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Optimization and Validation of β-lactamase Production by *Bacillus cereus* VITMUT using Response Surface Method and Artificial Neural Network

M. Rameshpathy[1], G. Jayaraman[1], A.S. Vickram[1], S. Venkatkumar[1], Raja Das[2] and T.B. Sridharan[1]*

[1]School of Bio Sciences and Technology, [2]School of Advanced Sciences, VIT University, Vellore - 632 014, India.

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The effect of various parameters like carbon sources, nitrogen sources, pH, temperature, NaCl and ampicillin on β-lactamase production by *Bacillus cereus* was investigated by one-factor-at-a-time method. Subsequently, Plackett– Burman method was employed to identify the significant variables. Of the different variables used, glucose, yeast extract and $(NH_4)_2HPO_4$ had significant influence on β-lactamase production. Further optimisation using response surface methodology (RSM) and artificial neural network (ANN) revealed that the medium containing 10 g/l glucose, 10 g/l yeast extract, and 2 g/l $(NH_4)_2HPO_4$ yielded 3,100 U/mg of β-lactamase. The higher values of coefficient of determination (0.9864_{RSM}, 0.9786_{ANN}) and lower average absolute deviation ($0.049\%_{RSM}$, $1.83\%_{ANN}$) indicated the applicability both RSM and ANN in predicting and validating the production parameters for β-lactamase by *B. cereus* VITMUT. The study, for the first time describes higher production of β-lactamase by non- pathogenic halotolerant organism.

Key words: Artificial Neural Network, β-lactamase, *Bacillus cereus,* Halotolerant, Response Surface Method.

β-lactamases (EC 3.5.2.6), are enzymes that catalyse the hydrolysis of β-lactam antibiotics such as penicillin, cephalosporin, ampicillin and others. There is substantial evidence in literature[1] that β-lactam resistant genes are transferred horizontally to other pathogenic microbes in the ecosystem thus rendering resistant to β-lactam antibiotics. Most of the microbes are being reported from clinical wastes and belong to diverse genera. On the other hand prediction of β-lactamases from non-pathogenic organisms has attracted much attention in recent years, as these enzymes find demanding clinical and medical applications. For example, β-lactamases conjugated with their monoclonal antibody is being used in Antibody directed enzyme prodrug therapy (ADEPT). This system interacts potentially with prodrug (cephalosporin carbamates) and hydrolyzes the prodrug to generate free nitrogen mustards, a cytotoxic chemotherapy agent[2,3]. It is also reported that during parenteral β-lactam antibiotics therapy, significant disruption of the indigenous micro flora occurs and therefore treatment with oral β-lactamase helps in the destruction of residual β-lactam antibiotics in the intestine thereby preserving colonization resistance[4,5]. Besides, β-lactamases have been the most common model protein when testing the efficiency of *B. subtilis* secretion vectors[6]. These enzymes are also used for the specific assay of penicillins, sterility tests of penicillins, treatment of penicillin sensitivity reactions, design of penicillin electrodes and also for drug design[7]. Such ranges of applications necessitate unravelling new non-pathogenic microbes and optimize the variables for β-lactamase production in designed medium so as to increase the yield and productivity.

* To whom all correspondence should be addressed.
Mob.: +91 9486677314;
E-mail: tbsridharan@vit.ac.in

Statistical approaches such as Plackett–Burman design and response surface methodology (RSM) have been extensively investigated for optimizing the fermentation process parameters that could result in significant improvement in the yield of the desired product[8,9]. Optimization of β-lactamase production by such statistical approaches is very obscure. The effect of glucose[6], temperature[10, 11], pH[12], and dissolved oxygen[13], on β-lactamase production was studied mostly at the level of secretion and induction. Also, the influence of various parameters like various carbon and nitrogen sources, pH and temperature on β-lactamase production and the bioprocess characteristics like oxygen transfer were studied in laboratory scale bioreactors using *B. licheniformis* 749/C[14]. In addition to the statistical optimisation procedures referred above, artificial neural network is gaining importance for its use in designing, validating and predicting the process variables.

The objective of the present study was to find the variables which could significantly affect the β-lactamase production by a novel halotolerant strain, *Bacillus cereus* VITMUT using Plackett-Burman method and to find the optimum levels of the selected variables using CCD and response surface analysis. Also, the interactions between the different variables were investigated using response surface method and artificial neural network (ANN).

