Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(12):83-91
(http://www.derpharmachemica.com/archive.html)

Optimization and Immobilization of Lipase from *Bacillus subterraneus* TNUS15: Comparison between Response Surface Methodology and Artificial Neural Network

Thyagarajan R*, Narendrakumar G, Ujjashini Bhuyan, Shruti Kumari, Ramesh Kumar V

Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama University, Chennai-600119, India

ABSTRACT

Lipase produced by different microorganisms, receives significant attention with the rapid development and application in various biotechnological industries. To optimize the maximum enzyme production by Bacillus subterraneus TNUS15 and to develop a comparative model using Response Surface Methodology (RSM) and Artificial Neural Network (ANN). Thirty-two strains of bacteria were isolated and screened for production of lipase using Rhodamine B agar medium. The maximum enzyme producing strain, Bacillus subterraneus TNUS15 was selected and an empirical model was developed for the enhanced yield of enzyme production by various combination of independent variables such as temperature, pH, inoculum size, substrate-olive oil and incubation time. A 2nd order polynomial model was established and improved lipase production (57.61 IU/g) was observed. The closeness of the predicted response (56.3 IU/g) with that of the experimental value showed that statistically optimized design can be used in order to improve the lipase production to meet the increasing demand. On the other hand, a multilayer ANN was structured, trained and stimulated by Levenberg-Marquardt algorithm (back propagation). The isolated enzyme was further characterized at different temperature and pH with respect to the incubation time. The culture supernatant containing crude enzyme was purified by various steps such as ammonium sulphate precipitation, dialysis and column chromatography resulting in partially purified enzyme. The crude, partially purified and standard enzyme were immobilized using sodium alginate and a comparative analysis of enzyme activity was assayed. Bacillus subterraneus TNUS15 was identified by 16S rRNA sequencing. The results of the ANN model indicate it is much more robust and accurate in estimating the values of response–enzyme production when compared with the response surface model. SDS PAGE was performed to determine the molecular weight (52 kDa) of the enzyme. The activity of the immobilized enzyme has been performed and its activity is compared with the standard enzyme.

Keywords: Lipase, Purification, RSM–ANN, Levenberg-Marquardt algorithm, Immobilization

INTRODUCTION

Lipases belong to the group of serine hydrolases (E.C.3.1.1.3) that catalyze the hydrolysis of triacylglycerols to liberate free fatty acid, diacylglycerols, monoacyl glycerols and glycerol [1,2]. Lipolytic enzymes are widely distributed in nature. Lipases are sourced from various animals, plants and microorganism. Microbial enzymes are often stable and their production is more convenient and safer [3]. Currently bacterial lipases are of great demand because of their potential industrial applications [4]. *Bacillus subterraneus* is a gram positive bacillus that has the capability to grow and produce extracellular lipase during the culture period [5].

The enzyme production from the microorganism was strongly influenced by the media components and the culture characteristics. In classical optimization method single factor variation is carried out while keeping the other factor constant. This method is not suitable for the multifactor constant. The statistical optimization abate time allows quick screening of large experimental domain and also reflect on the individual components for an exemplary production of products [6]. The Response surface methodology is widely used for the optimization of physical and culture growth parameters for the production of enzymes and metabolites [7,8]. Artificial neural networking is a tool used for predicting the quality of enzyme production from any variable for which the system was trained and stimulated.

Microbial lipases are mostly produced by submerged culture, but solid-state fermentation methods [9,10] can be also used. Immobilized cell culture has been used in only few cases [11]. Many studies were done to define the optimal culture and nutritional requirements for the lipase production by submerged culture. Also the Lipase production is influenced by the type and concentration of carbon [12] and nitrogen sources, the culture pH, growth temperature, and the total dissolved oxygen concentration.

Lipidic carbon sources seem to be essential for obtaining a high yield of lipase; however, some strains of bacteria [13] have produced good yields in the absence of fats and oils.

The present paper focuses on screening for a potential lipase producing strain of *Bacillus* sp. Optimization of nutrient requirement and cultural condition by statistical methods like RSM–CCD and comparison with ANN for the optimal production of enzyme activity and the enzyme stability of different pH and temperature were studied. The extracted enzyme was subjected for immobilization to study it activity and life time.

