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CELL GROWTH PREDICTION FOR *BACILLUS LICHENIFORMIS* THROUGH ARTIFICIAL NEURAL NETWORK AT SIMULTANEOUS MULTIPLE VARIATION IN CONCENTRATION OF NUTRIENTS IN MEDIA

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ABSTRACT
In the cell growth and metabolite production, the selection of nutrients and determination of its concentrations in the cultivation media is very important step for commercially viable products. Formulation of media requires lot of experiments and so time consuming and tedious. The conventional methods also involves errors. To eliminate the error involved and to reduce the number of experiments a new has been tried in the media formulation and optimization. The application of Artificial Neural Network for the prediction of effect of nutrients in the media on cell growth of *Bacillus licheniformis* has been presented in this work. Ten different medias used were prepared by simultaneous multiple and randomly variation of the concentration of the components in the selected range. The medias were composed of starch, peptone and various selected salts. The cell concentrations were determined at various media composition. An Artificial Neural Network was prepared to use the nutrient concentrations as signal input and cell concentrations as output. Once the network was trained, the results showed its ability to model biochemical nonlinear processes and could be used for the selection and optimization of media composition.

Key words: Artificial Neural Network,Bacillus Licheniformis, Nutrients, Growth media

INTRODUCTION
Useful metabolites produced from the microbial, plant and animal cells has touched almost the every aspect of requirement in human life and is increasing day by day. In cell growth and metabolite production, the selection of the nutrients and determination of its concentrations in the cultivation media is a very important step. For commercially viable products, quest of cheaper processes are essential. A lot of variations are made in media formulation for the reduction in cost of the media, maximization of the cell growth and/or product formation and easy separation of the products. Finding out of the cheaper sources of raw materials are particularly important for the products which are required in large quantities.

Thermostable α-amylases enzyme made by Bacillus spp. are produced in large quantities and have many commercial applications in textile and paper industry, starch liquefaction, food adhesive and sugar production. To meet the demand of these industries, low cost media is required for the production of alpha amylase. The contents of the media such as nutrient broth,

soluble starch as well as other components are expensive and have to be utilized optimally for the cell growth and enzyme production.

Several studies have showed that these organisms can grow on a wide variety of complex and synthetic medias. Variations had been studied in using carbon sources, [1-6] nitrogen sources [3-4,6-9] and numerous salts [1,3,8,10-11] and their concentrations as well. Final cell concentrations and product concentrations have also varied. From the results it became really difficult to find out the best composition of media and to isolate the effect of individual nutrients.Formulation of media required a lot of experiments and so time consuming & tedious. In the conventional method, the concentration of the components are varied individually to see its effects keeping the others constant. Each time the best results are selected and the media compositions are changed accordingly for the next set of experiments. The net results of the microbial growth/ product output are in fact a combination of multiple metabolic reactions within the cells. So the best composition of nutrients reached in the media formulation may not be true because each best results were selected at different conditions.

To eliminate the error involved and to reduce the number of experiments, a new method has been tried in the present work for the media formulation and optimization.The application of Artificial Neural Network for prediction of the effect of nutrients in the media on cell growth has been presented in this work.

MATERIALS AND METHODS

The microorganism in the study was Bacillus licheniformis obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh. The organism was maintained the nutrient agar medium containing (g/L): beef extract, 1.0; yeast extract, 2.0; peptone, 5.0; NaCl, 5.0; and agar, 15.0. The pH of the nutrient broth was 5.5. The culture was stored at 20^0 C and subcultured every two weeks. The inoculum was gorwn in the liquid media containing (g/L): Beef extract, 3.0; peptone, 5.0; and glucose, 1.0 and iuital pH of the medium was 7.0.

Studies for the cell growth were done in medias composed of starch, peptone and various selected salts NH_4Cl, $CaCl_2$, $MgSO_4$, Sodium citrate, NaCl, and $FeSO_4$. Ten different medias used were prepared by simultaneous multiple & randomly variation of the concentrations of the components in the selected range. The variations in the concentrations were in the range (g/L) starch 10.0-15.0; peptone 3.0-5.0; NH_4Cl 1.0-2.5; $CaCl_2$ 0.2-0.45; $MgSO_4$ 0.1-0.5; Sodium citrate 1.0 (constant); NaCl 1.0-3.0; $FeSO_4$ 0.025-1.0. The compositions of the different mediums used in the studies were (all quantities are in g/L).

