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Kinetic study of a low molecular weight protease from newly isolated *Pseudomonas* sp. using artificial neural network

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A soil isolate, identified as *Pseudomonas* sp. produced an extra-cellular protease enzyme of 14.4 kDa molecular weight. The kinetic properties of the purified fraction of the bacterial protease were studied experimentally and the rate of casein hydrolysis was predicted by a model based on artificial neural network. The various kinetic factors studied were incubation time, initial enzyme concentration, initial substrate concentration, *p*H and temperature. The prediction error in simulating casein hydrolysis was less than 1%.

Keywords: *Pseudomonas* sp., protease, kinetics, simulation, artificial neural network

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Introduction

Proteases find enormous industrial applications in detergents, leather, baking, brewery, dairy, photographic industries, etc[1]. Purification and characterization of protease, therefore, play an important role in the success of its commercial production. There are many types of conventional purification techniques like gel-filtration, ion exchange chromatography, affinity chromatography, high performance liquid chromatography, ultrafiltration and electrophoresis used for purification[2]. In most cases satisfactory purification is obtained by using many of these techniques in combination.

Kinetic study of purified enzyme constitutes the information pertaining to rates of activation and inactivation of enzymes and actually gives the rate at which a process occurs[3]. The kinetic parameters controlling the rates of enzyme catalyzed reaction are: (i) incubation time, (ii) initial substrate concentration, (iii) initial enzyme concentration, (iv) *p*H, and (v) reaction temperature. The dependence of the reaction rate on these parameters is in general non-linear in nature and there can be considerable amount of cross-coupling between the different parameters. Modeling of this integrated behaviour is difficult to capture within a set of empirical equations. Artificial neural network (ANN) can solve such problems efficiently.

There is a report on ANN based computations being applied for developing predictive models as a result of combined effects of three environmental factors (temperature, *p*H and water activity) on thermal inactivation rate of *Escherichia coli*[4]. In this paper, the modelling ability of ANN algorithm compared to that of response surface methodology (RSM) method showed the superiority of the accurate prediction by ANN-based approach. Similarly, ANN and RSM have been used to build up a predictive model of the joint effect of NaCl concentration, *p*H level and storage temperature on kinetic parameters of the growth curve of *Lactobacillus plantarum*. In the present study also ANN showed much better results than RSM technique[5]. Static and dynamic neural networks were also used for on-line estimation of biomass concentration during batch cultures of *Streptococcus salivarius* ssp. *thermophilus* 404 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 398 at controlled *p*H and temperature[6].

This paper reports the purification and kinetic study of extra-cellular protease enzyme from a newly isolated *Pseudomonas* sp. RAJR 044 along with its parametric behaviour. The results were integrated into an ANN based model that can be used for extensive simulation studies.

Materials and Methods

Microorganism

A new soil isolate from Indian Institute of Technology (IIT) Kharagpur Campus, identified as *Pseu-*

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domonas sp. RAJR 044 producing extracellular protease enzyme within 24 hrs was used for the subsequent experiments. The strain was maintained on 2% nutrient agar slants at 4°C.

Chemicals

All the chemicals used for the following experiments were of analytical grade. The wheat bran used in the medium was purchased from the local market.

Composition of Media

Nutrient agar (2%), Czapek-dox[7]: $NaNO_3$ 2.5 g/l; KH_2PO_4 1.0 g/l; $MgSO_4.7H_2O$ 0.5 g/l; KCl 0.5 g/l.

Inoculum Preparation

For inoculum preparation, 10 ml of sterile distilled water was added to the slant grown on nutrient agar plate for 3 days at 30°C and scrapped with inoculating loop aseptically. This suspension having spore concentration approx. 3×10^7cells/ml was used as inoculum for subsequent fermentation.

Assay of Protease Activity

Protease activity was determined by caseinolytic method[8]. The protease activity was assayed by taking casein as a substrate. One unit (U) of protease activity is defined as the amount that liberates peptide fragments equivalent to 1 mg of bovine serum albumin under the assay condition[7].