MATERIALS AND METHODS

Chemicals

Chemicals used for the experiments are peptone, beef extract, tributyrin, agar-agar, nutrient agar, olive oil, ethanol, thymolphthalein, crystal violet, ammonium sulphate, sodium hydroxide, bovine serum albumin, sodium carbonate, copper sulphate, sodium potassium tartarate, folin's phenol reagent, tween-20/80, sodium alginate, calcium chloride, tris-HCl, silica gel, and glass wool.

Maintenance of Aseptic Environment

Glassware such as pipettes, conical flask, Petri plates, test tubes and beakers were washed with detergents and rinsed under running tap water several times to wash away the detergents properly. The culture vessels were kept in the hot air oven at 160-180°C for 2-4 h. The forceps, blades and spatula were sterilized by autoclaving at 121°C at 15 lbs pressure for 15-20 m.

Collection and isolation of microorganism

Soil sample was collected from Sathyabama University (12.8729°N, 80.2219°E), serially diluted and pour plate technique was carried out to isolate the microorganism from the soil. The organism has been cultured in the nutrient agar plates. The organism was sub-cultured and growth preserved in the refrigerator at 4°C for further use.

Screening for lipase production

The identified organisms were tested for lipase production on Rhodamine B Agar. After 3 days of incubation at 30^{o}C the lipolytic activity was confirmed by the halos around the colony. An orange fluorescent halo was observed upon the irradiating plates with UV at 350 nm [13,14].

Identification of the isolated microorganism

The lipase producing bacteria strain was identified using Gram staining and biochemical test was performed. For further confirmation the sample was submitted for 16S rRNA sequencing, which shows 99% similarity with *Bacillus subterraneus* in BLAST program and the sequence subsequently was submitted in Genbank.

Production of the enzyme by submerged fermentation

The effect of media component and physical parameter such as pH, temperature, agitation, inoculum size and incubation time on the production of lipase by the isolated *Bacillus subterraneus* TNUS15 was studied in submerged fermentation. The preparation media composed of peptone 1.25 g, beef extract 0.75 g, Olive oil 0.01 ml and glucose 0.75 g was sterilized using an autoclave.

Extraction of the enzyme

The enzyme has been extracted from production media by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant containing the enzyme is collected. The enzyme activity has been assayed for 3 days and also the protein has been estimated respectively.

Assay for lipase activity

Fadıloglu and Soylemez [15] determined lipase activity using olive oil as substrate. Reactions were carried out in 100 ml conical flasks at 40°C by immersion in a water bath. The reaction mixture, consisting of 2 ml of 0.1 M potassium phosphate buffer, pH 7.0, 1 ml of olive oil and 1 ml of culture supernatant was incubated at 40°C for 30 min. The enzyme reaction was terminated by the addition of 5 ml of 96% ethanol and followed by titration with 0.01 N NaOH solution using phenolphthalein as the indicator [16]. One unit of lipase activity was defined as the amount, which liberated 1 µmol of fatty acid per min at 40ºC.

Optimization of production media and various physio-chemical parameters by using RSM

The convolution in optimization of culture conditions is the presence of interactive effects of medium compositions and culture condition factors. Statistical experimental design, which is a collection of statistical techniques applicable to experimental design, model building, evaluating the effect of factors, and screening optimum conditions of factors for desirable responses, can overcome these problems. Statistical experimental design methods such as Response Surface Methodology (RSM) can provide a methodological and resourceful plan for experimentation to achieve certain target, so that several control factors are simultaneously studied. The statistical software package Design Expert-Version 7.0 was used for analyzing the experimental data [17].

Artificial Neural network modeling

The multi-layered perceptron (MLP) architecture of ANN approximates non-linear relationships existing between multiple causal (input) process variables and the corresponding dependent (output) variables. In the MLP architecture, data flow from input layer to the output layer, through the hidden layer. The input layer introduces scaled input data to the hidden layers via the weights and bias which are the network numerical parameters. The neurons in the output layer produce an output based on a similar procedure as the hidden layer. In the training phase, an error value is produced based on the difference between the predicted network output and the experimental value. ANN modelling was done by MATLAB R2011b. This topology of neural network was used in this study for the prediction of the lipase activity under various cultural conditions [18]. The Levenberg-Marquardt (LM) algorithm [19] is the most widely used optimization algorithm.