Medium 1 (M1): Starch, 15.0; peptone, 5.0; NH_4Cl, 2.5; $CaCl_2$, 0.45; $MgSO_4$, 0.5; sodium citrate, 1.0; NaCl, 3.0; $FeSO_4$, 1.0.

Medium 2 (M2): Starch, 15.0; peptone, 2.0; NH_4Cl, 1.0; $CaCl_2$, 0.2; $MgSO_4$, 0.1; sodium citrate, 1.0; NaCl, 1.0; $FeSO_4$, 0.025.

Medium 3 (M3): Starch, 14.0; peptone, 4.0; NH_4Cl, 2.0; $CaCl_2$, 0.3; $MgSO_4$, 0.3; sodium citrate, 1.0; NaCl, 2.5; $FeSO_4$, 0.0625.

Medium 4 (M4): Starch, 14.0; peptone, 3.0; NH_4Cl, 1.5; $CaCl_2$, 0.2; $MgSO_4$, 0.2; sodium citrate, 1.0; NaCl, 1.5; $FeSO_4$, 0.25.

Medium 5 (M5): Starch, 13.0; peptone, 5.0; NH_4Cl, 1.0; $CaCl_2$, 0.4; $MgSO_4$, 0.4; sodium citrate, 1.0; NaCl, 2.0; $FeSO_4$, 0.125.

Medium 6 (M6): Starch, 13.0; peptone, 4.0; NH_4Cl, 2.5; $CaCl_2$, 0.45; $MgSO_4$, 0.3; sodium citrate, 1.0; NaCl,1.5; $FeSO_4$,0.5.

Medium 7 (M7): Starch, 12.0; peptone,2.0; NH_4Cl, 1.5; $CaCl_2$, 0.3; $MgSO_4$, 0.5; sodium citrate, 1.0; NaCl, 2.0; $FeSO_4$, 0.5.

Medium 8 (M8): Starch, 11.0; peptone, 4.0; NH_4Cl, 2.0; $CaCl_2$, 0.40; $MgSO_4$, 0.2; sodium citrate, 1.0; NaCl, 2.5; $FeSO_4$, 0.0625.

Medium 9 (M9): Starch, 10.0; peptone, 5.0; NH_4Cl, 1.0; $CaCl_2$, 0.2; $MgSO_4$, 0.1; sodium citrate, 1.0; NaCl, 1.0; $FeSO_4$, 0.025.

Medium 10 (M10):, Starch, 10.0; peptone, 3.0; NH_4Cl, 2.5; $CaCl_2$, 0.45; $MgSO_4$, 0.5; sodium citrate, 1.0; NaCl, 3.0; $FeSO_4$, 1.0.

Batch cultivation of the cells were carried out in the shake flask study. 200 ml medium was taken in 500 ml flask. The flasks were inoculated with 10 % (volume/volume) inoculum grown for 12 hours in inoculum media. The pH of the medium was adjusted to 7.0 during the time of inoculation and was not maintained after that. After the inoculation the cells were grown at constant temperature 40^0 C with shaking speed of the incubator at 200 rpm in incubator.

The cell concentration in the broth was measured from the standard plot of dry cell concentration (g dry cell weight/L) against optical density of the cell suspension. For the measurement of the dry cell weight, the cells were pelleted after centrifuging 25 ml of the broth at 10,000 rpm for 15 minutes, resuspended in 10 ml distilled water and centrifuged again. They were then transferred to preweighted aluminium foil cup, dried at 90^0 C overnight and weight for dry cell weight measurement. The optical density of the cell suspension was read in spectrophotometer at 600 nm.

RESULTS AND DISCUSSION

To observe the cell growth concentrations with time and final yield of the cells in the different semisynthetic medias, ten different medias were inoculated in the shake flask. The samples were withdrawn from the two of the inoculated medium (M2 and M6) and analysed for the cell growth at regular interval of time. The cell growth in both the medias continued to increase upto 70 hours and then stopped. Figure 1 shows the variation of cell concentration with time in the media M2. The measurement of the optical density allowed the determination of cell concentration in the media. The growth curve showed that cells started to grow after around three hours of inoculation and exponential phase continued upto 48 hours. The cell concentration after 70 hours reached to constant.Also by observing the growth of the two samples we were able to extrapolate the time when the nutrient in all the other broths would be consumed.

