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Application of Artificial Neural Network Technique for the Production of Biotoxin from the Locally Isolated Strain of *Bacillus thuringiensis Bt.* used as Biotoxin against Dengue Vector

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Summary: In this study. a locally isolated strain of *Bacillus thuringiensis* that has insecticidal activity against dengue vector (larvae of *Aedes aegypti*), was cultivated. Different carbon and nitrogen sources were screened for enhanced bacterial growth. The factors affecting *Bacillus thuringiensis*'s biomass production like concentration of carbon, nitrogen, pH and temperature were optimized by one parameter at a time technique. The optimal levels of the selected parameters were also obtained by using an Artificial Neural Network (ANN). Peptone and molasses were selected as the best nitrogen and carbon sources respectively. The optimal levels obtained for nitrogen, carbon, pH and temperature by using the one parameter at a time technique were 1%, 0.25%, 8, and 37 ℃ respectively with 0.53 mg/mL biomass production. The ANN predicted levels were 1% for nitrogen, 0.25% for carbon, 9 pH and 31 ℃ for temperature with the predicted value of biomass being 0.85 mg/ml. The biomass produced at predicted optimum levels of variables was 0.82 mg/ml, very close to the predicted value of 0.85 mg/ml.

Keywords: Dengue; *Bacillus thuringiensis*; Optimization; ANN; biotoxin.

Introduction

The biopesticides have been recognized to be a harmless way to remove the target organisms from the natural environment and have no damage to the non-target organisms [1]. Bio-pesticides have attracted increased attention due to the toxic hazardous nature of chemical pesticides for the environment. *Bacillus thuringiensis* has the ability to be used as a bio-pesticide. Some strains of it are very effective against *Aedes aegypti* and are used in many countries in order to control the danger of dengue epidemic [2, 3]. *Aedes aegypti* is known as the main reason behind the spread of dengue virus and it has caused devastating damages to the health of people and the economy of countries. It may cause dengue fever and other diseases like hemorrhagic fever or dengue shock syndrome.

Therefore the cost effective production of *B. thuringiensis* is focused at present. For commercial scale production of *B. thuringiensis* analyzing the optimal fermentation (growth) conditions is crucial to ensure maximum bio toxin production from the bacterial culture [4].

The major ingredients of microbial growth media are carbon and nitrogen sources along with some trace elements [5]. Carbon is a very important nutrient required by the bacterial cell for the development of its main cellular material. Bacterial cell depends on carbon source for energy and cell development [6]. The selection of carbon source requires great care, as many bacteria do not show a positive growth rate in the presence of the particular sugar. All the larvicidal activity of *Bt*. is dependent on the toxic crystal proteins. The protein structure and the formation of the nucleic acids require nitrogen. Although 50% of the bacterial cell comprises carbon and require it essentially but the cell also needs nitrogen for the synthesis of important growth factors [6]. Beside the nutritional requirements, the two most important growth factors that show prominent effects on the bacterial growth are pH and temperature. These parameters strongly effect the growth of bacteria and thus need to be optimized for providing best growth conditions in order to get maximum yield [7].

When there is a set of variable conditions like in fermentation then there is need to perform many experiments for obtaining optimum parameters. This practice is laborious, time consuming and also expensive. So this issue can be solved by using

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Artificial Neural Network (ANN), which is a mathematical model that has been successfully used in empirical modelling, system designing and prediction/forecasting. By using the universal approximation capability of ANN, we can approximate non-linear and non stationary functions. In most of the studies it was found better technique to optimize a system as compared to the others [8-10].

The objective of this study was to use ANN modeling for the optimization of fermentation conditions and compare them with conventional one parameter at a time technique, for the production of biomass of *Bacillus thruingiensis* to be used as bio insecticide against dengue vector. According to our knowledge, the ANN modelling has not been used in any previous study for the optimization of biotoxin production against dengue vector. The input variables were carbon, nitrogen, temperature and pH, while the output was yield of maximum bacterial biomass which results in the production of considerable amount of bio-toxin. This makes the employment of this environmental friendly tool quite applicable to Fight against the dengue vector.

