J Biochem Tech (2009) 2(1):138-143
ISSN: 0974-2328

Fuzzy rule-based prediction of lovastatin productivity in continuous mode using pellets of *Aspergillus terreus* in an airlift reactor

Kamakshi Gupta, Bodhisatta Maiti, P K Mishra, Pradeep Srivastava*

Received: 27 November 2009 / Received in revised form: 18 January 2010, Accepted: 11 February 2010, Published online: 23 March 2010

Abstract

Lovastatin production using pellets of *Aspergillus terreus* was investigated in an airlift reactor. A fuzzy system has been developed for predicting the lovastatin productivity. Analysis of the effect of dilution rate and biomass concentration on the productivity of lovastatin was carried out and hence these were taken as inputs for the fuzzy system. The rule base has been developed using the conceptions of developmental processes in lovastatin production. The fuzzy system has been constructed on the basis of experimental results and operator's knowledge. The values predicted for lovastatin productivity by the fuzzy system has been compared with the experimental data. The R squared value and mean squared error has been calculated to evaluate the quality of the fuzzy system. The performance measures show that the rule-based results of the fuzzy system is in accordance with the experimental results. The utilization of fuzzy system aided in the increase of lovastatin productivity by about 1.3 times when compared to previous empirical experimental results.

Keywords: lovastatin, airlift reactor, fuzzy rule-based system, *Aspergillus terreus*, continuous fermentation, pellets.

Introduction

Lovastatin is a secondary metabolite which is a highly powerful therapeutic agent for the treatment of hypercholesterolemia. Lovastatin (mevinolin) is a strong inhibitor of hydroxymethylglutaryl- coenzyme A (HMG-CoA) reductase, which is the rate limiting enzyme in the cholesterol biosynthesis pathway. *Aspergillus terreus* has been used to produce lovastatin which aids in lowering the plasma cholesterol level in human and animals (Alberts et al. 1980).

Our present work deals with the continuous production of lovastatin with pellets of *Aspergillus terreus* using an optimized production medium in an air lift reactor (ALR). The production of lovastatin is a complex and ambiguous process. Often the data obtained from experiments were vague and indefinite. To deal with such imprecise biological process, a fuzzy system has been developed to analyze the effect of dilution rate and biomass concentration on lovastatin productivity in an air lift reactor (ALR). The submerged cultivation process was carried out for 72 hours in batch mode for the formation of biomass for lovastatin production and then it was shifted to continuous mode. The process was carried out by submerged cultivation in continuous mode. The fuzzy rule-based system functions on the basic principles of fuzzy set theory (Zadeh 1965). The effect of dilution rate on the bioproductivity has been studied with the aid of fuzzy logic (Vassileva and Tzvetkova 2003).

The utilization of fuzzy logic to understand the lovastatin production system is more advantageous when compared with artificial neural networks and quadratic polynomial models. The fuzzy system gives a notion of the causes responsible for the change in the characteristics of lovastatin productivity, which can't be explicated with artificial neural networks. The regression equation from curve fitting results doesn't portray a clear picture of the ambiguous nature of biological system. Several advantages of fuzzy logic can be taken from the Mahabir et al. 2006 article.

The objective of this present study is to provide an insight into the working methodology of fuzzy logic which had been applied for optimization of our previous experiments on lovastatin production (Srivastava et al. 2009). We have also portrayed the performance measure of the system and tested its predicting capabilities.

Kamakshi Gupta, Bodhisatta Maiti, Pradeep Srivastava*

School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi-221005, India

*Tel: 0091 542 2307076, Fax: 0091 542 2368428
Email: drpradeep19.itbhu@gmail.com

P. K. Mishra

Department of Chemical Engineering and Technology, Institute of Technology, Banaras Hindu University, Varanasi-221005, India

Materials and Methods

Organism and Fermentation Conditions

The production of lovastatin was carried out using ***Aspergillus terreus*** (NRRL 255). The strain was obtained from Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research Peoria, Illinois USA. It was maintained on PDA (potato dextrose agar) incubated at 28^{o}C for 4-5 days and stored under refrigeration at 5-10^{o}C.

