optimization assists in accurate optimization of the process of interest while reducing the number of experiments as compared to conventional technique, therefore, immense saving in time and cost can be achieved. [24] Recently, artificial neural network (ANN) has become an attractive tool for development of non-linear empirical models, especially in case of impracticality of conventional or phenomenological empirical models. [25] ANN is a simulation of the human brain which is able to use the trained data in the range of an investigation to estimate the response. [26]

The focus of this study was on optimization of CGTase biosynthesis by recombinant *L. lactis* NZ:NSP:CGT using ANN as the main tool. To the best of our knowledge, there is no literature available on either optimization of CGTase production using ANN or CGTase biosynthesis by *L. lactis* strain. Therefore, this study can be considered the first attempt at both.

## Materials and methods

### Strain and inoculum preparation

Recombinant *Lactococcus lactis* NZ:NSP:CGT, which carries the CGTase gene originally from *Bacillus* G1, was employed as the CGTase producer. [1] Bacterial culture in the form of glycerol stock, maintained at  $-20^{\circ}\text{C}$ , was used for inoculum preparation. For this purpose, the stock culture was thawed and streaked on M17 agar (Merck, Germany) plate followed by incubation at  $30^{\circ}\text{C}$  for 24 h. A single colony was inoculated in 10 mL of M17 broth and incubated overnight followed by a sub-culture in fresh M17 broth and incubation at the same conditions. A culture with optical density (OD) of 0.5 was used in order to inoculate the fermentation broth.

### Experimental design

#### Plackett–Burman design

Initially, a screening step was performed using Plackett–Burman Design (PBD) (Design Expert Software 6.06 (Stat Ease, USA)) in order to determine the significant factors out of all the parameters selected. The factors to be screened were initial pH, temperature, soluble starch concentration, yeast extract concentration, inoculum size and Na- $\beta$ -glycerophosphate concentration based on published reports and M17 medium compositions. PBD suggested 12 experiments as presented in Table 1. The model

Table 1. Plackett–Burman design matrix and corresponding results (CGTase activity and biomass concentrations).

Stn	A	B	C	D	E	F	Max. enzyme activity (U/mL)	Max. biomass (g/L)
1	(+1)8	(-1)25	(+1)3.5	(-1)1	(-1)5	(-1)0.05	6.02	38.47
2	(+1)8	(+1)35	(-1)0.5	(+1)5	(-1)5	(-1)0.05	3.73	52.15
3	(-1)5	(+1)35	(+1)3.5	(-1)1	(+1)10	(-1)0.05	0.00	29.51
4	(+1)8	(-1)25	(+1)3.5	(+1)5	(-1)5	(+1)2.00	9.18	52.63
5	(-1)5	(+1)35	(-1)0.5	(+1)5	(+1)10	(-1)0.05	5.88	47.91
6	(+1)8	(+1)35	(+1)3.5	(-1)1	(+1)10	(+1)2.00	2.01	32.81
7	(-1)5	(+1)35	(+1)3.5	(+1)5	(-1)5	(+1)2.00	7.46	34.23
8	(-1)5	(-1)25	(+1)3.5	(+1)5	(+1)10	(-1)0.05	8.89	47.91
9	(-1)5	(-1)25	(-1)0.5	(+1)5	(+1)10	(+1)2.00	7.46	37.53
10	(+1)8	(-1)25	(-1)0.5	(-1)1	(+1)10	(+1)2.00	2.01	15.35
11	(-1)5	(+1)35	(-1)0.5	(-1)1	(-1)5	(+1)2.00	1.15	17.24
12	(-1)5	(-1)25	(-1)0.5	(-1)1	(-1)5	(-1)0.05	0.29	16.30

Note: A, B, C, D, E and F are the initial pH, temperature, soluble starch concentration, yeast extract concentration, inoculum size and Na- $\beta$ -glycerophosphate concentration.

of the system is expressed as follows:

$$Y = \beta_0 + \beta_1 X_1 - \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 - \beta_5 X_5 + \beta_6 X_6, \quad (1)$$

where  $Y$  represents the response variable and  $\beta_0$  is the interception coefficient;  $\beta_1, \beta_2, \beta_3, \beta_4, \beta_5$  and  $\beta_6$  are coefficients of the linear effects for the six independent variables ( $X_1 = \text{pH}, X_2 = \text{temperature}, X_3 = \text{soluble starch concentration}, X_4 = \text{yeast extract concentration}, X_5 = \text{inoculum size}$  and  $X_6 = \text{Na-}\beta\text{-glycerophosphate concentration}$ ).