It outperforms simple gradient descent and other conjugate gradient methods in a wide variety of problems. The problem for which the LM algorithm provides a solution is called *Nonlinear Least Squares Minimization*. This implies that the function to be minimized is of the following special form

$$f(x) = \frac{1}{2}\sum_{j=1}^{m} r^2{}_j(x)$$

Where $x=(x1; x2; : : : ; xn)$ is a vector, and each $r\,j$ is a function from $\Re^n$ to $\Re$. The $r\,j$ are referred to as a *residuals* and it is assumed that $m \geq n$ (Figure 1).

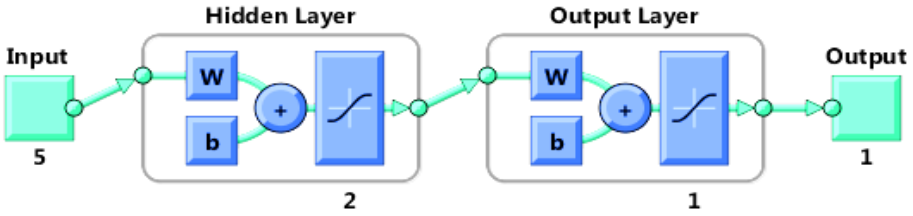

Figure 1: ANN modelling showing the 5 independent variables, hidden layer, output layer and the final output– response.

Purification

Ammonium sulphate precipitation

Solid ammonium sulphate was added to 20 ml of the enzyme at 10-100% saturation range and allowed to stand for 4 h. Precipitate was obtained at 70%, 80% and 90%. The precipitates were suspended in 2 ml of 50 mM of Tris-buffer [20].

Dialysis

The diluted enzyme was dialysed in a dialysis membrane. In 500 ml of 25 mM Tris buffer (pH 8) the dialysis bag was submerged overnight. The dialysis bag was removed and the enzyme collected. The protein content of the dialysate was estimated by Lowry's method and also assayed for enzyme activity.

Column chromatography

The column was prepared using glass wool of 1 cm and silica gel of 20 cm and kept for two days and allowed to pack properly. After washing the column with 25 mM of Tris HCl buffer 2 ml of enzyme solution was poured and 27 ml of Tris HCl buffer was added from the top. Aliquots of 2 ml were collected in 20 different fractions. Then, the optical density of the aliquots at 280 nm was taken. The enzyme assay and protein estimation was performed on the aliquots.

Determination of molecular weight by SDS-PAGE

The molecular weight of the lipase purified by Column chromatography were analysed using Sodium Dodecyl Sulphate –Poly Acrylamide Gel Electrophoresis.

Immobilization using sodium alginate

Immobilization was performed for crude, partially purified and standard lipase. An equal volume of enzyme solution and sodium alginate solution was mixed to give a 4% (w/v) final concentration of sodium alginate solution in the mixture. The mixture obtained was extruded drop wise through a Pasteur pipette (1 mm diameter) into four different beakers (0.5-2 ml of equal amount of enzyme and sodium alginate solution) containing 2% of calcium chloride solution for two h to give accurate beads size. Then the lipase assay was performed with the crude, partially purified and the standard enzyme separately with the obtained beads. The activity of the crude enzyme, partially purified enzyme and the standard enzyme, after and before immobilization has been compared and also the protein has been estimated by Lowry's method [21].

Characterization of lipase

Effect of pH on the activity and stability of lipase

The activity of lipase was examined in the pH range of 3.5-9.0 [22]. The effect of pH on lipase stability was determined by incubating the lipase in desired buffer at different time intervals. After incubation 100 µl aliquots of the buffered enzyme solution was taken and the lipase activity was assayed by Fadiloglu and Soylemez method [15].

Effect of temperature on the activity and stability of lipase

The activity of lipase was examined within the temperature range of 20-80°C. The effect of temperature on lipase stability was determined by incubating the lipase in desired temperature at different time intervals. After incubation 100 µl aliquots of the buffered enzyme solution was taken and the lipase activity was assayed by Fadiloglu and Soylemez method [15].

RESULTS AND DISCUSSION

Culturing of the isolated organism

The organism that has been isolated from the soil has been cultured in the nutrient agar medium without any contamination.

Screening for Microorganism

Thirty-two strains of bacteria were screened for the production of lipase from which maximum enzyme producer was identified as *Bacillus subterraneus* TNUS15. The identification was performed using Rhodamine B agar medium. The area of discoloration appeared after 24 h incubation and depending upon the area of the discoloration *Bacillus* sp. that showed maximum activity was selected for further analysis [23]. The confirmed stains were stored in nutrient agar slants [24].