Growth medium (Samiee et al. 2003) was prepared using glucose (45 g/l), KH_2PO_4 (5g/l), K_2HPO_4 (5g/l), monohydrate sodium glutamate (12.5 g/l), H_3BO_3 (11mg/l), $FeSO_4.7H_2O$ (0.2 g/l), $MnSO_4.4H_2O$ (0.1 g/l), $ZnSO_4.7H_2O$ (0.2 g/l), $MgSO_4.7H_2O$ (0.1 g/l), $CaCl_2.2H_2O$ (20 mg/l), $CuCl_2.2H_2O$ (5 mg/l) and $(NH_4)_6Mo_7O_{24}.4H_2O$ (5 mg/l). The fermentation was carried out at 28^{o}C for 3 - 4 days on a rotary shaker at 180 rpm. The culture was grown in 500 mL shake flask containing 100 mL medium. Large numbers of pellets attaining the size of less than 1 mm to several millimeters were formed. The average size of the pellet ranging from 1.8 to 2 mm was used to inoculate the production media for lovastatin.

Production medium was prepared with glucose (40 g/l), milk powder (15 g/l), soybean meal (5.5 g/l), malt extract (0.5 g/l), sodium acetate (1.0 g/l), peptone (1.0 g/l), NaCl (0.2 g/l), $CaCO_3$ (1.5 g/l), KH_2PO_4 (0.05 g/l), $MgSO_4.7H_2O$ (0.05 g/l) and the pH was adjusted to 5.8 - 6.0 using HCl. Silicone oil was used as the antifoam agent. For the optimization experiments the production medium containing flasks were incubated on a rotary shaker at 250 rpm, 28^{o}C for 10 days.

Experimental set up

A 5.0 liter internal loop airlift reactor (ALR) was indigenously designed to study the lovastatin production process. The fundamental design of the lab scale ALR was determined from the conceptions of earlier experiments (Srivastava and Kundu 1998). The construction of ALR was done using borosilicate glass and the dimensions were restricted to ease the sterilization process in the lab autoclave. An air filter was utilized in order to maintain a constant air flow rate and a rotameter was used to measure the flow rate. All the ports of ALR were aseptically sealed with rubber corks after inoculation. Sterile air was sparged co-currently through a single sparger and air flow rate was maintained. The dimensional details of the ALR have been given in table 1.

Table 1: The dimensions of the air lift reactor

Volumetric capacity of the reactor	**5 L**
Working volume	4 L
Column diameter	80 millimeters
Column height	800 millimeters
Ungassed liquid height	200 millimeters
Draft tube height	500 millimeters
Draft tube diameter	56 millimeters
Height of draft tube above the fermenter base	100 millimeters
Single sparger nozzle	1 millimeter

Continuous operation

The process was operated initially for 72 hours in batch mode after which it was shifted to a continuous mode keeping aseptic conditions. A peristaltic pump was used to collect the overflow. The reactor was fitted with a two-channel peristaltic pump which provided both substrate feeding and exhausted effluent withdrawal. The feed was continuously supplied at the bottom. Feed rates were varied and the output collected at the top was analyzed for biomass, substrate and lovastatin concentration. The foaming was controlled by using silicone oil and the different parameters for continuous cultivation were observed.

Analytical Methods

Batch fermentation runs were conducted for 10 days of growth and lovastatin production. Various samples were collected intermittently every 24 hours and every run was done in triplicate. Samples were centrifuged and extracted to separate cell mass for further analysis. Biomass was determined by dry cell weight (DCW) method. Reducing sugar estimation was estimated by 3, 5-Dinitrosalicylic acid (DNS) method (Miller 1959). Total sugar estimation was done by phenol sulfuric acid method (Dubois et al. 1956).