Experimental

Materials

The medium used was of Rapid Labs, UK and all other chemicals used in this study were of analytical grade.

Microbial Strain

The potent bacterial strain of *Bacillus thuringiensis,* was taken from Center for Environment Protection Studies (CEPS), Pakistan Council of Scientific and Industrial Research Laboratories Complex (PCSIR Laboratories), Lahore, Pakistan. The strain CEPS-101 of *Bacillus thuringiensis* was isolated and identified in CEPS, PCSIR labs and was used for optimization and scale up studies.

Culture medium preparation

The liquid nutrient medium (DIFCO) was used and it was prepared as per instructions given on the media bottle (8g /L) in a 250 ml Erlenmeyer flask. It was then autoclaved and then inoculated with CEPS-101 and placed on shaker at 35℃ temperature, the biomass obtained was used as a standard culture for further studies.

Screening of Carbon and Nitrogen sources

Different cheaper and easily available carbon sources including molasses, glycerol, sucrose and glucose were used for the screening experiments to find the best from them. Organic and inorganic nitrogen sources like casein, peptone, tryptone, and urea and ammonium sulphate were used to sort out the best nitrogen source for the maximum cell growth. The concentrations used for the nitrogen and carbon sources in these experiments were 0.25 %, pH of the media was maintained at 7 and autoclaved for sterilization before use. The media inoculated with CEPS-101 culture (1%) were incubated at 37 ℃ for 72 hours.

Optimization of carbon and nitrogen concentration

The fermentation conditions were initially optimized by one parameter at a time technique. For the selection of best concentration of carbon or nitrogen the varying amounts (i.e. 0.25 %, 0.50 %, and 1 %) of both nutrients were studied. Each concentration of respective source was added in 100 ml water, substituted with 0.1% sodium chloride and then autoclaved. Then these flasks were incubated for 72 hours after inoculating each with one mL of prepared culture and placed in a shaking incubator. Samples were withdrawn from each concentration after regular interval of time i.e., for four hours and the Cell Dry weight was calculated. The other conditions of bacterial growth like pH, temperature, and shaking speed were kept constant (37 ℃, 7 and 120 respectively). For optimization each experiment was repeated thrice in order to get accurate results.

Optimization of temperature and pH

For the optimization of temperature, the growth was measured at different temperatures of 20, 25, 30, 35, 37 and 40 ℃. The studied range was from acidic conditions of pH to basic, i.e., from pH 4, 6, 7, 8, 9 and11. In each flask the pH was adjusted accordingly and then all the flasks were inoculated by the culture of CEPS-101 and the cell dry weight (CDW) was estimated after regular intervals of time.

Artificial Neural Network Model

Different regression-based networks were constructed from the data, in software (STATISTICA 7th version) using its intelligent problem solver application. Trained networks were studied in about 60 numbers and best was selected based on selection error and correlation determination coefficient (R^2). A network with lowest selection error and highest value of R^2 was considered the best network. A perception network with multi layers (MPN) was selected according to the above criteria. The important algorithms used for the training of the network were conjugate gradient descent (CG) and back propagation (BP) mainly based on variation of data sets in input/output pair. The data experiments that were used for the testing (3), selection (4) and training (8) are represented in Table-1. The network's topology consisted of three layers (one hidden, one input and one output layers). The input and output layers have 4 and 3 neurons respectively.

Estimation of Cell Dry Weight (CDW)

For the estimation of cell dry weight, 10 mL sample of bacterial culture was taken in plastic tubes of 2mL capacity used for centrifugation. Tubes were then centrifuged at one thousand rpm for fifteen minutes. Then the supernatant was decanted in small plastic bottles for further studies and the pellet produced at the bottom of centrifuge tubes was dried in an oven at 80 ℃. Each of the tubes was weighed on analytical balance before and after drying and cell dry weight was obtained in mg/ml.