MATERIALS AND METHODS

Microorganism and materials

The organism used in this study was a halotolerant *Bacillus cereus* VITMUT (GenBank: JX915753), isolated from Muttom (8.13°N 77.32°E) coastal region in Tamilnadu, India. The isolate was initially grown in Luria Bertani (LB) medium and maintained in LB agar stab culture. Unless otherwise stated all chemicals, antibiotics and medium were purchased from Hi Media (Mumbai). All experiments were performed in triplicate and the graphs were generated using either Sigma Plot V-10 and or Graphpad prism 5.

Synthetic Production medium

The synthetic production medium[14] for β-lactamase production used in this study contained (g/l) glucose, 10; $(NH_4)_2HPO_4$, 1.18; Yeast extract, 8, $MgSO_4.7H_2O$, 0.25; $FeSO_4.7\ H_2O$, 1.0 x 10^{-3}; $ZnSO_4.7H_2O$, 1.0 x 10^{-3}; $MnSO_4.H_2O$, 7.5 x 10^{-5}; $CuSO_4.5H_2O$, 1.0 x 10^{-5} in sodium phosphate buffer (50mM), pH 7.2.

β-lactamase activity

Fresh culture (36h) from synthetic production medium was centrifuged at 10,000 rpm (10 min, 4ºC). 0.1 ml of the supernatant was added to 0.1% (w/v) ampicillin in 0.1 M phosphate buffer, pH 7.0. β-lactamase activity was determined spectrophotometrically by monitoring the hydrolysis of ampicillin at 232 nm[15]. One unit of enzyme activity is defined as the amount of enzyme that could hydrolyze 1 µmol of ampicillin at 37ºC and pH 7.0 in 1 hour. The protein concentration in solution was assayed by the Lowry's method[16], with bovine serum albumin (BSA) as standard.

Effect of various parameters on β-lactamase production

The classical one-factor-at-a-time (OFAT) approach was employed to evaluate the possible optimum levels of the medium components. For various carbon sources like glucose, sucrose, glycerol, starch and maltose different concentration ranges was studied (2.0 to 10.0 mg/ml), while 2.0-12.0 mg/ml and 0.1–5.0 mg/ml were investigated for various organic (yeast extract, peptone, caseaminoacid) and inorganic nitrogen sources (ammonium chloride, ammonium hydrogen phosphate, ammonium nitrate), respectively. Effect of other parameters like pH (4.0, 6.0, 6.5, 7.0 and 8.0), temperature (29, 32, 37 and 42ºC), NaCl concentration (0, 1, 3, and 5%), and ampicillin concentration (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) on β-lactamase production was also studied. The synthetic production media mentioned above was used in all these experiments. The enzyme activities were tested in 36 h culture incubated at 37°C.

Plackett- Burman method

The most significant variables that effected the β-lactamase production, as inferred from one-at-a-time experiments, were studied by Plackett- Burman method. Eight main variables which includes glucose, yeast extract, $(NH_4)_2HPO_4$, pH, temperature, NaCl, ampicillin and $ZnSO_4$. In addition, three dummy variables were included in this study. Based on Plackett–Burman factorial design, each variable was examined at two levels: "1 for low level and +1 for high level, and a centre point was run to evaluate the linear and curvature

effects of the variables[8]. β-lactamase activity assay was carried out in triplicates and the average of these values was taken as response. The quality of the fit of the model equation was determined by the coefficient R^2 and the statistical significance by F-test. Since this model does not describe interaction among variables and also the optimum concentration of each variable, further optimization was carried out by response surface methodology (RSM).

Central composite design (CCD) and response surface analysis

To locate the true optimum concentrations of glucose, yeast extract and $(NH_4)_2HPO_4$ for enzyme production, a Central composite design (CCD) with five coded level was conducted, in the subsequent phase of the statistical approach. This trial was conducted using a full 23 factorial design with six axial points and six replications of the centre point, resulting in a total number of 20 experiments. By using a second order polynomial equation, β-lactamase production was analyzed and fitted into the equation by the multiple regression procedure. The quality of fit of the second-order model equation was expressed by the coefficient R^2, and its statistical significance was determined by an F-test. The significance of the effect of each variable on enzyme production was measured using a t-test.