Production of the enzyme by submerged fermentation

The lipase activity was observed as shown in Table 1. Table 1 the enzyme has been extracted and its lipase activity has been noted for 3 days:

Table 1: Lipase Activity

Hours	Lipase activity (units/ml)
24	19.8
48	31.9
72	46.1

16S rRNA Sequencing

The following partial sequence data (~ 500 bp) has been obtained
>Bacillus_395
TTAGCTTGCTTTTGAGATCAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGG
GAAACCGGAGCTAATACCGGATAATCCTTTCCCTCACATGAGGGAAAGCTGAAAGACGGTTTCGGCTGTCACTTACAG
ATGGGCCCGCGGCGCATTAGTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAA
AGTCTGACGGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTCAGGGAAGAACAAGTAC
CGGAGTAACTGCCGGTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCC
CGGCTCAACCGGGGAGG

Identification of the Bacillus sequence

The sequence obtained from the 16S rRNA has been given for BLAST and following phylogram has been obtained (Figure 2).

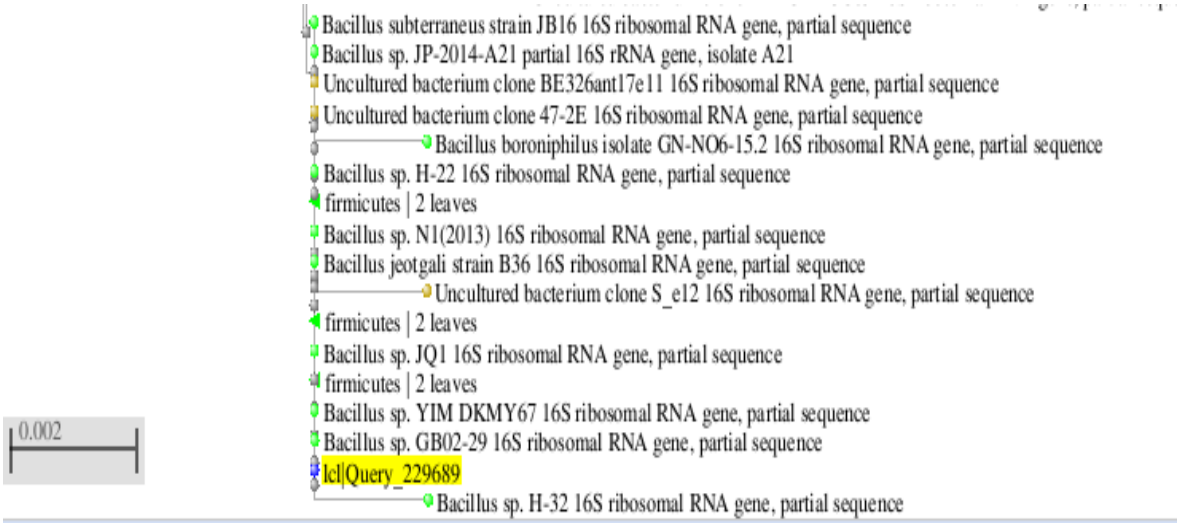

Figure 2: Phylogenetic tree

Clans et al. [25] identified the strain as *Bacillus* sp. based on characteristics such as aerobic growth, gram positive, bacilli shaped, motile, spore forming and catalase positive. It did not require sodium chloride and potassium chloride for growth. It was oxidase negative in nature and it did not produce any gas with glucose. *Bacillus subterraneus* TNUS15, was straight bacilli, occurred singly and rarely in pairs or in chains [26].

Phylogram

The lipase producing bacterial strain was submitted for 16S rRNA sequencing, which shows 99% similarity with *Bacillus subterraneus* and therefore would be labelled as *Bacillus subterraneus* TNUS15. The sequence was submitted to Genbank with the accession number KP 835181.

Optimization by Central Composite Design (CCD) and Statistical Analysis

Central Composite Design was used to determine the optimal levels of the five significant factors (pH, temperature, Time of incubation, Inoculum size, Substrate concentration) that affect the lipase production. The respective low and high level of the factors and each variable with the coded levels of the factors and the activity of lipase in terms of predicated and experiment values are given in Table 2. According to ANOVA of RSM, Table 2 the model terms A, B, C, D, AB, AC, AD, CD, A^2, B^2, C^2 D^2 were significant ($p<0.05$) the regression equation co-efficiencies were studied by substituting the data in the 2^{nd} order polynomial equation (Figure 3). Essamri et al. [27] estimated the effect of solvent along with varied temperature and pH.