Assay of Protein

The total protein in the sample was determined by Lowry's method[9] using bovine serum albumin as a standard. The O.D. was taken at 750 nm.

Experimental Design and Protease Production

The novel *Pseudomonas* strain was cultured under submerged fermentation for extra-cellular protease production in 250 ml Erlenmeyer flasks containing 100 ml of sterilized wheat bran and Czapek-dox medium (in 1:10 ratio) inoculated with the freshly prepared bacterial suspension (as discussed earlier) for 3 days at 30°C. After fermentation, the cell-free supernatant was obtained by centrifugation at 10,000 rpm and the extra-cellular protease activity of the fermented broth was determined.

Purification

In order to purify the protease enzyme, to be used further for the study of protease kinetics, the culture broth was first centrifuged at 10,000 rpm for 10 minutes to separate the insoluble and then two volumes of cold acetone (4°C) was added to one volume of the supernatant and kept at 0°C for 3 hours. After acetone precipitation, the enzyme sample was dissolved in minimum buffer solution and further purified by carboxy-methyl (CM) Sephadex ion exchange chromatography. The molecular weight of the purified fraction of the enzyme was then determined by Laemmli's sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method[10].

Protease Kinetics

Effect of Different Substrates on Hydrolytic Action of Protease

To study the effect of various substrates, 0.02 ml of the enzyme (of total protein concentration of 3.1 mg/ml) of the wild strain was incubated with 2 ml of each of these substrates (of 1 mg/ml concentration) for 30 min at 37°C separately. The reaction volume was adjusted to 2.5 ml by buffer. After the required incubation time the enzyme activities were determined.

Effect of Incubation Time on Casein Hydrolysis

To investigate the effect of incubation time on casein hydrolysis, 0.02 ml of the enzyme solution (of total protein concentration of 3.1 mg/ml) of wild strain was taken in 2 ml of 2% casein stock solution and incubated from 5-30 min at 37°C in 2.5 ml of reaction mixture and then enzyme assay was done.

Effect of Initial Enzyme Concentration on Casein Hydrolysis

The rate of enzyme-catalyzed conversion of substrate to product is, under assay conditions, directly proportional to enzyme concentration (i.e. $v_o= k\ [E]_o$ at constant conditions of substrate concentration, *p*H, temperature etc.). Although the rate of hydrolysis increases with increase in enzyme concentration, its characteristic differs for each enzyme. Accordingly, the reaction mixtures were prepared varying the enzyme concentration from 0.02-0.1 ml. Substrate concentration of 2 ml was taken to which each of the above enzyme concentrations were added and finally total volume was made up to 2.5 ml with glycine-NaOH buffer, then these were incubated at 37°C for 15 min for the wild strain.

Effect of Initial Substrate Concentration on Casein Hydrolysis

One of the most fundamental factors affecting the enzyme activity is substrate concentration. In this experiment reaction mixtures were designed varying the substrate concentration from 0.05 to 2 ml from 2% casein stock solution to which 0.06 ml enzyme solu-

tion for the wild strain was added. The volume of the reaction mixture was made to 2.5 ml by buffer and each sample was incubated at 37°C for 15 min and then protease activity was determined.

Effect of pH on Casein Hydrolysis

To evaluate casein hydrolysis at different *p*H (ranging from 3-11), the optimum enzyme concentration of 0.06 ml for the wild strain was added to the optimum substrate concentration of 0.5 ml for the wild strain and the final volume made up to 2.5 ml with buffer by adjusting the *p*H from 3-11. For *p*H 3-6, glycine-HCl buffer and for *p*H 8-11, 0.2 M glycine-NaOH buffer was selected. Phosphate buffer of similar ionic strength was used for *p*H 7. The reaction mixtures were incubated for 15 min at 37°C and then the enzyme activities were determined.