Artificial neural network

Artificial Neural Network (ANN) is an advanced computing tool that processes information using neurocomputing technique. ANN is composed of layers of neurons. The input layer of neurons is connected to the output layers of neurons through one or more hidden layers of neurons. Initially, ANN was trained and tested with experimental data (obtained from the RSM) to reach an optimum topology and weights. During the training process, ANN adjusts its weights to minimize the errors between the predicted result and actual output by using back-propagation algorithm. The output of ANN was determined by giving the inputs and computing the output from various nodes activation and interconnection weights. The output was compared to the experimental output and Mean Squared Error was calculated. The error value was then propagated backwards through the network and changes were made to the weights at each node in each layer. The whole process was repeated, in an iterative fashion, until the overall error value drops below a predetermined threshold. At this point, it is said that the ANN has learnt the system to asymptotically approach the ideal function but never exactly learn the ideal function.

A schematic representation of the Back-Propagation Neural Network (BPNN) with n input nodes, r output nodes and a single hidden layer of m nodes are shown in figure.1. Each interconnection between the nodes has a weight associated with it. The input nodes have a transfer function of unity and the activation function of the hidden and output nodes are sigmoidal S(•) and linear, respectively. According to Figure1, the net input to the j^{th} hidden neuron is given by

$$y_j(x) = \sum_{i=1}^{n} wl_{ji}x_i + bl_j$$

Where wl_{ji} is the weight between the i^{th} node of input layer and j^{th} node of hidden layer and bl_j is the bias at j^{th} node of hidden layer. The output of the j^{th} hidden node is defined by

$$z_j(x) = \frac{1}{1 + \exp(-y_j(x))}$$

Given an input vector x, the output, value $o_k(x)$ of the k^{th} node of output layer is equal to the sum of the weighted outputs of the hidden nodes and the bias of the k^{th} node output layer, and is given by

$$o_k(x) = \sum w2_{kj}z_j + b2_k$$

Where $w2_{kj}$ is the weight between the j^{th} node of hidden layer and k^{th} node of output layer, $b2_k$ is biasing term at the k^{th} output node; $b2_k$ is the biasing term at the k^{th} node of output layer.

RESULTS AND DISCUSSION

Effect of various parameters on β-lactamase production

The effect of various parameters on β-lactamase production by *B. cereus* VITMUT was

studied in the synthetic production medium by OFAT method. Table 1 summarises the varied concentrations of different carbon and nitrogen sources, pH, temperature, NaCl and ampicillin used for inferring the conditions for maximal production of β-lactamase and "the detailed graphical representation are given in the online resource 1". It could be observed that both glucose and sucrose enhances β-lactamase production whereas glycerol, starch and maltose have less effect on β-lactamase production. Among all the carbon sources used, glucose (10 mg/ml) gave the highest specific activity of 2020 U/mg above which the activity decreases. Similar type of response was found with *B. licheniformis* 749/C in which increase in glucose concentration, upto 8 mg/ml, increases the biomass and β-lactamase production above which the production decreases[14].

Among the various nitrogen sources used, yeast extract (10 mg/ml) and $(NH_4)_2HPO_4$ (2 mg/ml) were found to be the best organic and inorganic nitrogen sources with maximum specific activity of 1700 and 800 U/mg respectively. The maximum specific β-lactamase activity of 2130 U/mg was obtained at pH 7.0 and the activity decreased beyond this pH value. β-lactamase activity from *B. lichenformis* was reported over a wide range of pH (5.5 to 7.5), however the secretion of enzyme into the extracellular region is reported to be favoured at pH 7.5 [12]. It was also reported that pH control was not beneficial for β-lactamase productivity[17].

NaCl significantly affected the β-lactamase production in the absence of NaCl, the activity was 2060 U/mg but increased to 2310 U/mg in the presence of 1% NaCl, and similarly in the absence of ampicillin the activity was 2060 U/mg and increased to 2410 U/mg in the presence of 0.1 mg/ml of ampicillin. Hence both NaCl and ampicillin was found to have an inducing effect on the β-lactamase production. The temperature for optimal production of β-lactamase was found to be 37 °C. The optimum temperature for β-lactamase production by *B. cereus* and *B. licheniformis* had been reported to be in the temperature range of 30 - 42 °C [10, 11, 14, 18].