Results and Discussion

Screening and optimization of carbon and nitrogen sources

Literature has the findings that the composition of growth medium is very important for the growth and sporulation of *BT* strains [9, 10]. In present study different carbon sources for growth medium, including molasses, glycerol, sucrose and glucose were used for screening experiments and the results were presented in Fig**.** 1. It was observed from the results that molasses showed the prominent increase in cell growth therefore it was selected to be used in further experiments. Molasses has been supported by other studies and [11] reported that it has a prominent effect on the production of bacterial biomass. In another study it was observed that glycerol produces maximum cell dry weight among various studied carbon sources and it is followed by the sugarcane molasses [12]. In a comparative study, molasses and soybean were compared as carbon source and it was found that molasses was a good carbon sources as compared to soybean and can produce a considerable quantity of bacterial cell biomass [13]. In this work, among other selected sources of carbon, glucose was also studied and it was found that glycerol and glucose gave a minimum yield of cell dry weight and this finding was also supported by literature [14] but was in contrast to another finding, who declared that glucose is a good carbon source for *Bacillus thuringiensis* [15]. Among all the selected sources, molasses was cheapest and easily available, so it was selected as a carbon source in the fermentation media for the growth of CEPS-101 to get maximum yield of bacterial cell mass.

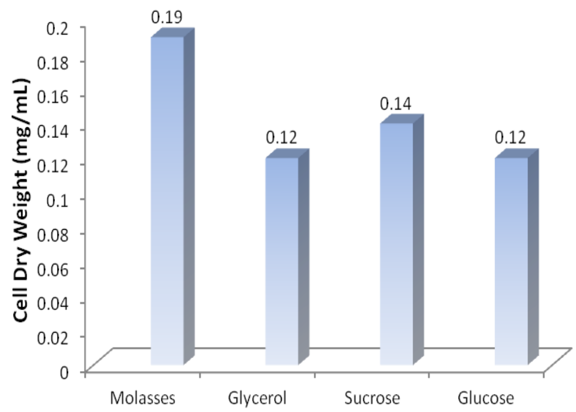

Fig. 1: Effect of different carbon sources on cell mass production of *Bacillus thuringiensis.*

For molasses, the optimization studies were conducted at three different levels of concentration i.e., 0.25, 0.5 and 1 %. It was observed that when the concentration of molasses was increased it caused a decrease in the amount of cell dry weight. So the least concentration (0.25 %) of molasses showed maximum growth of bacteria and after addition of more amounts of molasses a decrease in bacterial growth was observed. By observing these findings, molasses was selected as a good carbon source that not only gives high yield but also is required in low amount. In another study [14], the same effect was noticed on increasing the concentration of molasses.

Nitrogen is also an essential nutrient required for bacterial growth and has prominent place in bacterial medium. In other research works different organic and inorganic sources of nitrogen have been used [15-18] like soya bean, poultry products, flours, bird feathers, animal extracts and ammonium salts etc. In this study two inorganic nitrogen sources i.e., ammonium chloride and urea were studied along with other organic sources, like tryptone, peptone, casein, and urea. It was observed (Fig 2) that ammonium chloride and urea were not supportive for the growth of this bacterial strain; however the organic nitrogen sources were showed good growth. The peptone was best and followed by tryptone and casein.

Table-1: Experiments conducted with different levels of four parameters for the cell mass production of *Bacillus thuringiensis* with the observed and predicted yield and residual values.

