Current Trends in Biotechnology and Pharmacy
Vol. 15 (6) 125 -130, 2021, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2021.6.22

Optimization Studies of Medium Components for Protease Production from *Pseudomonas thermaerum* GW1

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Abstract

A *Pseudomonas* strain was isolated from soil of a regular feather dumping site of Ghazipur poultry processing plant, Ghaziabad, India. Strain was identified as *Pseudomonas thermaerum* GW1 after biochemical and 16S rDNA sequencing studies and was found to produce protease extracellularly in the media. The present investigation was carried out to study the application of *Resilient Backpropagation (RPROP)* algorithm to forecast constituent's viz., Casein, NaCl and Glucose for optimized production of protease. Variation of three factors and three levels according to Box-Behnken design was used for optimization procedure. Casein according to *RPROP* was observed as an insignificant variable and does not have any effect on protease production. However, Glucose and NaCl had a profound effect on protease for enhanced yield. Optimum production of protease forecasted according to ANN studies could be generated when factors Glucose is 3.75 gms, NaCl is 0.1875 gms and Casein is 1.25 gms. These combinations yielded a specific activity of 4.6 units /mg protein. This predicted medium composition can be used for maximum protease production by the new strain GW1. We report validation of the predicted values generated by ANN studies.

Keywords Protease, Caesin, *Pseudomonas thermaerium* GW1, *Resilient Backpropagation (RPROP)*

Introduction

Proteolytic enzymes as a class catalyze the hydrolysis of peptide bonds in protein molecules. Proteases (EC 3.4) are enzymes that hydrolyze proteins to short peptides or free amino acids. Protease has wide range of industrial application and it is reported that proteases count for nearly 65% of the world enzyme market (21). Its wide range of applications is in food, detergent, leather, pharmaceutical and chemical industries. Commercial proteases are mostly produced from various bacteria and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources (4).

Plants, animals and microbes are the main sources for protease production. The preferred sources of proteases are microbes because of their rapid growth and the ease with which they can be genetically manipulated to generate new enzymes with altered properties. Proteases have been purified Ingenious ways have been exploited by scientists to improve protease yield for commercial use, some of these techniques involve screening of strains, fed batch, and chemostat fermentation, cloning and overexpression, strain improvement for hyperproduction of proteases and also use of different statistical approaches, such as RSM for the optimization of different media and growth conditions (22).. Studies on medium optimization for protease production are useful techniques for multifactor experiments and they should be less time consuming and capable of detecting the true optimum factor. Conventional methods for optimization of protease parameters are extremely time-consuming and expensive . Medium optimization is very important to maximize the yield and productivity and also minimizes the production cost (9,10,11). Alkaline proteases have found application in detergentindustry as a cleaning additive (1,2,12,14,27) Various generic species of microorganism produce alkaline protease and it would be economically viable if the process parameters for optimization of media components are managed scientifically so that the above mentioned objectives are met.This study reports optimum parameters (Glucose, NaCl and Caesin) for protease enzyme secretion by *Pseudomonas thermareum* GW1 isolated from our lab (13) using artificial neural network that uses a data driven modeling approach and the principle of artificial intelligence is applied.

The network is first trained on a given set of data; here data refers to the specific activity of protease produced by *Pseudomonas thermaerum* in various media composition containing (Glucose, NaCl and Casein). The trained network can now predict new data points thus providing a mathematical alternative to quadratic polynomial required for data derived from statistically designed experiments (17) In the present work we use Resilient back propagation- *RPROP*, a very promising algorithm for feed-forward neural networks, introduced by Neeraj Wadhwa Besides fast convergence, one of the main advantages of *RPROP* lies in the fact that for many problems the choice of at most one parameter is needed to obtain optimal or at least nearly optimal convergence for the prediction of yield.

Materials and Method

Microorganism

Pseudomonas thermaerum GW1 isolated from soil of a regular feather dumping site of Ghazipur poultry processing plant, Ghaziabad, India. The isolate GW1 was identified originally as a strain of *Pseudomonas* by our laboratory based on Morphological, Physical, Biochemical characteristics and confirmed by partial 16S rDNA sequence analysis by Bangalore Genei India as *Pseudomonas thermaerum* GW1, GenBank accession no.

GU95151. It was maintained on LB agar plates.