Plackett- Burman design

Based on the above results of the OFAT method, glucose, yeast extract, $(NH_4)_2HPO_4$, pH, NaCl, temperature, ampicillin were chosen as the significant variables for further optimization by Plackett- Burman design. In addition, $ZnSO_4$ was used as an additional parameter in the design as it has been reported that its concentration in the medium significantly influences β-lactamase production due to its catalytic dependence[19]. Of the parameters used, glucose, yeast extract and $(NH_4)_2HPO_4$ were the most important variables that

Table 1. Optimum values of the components used in the one-factor-at-a-time (OFAT) method and the respective specific activity

Components		Range	Optimum concentration	Specific activity (U/mg)
Carbon sources	Glucose		10.0	2020
(mg/ml)	Sucrose		8.0	1620
	Glycerol	2.0-12.0	8.0	690
	Starch		4.0	360
	Maltose		4.0	480
Organic nitrogen	Casaminoacid		4.0	440
sources	Yeast extract	2.0-12.0	10.0	1700
(mg/ml)	Peptone		6.0	1350
Inorganic	NH_4Cl		0.5	410
nitrogen sources	$(NH_4)_2HPO_4$	0.5 to 4.0	2.0	800
(mg/ml)	NH_4NO_3		2.0	640
pH		4.0 to 8.0	7.0	2130
Temperature (°C)		32.0 to 42.0	37.0	2110
Nacl (%)		0.0 to 3.0	1.0	2310
Ampicillin (mg/ml)		0.0 to 0.6	0.1	2410

affect β-lactamase production at positive levels and ampicillin, NaCl at negative levels (Table 2). The adequate precision value (measure of signal to noise ratio) of the model is 17.395 which is well above the required level of 4. The regression coefficient R^2 of the model was 0.989, indicating that upto 98.9% of the data variability could be explained by the model. The statistical significance of the model was evaluated by F-test analysis of variance (ANOVA). Additionally, the probability value of 0.0005 for the model revealed that this regression was significant. Also, the influence by glucose, $(NH_4)_2HPO_4$, yeast extract, pH and ampicillin on β-lactamase production was found to be statistically significant.

Table 2. Statistical details for the different variables used in the media optimization by Plackett- Burman method

Variables	Effect	F- value	P-value
Glucose	0.31	95.39	0.0023*
Yeast extract	0.24	57.18	0.0048*
$(NH_4)_2HPO_4$	0.19	34.59	0.0098*
pH	0.12	13.51	0.0349*
NaCl	-0.047	2.16	0.2378
Temperature	0.023	0.54	0.5155
Ampicillin	-0.23	52.51	0.0054*
$ZnSO_4$	0.09	8.04	0.0659

* indicates statistically significant variable

Response surface method (RSM)

The three variables glucose, yeast extract and $(NH_4)_2HPO_4$ identified by Plackett- Burman method, which significantly influenced β-lactamase production were further investigated for their optimum concentrations by RSM. The interaction of these variables within a range of -1 to +1 of their midvalue, in relation to β-lactamase production was studied using central composite design. The design matrix and the corresponding experimental and predicted response are given in Table 3. The predicted response, the specific activity of the β-lactamase produced, for all 20 runs were obtained from the second-order polynomial equation,

$$Y = -3.4980 + 0.8091\,A + 0.3500\,B + 1.0466\,C - 0.0003\,AB - 0.0288\,AC - 0.0263\,BC - 0.0364\,A^2 - 0.0138\,B^2 - 0.2324\,C^2$$

This equation above shows the empirical relationship between the β-lactamase production

Table 3. Comparision of the experimental response and the predicted response (specific activity of β-lactamase) by RSM and ANN for different parameters used in the media optimization