Sr. No	Peptone (%)	Molasses (%)	Temp (°C)	pH	CDW (mg/ml) Observed	CDW (mg/ml) Predicted by ANN	Residuals
1	*0.25*	*0.25*	*37*	*8*	*0.08*	*0.082*	0.001724
2	0.5	0.25	37	8	0.32	0.144	-0.176220
3	1	0.25	37	8	0.48	0.478	-0.001567
4	*1*	*0.25*	*37*	*8*	*0.44*	*0.478*	0.038433
5	1	0.5	37	8	0.402	0.446	0.043715
6	*1*	*1*	*37*	*8*	*0.411*	*0.415*	0.003544
7	*1*	*0.25*	*25*	*8*	*0.306*	*0.310*	0.004692
8	1	0.25	35	8	0.581	0.631	0.050171
9	1	0.25	37	8	0.72	0.478	-0.241567
10	*1*	*0.25*	*40*	*8*	*0.193*	*0.201*	0.008909
11	*1*	*0.25*	*37*	*6*	*0.31*	*0.314*	0.004588
12	*1*	*0.25*	*37*	*7*	*0.41*	*0.373*	-0.036114
13	1	0.25	37	8	0.73	0.478	-0.251567
14	*1*	*0.25*	*37*	*9*	*0.62*	*0.606*	-0.013361
15	*1*	*0.25*	*31*	*9*	*0.87*	*0.85*	*0.02*

*The bold numbers represent to select, italic for training and normal for testing.
*Experiment 15 represents the optimum levels of parameters predicted by ANN, the observed and predicted results on these levels and residual values.

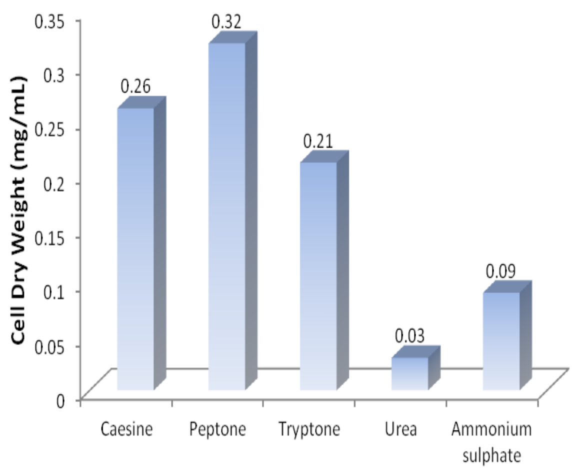

Fig. 2: Effect of different nitrogen sources on cell mass production of *Bacillus thuringiensis.*

Peptone is an excellent natural source of nitrogen and it promotes the growth of many bacteria. It is easily available commercially and gave the best results. Casein also produced good results and yield considerable amount of biomass, such findings are also supported by literature [15]. In this study two inorganic sources of nitrogen i.e., urea and ammonium chloride was tried for growth studies and it was found that both have no positive impact on bacterial growth. In another comparative study [16] it was investigated that inorganic sources of nitrogen like urea and ammonium chloride are good sources of nitrogen as compared with organic sources. This contradiction could be satisfied by the fact that in order to support the growth of *Bacillus Thruingiensis,* only inorganic source is not enough because the bacteria required some nutrients like peptide and amino acids that are present in organic source of nitrogen.[19] The maximum growth was observed with peptone, so it was selected for further optimization studies. Peptone was studied at same three concentration levels that were selected for molasses i.e., 0.25, 0.5 and 1 %. It showed maximum bacterial growth at highest selected concentration of 1%, However it gave minimum growth at 0.25%.

Effect of pH on growth of CEPS-101

pH has very prominent effect on bacterial growth, so the isolated strain (CEPS-101) was studied at different pH values i.e., from acidic to basic and the selected pH range was 4, 6, 7, 8, 9 and 11. The maximum growth was observed at alkaline conditions of pH i.e. at 7 (Fig. 3). The variation in pH was measured during the whole experiment and it from first it become acidic and then basic and maintained itself around pH 7 to 8. Similar findings of pH change were also observed by others during growth of *Bacillus Thuringiensis* [20, 21].