Effect of Reaction Temperature on Casein Hydrolysis

Enzyme activity is also strongly affected by temperature. Experiments were designed as described above except that the *p*H was fixed at 8 for the wild strain and the reaction was carried out at temperatures varying from 4-80°C and then enzyme assay was performed.

Mathematical Modelling

Mathematical models also play important role in designing appropriate controller for a non-linear process. Mathematical modelling can be done in several ways, (i) in dynamic form by a set of differential equations, (ii) by empirical algebraic models, (iii) by ANNs.

The rate of hydrolysis not only depends on several parameters in a non-linear way but also there may be considerable amount of interdependencies between the environmental parameters. Thus, neural network is taken as an appropriate simulation tool for this purpose.

ANN

Fig. 2—Weight vectors in the MLP feed-forward network, v_{ij} – weight from input to hidden layer, w_{pq} - weight from hidden layer to output layer, a – input vector, b – response at the hidden node, c – output vector.

A good background of ANN is available in literature[11-13]. The most popular model is the multilayer perceptron (MLP) model having feed-forward structure. A representative MLP feed-forward network is given in Fig. 1. Each node represented by the box is called a perceptron. The most critical part of an ANN-based model is to train the network. The problem of neural network training is to devise a method of updating the representative weights that minimizes the error. It is essentially an optimization problem. The updating of the weights has been done here by Levenberg-Marquardt algorithm. Levenberg-Marquardt algorithm performs much better with some knowledge of the process so that quick convergence is obtained with a very low error. The network used is a feed-forward one with one input layer, one output layer and a hidden layer.

The Network

The structure of the network is given in Fig. 2 where v_{ij} and w_{pq} denote the weights for the successive layers. The basic purpose of training a network is to optimize v_{ij} and w_{pq} corresponding to a particular set of input-output training pattern. The responses at the hidden nodes b_j (b_1 to b_p, $j = 1,2,\ldots p$) are calculated by evaluating the contributions from all the input nodes through a nonlinear mapping function.

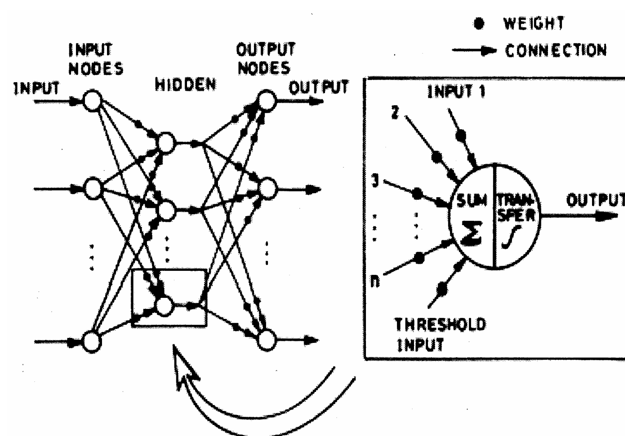
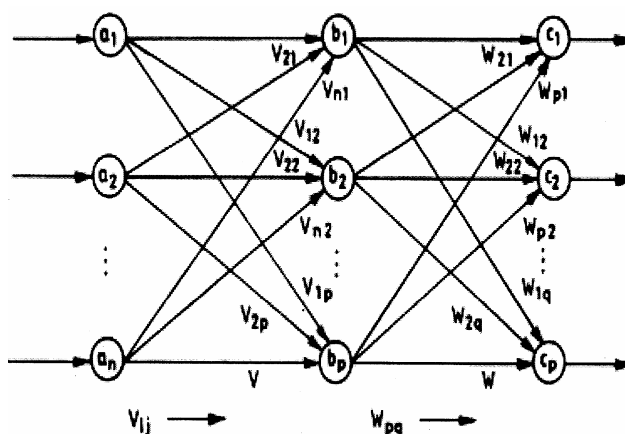


Fig. 1—Multilayered perceptron model



$$b_j = f\left[\sum_{i=1}^{n} a_i v_{ij} + \theta_j\right]$$

where f(x) is the 'tansig' function of MATLAB[14] and given by

2 ./ (1 + exp(-2*n)) – 1

θ_j is the bias at the jth hidden layer node and a_i is the input vector.
Similarly, c_k is calculated using

$$c_k = f\left[\sum_{j=1}^{n} b_j w_{jk} + \tau_k\right]$$

where, τ_k is the bias at the kth output layer node.