Extraction

The broth was adjusted to pH 3.5 with concentrated HCl followed by addition of an equal volume of ethyl acetate to the whole fermentation broth. Extraction was carried out on a rotary shaker at 220 rpm in an ambient temperature for 6 hours. The samples were subsequently centrifuged at 10000 rpm for 15 minutes and the organic phase was collected for further steps (Samiee et al. 2003; Kysilka and Kren 1993).

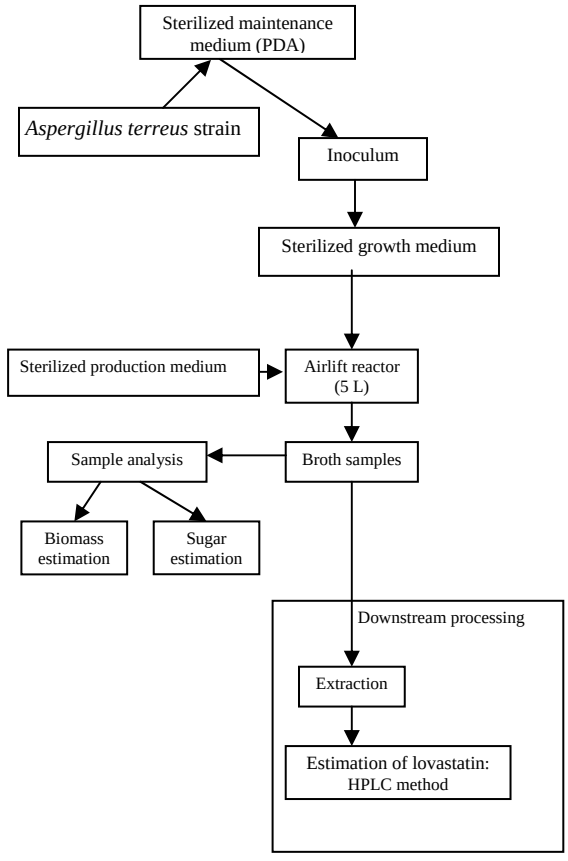

Figure 1: Schematic representation of experimental set up

Estimation of lovastatin

Lovastatin was estimated by reversed phase chromatography, which was performed in Perkin Elmer (Waters) C18 column of size 4.6 mm * 250 mm and was quantified using UV absorbance at 235 nm. Acetonitrile and water (acidified with orthophosphoric acid to the concentration of 0.1%) (60:40, v/v) was used as the mobile phase. The flow rate was maintained at 1.5 mL/min (Friedrich et al. 1995). The reference solution was prepared using the standard lovastatin obtained from Lupin Limited, India.

The schematic representation of the whole experimental set up has been shown in figure 1.

Figure 2 shows the schematic presentation of a fuzzy inference system which comprises of a fuzzifier, which is used to convert crisp inputs into fuzzy values and assigning them to fuzzy sets with the help of membership functions. It comprises of a database