Equation

$$Y=a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_5X_5 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{55}X_5^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{15}X_1X_5 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{24}X_2X_4 + a_{34}X_3X_4 + a_{35}X_3X_5 \quad (1)$$

where Y is the predicted response; X_1, X_2, X_3, X_4, X_5, the independent variables, a0 the offset term, a_1, a_2, a_3, a_4, a_5 the coefficients of linear effects; a_{11}, a_{22}, a_{33}, a_{44}, a_{55} coefficients of squared effect, a_{12}, a_{13}, a_{14}, a_{23}, a_{24}, $a_{25,}$ $a_{34,}$ $_{35}$ coefficients of interaction terms.

Response

$$R=52.280-1.223A-8.660B-1.383C+1.271D+1.636E-1.881AB+2.779AC+1.053\ AD+127AE-1.892BC+1.107BD-0.476\ BE-2.549\ CD+0.324\ CE+0.439\ DE-2.004A^2-3.606B^2-1.815C^2-3.154D^2-0.472E^2 \quad (2)$$

Table 2: Experimental design for five independent variables for the production of lipase from *Bacillus subterraneus* TNUS15

Std	Coded Value					Actual Value					Response	
	A	B	C	D	E	pH	Substrate	Temp	Incu. time	Ino. size	Actual	Predicted
23	-1	1	1	-1	1	3	15	40	24	4	31.51	28.48
18	1	-1	-1	-1	1	7	8	20	24	4	40.25	41.69
8	1	1	1	-1	-1	7	15	40	24	2	30.58	30.18
15	-1	1	1	1	-1	3	15	40	72	2	30.46	29.87
7	-1	1	1	-1	-1	3	15	40	24	2	28.65	33.19
29	-1	-1	1	1	1	3	8	40	72	4	40.52	43.00
46	0	0	0	0	0	5	11.5	30	48	3	51.26	52.28
16	1	1	1	1	-1	7	15	40	72	2	32.25	31.08
26	1	-1	-1	1	1	7	8	20	72	4	52.58	50.10
44	0	0	0	0	0	5	11.5	30	48	3	55.58	52.28
19	-1	1	-1	-1	1	3	15	20	24	4	35.04	34.84
10	1	-1	-1	1	-1	7	8	20	72	2	49.25	51.94
3	-1	1	-1	-1	-1	3	15	20	24	2	39.58	40.85
31	-1	1	1	1	1	3	15	40	72	4	24.25	26.92
5	-1	-1	1	-1	-1	3	8	40	24	2	52.46	51.80
20	1	1	-1	-1	1	7	15	20	24	4	18.65	21.23
38	0	0	1	0	0	5	11.5	53.7	48	3	38.56	38.72
1	-1	-1	-1	-1	-1	3	8	20	24	2	51.25	51.89
40	0	0	0	1	0	5	11.5	30	105.5	3	39.24	37.46
45	0	0	0	0	0	5	11.5	30	48	3	50.25	52.28
28	1	1	-1	1	1	7	15	20	72	4	32.45	34.07
42	0	0	0	0	1	5	11.5	30	48	5.3	47.95	45.71
22	1	-1	1	-1	1	7	8	40	24	4	51.37	54.01
11	-1	1	-1	1	-1	3	15	20	72	2	48.56	47.72
39	0	0	0	-1	0	5	11.5	30	-9	3	32.24	31.41
21	-1	-1	1	-1	1	3	8	40	24	4	49.59	48.99
33	-1	0	0	0	0	3	11.5	30	48	3	45.24	43.85
25	-1	-1	-1	1	1	3	8	20	72	4	50.89	51.99
6	1	-1	1	-1	-1	7	8	40	24	2	57.61	56.31
14	1	-1	1	1	-1	7	8	40	72	2	49.58	52.77
36	0	1	0	0	0	5	11.5	30	48	3	13.68	11.28
32	1	1	1	1	1	7	15	40	72	4	27.56	28.62
27	-1	1	-1	1	1	3	15	20	72	4	42.25	43.47
41	0	0	0	0	-1	5	11.5	30	48	0.62	53.87	53.50
47	0	0	0	0	0	5	11.5	30	48	3	52.69	52.28
50	0	0	0	0	0	5	11.5	30	48	3	52.49	52.28
34	1	0	0	0	0	7	11.5	30	48	3	39.25	38.03
43	0	0	0	0	0	5	11.5	30	48	3	52.15	52.28
49	0	0	0	0	0	5	11.5	30	48	3	50.97	52.28
4	1	1	-1	-1	-1	7	15	20	24	2	27.25	26.73
2	1	-1	-1	-1	-1	7	8	20	24	2	46.89	45.29
9	-1	-1	-1	1	-1	3	8	20	72	2	53.25	54.33
24	1	1	1	-1	1	7	15	40	24	4	25.85	25.98
17	-1	-1	-1	-1	1	3	8	20	24	4	46.58	47.79
48	0	0	0	0	0	5	11.5	30	48	3	54.56	52.28
35	0	-1	0	0	0	5	8	30	48	3	52.68	52.47
30	1	-1	1	1	1	7	8	40	72	4	54.58	52.23
12	1	1	-1	1	-1	7	15	20	72	2	36.25	37.81
13	-1	-1	1	1	-1	3	8	40	72	2	46.85	44.04
37	0	0	-1	0	0	5	11.5	6.2	48	3	48.07	45.30