Medium and Culture Conditions

Strain GW1 was maintained on the Basal media for protease production composed of
(g $^{L-1}$): Peptone, 5; Glucose, 10; NaCl, 0.5; $CaCl_2.2H_2O$, 0.1; K_2HPO4, 0.3; KH_2PO4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.1; yeast extract, 5 and casein, 10. The pH was maintained at 7.5. Microbes were allowed to grow in 500 ml conical flask containing 50 ml of the culture media that was maintained at 37°C at 140 rpm. 5% (v/v) of the 20 hrs old culture was inoculated in cultivation media (13).

Experimental design and protease production

Fifteen experiments comprising of different initial values of glucose, NaCl, casein were set up in 250 volume flask having the woking volume of 50 ml. The range of the three variables is mrntioned in table 1. Erlenmeyer flask had final basal minimal medium (pH 7.5) that was used for protease production contained (g/L): casein (variable), NaCL(variable)and glucose (variable) along with; KH_2PO_4, 0.3; K_2HPO_4,0.4; . The production medium (50 ml in a 250-ml Erlenmeyer flask) .designated for each set was inoculated for further 48 hours with the 5%(v/v) 20 hrs old culture grown in cultivation media and incubated at 37°C under shaking 140 rpm) in New Brunswick Scientific Shaker (Edison, NJ, USA).The cell-free supernatant was obtained by centrifugation at 10,000 rpm for 10 min °C at 4°C and the protease production was determined in the cell-free supernatant.

Protein concentration

Protein concentration was determined by the method of Bradford MM 1976 with bovine serum albumin as standard.

Determination of protease activity

Protease activity was assayed by a modified method of Tsuchida et al. 1986 by using casein as substrate. 100 µl of enzyme solution

Variable	Range	Code	Range	Code	Range	Code
Glucose	1.25g	**-1**	2.5g	**0**	3.75g	**+1**
NaCl	0.0625g	**-1**	0.125g	**0**	0.1875g	**+1**
Casein	1.25g	**-1**	2.5g	**0**	3.75g	**+1**

Experimental factors and coded levels in the three factor three-level design called the nodes used for optimizing the protease production. These codes were used to train the artificial neural network combination of .ranges were used to set up the experiment and the specific activity of enzyme obtained was the input data points.

was added to 900 µl of substrate solution (2 mg/ml (w/v) casein in 10 mM Tris–HCl buffer, pH 8.0). The mixture was incubated at 45°C for 30 min. Reaction was terminated by the addition of an equal volume of 10% (w/v) chilled trichloroacetic acid then the reaction mixture was allowed to stand in ice for 15min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 10,000 rpm for 10 min at 4°C; the acid soluble product in the supernatant was neutralized with 5 ml of 0.5 M Na_2CO_3 solution. The colour developed after adding 0.5 ml of 3-fold-diluted Folin–Ciocalteau reagent was measured at 660 nm.

All assays were done in triplicate. One protease unit is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the above assay conditions. The specific activity is expressed in the units of enzyme activity per milligram of protein.

The algorithm Resilient Backpropagation (RPROP)

The algorithm *Resilient Back-propagation (RPROP)* is a local adaptive learning scheme, performing supervised batch learning in feed-forward neural networks. M. Riedmiller introduced it in 1993. The basic principle of *RPROP* is to eliminate the harmful influence of the size of the partial derivative on the weight step. As a consequence, only the sign of the derivative is considered to indicate the direction of the weight update. To achieve this, he introduces for each weight w_{ij} its individual *update-value* $\Delta_{ij}(t)$, which solely determines the size of the weight-update (24).It is introduced a second learning rule, which determines the evolution of the update-value $\Delta_{ij}(t)$. This estimation is based on the observed behavior of the partial derivative during two successive weight-steps:

In words, the adaptation rule works as follows. Every time the partial derivative of the corresponding weight w_{ij} changes its sign, which indicates that the last update was too big and the algorithm has jumped over a local minimum, the update-value $\Delta ij^{(t)}$ is decreased by the factor η^-. If the derivative retains its sign, the update- value is slightly increased in order to accelerate convergence in shallow regions.

$$\Delta_{ij}(t)=\begin{cases}\eta^+ \cdot \Delta_{ij}(t-1), & if \ \frac{\partial E}{\partial w_{ij}}(t)\cdot\frac{\partial E}{\partial w_{ij}}(t-1)>0\\ \eta^- \cdot \Delta_{ij}(t-1), & if \ \frac{\partial E}{\partial w_{ij}}(t)\cdot\frac{\partial E}{\partial w_{ij}}(t-1)<0\\ \Delta_{ij}(t-1), & else\end{cases}$$

where $0<\eta^-<1<\eta^+$.