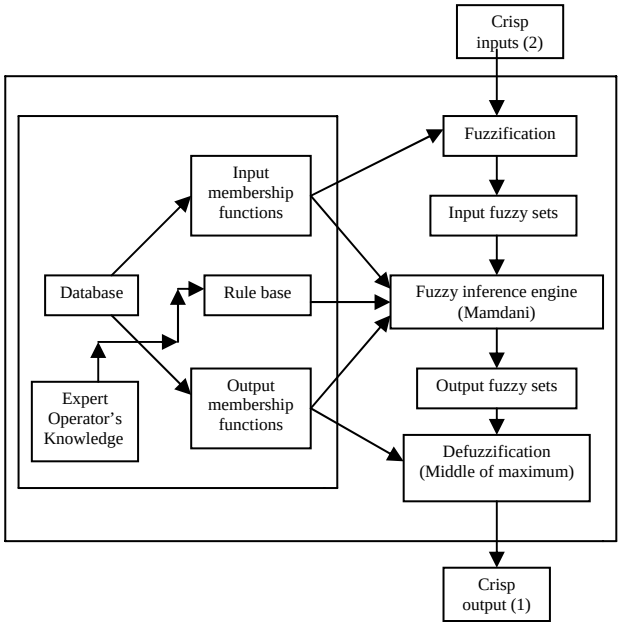

Figure 2: Schematic presentation of a fuzzy inference system

consisting of input and output membership functions. The rule base has been developed using the conceptions of developmental processes in lovastatin production and operator's knowledge. The fuzzy input values entering the inference engine are converted to output fuzzy values after the evaluation of rules in the rule base and also taking membership functions into consideration. The output fuzzy values are defuzzified using middle of maximum method to crisp output values. Figure 3 shows the fuzzy inference system (Mamdani) for predicting lovastatin productivity in MATLAB software.

The work has been carried out in fuzzy logic toolbox in MATLAB version 7.3 (The MathWorks Inc., USA). The mamdani type of fuzzy inference has been implemented in our work. Gaussian membership function has been used for all the fuzzy sets in both input variables (dilution rate and biomass) and output variable (productivity). The Gaussian membership function has been chosen over triangular membership function because the three dimensional plot obtained by using triangular membership function is uneven and irregular with the protrusion of sharp points. The plot doesn't portray properly the true characteristics of the biological system. On

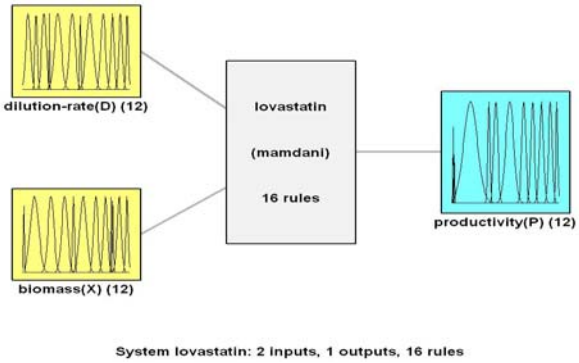

Figure 3: Fuzzy inference system in MATLAB software

the other hand the plot obtained upon using gaussian membership function is quite even, regular and till a large extent it is free from undesirable projections or indentations. The plot gives a coherent scenario of the behavior of productivity with respect to changes in dilution rate and biomass concentration.

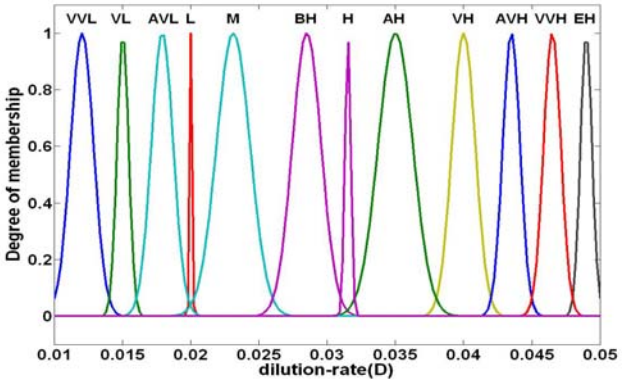

Figure 4.A: Membership function plots for input variable dilution rate

The input variable D (dilution rate) has a range ([0.01; 0.05] h^{-1}) and it has been divided into twelve fuzzy sets VVL (very very low), VL (very low), AVL (above very low), L (low), M (medium), BH (below high), H (high), AH (above high),VH (very high), AVH (above very high) , VVH(very very high) and EH (extremely high). Figure 4.A shows the membership function plots for D. The fuzzy sets and ranges for D are as follows:

VVL	(0.0008493 0.012)
VL	(0.0004247 0.015)
AVL	(0.0008069 0.0179)
L	(8.493E-05 0.02)
M	(0.001232 0.0231)
BH	(0.001062 0.0285)
H	(0.0002123 0.0315)
AH	(0.001274 0.035)
VH	(0.0008493 0.04)
AVH	(0.000637 0.0435)
VVH	(0.000637 0.0465)
EH	(0.0004247 0.049)