**Steepest ascent**

The operating regions of optimization for significant parameters in the CGTase production process were determined using the steepest ascent method (Table 2). For this purpose, the first-order model generated by Plackett–Burman design (Equation (1)) was employed. The experimental values were calculated using various coded values. Coded value determines the direction and scale of changes of each parameter.

**Artificial neural network**

The final optimization step was performed to determine the optimum level of key variables and their interaction effects on CGTase production as the main response. The experiments were designed using central composite design (CCD). According to CCD, a total of 19 experimental runs of fermentation (eight factorial points, six axial points ( $\alpha = 1.682$ ) and five centre points) were conducted as shown in Table 3.

The experimental data were divided into training sets (14 items of data) and testing sets (5 items of data) with three input variables (temperature, soluble starch concentration and yeast extract concentration) and two output responses (CGTase activity and biomass concentration).

Table 2. Steepest ascent/descent experiments and the corresponding results (CGTase activity and biomass concentrations).

Coded Value	Temperature (°C)	Soluble starch (%)	Yeast extract (%)	Max. CGTase activity (U/mL)	Max. biomass (g/L)
1.50	13	7.36	6.0	0.00	4.50
1.00	19	5.57	5.0	24.27	9.12
0.75	21	4.68	4.5	11.00	14.31
0.50	24	3.79	4.0	8.91	22.90
0.25	27	2.89	3.5	4.91	25.17
0.00	30	2.00	3.0	4.13	21.20
-0.50	35	1.11	2.5	5.43	0.30

Table 3. Experiments designed by CCD and the corresponding results (CGTase activity and biomass concentrations).

No.	Temperature (°C)	Soluble starch (%)	Yeast extract (%)	Max. CGTase activity (U/mL)	Max. biomass (g/L)
1	17	4.5	4.5	0.0	4.407
2	21	4.5	4.5	13.1	18.303
3	17	6.5	4.5	0.0	4.312
4	21	6.5	4.5	10.8	17.051
5	17	4.5	5.5	0.0	5.869
6	21	4.5	5.5	3.9	19.552
7	17	6.5	5.5	0.0	7.332
8	21	6.5	5.5	6.2	22.524
9	16	5.5	5.0	0.4	6.813
10	22	5.5	5.0	3.9	22.477
11	19	3.8	5.0	20.0	11.434
12	19	7.2	5.0	20.8	11.859
13	19	5.5	4.2	26.0	12.284
14	19	5.5	5.8	15.6	13.416
15	19	5.5	5.0	14.4	14.926
16	19	5.5	5.0	11.2	16.530
17	19	5.5	5.0	13.6	16.577
18	19	5.5	5.0	17.6	12.000
19	19	5.5	5.0	11.1	16.271

The data were used to construct the regression-based networks by intelligent problem solver in Neural Power software (version 2.5 (CPS-X softwares, USA)). The training and testing processes were performed in order to determine the coefficient of correlation ( $R^2$ ), root minimum square error (RMSE) and absolute average deviation (AAD) values for the model:

$$R^2 = \frac{\sum_{i=1-n} (X_i - Y_i)^2}{\sum_{i=1-n} (\bar{Y}_i - Y_i)^2}, \quad (2)$$

where  $X$  is the predicted CGTase activity,  $Y$  is the observed CGTase activity and  $\bar{Y}$  is the average observed CGTase activity;

$$\text{RMSE} = \sqrt{\frac{\sum (y_{i, \text{exp}} - y_{i, \text{cal}})^2}{n}}, \quad (3)$$

where  $y_{i, \text{exp}}$  is the experimental response,  $y_{i, \text{cal}}$  is the calculated response and  $n$  is the number of experiments;

$$\text{AAD} = \left\{ \left[ \sum_{i=1}^p (|y_{i, \text{exp}} - y_{i, \text{cal}}| y_{i, \text{exp}}) \right] / P \right\} \times 100, \quad (4)$$

where  $y_{i, \text{exp}}$  and  $y_{i, \text{cal}}$  are the experimental and calculated responses, respectively, and  $P$  is the number of experiments.