Once the update-value for each weight is adapted, the weight-update itself follows a very simple rule: if the derivative is positive (increasing error), the weight is decreased by its update-value, if the derivative is negative, the update-value is added:

However, there is one exception. If the partial derivative changes sign that is the previous step was too large and the minimum was missed,

$$\Delta w_{ij}(t)=\begin{cases}-\Delta_{ij}(t), & if \ \frac{\partial E}{\partial w_{ij}}(t)>0\\ \Delta_{ij}(t), & if \ \frac{\partial E}{\partial w_{ij}}(t)<0\\ 0, & else\end{cases}$$

$$w_{ij}(t+1)=w_{ij}(t)+\Delta w_{ij}(t)$$

the previous weight-update is reverted

Due to that 'backtracking' weight-step, the derivative is supposed to change its sign once again in the following step. In order to avoid a double punishment of the update-value, there should be

$$\Delta w_{ij}(t) = -\Delta w_{ij}(t-1), \quad if \quad \frac{\partial E}{\partial w_{ij}}(t) \cdot \frac{\partial E}{\partial w_{ij}}(t-1) < 0$$

no adaptation of the update- value in the succeeding step. In practice this can be done by setting

update –rule above. The partial derivative of the total-error is given by

$$\frac{\partial E}{\partial w_{ij}}(t-1) = 0$$ in the Δ_{ij} Hence, the partial derivatives of the errors must be accumulated

$$\frac{\partial E}{\partial w_{ij}}(t) = \frac{1}{2}\sum_{p=1}^{P}\frac{\partial E_p}{\partial w_{ij}}(t)$$ patterns. This m sentation of all tr

The weight-decay parameter is also introduced .This parameter determines the relationship of two goals, namely to reduce the output error (the standard goal) and to reduce the size of the weights (to improve generalization). The composite error function is:

Note that the weight-decay parameter α denotes the exponent, to allow comfortable input of very small values

Hence, a choice of α =4 corresponds to a ratio of weight decay term to output error of 1:10000.

We shall use this version of RPROP in this paper.

Result and discussion:

Using Artificial neural network provides for a mathematical alternative to quadratic polynomial for representing data derived from designed experiments. It can handle large amount of data easily

$$E = \frac{1}{2}\sum_{p=1}^{P}\sum_{j=1}^{N_O}(d_{pj} - a_{pj})^2 + \frac{1}{10^{\alpha}} \cdot \sum_{i,j} w_{ij}^2$$

and hence is suitable for medium optimization (16).Optimization and scale up of industrial fermentation process is a never ending task and every optimization technique has its advantage and disadvantage. Replacing one factor at a time was used earlier followed by response surface methodology for optimization of alkaline protease production (19,20,26). Effect of substrate on protease production The highest production of 32units/mg was reported when basal media was supplemented with 1% casein on second day of growth at pH7.5. In this experiment casein at 1.25 g/L,2.5g/L,3.75g /L was tested and results show that maximum protease was produced when 3.75 g/L of casein was used in the production media. Effect of NaCl on protease productionSome bacteria are intolerant to high salt concentration and this affects enzyme production The study of effect of salt concentration protease activity is important in dairy industry where. it is known that adding salt to milk or casein systems promotes dissociation of calcium and phosphate from within casein micelles into solution

Table 2- Specific activity of Protease produced by Pseudomonas thermaerum GW1

.	Glucose	NaCl	Casein	Data points used for training ANN Specific activity	Prediction of Specific activity by ANN	Experimental revalidation of predicted values
1	0	0	0	**3.3**	328	
2	0	0	1		0.6	**0.618**
3	0	1	0	**1.8**	1.78	
4	0	1	1		0.858	**0.868**
5	1	0	0	**1.544**	1.5	
6	1	0	1		0.746	**0.76**
7	1	1	0		3.277	
8	1	1	1	**4.25**	4.24	
9	0	0	-1		2.3	**1.558**
10	0	1	-1		4.18	
11	1	0	-1		3.82	
12	1	1	-1		4.6	
13	0	-1	0		1.46	**2.07**
14	0	-1	1		0.89	
15	1	-1	0		3.86	
16	1	-1	1		1.17	
17	-1	0	0	**0.6355**	0.756	
18	-1	0	1		0.54	
19	-1	1	0		3.29	
20	-1	1	1		0.533	
21	0	-1	-1	**1.942**	1.937	
22	1	-1	-1		3.176	
23	-1	-1	0	**2.22**	2.24	
24	-1	-1	1		2.01	
25	-1	0	-1	**1.54**	1.52	
26	-1	1	-1		3.13	
27	-1	-1	-1	**2.725**	2.73	

Experimental data used in ANN studies was specific activity of the enzyme measured when different

combination of three variables (glucose, NaCl, casein) was used for production of enzyme. Table shows t

observed and predicted values of protease production followed by the validation studies. Results depicts that the

ANN predicted values for protease produced by *Pseudomonas thermaerum* has specific activity comparable to

the validated values.