The input variable X (biomass) has a range ([0; 11] gl^{-1}) and it has been divided into twelve fuzzy sets VVL (very very low), BVL (below very low), VL (very low), BL (below low), L (low), AL (above low), M (medium), BH (below high), H (high), AH (above high), VH (very high) and VVH (very very high). Figure 4.B shows

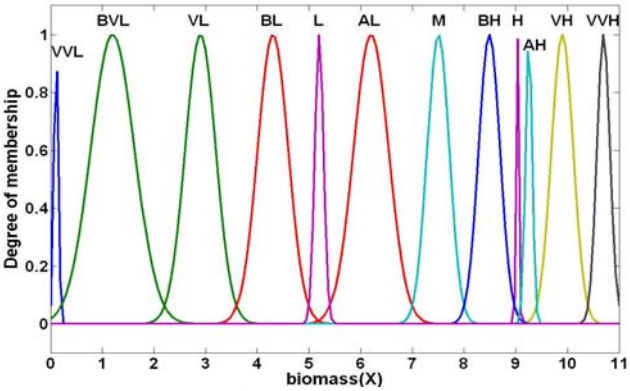

Figure 4.B: Membership function plots for input variable biomass concentration

the membership function plots for X. The fuzzy sets and ranges for X are as follows:

VVL	(0.04247 0.1)
BVL	(0.4247 1.2)
VL	(0.2973 2.9)
BL	(0.2973 4.3)
L	(0.08493 5.2)
AL	(0.3397 6.2)
M	(0.2123 7.5)
BH	(0.2081 8.49)
H	(0.02548 9.04)
AH	(0.0637 9.25)
VH	(0.2123 9.9)
VVH	(0.1274 10.7)

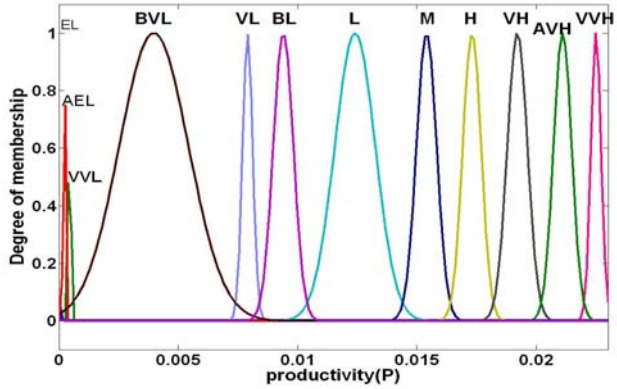

Figure 4.C: Membership function plots for output variable lovastatin productivity

The output variable P (lovastatin productivity) has a range ([0; 0.023] $gl^{-1}h^{-1}$) and it has been divided into twelve fuzzy sets EL (extremely low), AEL (above extremely low),VVL (very very low), BVL (below very low),VL (very low), BL (below low), L (low), M (medium), H (high), VH (very high), AVH (above very high) and VVH (very very high). Figure 4.C shows the membership function plots for P. The fuzzy sets and ranges for P are as follows:

EL	(2.336E-05 5.5E-05)
AEL	(4.671E-05 0.00022)
VVL	(4.671E-05 0.00044)
BVL	(0.0014540 0.003975)
VL	(0.0002123 0.0079)
BL	(0.0004247 0.0094)
L	(0.0008493 0.0124)
M	(0.0004247 0.0154)
H	(0.0003822 0.0173)
VH	(0.0004247 0.0192)
AVH	(0.0003822 0.0211)
VVH	(0.0002123 0.0225)

Sixteen rules have been developed for predicting the lovastatin productivity and the MOM (middle of maximum) defuzzification method has been implemented in order to defuzzify the output membership functions.