Table 3: Analysis of Variance (ANOVA) of the central composite design

Source	Sum of Squares	Df	Mean Square	F-Value	p-value Prob>F	
Model	5633.70	20.00	281.68	49.11	<0.0001	Significant
Pure Error	22.83	7.00	3.26	-	-	-
Total	5800.03	49.00	-	-	-	-
R^2=0.971, Adj R^2=0.952, Pred R^2=0.905, PRESS=552.070						

The regression analysis as given in Table 3 showed that R^2 was 0.971 which means the model could explain 97.10% of the variation in response which indicated the fitness of the model. The predicted R^2 of 0.952 was in reasonable agreement with Adjusted R^2 of 0.9594 indicating that the regression model could be used to analyze trends of responses. The high model F-value is 0.1756. The lowest value of co-efficient CV=5.608 which was similar to the response obtained by Shabbiri and Adnan [28].

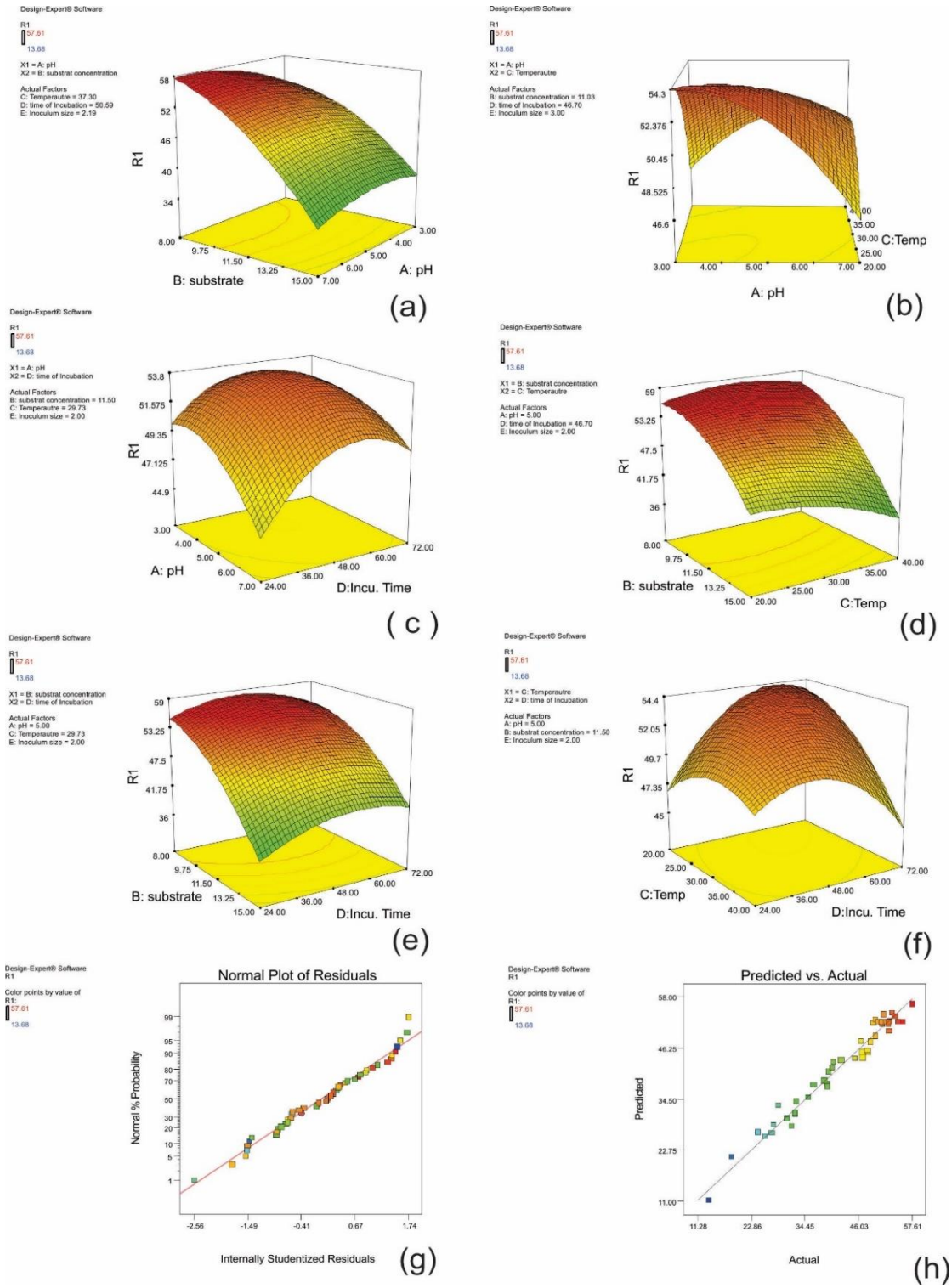

Figure 3: Statistical optimization of Lipase production using RSM: A: pH, B: Substrate concentration, C: Temperature, D: Incubation time, E: Inoculum size (a-f) Contour plot for two different independent variables with respect to the response. (g) Normal plot of residualsand, (h)–Predicted vs. Actual.

Artificial Neural Network model

The Network was trained using the implemented Levenberg-Marquardt algorithm or back-propagation method (Figure 1). The training was carried out by adjusting the weight connections between neurons with the aim of reducing the Mean Square Error (MSE) between the predicted and the experimental outputs below an acceptable threshold, thus minimizing the performance of MSE function [29]. The input sensitivity shows how much lipase production changes when the inputs are varied within the experimental range. The inputs were all set to the median values and then each in turn was varied from the lowest to the highest value.

In decreasing order of sensitivity was temperature, pH, substrate concentration, inoculum size and incubation time. The accuracy of the neural network prediction is dependent on the training patterns as well as the structure of the neural network (Figures 4 and 5) [30]. These observations imply that lipase production by *Bacillus subterraneus* TNUS15 will be greatly influenced by substrate, inoculum size and process time.