ANN Applied to Modelling of Hydrolysis

ANN model gives a proper representation of the non-linearity and interdependencies within one framework for different bioprocesses[4-6,15]. A 5-4-1 feed-forward network has been chosen for the present application with 4 nodes in the hidden layer. Before starting the training, training pattern is normalized by a scale factor to limit the patterns within 1. Five inputs fed to the five nodes are *p*H, temperature, enzyme concentration, incubation time and substrate concentration. The output taken from the single output node is the rate of hydrolysis. Training process has been carried out till the sum of square error is less than 1.e-5. Even such a low error criterion has been satisfied by Levenberg-Marquardt algorithm[14] within 300 number of iterations, whereas back propagation with momentum was taking more than 10,000 iterations.

Results and Discussion

Purification Methods

Table 1 shows the different steps of purification for the wild strain with % recovery and fold purification. The homogeneity of the purified fraction from the wild strain was proved by the appearance of a single band corresponding to 14.4 kDa in SDS-PAGE as shown in Fig. 3.

Effect of Different Substrates on Hydrolytic Action of Protease

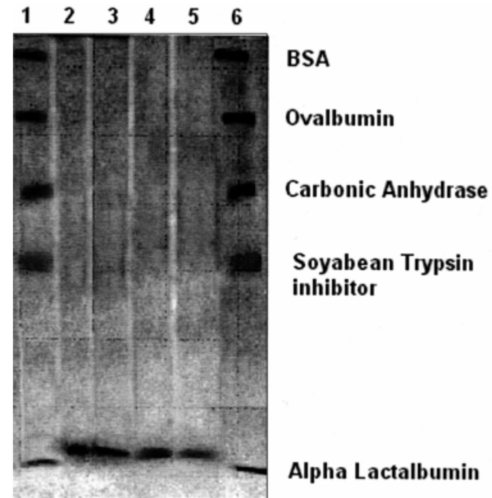


Fig. 3— SDS-PAGE Electrophoresis analysis; Lane 1,6: Standard Protein Marker: From top: Bovine serum albumin- 67 kDa; Ovalbumin-43 kDa; Carbonic anhydrase- 30 kDa; Soyabean trypsin inhibitor- 20.1 kDa; Lactalbumin- 14.4 kDa, Lane 2,3,4,5: Purified fraction of wild strain.

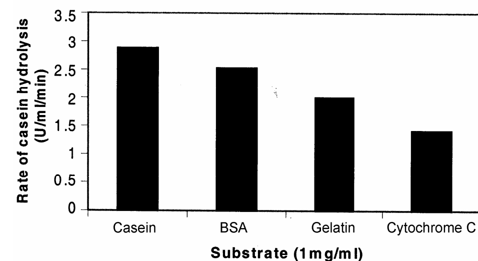


Fig. 4—Effect of substrates on hydrolytic action of protease; 0.02 ml of the enzyme (of total protein concentration of 3.1 mg/ml) was incubated with 2 ml of each of the substrates (of 1 mg/ml concentration) for 30 min at 37°C.

Among the various substrates in Fig. 4, casein was found to be the best for the study of hydrolytic action of protease. This is an important animal milk protein on which protease

Table 1— Protease purification by various methods for the wild strain RAJR 044

Purification steps	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (u/mg)	Protease recovery (%)	Fold purification
Crude extract	50	2000	525	3.81	100	1.0
Acetone treatment	3.0	912	59.9	15.23	45.6	4.0
CM-Sephadex	15.0	348	18.3	19.02	17.4	5.0

acts to give the maximum rate of hydrolysis due to which further study of enzyme kinetics was done with casein.