Fuzzy rules for predicting lovastatin productivity are as follows:

Rule 1: if (D is VVL) and (X is VVH) then (P is L)
Rule 2: if (D is VL) and (X is VH) then (P is H)
Rule 3: if (D is L) and (X is H) then (P is VVH)
Rule 4: if (D is M) and (X is M) then (P is VH)
Rule 5: if (D is BH) and (X is AL) then (P is H)
Rule 6: if (D is AH) and (X is VL) then (P is VL)
Rule 7: if (D is VH) and (X is VVL) then (P is VVL)
Rule 8: if (D is EH) and (X is VVL) then (P is EL)
Rule 9: if (D is M) and (X is H) then (P is VH)
Rule 10: if (D is AVH) and (X is VVL) then (P is AEL)
Rule 11: if (D is VH) and (X is BVL) then (P is BVL)
Rule 12: if (D is AH) and (X is BL) then (P is BL)
Rule 13: if (D is VVH) and (X is VVL) then (P is EL)
Rule 14: if (D is AVL) and (X is AH) then (P is AVH)
Rule 15: if (D is AVL) and (X is H) then (P is VH)
Rule 16: if (D is H) and (X is L) then (P is M)

Results and discussion

The experimental results for lovastatin production in continuous mode using pellets of *Aspergillus terreus* in an airlift reactor are given in table 2.

Table 2: The experimental results for lovastatin production in continuous mode using pellets of *Aspergillus terreus* in an airlift reactor

D Dilution rate (h^{-1})	X Biomass (gl^{-1})	S Substrate (gl^{-1})	P Productivity ($gl^{-1}h^{-1}$)
0.01	11	0	0.012
0.015	10	0.6	0.0169
0.02	9	1.2	0.022
0.025	7.3	2.2	0.019
0.03	6	3	0.017
0.035	2.5	4.2	0.008
0.04	0	5.4	0.0004
0.045	0	13	0.0001
0.05	0	20	0

The fuzzy rule-based system was simulated using the fuzzy logic toolbox in MATLAB, in which D (dilution rate) and X (biomass concentration) have been considered as input variables and P (lovastatin productivity) as output variable.

The fuzzy logic toolbox facilitates the users with a rule viewer shown in figure 5. The rule viewer shows the sixteen rules used for the construction of the system. It also shows the numerical ranges of the input variables (D and X) and the output variable (P). The rule viewer provides a platform for the modelers where one can enter the crisp input values and obtain a crisp output value. Table 3 portrays the fuzzy predicted values of productivity alongside experimental

Table 3: Comparison of fuzzy predicted productivity values with experimental productivity values at different dilution rate and biomass concentration

D Dilution rate (h^{-1})	X Biomass (gl^{-1})	P Productivity ($gl^{-1}h^{-1}$)	
		Experimental	Predicted
0.01	11	0.012	0.0124
0.015	10	0.0169	0.0174
0.02	9	0.022	0.0225
0.025	7.3	0.019	0.0192
0.03	6	0.017	0.0173
0.035	2.5	0.008	0.00794
0.04	0	0.0004	0.00046
0.045	0	0.0001	0.000115
0.05	0	0	0

productivity values at different dilution rates and biomass concentration.

Figure 6 shows the surface plot of output (lovastatin productivity) with respect to the input variables. We can deduce from the surface generated upon simulation and from table 3 that the productivity increases rapidly, approximately by about 1.81 times when the