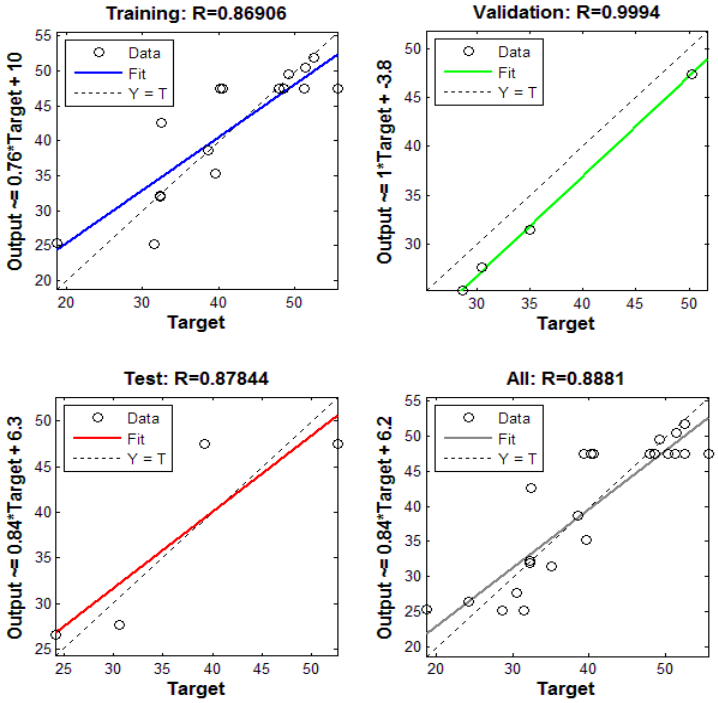

Figure 4: Training and Validation for ANN

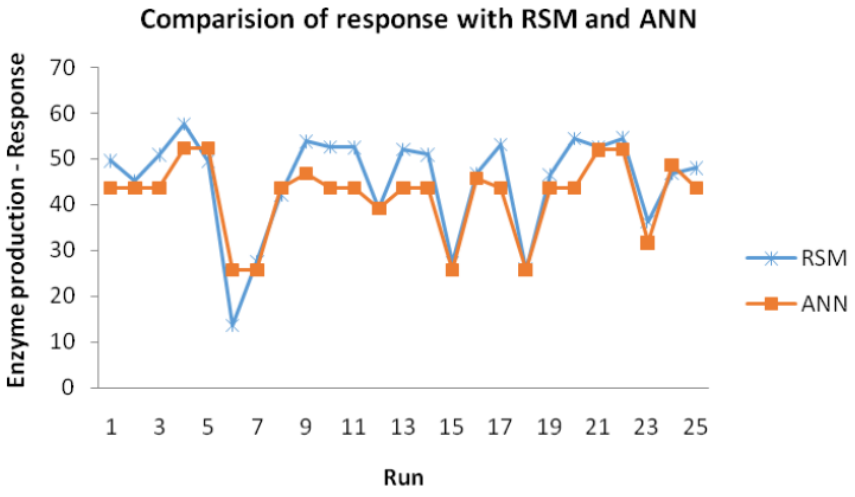

Figure 5: Comparative validation of optimum process conditions as determined by ANN and RSM

Purification of lipase enzyme

The purification of lipase was done by column chromatography, the fraction of 19-33 showed maximum activity which was about 17.76 times higher [31].

Molecular weight determination

The molecular weight was determined using SDS-PAGE and the purified protein was about 52 kDa.

Characterization of lipase enzyme

The purified lipase hydrolyzed olive oil as substrates appreciably over a relatively broad pH range from 3.5 to 9.0 (Figure 6). The enzyme had more activity on pH 7.0. At acid and alkaline pH the activity of the enzyme decreased gradually [32]. Izumi et al. [33] and Xia and Lee [34] reported that the stability of the crude enzyme in the culture medium decreased with either increasing or decreasing pH value. The