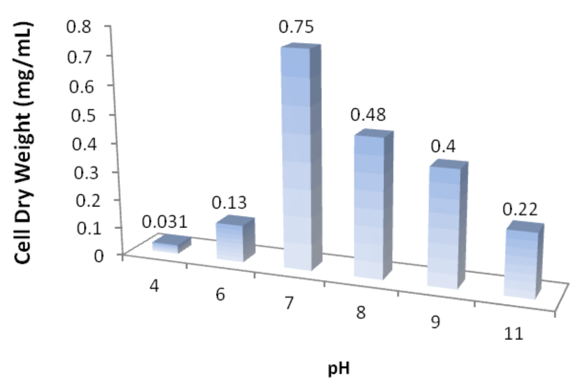

Fig. 3: Effect of different pH on cell mass production of *Bacillus thuringiensis.*

In present work pH 8 was observed to be the best pH for the growth of CEPS-101 and the findings were similar in trend with other studies described in literature [14, 16, 21-23] that indicated that the pH varied and becomes slightly basic from neutral.

Bacterial growth at different temperatures

Temperature of bacterial growth media has a prominent effect on the production of biomass and this parameter has critical effect on the toxin production as well. A safe range of temperature is required for the good bacterial growth, above and below of that range it goes in the stress condition and does not show required results.

In this optimization study, the selected temperatures were 20, 25, 30, 35, 37 and 40 ℃. The maximum growth of bacteria was recorded to be at 37 ℃ (Fig. 4) above and below this temperature (37 ℃) a prominent decrease in the yield of bacterial biomass was observed.

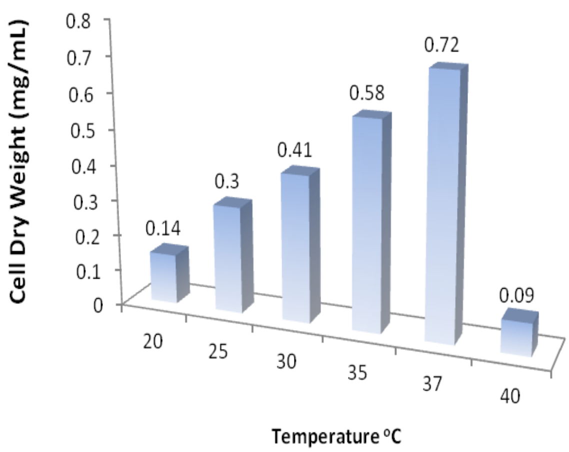

Fig. 4: Effect of different temperatures on cell mass production of *Bacillus thuringiensis*.

In another study [14] the growth of *Bt.* was observed at variable temperatures and found best bacterial growth at 30 ℃ but in this study 37 ℃ temperature gave maximum growth of CEPS-101. These findings have similar results that are produced by another study [16] conducted in order to optimize the temperature and other growth conditions for the production of PHB. Our finding is also supported by another study that synthesize poly- β -hydroxybutyrate from *Bacillus thuringiensis* [12].

Optimization using ANN

ANN is a modern technique to optimize a system with maximum accuracy. It was found the best bio process optimization technique in many studies as compared to the conventional and other statistical methods [8-9]. One of the benefits of ANN is that it can be applied on any type of data statistically designed or from the conventional optimization data. The ANN is a black box model that select some experiments for the construction of ANN model used some other experiments to train the model and the remaining experiments for the training of the model. Different types of propagation and algorithms were used for the training of the model. The model of ANN is considered good if it has a good correlation coefficient.

In the present study, 60 different ANN models were generated by the software for the data used. The best model was selected on the basis of correlation coefficient. The MPN model was selected from them and trained by using the BP and CG algorithms. The topology of the ANN model selected is represented in Fig. 3. It is consisted of three layers. First layer of 4 neurons represents the input of 4 parameters used in the study. One hidden layer of 8 neurons represents the black box processing by the selected ANN model and a one neuron outer layer of one neuron showing the output that is cell mass in the present work. The Table-2 represents the statistical analysis of the selected ANN. Three experiments of the data were selected for the ANN model generation, 8 were used for the training of the model and three were used for the testing. It can be observed from the results that the correlation coefficients for the selected, training and testing experiments were very close to 1, representing the accuracy of model used.