The final cell concentrations were measured after 70 hours for all the medium and are shown in the Table-1.The final concentrations in medias has varied widely depending upon the media compositions. Since the multiple nutrient concentrations were changed arbitrarily and simultaneously at a time, it was not possible to correlate the results with any individual in simple way.

The situation here is dependant variable is function of multiple variable. The general for of the equation could be

$$y = a_0 + a_1x_1 + a_2x_2 + ... + a_nx_n$$

The method of multiple linear regression analysis was used for determining the cell growth in the culture with respect to different nutrient concentrations in the media. The equation for cell growth becomes $y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x + a_7x_7 + a_8x_8 + a_9x_9$ where x_1 = starch , x_2 = peptone, $x_3 = NH_4Cl$, $x_4 = CaCl_2$, $x_5 = MgSO_4$, x_6 = Sodium citrate, x_7 = NaCl, $x_8 = FeSO_4$. All the variables are expressed as concentration units in g/l.
The equation obtained for cell dry weight.

$$Y = -1.088756 + 0.208739\ X_1 + 0.187348\ X_2 + 1.622863\ X_3 - 0.782136\ X_4 + 0.42863\ X_5 - 2.563202\ X_6 + 6.613622\ X_7 - 5.098946\ X_8$$

The developed equation was validated with the available data. In this case it was found that the modelled equation gave large differences with the calculated and actual outputs. So it proved the incapability of the method of multiple linear regression to model the observed phenomena.
To predict the nonlinear behavior of the cell growth with nutrient concentration in the media, the method of forward feed Artificial Neural Network was tried.An Artificial Neural Network was prepared to use the nutrient concentrations as signal input and cell concentrations as the output. First the initial population of neural network was created with random weights and bias values. Then specific neural network architecture was selected having 8 input neurons, 10 hidden neurons and one output neuron. The architecture of the neural network is shown in the Figure-2.Neural network structure was made to learn the outputs (cell concentrations) for the given inputs (starch, peptone and salt concentrations). The estimated values were compared with the target values and if the error was larger than a permitted threshold value, the training algorithm readjusted the weights by delta rule. In a complete random way, training and testing data sets were taken up for the learning algorithm.

Variables (salt concentrations and cell mass produced) were scaled in the rank [0.1, 0.9] due to their measurement ranges and to avoid saturation problems in the sigmoidal function of the network. The scaled variables were obtained as follows:

$$X = 0.8\frac{X - X_{\min}}{X_{\max} - X_{\min}} + 0.1$$

The output values of Y* were estimated using a linear function. These values were de-scaled by:

$$Y = \frac{(\max Y - \min Y)(Y^* - 0.1)}{0.8} + \min Y$$

The theoretical architecture of the network is given in Figure 1. The RMS error of prediction is a relative standard and was obtained by the following expression

$$\text{RMS Error}=\sqrt{\frac{\sum_{i=1}^{n}(Y_i - Y^*_i)^2}{n}}$$

Where Y and Y* are the observed and the predicted values of the cell concentration, respectively; $\overline{Y}$ is its average value; and n the number of data points used.
The input and corresponding output data sets were selected randomly and were used to train the selected neural network. The rest data sets were used for testing the neural network.

The presented Artificial Neural Network was trained for 10000 epochs. The Learning rate used was 0.005 and the execution time for prediction was 16.5 seconds, Root mean square training error 0.3586 and Root means square error 1.8905.The best network obtained after training, was then used for predicting the dependence of cell mass concentrations on the variation of each individual nutrient concentrations in the media, keeping all other nutrient concentrations constant and is shown in Figure-3.

The variations were made based on the best result obtained experimentally. So the base composition was taken as the composition of the medium 7 (M7). Changes in the trend of the curves with different concentration of nutrients shows the significance. Results showed that increase in the concentration of starch, peptone, $CaCl_2$ and $FeSO_4$ should have negative effect and MgSO4 should have positive effect on the cell concentrations. The curve of cell concentration versus NH_4Cl gives an interesting result showing optimum value of concentration. Comparison of the results with literature values could not be done because earlier studies were focused with the production of enzyme and not cell growth. The effect of concentration nutrients on cell growth is not available.