Table 3 - Network training parameters

Training parameters	Range of values	Best value
Nos of neurons in input layer	3	3
Nos of neurons in hidden layer	6-12	9
Nos of neurons in output layer	1	1
Learning rate	0.2-0.8	0.2
Activation function	Tangent Sigmoid, Log Sigmoid, Pure Linear	Hidden layer -Tangent Sigmoid Output layer - Pure linear
Performance	1-10	4.53
Epoch size	150-400	341
Nos of training datasets	10-20	10
Gradient and related parameters		Gradient $7.5e^{-11}$ Minimum gradient 1 e^{-010} increment 1.2 decrement 60.5 maximum 50.

Table 4 -Weights and Bias terms obtained from the trained network

4a-Input to hidden layer weights

	Glucose	NaCl	Casein
1	2.6635	2.2789	0.64292
2	1.694	1.6539	0.74673
3	0.31688	2.5222	-0.097541
4	1.6305	1.4648	-0.96307
5	1.9083	2.1606	3.4176
6	-2.6651	1.308	1.8951
7	-13.7325	-12.97752	1.32
	1.4445	0.72132	1.8719
9	22.2075	22.5382	3.5708

4b-Hidden to Output to layer Weight (specific activity of enzyme in units/mgprotein)

1.4529	0.72074	-1.007	0.74781	-1.9334	0.41571	-1.1397	-0.71087	0.71118

4c-Bias terms

b1	-4.4227
b2	-3.3532
b3	1.7466
b4	-1.4771
b5	-1.5785
b6	0.81014
b7	-10.8073
b8	2.7835
b9	2.4137
c1	0.35876

(6,12,9). In *Bacillus subtilis* (Natto) maximum milk clotting activity was observed at the concentration of 5 g/L, the milk-clotting activity decreased gradually and it disappeared completely when NaCl concentration approached 70g/L Similar results were seen by *B. sphaericus* (10). The results showed that rennet clotting activity and gel firmness decreased with increasing NaCl concentration in milk In our earlier report (13) we had added 0.5 g/L of NaCl for production of enzyme. On varying the salt concentration, 0.0625g/L,0.1875g/L and 0.1825g/L in our experimental set up we report that for maximum production of enzyme NaCl levels in the culture medium do effect the production as seen by our experimental studies where 0.1875g/L NaCl produced 4.25 units and by the prediction by ANN these results were also

confirmed see S. No. 12, 15, 8 in table 2 where we report 4.6 units of enzyme.

Effect of Glucose on protease production

Glucose source alone cannot effect the production of protease when maximum concentration 3.75 g/L was used only 1.544 units were recovered whereas Glucose along with NaCl at 0.1875g/L see S.No. 7, 12 of table 2 where 4.6 units were recovered. The concentration of Glucose and NaCl affect the production where as the level of substrate-Casein does not seem to have affect on the production of protease by the *bacillus Pseudomonas-thermaerum*.

Enzyme activity prediction by ANN

The predicted optimum levels of tested variables Glucose, NaCl, and Casein were obtained by Resilient Back Propogation prediction model for ANN. The optimum levels for the variables were as follows (Glucose ; 3.75 g/L, NaCl ; 0.1875 g/L and Casein ; 1.25 g/L) these values are recommended for one liter of the media. Ten experimental values of specific activity at various concentrations of variables were initially provided for training ANN and this was used to predict new data points. These new data points generated by the trained network is the output (Specific activity of enzyme). The predicted values were validated again experimentally. To validate this model, experiments were conducted at data points which were not used in training and these were picked up randomly. Our validation result is concurrent with the predicted values obtained by the ANN. The developed model can be used for prediction of enzyme production.

Conclusion

Nutritional factors have been studied for the production of alkaline protease by *Pseudomonas thermaerum* The ANN optimized shaker flask cultivation medium composition is (Glucose is 3.75 g/L , NaCl is 0.1875 g/L and Casein is 1.25 g/L) Enzyme activity prediction by ANN model was validated with the experimental studies In future work, we intend to make a comparison between our approach using neural networks ,classical predictions and use fuzzy logic for prediction as it has ability to tolerate highly variable data

Acknowledgement

We are thankful to Jaypee Institute of Information Technology University, Noida, India for providing the infra structure facilities for this study.

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