In the present study the ANN method was applied on a data obtained from a conventional one parameter at a time method. The optimal levels obtained from the data were 1% for nitrogen source, 0.25% for carbon source, pH 8, and 37 ℃ temperature with 0.73 mg/mL biomass production (Table 1). While the optimal levels obtained from the ANN model were 1% peptone, 0.25% molasses, pH 9 and temperature 31℃ with a predicted cell mass of 0.855 mg/ml (Fig 5-9), response graphs showing optimal levels). The observed cell mass obtained on these levels was 0.823 mg/ml, very close to the predicted level and better then that obtained by the conventional method. Table-3 shows the sensitivity analysis of the four parameters used for the optimization. Rank 1 was given to the nitrogen source due to the highest ratio value, representing that the maximum effect is from nitrogen source for biomass production. Next is the temperature that is followed by pH and carbon sources source.

Table-2: Statistical values calculated by ANN for the four parameters used in the experiments.

Parameters	Training	Selection	Test	Overall
Data Mean	0.346250	0.590000	0.487667	0.428786
Data S.D.	0.153824	0.190962	0.073277	0.180806
Error Mean	0.001552	-0.223118	0.030773	-0.040330
Error S.D.	0.019617	0.033412	0.023019	0.099085
Abs E. Mean	0.013921	0.223118	0.031818	0.062584
S.D. Ratio	0.127528	0.174968	0.314136	0.548022
Correlation	0.991857	0.999771	0.960075	0.836972

Table-3: Rank and ratio values calculated by ANN for the four parameters used in the experiments.

Parameter	Nitrogen source	Carbon source	temperature	pH
Ratio	1.543244	1.114106	1.458320	1.270511
Rank	1.000000	4.000000	2.000000	3.000000

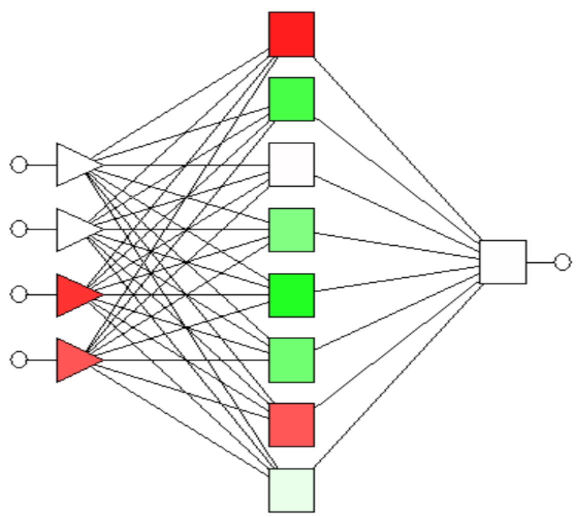

Fig. 5: Topology of the used ANN for the optimization of *Bacillus thuringiensis* growth

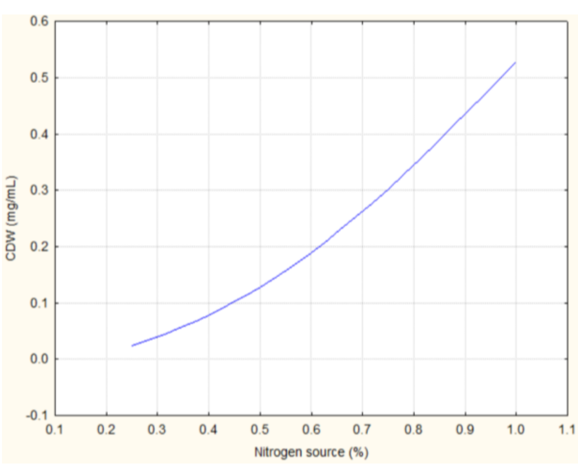

Fig. 6: Surface graph of nitrogen source showing the optimal level for the maximum cell growth.