CONCLUSIONS

The above study conducted shows the inability of the method of multiple linear regression to model the observed phenomena. The trained Artificial Neuron Network was then used for the estimation of the variation of cell concentration with salt concentrations in different individual media. The training error obtained was 0.3586, and the corresponding testing error was 1.8905. The Artificial Neuron Network approach showed the ability of it to model biochemical process, which may be used for the optimization, and selection of the best media composition. Further work is needed to confirm these preliminary result, which could be economic significance on the production scale.

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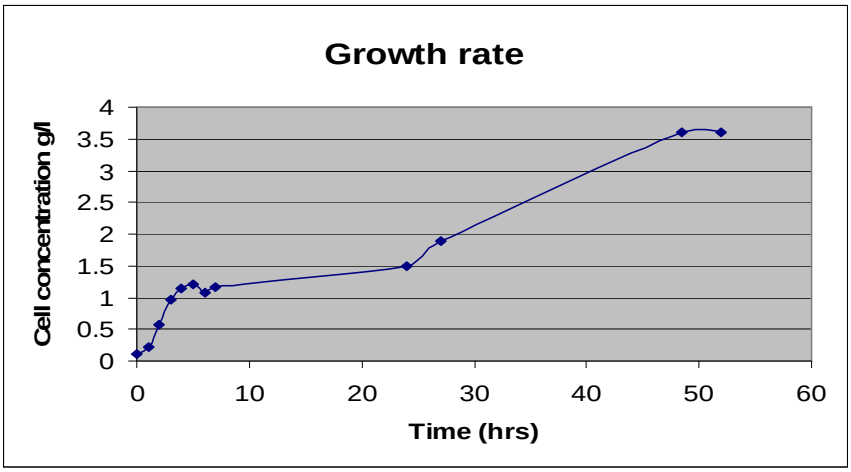

Figure 1: Variation of Cell Concentration with Time in the media.

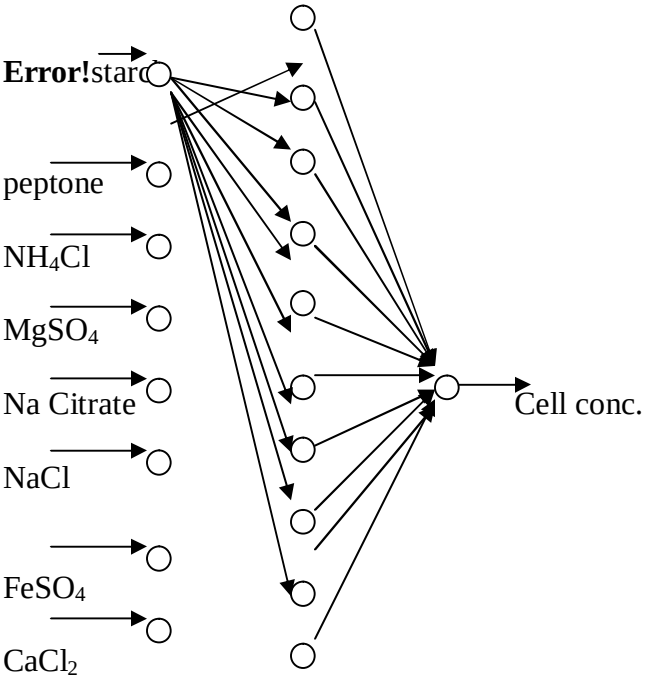

Figure 2. Neural Network Architecture

Table 1: Ultimate cell concentration in different media.