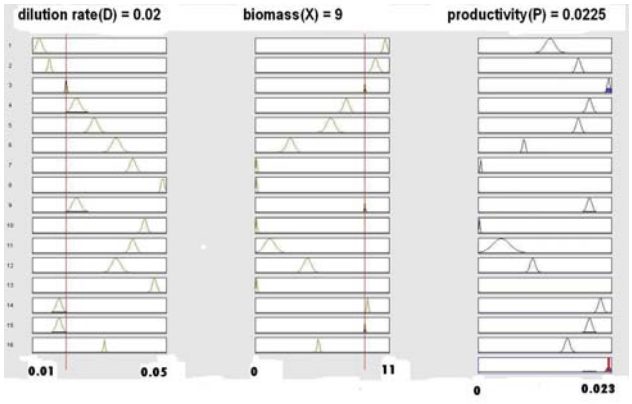

Figure 5: A fuzzy rule viewer for predicting lovastatin productivity

biomass concentration decreases from 11 gl^{-1} to 9 gl^{-1} and the dilution rate increased from 0.01 h^{-1} to 0.02 h^{-1}. The experimental findings show that the productivity increases by about 1.83 times. The surface plot shows that the productivity increases from 0.0124 $gl^{-1}h^{-1}$ to 0.0225 $gl^{-1}h^{-1}$ as the biomass concentration decreases from 11 gl^{-1} to 9 gl^{-1}, we can infer from this observation that lovastatin production is not growth-associated.

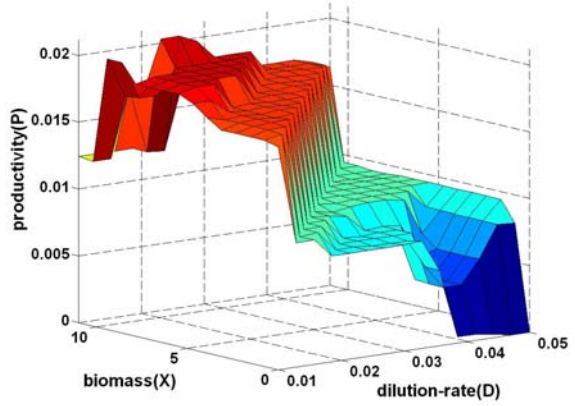

Figure 6: Surface plot demonstrating the effect of dilution rate and biomass concentration on lovastatin productivity

The fuzzy system predicts an maximum productivity value of 0.0225 $gl^{-1}h^{-1}$, at a dilution rate of 0.02 h^{-1} and biomass concentration of 9 gl^{-1}, which is in good accordance with the experimental maximum productivity value (0.022 $gl^{-1}h^{-1}$). After this, the productivity level is predicted to fall rapidly (from plot) when the dilution rate is increased from 0.02 h^{-1} to 0.035 h^{-1}. The fuzzy system predicts that the decrease in productivity is about 2.83 times, however, the experimental results show that the productivity decreases approximately by about 2.75 times. During washout phenomena (biomass concentration=0 gl^{-1}) at dilution rate 0.04 h^{-1}, the fuzzy system predicts a productivity of 0.00046 $gl^{-1}h^{-1}$, whereas the experimental findings show that the productivity is 0.0004 $gl^{-1}h^{-1}$. Upon further increase of dilution rate to 0.05 h^{-1}, the fuzzy predicted productivity is 0 $gl^{-1}h^{-1}$ which coincides with the experimental productivity.

In order to assess the predicting capabilities of the fuzzy system, the mean squared error (MSE) has been calculated and R-squared value obtained from MS-Excel plot (MS Excel, Microsoft Inc., USA) (figure 7) between fuzzy predicted productivity values versus experimental productivity values. The MSE value obtained is 8.86E-08. The R-squared value obtained from the plot is 0.9998.

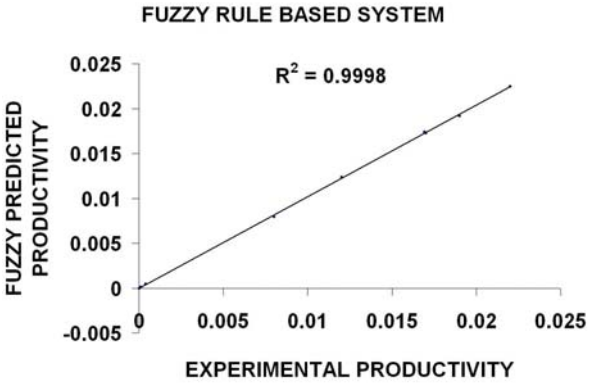

Figure 7: Plot between experimental productivity values and fuzzy predicted productivity values

The middle of maximum defuzzification method has been chosen because it gives the highest coefficient of determination value (R-squared value) and lowest mean squared error value when compared with other defuzzification methodologies (table 4).