Effect of Different Environmental Parameters on Casein Hydrolysis

On enzyme assay, it was found that there was increase in the rate of hydrolysis in the early phase of incubation time and became maximum at 15 min for the wild strain but thereafter the hydrolysis rate did not change with time as shown in Fig. 5. Variation of the rate of casein hydrolysis with time in Fig. 5 indicated that at initial stage, substrate to enzyme ratio plays an important role. This ratio is maximum at the beginning, with time the substrate is hydrolyzed and the ratio arrives at an optimum value when hydrolysis rate also becomes maximum. For hydrolysis, active site of enzyme must bind to appropriate position of substrate. Faster the matching occurs, quicker is the product formation resulting in higher reaction rate[16].

From the study of the effect of initial enzyme concentration it was clear that at low concentration the rate of hydrolysis increased linearly and finally the curve became asymptotic at higher enzyme concentration, thereby indicating a substrate-limiting condition. It was observed from Fig. 6 that 0.0 6 ml (of total protein concentration of 3.1 mg/ml) was optimum for the wild strain. This may be due to the limitation of availability of substrate for the active sites of enzyme and for which, further addition of the enzyme could not enhance the rate of product formation[17,18].

The study of the effect of initial substrate concentration (Fig. 7) showed that there was an increase in substrate hydrolysis up to 0.5 ml in the wild strain and thereafter there was no change in the hydrolysis rate.

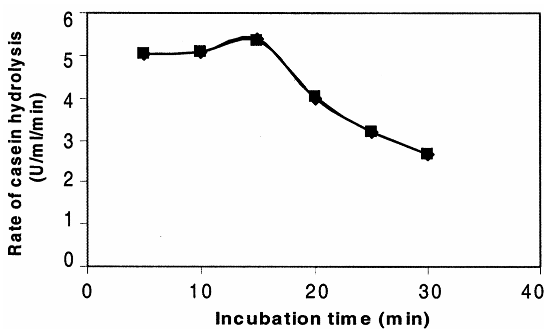


Fig. 7—Simulated and experimental rate of hydrolysis for wild strain with varying initial substrate concentration (- ♦- experimental for wild, ■- simulated for wild); 0.06 ml of enzyme solution was added separately to varying substrate concentrations of 0.05 to 2 ml from 2% casein stock solution and incubated at 37°C for 15 min. Fig. 5—Simulated and experimental rate of hydrolysis for wild strain with varying incubation time (- ♦ - experimental for wild, ■- simulated for wild) 0.02 ml of the enzyme solution (of total protein concentration of 3.1 mg/ml) was taken in 2 ml of 2% casein stock solution and incubated from 5-30 min at 37°C.

From Michaelis-Menten correlation it is seen that the rate of enzymatic reaction is directly proportional to the substrate concentration when the magnitude of the latter is low. But at higher magnitudes the reaction rate is not influenced by substrate concentration[19].

The *p*H affects the ionization of amino acids, which dictate the primary and secondary structure of enzyme and hence control its activity. A typical bell-shaped curve is obtained in Fig. 8 with the maximum protease activity at *p*H 8 for the wild strain. A fall in the hydrolysis rate on either side of the optimum values is due to decrease in affinity of enzyme for the substrate[19].

The study of the effect of temperature in Fig. 9 showed that the rate increased with increase in temperature up to 45°C for wild strain but thereafter decreased. Increase in temperature above optimum level affects important factors like protein denaturation, protein ionization state and solubility of species in solution reducing enzyme activity[20]. The experimental and simulated rate of hydrolysis obtained from ANN model has also been shown in the Figs 5-9.