Medium Number	Cell concentration, g/l
M1	4.6322
M2	3.7724
M3	2.9774
M4	2.9396
M5	4.6372
M6	2.8480
M7	5.6974
M8	4.9272
M9	3.1910
M10	2.8448

Table 6: Comparison of actual and predicted Outputs (*denotes testing sample)

Medium Number	Cell conc., g/L (Actual)	Cell conc., g/L (Predicted)
M1	4.6332	4.2179
M2	3.7724	3.7777
M3	2.9774	3.1343
M4	2.9396	2.830
M5	4.6372	3.9659
M6	2.8480	2.947
M7	5.6974	5.6271
M8*	4.9272	4.9804
M9*	3. 1910	3.2097
M10*	2.8448	3.0251

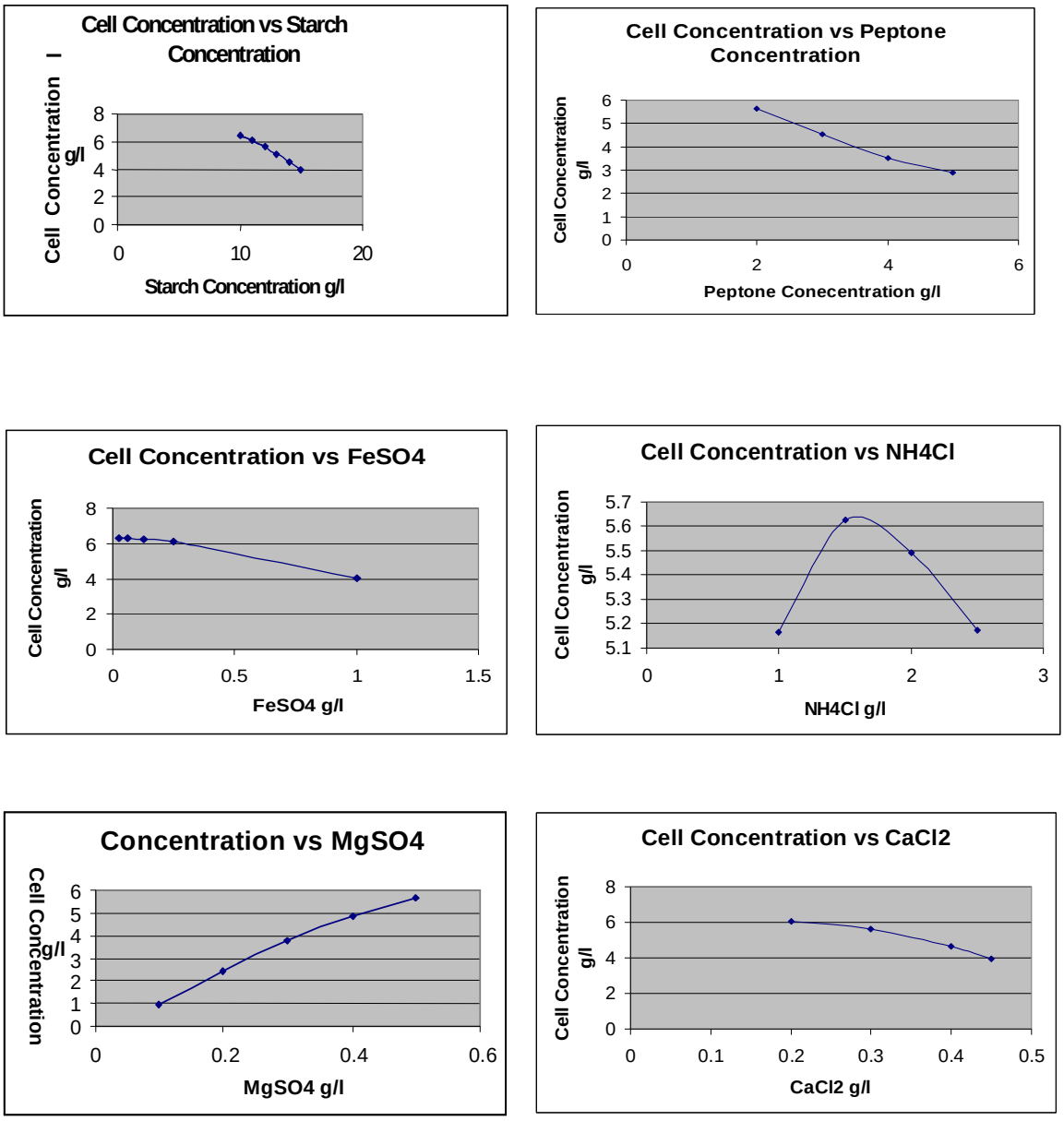

Figure 3:Variations in cell concentrations obtained with respect to variations variation of individual nutrient concentration.