A multilayer normal feed-forward network structure was used for model development, which was trained by incremental back propagation (IBP). The network structure consisted of one input layer (three nodes), one output layer (two nodes) and one hidden layer (nine nodes). The learning algorithms and number of neurons were involved in ANN parameters adjustments. Hyperbolic tangent transfer function was used, while other parameters for the network were set as default values.

### Analytical procedures

The dry-cell-weight technique was used to measure the biomass concentration in the cultivation samples. Cell-free supernatant was used for substrate consumption determination. Starch-iodine assay was employed to measure the starch concentration in the samples.[27] An appropriately diluted sample (1 mL) was mixed with 0.1 mL of iodine solution (0.2 g of I<sub>2</sub> and 2.0 g of KI in 100 mL of distilled water). The mixture was then filled up to 10 mL using distilled water and the absorbance was measured at 590 nm.

Kaneko's method, with minor modifications, was employed for the CGTase assay.[28] The reaction mixture containing 40 mg of soluble starch in 1.0 mL of 0.1 mol/L sodium phosphate buffer (pH 6.0) and 0.1 mL of crude enzyme was incubated at 60 °C for 10 min. The reaction was stopped by the addition of 3.5 mL of 30 mmol/L NaOH and 0.5 mL of 0.02% (w/v) phenolphthalein in 5 mmol/L Na<sub>2</sub>CO<sub>3</sub> solution. The colour intensity was then measured at 550 nm using a spectrophotometer. One unit of the enzyme activity is defined as the amount of enzyme that forms 1 μmol of β-cyclodextrin per minute.

## Results and discussion

### Plackett–Burman design

The PBD is considered a useful and powerful tool for quick selection of key factors in multivariable studies.[29] Therefore, PBD was employed for screening in this study. The experimental matrix designed and the corresponding results are presented in Table 1.

The activity of the CGTase enzyme produced by *L. lactis* NZ:NSP:CGT varied from 0.00 U/mL to 9.18 U/mL in the 12 experiments due to the strong influence of some parameters on CGTase enzyme production. The low *p*-values (<0.05) of temperature, carbon source concentration and nitrogen source concentration show that these three parameters are significant factors for optimization of CGTase biosynthesis by *L. lactis* NZ:NSP:CGT. Based on the results, the lower limit of temperature (25 °C) and the upper limit of nitrogen source concentration (5 g/L) were more suitable for CGTase biosynthesis by *L. lactis* NZ:NSP:CGT, while the carbon source concentration was not

found to influence the biosynthesis process. Further study is needed to determine the effects of these significant parameters on the responses with greater precision. A first-order model was fitted based on the results from 12 experiments and the equation was as follows:

$$Y = 4.5 + 0.3A - 1.14B + 1.09C + 2.59D - 0.13E + 0.37F, \quad (5)$$

where *Y* is the CGTase activity and *A*, *B*, *C*, *D*, *E* and *F* are pH, temperature, soluble starch concentration, yeast extract concentration, inoculum size and Na-β-glycero-phosphate concentration. The positive coefficients of soluble starch concentration (1.09) and yeast extract concentration (2.59) indicated the suitability of high concentrations of soluble starch and yeast extract for improvement of the CGTase production. As stated previously, soluble starch and yeast extract have induction effects on CGTase production.[30,31] In contrast, the negative coefficient of temperature (−1.14) suggested that low temperature is more favourable for CGTase biosynthesis.