Run	A:Glucose (mg/ml)	B:Yeast Extract (mg/ml)	C:$(NH4)_2HPO_4$ (mg/ml)	Experimental response (U/mg)	RSM response (U/mg)	ANN response (U/mg)
1	10.0	10.0	2.0	3110	3100	3050
2	15.0	15.0	1.0	2600	2510	2540
3	10.0	1.5	2.0	2050	1970	2050
4	1.5	10.0	2.0	450	360	0.30
5	15.0	5.0	3.0	1050	1180	1420
6	10.0	10.0	0.3	2990	3170	2950
7	18.4	10.0	2.0	700	690	700
8	10.0	10.0	2.0	3100	3100	3050
9	10.0	10.0	2.0	3130	3100	3050
10	10.0	10.0	2.0	3120	3100	3050
11	5.0	15.0	3.0	1050	1190	1040
12	10.0	10.0	2.0	3080	3100	3050
13	15.0	5.0	1.0	2150	2070	2190
14	10.0	18.4	2.0	2300	2280	2520
15	15.0	15.0	3.0	1000	1090	660
16	10.0	10.0	2.0	3070	3100	3050
17	5.0	15.0	1.0	2100	2030	2130
18	10.0	10.0	3.6	2000	1710	1970
19	5.0	5.0	1.0	1600	1570	1520
20	5.0	5.0	3.0	1100	1250	1140

(Y) and the three screened variables, viz., glucose (A), yeast extract (B) and $(NH_4)_2HPO_4$ (C). The R^2 and adjusted R^2 values of 0.9865 and 0.9743, respectively, denotes good correlation between the actual and predicted responses, suggesting that 98.65% and 97.43% variation could be accounted by the model equation. The results revealed that all the three linear coefficients A, B, C; two interactive coefficients AC, BC and all three quadratic coefficients A^2, B^2, C^2 are statistically significant with $P < 0.0001$ (Table 4). Of these variables, the contribution of $(NH_4)_2HPO_4$ appeared to be the most significant, both individually and also with respect to its interaction with the other two parameters.

The 3D graphical representation of the regression equation (response surface plot) was used to investigate the interaction among variables and to determine the optimum concentration of each variable for maximum β-lactamase production (Figure 2). These response surface curves representing the production of β-lactamase as a function of concentration of two variables at the optimum level of the third variable. The elliptical or saddle nature of the contour plots suggests

Table 4. P-value for different variables obtained for the media optimisation by RSM

Variable	P-Value (Prob > F)
Model	< 0.0001*
A (Glucose)	0.0320*
B (Yeast Extract)	0.0437*
C $[(NH_4)_2HPO_4]$	< 0.0001*
AB	0.9079
AC	0.0212*
BC	0.0319*
A^2	< 0.0001*
B^2	< 0.0001*
C^2	0.0001*

* Significant variable

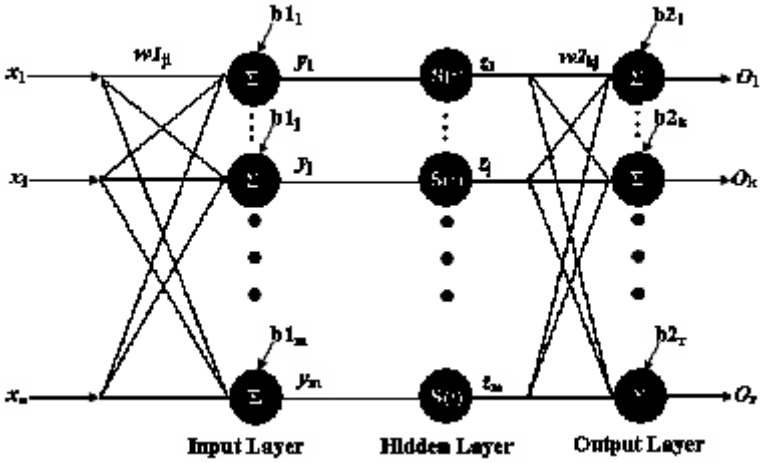

Fig. 1. Back-Propagation Neural Network (BPNN) indicating the inputs and output of neural network model