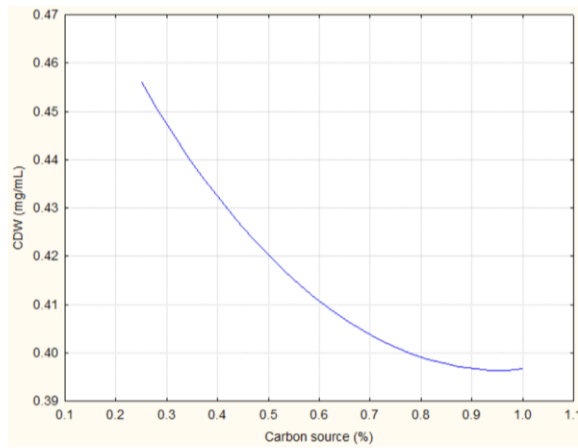

Fig. 7: Surface graph of Carbon source showing the optimal level for the maximum cell growth.

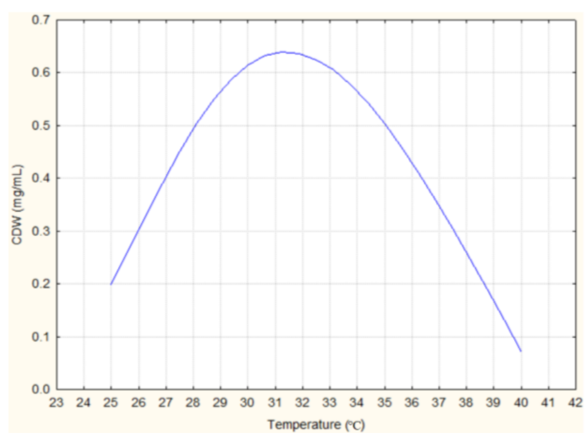

Fig. 8: Surface graph of Temperature showing the optimal level for the maximum cell growth.

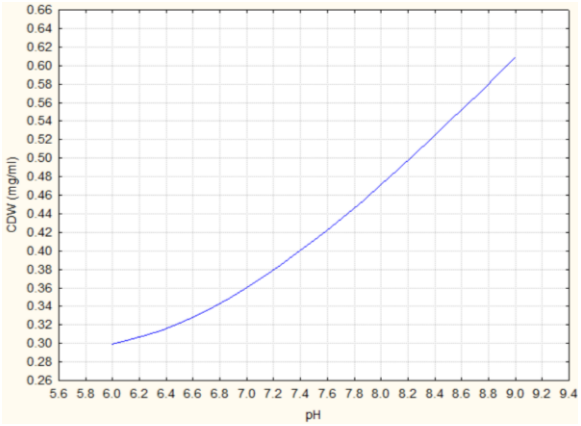

Fig. 9: Surface graph of pH showing the optimal level for the maximum cell growth.

Conclusion

The present research work was planned to obtain the maximum cell biomass of locally isolated strain of *Bacillus thruingiensis* which is nammed as CEPS-101. The cell biomass contains biotoxin, which is to be used as insecticide against dengue vector i.e. three instar larvae of *Aedes Aegypti*. For that purpose molasses and peptone was found the best carbon and nitrogen sources respectively. The optimum pH and temperature conditions for this strain of bacteria were 8 and 37 °C respectively. The optimal levels of four influencing parameters including peptone, molasses, temperature and pH were optimized by one parameter at a time and ANN methods. The optimal levels obtained from ANN model gave better biomass production as compared to the other method. Therefore it was concluded that ANN method may gave better results than the conventional methods and can be used successfully for the optimization of microbial biomass production system.

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