Therefore, the three non-significant factors were eliminated from the formula and a new equation was formed as follows:

$$Y = 4.51 - 1.14B + 1.09C + 2.59D. \quad (6)$$

According to the results, there was a negative relationship between temperature and CGTase biosynthesis by *L. lactis* NZ:NSP:CGT, while carbon source and nitrogen source concentrations showed a direct positive relationship. These three parameters are normally among the ones used for CGTase production optimization. For example, carbon and nitrogen sources, as medium components, have been employed in CGTase optimization studies by Ibrahim et al.[30], Ravinder et al. [19], Sivakumar and Shakilabanu [32] and Ayadi-Zouari et al. [33]. On the other hand, temperature is considered a culture condition parameter, which has been studied by various researchers, e.g. Illias et al. [10], Menocci and Goulart [21] and Ravinder et al. [19], in order to improve CGTase biosynthesis by various producers.

On the contrary, initial pH and inoculum size are among the parameters optimized by Ravinder et al. [19], Illias et al. [10], Menocci and Goulart [21] and Sivakumar and Shakilabanu [32] for CGTase production improvement, whereas these parameters did not show any significance in our study. These dissimilarities could be due to different characteristics of various CGTase producers in different environmental conditions and medium compositions.

The three significant factors (temperature, carbon source concentration and nitrogen source concentration) were subjected to further optimization to determine the

optimum values for achieving maximum CGTase production.

### Steepest ascent

In the steepest ascent technique, different values of three significant factors were tested to obtain the best range of optimization for each. For this purpose, Equation (6) was used. Based on the coefficients, the path was mostly moved along the direction in which soluble starch and yeast extract concentrations increased, while temperature decreased. The experiments performed and the corresponding results are presented in Table 2.

By increasing the coded value to more than one, no CGTase production was detected. This might be due to the very low temperature (13 °C), which can reduce the cell growth. The negative coded value resulted in a very low biomass concentration as well, which was most probably due to low concentrations of carbon and nitrogen sources. The highest CGTase enzyme activity of 24.27 U/mL was observed at 5.57% (w/v) soluble starch concentration, 5% (w/v) yeast extract concentration and temperature of 19 °C. According to the results, the aforementioned values are in the region of maximum CGTase production; therefore, they were used as centre points for further optimization. Thus, the centre point for temperature was chosen to be 19 °C and its range was set from 17 °C to 21 °C. The soluble starch concentration's centre point was 5.5% (w/v) and the upper and lower limits were 4.5% and 6.5% (w/v), respectively, while the yeast extract concentration was set between 4.5% and 5.5% (w/v). Since maximal CGTase activity can be possibly obtained in a specific range of carbon and nitrogen concentrations, the significant parameters and their corresponding spans determined so far were used for more accurate optimization.

### Artificial neural network

CGTase biosynthesis by *L. lactis* NZ:NSP:CGT was further optimized using ANN. The experimental matrix designed by CCD and the corresponding responses are presented in Table 3.

The architecture of ANN is demonstrated by its topology.[34] The network's topology comprised of three layers (3:9:2): an input layer of three fermentation variables; a middle, hidden layer, consisting of nine neurons and an output layer with two neurons for responses (Figure 1).

The network structure used for data was the multilayer full feed-forward IBP. Generally, feed-forward neural networks containing a hidden layer with a suitable number of neurons show capability of providing an accurate approximation.[35] In the optimization of the neural network structure (number of hidden layers and neurons present, transfer function, learning rate, number of iterations and the

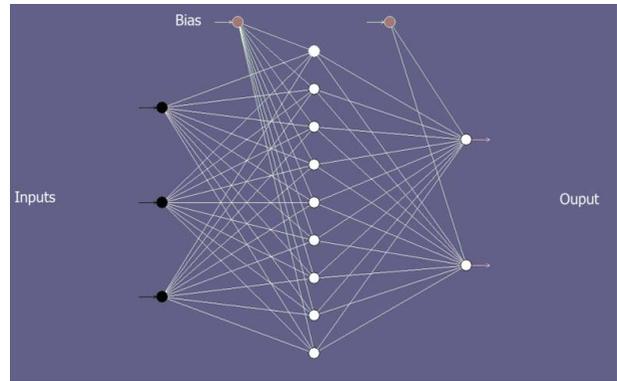


Figure 1. Topology of the neural network for the estimation of CGTase production. Note: Black circles represent the inputs (neurons added for ANN processing): temperature, soluble starch concentration and yeast extract concentration. White circles represent the hidden and output layer (neurons generated during ANN processing). Red circles represent the bias.