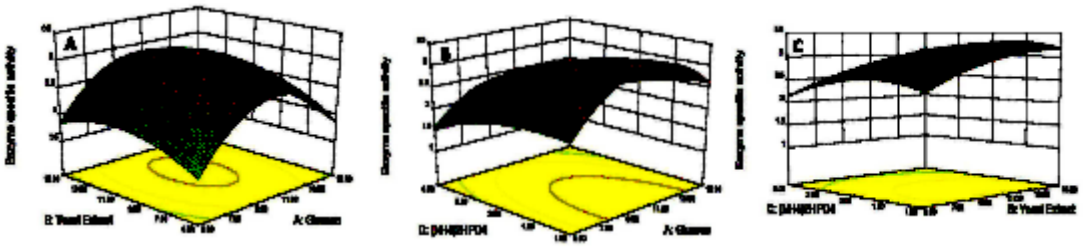

Fig. 2. Three-dimensional response surface plot of variables (a) glucose and yeast extract and its effect on â-lactamase production with $(NH_4)_2HPO_4$ in fixed level of 2 g/l; (b) glucose and $(NH_4)_2HPO_4$ and its effect on â-lactamase production with yeast extract in fixed level of 10 g/l; (c) yeast extract and $(NH_4)_2HPO_4$ and its effect on β-lactamase production with glucose in fixed level of 10 g/l

significant interaction between variables whereas the circular contour plots suggest the insignificant interactions[20,21]. The circular contour plot representing the interaction between glucose and yeast extract (Figure 2a) with the corresponding P-value of 0.9079 shows less interaction between these two variables. The elliptical contour plots of the other two interactions (Figures 2b and 2c) and the corresponding P-values of 0.0212 and 0.0319, respectively, indicate positive interaction between the two variable pairs: (a) glucose and $(NH_4)_2HPO_4$ and (b) yeast extract and $(NH_4)_2HPO_4$ respectively. The β-lactamase activity in the synthetic production medium before optimization was 2020 U/mg and was found to be 3100 U/mg after optimization by RSM. The optimum concentrations of the three components thus identified were glucose 10 g/l, yeast extract 10 g/l and $(NH_4)_2HPO_4$

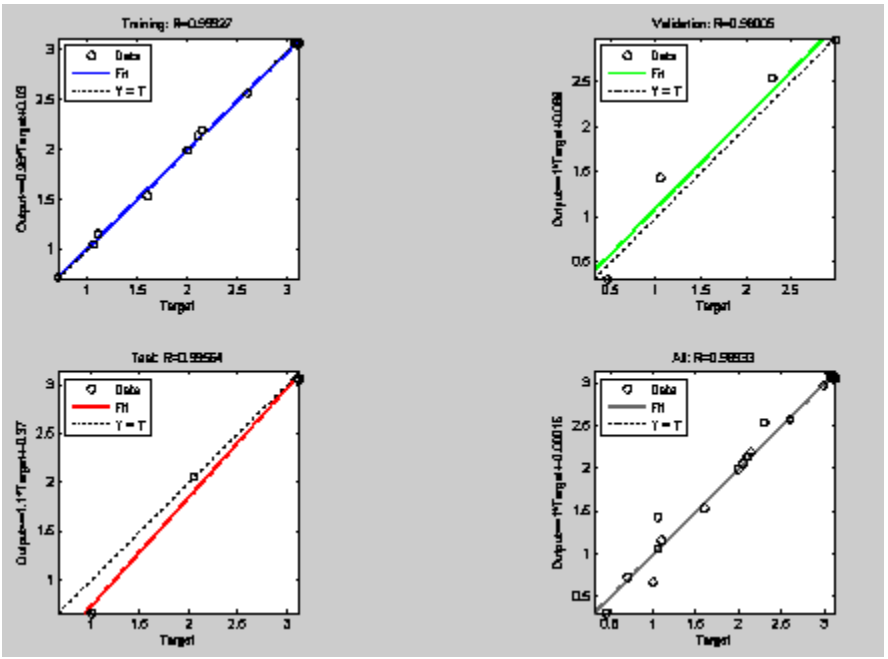

Fig. 3. The regression coefficient value for training, validation, testing and ANN was found to be 0.99, 0.98, 0.99, 0.98 respectively indicating more closeness of fit