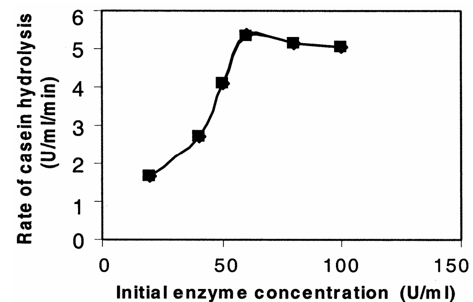
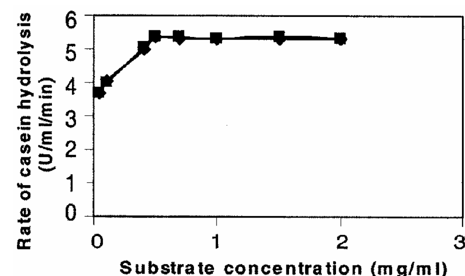


Fig. 6—Simulated and experimental rate of hydrolysis for wild strain with varying initial enzyme concentration (- ♦- experimental for wild, ■- simulated for wild); 2 ml of casein solution was added to each of the varying enzyme concentrations of 0.02 ml-0.1 ml separately and then incubated at 37°C for 15 min.



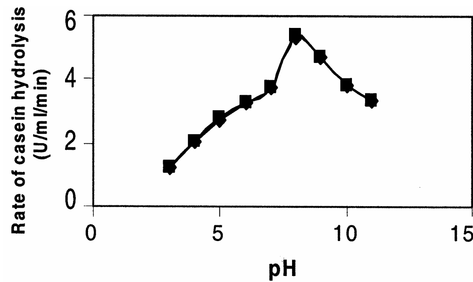


Fig. 8—Simulated and experimental rate of hydrolysis for wild strain with varying *p*H (- ♦- experimental for wild, ■- simulated for wild). The optimum enzyme concentration of 0.06 ml was added to the optimum substrate concentration of 0.5 ml at *p*H ranging from 3-11 for 15 min at 37°C.

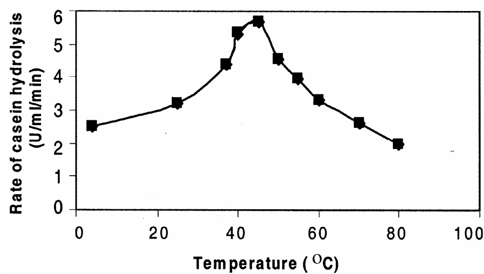


Fig. 9—Simulated and experimental rate of hydrolysis for wild strain varying temperature (- ♦- experimental for wild, ■- simulated for wild); 0.06 ml of enzyme was added to 0.5 ml of substrate at *p*H 8 with temperature varying from 4°C-80°C for 15 min.

Response of ANN Model to Unknown Data Sets (for Wild Strain)

Data set 1: [6, 40, 60, 15, 0.5] – simulated rate of hydrolysis -3.16, experimental- 3.22
Data set 2: [8, 40, 60, 15, 0.5] – simulated rate of hydrolysis -3.90, experimental- 3.84
Data set 3: [8, 70, 60, 15, 0.5] – simulated rate of hydrolysis -2.7, experimental- 2.65

The chosen neural network has represented the system nonlinearities in an excellent manner. Average error in the predicted and actual data is less than 0.1%, whereas maximum error is less than 1%. Usually neural network is good at interpolation, however in this case it is found that the network is even successful in extrapolation beyond 20% of the training range.

Conclusion

Enzyme kinetics continues to be the most fundamental aspects of enzymology. From industrial viewpoint it is desirable to choose an enzyme, which will have the fastest reaction rate per unit amount of enzyme as this indicates the maximum effect for minimum amount of added catalyst.

In this study, the ANN based model has been applied to represent the behaviour of the enzyme kinetics. This model can further be used to optimize the working condition for maximizing the rate of hydrolysis. Neural network is an excellent tool for representing system non-linearities but it is very difficult to quantify the order of the non-linearity in the conventional sense, i.e., whether dependency is quadratic, cubic, exponential or logarithmic. In many non-linear systems, however, the dependency is a mixed type whose mathematical form is not known. Conventional empirical non-linear models simulate such systems, often tolerating a large error. In such cases use of neural network is more than justified.

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