momentum coefficient) in the learning and testing process, it is significant to obtain the best combinations and applications. The optimum number of hidden layers and their neurons are normally selected by the trial and error technique performed by the software itself. This optimization prevents the neural network from learning the database's noise. The number of iterations used in this study was between 49,000 and 57,000, with one hidden layer applied in the feed-forward network design. Increasing the numbers of iterations and hidden layers could lead to over-fitting.[36]

The RMSE and determination coefficient for the testing set configuration was 0.234 and 0.967, respectively. ANN has the capability to rank the variables in terms of their importance based on responses (Figure 2).

In this study, temperature showed the greatest importance ( $\approx 45.4\%$ ) for CGTase production optimization. Soluble starch concentration and yeast extract concentration represented the second ( $\approx 31.2\%$ ) and the third factor ( $\approx 23.4\%$ ) in terms of significant effects.

Figure 3 shows the three-dimensional plots of temperature, soluble starch concentration and yeast extract concentration on CGTase biosynthesis by *L. lactis* NZ:NSP:CGT. The response surface shown in Figure 3(a) represents the interaction effect between temperature and soluble starch concentration. High CGTase production by *L. lactis* NZ:NSP:CGT was achieved at low concentrations of soluble starch and temperatures between 19 °C and 20 °C. At higher concentrations of soluble starch, CGTase production was lowered. Even though from a concentration of 6.7% (w/v) onwards, CGTase production started to increase again, did not reach the maximum obtained at low concentrations, such as 3.6% (w/v). Temperatures other than the optimum point lead to a tremendous decrease in CGTase biosynthesis. Figure 3(b) illustrates the maximum CGTase production as an interaction between temperature and yeast extract concentration. The

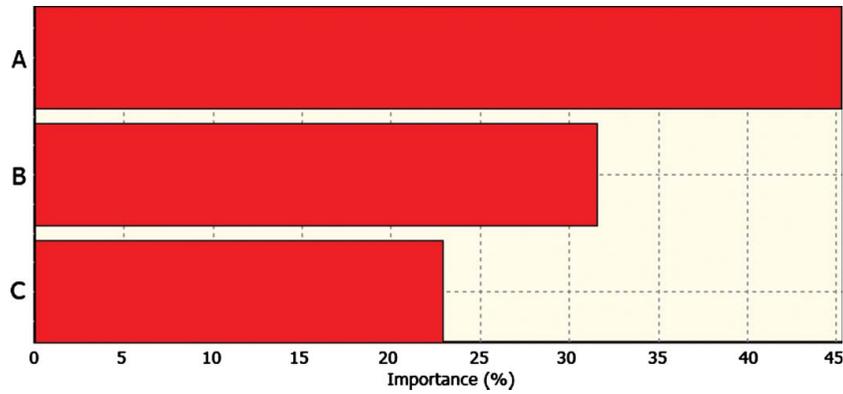


Figure 2. Importance of optimization parameters on CGTase production by *Lactococcus lactis* NZ:NSP:CGT. Note: A: temperature; B: soluble starch concentration; and C: yeast extract concentration.

trend of temperature effects on CGTase production is the same as in Figure 3(a). Yeast extract concentration did not result in tangible differences but slightly higher CGTase biosynthesis was detected at higher yeast extract concentrations.

The final optimum values of the three variables were determined and the values were found to be: 20 °C for temperature and 3.82% and 5.67% (w/v) for the concentrations of soluble starch and yeast extract, respectively. A validation run using the optimum values was conducted to verify the obtained results. The obtained CGTase activity was 22.09 U/mL, while the predicted CGTase activity was determined as 24.17 U/mL. The predicted and the observed values differed by about two units. Generally, however, the exact same results were not obtained in any replicate of fermentations.