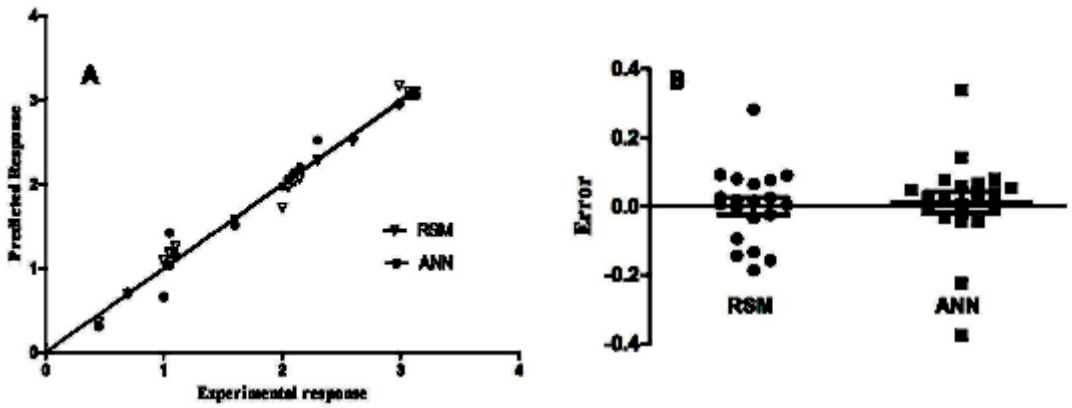

Fig. 4. (a) Correlation between the experimental and predicted response used in RSM and ANN shows all the points are on the line or close to the line of prefect prediction indicating higher; (b) the graph representing the error for the model, calculated as the difference between the experimental findings and predicted values by RSM and ANN

2 g/l. Similar type of response was reported for *B. licheniformis* 749/C with highest volumetric activity of 270 U/ml obtained in the medium containing glucose 10 g/l, yeast extract 8 g/l and $(NH_4)_2HPO_4$ 1.18 g/l [14], where as the volumetric activity in the present study is 372 U/ml.

Prediction and Validation by Artificial Neural Network

In this study, the process parameters glucose, yeast extract and $(NH_4)_2HPO_4$ were taken as the inputs and specific activity of the enzyme is taken as output for analysis by artificial neural network. Of the 20 experimental data points, twelve were used as training data sets, four for validating and the other four for testing the neural network. Initially the ANN was trained to reach the error goal of 0.1. The performance of the neural network model was studied with special attention to their generalization ability and training time.

For the best performance of the BPNN, the proper number of nodes in the single hidden layer or double hidden layers was selected through a trial and error method based on mean square error (MSE) values. "It was observed that the network performed well with three nodes in the single hidden layer and the best validation performance of BPNN model for specific enzyme activity met at 171 epochs (online resource 2)". The accuracy of the results was measured in terms of mean absolute error which was 0.037, 0.064, and 0.120 for training, validation and testing, respectively. This was supported by higher corresponding R^2values of 0.999, 0.980 and 0.995 (Figure 3).

The nonlinear behaviour of the variables is well documented with ANN than RSM[22]. Correlation between the experimental and predicted response obtained by RSM and ANN is shown in Figure 4a. Higher coefficient of determination of 0.9864 and 0.9786 by both RSM and ANN, respectively indicate higher modelling ability of both these methodology. The error for the model, calculated as the difference between the experimental findings and predicted values by RSM and ANN is given in figure 4b. The average absolute deviation (AAD) calculated[23], for RSM and ANN was 0.049% and 1.83% respectively indicating less deviation in prediction by both these methods from experimental data.

The superiority of ANN model over RSM model has been also reported for L-glutaminase production by *B. cereus* MTCC 1305 [23], ethanol production by *Clostridium autoethanogenum*[24], L-asparaginase production by *Enterobacter aerogenes* MTCC 2823 in submerged fermentation[25], optimization of medium variables for the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] by *Azohydromonas lata* MTCC 2311[26]. However, the results of the present study reveal no significant difference between both models indicating less non-linear relationship between the variables used in the β-lactamase production by *B. cereus* VITMUT.

CONCLUSION

The present study for the first time unveils the process parameters required for maximal production of β-lactamase by a non-pathogenic and halotolerant *B. cereus* VITMUT, isolated from the Indian coastal area. In addition, both RSM and ANN indicate the linear relationship between the process parameters used in the study and also reveal interaction between these parameters. The validated model indicates higher production (3,100 U/mg) of β-lactamase in the designed medium.

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