The use of fuzzy system also has helped in increasing the lovastatin productivity by approximately 1.3 times. Before implementing the fuzzy system, the maximum productivity obtained from randomly empirical experiments was 0.017 $gl^{-1}h^{-1}$ at a dilution rate of 0.03 h^{-1} and biomass concentration of 6 gl^{-1}. The productivity increased by about 1.3 times to 0.022 $gl^{-1}h^{-1}$ at a dilution rate of 0.02 h^{-1} and biomass concentration of 9 gl^{-1} after the implementation of fuzzy system.

Table 4: Comparison of different defuzzification methodologies based on coefficient of determination value and mean squared error value

Defuzzification methodology	R-squared value	Mean squared error
Centroid	0.9828	1.52E-06
Bisector	0.9844	1.43E-06
Middle of maximum	0.9998	8.86E-08
Largest of maximum	0.9953	8.27E-07
Smallest of maximum	0.9964	2.73E-07

Conclusion

The developed fuzzy system has good predicting capabilities as it gives an R-squared value of 0.9998 and mean squared error of 8.86E-08 when compared with the experimental findings. It gives a maximum productivity of 0.0225 $gl^{-1}h^{-1}$, which is in good accordance with the experimental maximum productivity (0.022 $gl^{-1}h^{-1}$). The utilization of fuzzy logic aided in the increase of lovastatin productivity by about 1.3 times when compared to previous empirical experimental results.

Acknowledgement

The authors acknowledge University Grants Commission, India for funding support vide Project No P 01/464. We also are grateful to NRRL, USA and Lupin laboratories ltd., India for providing *Aspergillus sp.* strains and standard lovastatin.

References

Alberts AW, Chen J, Kuron G et al (1980) Mevinolin: A Highly Competitive Inhibitor of Hydroxymethylglutaryl-Coenzyme A Reductase and a Cholesterol Lowering Agent. Proc Natl Acad Sci USA 77 (7):3957-3961

Dubois M, Gilles KA, Hamilton JK et al (1956) Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem 28:350-356

Friedrich J, Zuzek M, Bencina M et al (1995) High Performance Liquid Chromatographic Analysis of Mevinolin as Mevinolinic Acid in Fermentation Broths. J Chromatogr A 704:363-367

Kysilka R, Kren V (1993) Determination of Lovastatin (Mevinolin) and Mevinolinic Acid in Fermentation Liquids. J Chromatogr A 630:415-417

Mahabir C, Hicks F, Fayek AR (2006) Neuro-Fuzzy River Ice Breakup Forecasting System. Cold Reg Sci Technol 46:100-112

Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 31:426-428

Samiee MS, Moazami N, Haghighi S et al (2003) Screening of Lovastatin Production by Filamentous Fungi. Iran Biomed J 7:29-33

Srivastava P, Gupta Kamakshi, Mishra PK (2009) Enhanced Continuous Production of Lovastatin Using Pellets and Siran Supported Growth of *Aspergillus Terreus* in an Airlift Reactor. Biotechnol Bioprocess Eng 14:207-212

Srivastava P, Kundu S (1998) A Comparative Evaluation of Cephalosporin C Production Using Immobilization Modes. J Gen Appl Microbiol 44:113-117

Vassileva Svetla, Tzvetkova Bonka (2003) Influence of the Dilution Rate on the Bioproductivity of Lactose-Utilizing Yeasts: Fuzzy Logic Modeling. Z Naturforsch C 58:381-385

Zadeh LA (1965) Fuzzy Sets. Inform Control 8**:**338-353