As mentioned earlier, the optimum concentration of soluble potato starch was determined as 3.82% (w/v). According to Burhan et al. [37], strong substrate inhibition of microbial growth might occur at high starch concentrations. This is in agreement with the low concentration of biomass obtained for those high CGTase activities presented in Table 3. Moreover, high concentrations of starch increase the viscosity of the medium and lead to catabolite repression.[6,38] Therefore, the optimal concentration of carbon source obtained seems to be suitable in order to improve the CGTase production and at the same time to prevent the catabolite repression, high viscosity and extensive growth inhibition. Menocci and Goulart [21] conducted a study on carbon source optimization for CGTase production by *Bacillus sp.* BACRP. The best carbon source was potato starch and the optimum concentra-

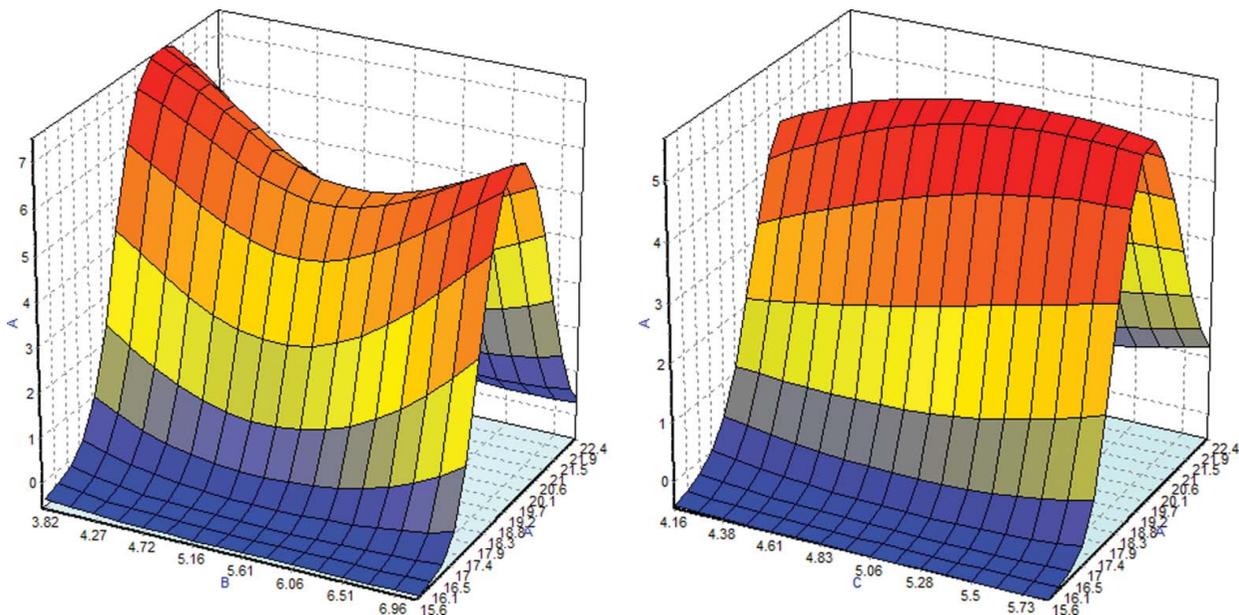


Figure 3. Three-dimensional plots of the ANN model for combined effects of temperature and soluble starch concentration (a) and temperature and yeast extract concentration (b) on CGTase production. Note: A: temperature; B: soluble starch concentration; and C: yeast extract concentration.

tion was determined as 4% (w/v). It is very similar with the results obtained in *L. lactis*.

The optimum yeast extract concentration was determined as 5.67% (w/v). As mentioned previously, yeast extract is a suitable nitrogen source for CGTase production by different species. Mahat et al. [31] studied the optimum yeast extract concentration for CGTase production by *Bacillus* sp. TS1-1 and found the best concentration to be 1.89% (w/v). In another similar study, Ravinder et al. [19] concluded that 0.5% (w/v) yeast extract concentration was the best for CGTase production by *Bacillus* sp. TPR71H. In contrast to these two studies, which employed *Bacillus* sp., as the enzyme producer, the present study used *L. lactis* as a CGTase producer and there is no information available about the optimum values for CGTase production by this strain.

There are similar studies on *L. lactis* for production of enzymes other than CGTase. Cheigh et al. [39] employed *L. lactis* for bacteriocin production. In their study, the optimal value of yeast extract concentration as nitrogen source was determined as 3%. It can be suggested that various bacterial enzyme producers have different nitrogen requirements.

The expression system used in the present study is the nisin-controlled expression (NICE) system [1,40]. Mierau et al. [40] conducted a study in order to optimize the *L. lactis* NICE system for industrial applications. They employed more than one nitrogen source (including yeast extract) in order to achieve the maximum expression of the NICE system and the total nitrogen sources concentration was 4.5% (w/v).

Recombinant CGTase-producing *Escherichia E. coli* was studied by Ravinder et al. [19] in order to optimize the nitrogen source. In their study, combinational nitrogen sources were used and the total concentration was 7.5% (w/v). It seems that in the mentioned cases, recombinant CGTase production, specially using the NICE system, requires high nitrogen content to achieve its maximum expression capacity. The inducer-like activities of yeast extract toward CGTase production [31] might be the reason for its high optimal concentration.

The optimum temperature for CGTase biosynthesis by *L. lactis* NZ:NSP:CGT was determined as 20 °C. As explained earlier, *L. lactis* NZ:NSP:CGT is a recombinant strain and the CGTase gene was originally cloned from *Bacillus* sp. G1 into *E. coli* and sub-cloned into *L. lactis* NZ:NSP:CGT. The optimum temperature for CGTase biosynthesis by *Bacillus* sp. G1 is 32 °C as reported by Illias et al. [10]. Kim et al. [41] studied the optimum culture conditions for the recombinant *E. coli*, carrying the CGTase gene from *Bacillus* sp. G1. They concluded that 20 °C is the best post-induction temperature to achieve the maximum CGTase activity. This is in good correspondence with the result obtained in this study. Normally, however, the optimum temperature for various producers of CGTase and for *L. lactis* producing different products

is higher than 25 °C. For example, Low et al. [42] reported 34.7 °C as the optimum temperature for CGTase biosynthesis by *E. coli*. Rosso et al. [43], Mahat et al. [31] and Higuti et al. [44] concluded that 37 °C was the optimum temperature for maximum CGTase production by various *Bacillus* strains. In the case of *L. lactis*, Mall et al. [45] and Sharma et al. [46] determined 30 °C and 35 °C as the best temperature to achieve maximum nisin and bacteriocin production, respectively.

It has been observed that cold shock leads to induction of a few genes.[47] Nisin induction is capable of acting in a vast range of temperatures, e.g. as low as 20 °C, but low temperatures might reduce the induction and heterologous protein production.[40] Cultivating the recombinant bacteria at low temperatures enhances the desirable heterologous protein production.[41] In this study, low temperature improved CGTase biosynthesis as well. Overall, CGTase biosynthesis by recombinant *L. lactis* NZ:NSP:CGT was successfully optimized and the enzyme activity was improved to 22.09 U/mL.

## Conclusions

In this study, optimization of CGTase biosynthesis by recombinant *L. lactis* NZ:NSP:CGT was performed using ANN as the main tool. Initially, Plackett–Burman Design (PBD) was used to determine the significant factors; followed by the steepest ascent method, which limited the operating region of optimization for significant parameters. The final optimization step was performed to determine the optimum level of key variables and their interaction effects on CGTase production as the main response. Temperature, carbon source concentration and nitrogen source concentration are three significant factors for optimization of CGTase biosynthesis by *L. lactis* NZ:NSP:CGT. At the end of this study, CGTase biosynthesis by recombinant *L. lactis* NZ:NSP:CGT was successfully optimized and the enzyme activity was improved to 22.09 U/mL.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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