enzyme was relatively stable at the pH 6.5-7.0 with initial activity remaining after 4 h at 30^{o}C. The enzyme activity decreased more sharply at high pH. The reason may be due to the degradation of lipase protein by proteases. Similar results have been observed by many workers in lipase optimizations experiments.

Effect of temperature on the activity and stability of lipase

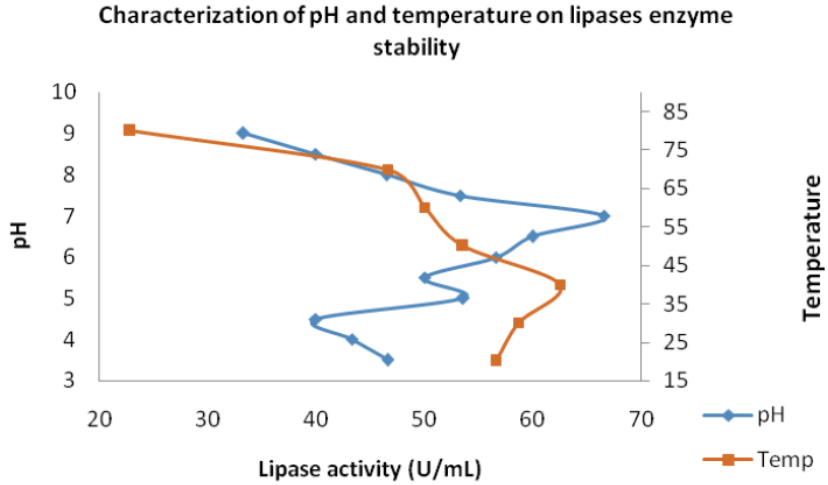

Figure 6: Characterization of pH and temperature on lipases enzyme stability

Table 4: Characterization of stability of lipases enzyme at 40ºC with respect to different incubation time

S. No.	Incubation time	Lipase activity (U/ml)
1	30	46.3
2	40	48.7
3	50	62.5
4	60	59.3
5	70	54.2
6	80	48.4
7	90	39.9

The effect of temperature on the activities of lipase enzyme was examined in the range of 20-80ºC (Table 4). Lipolytic activity of these enzymes was found to be profoundly affected by temperature. The activity of lipase gradually increased with rise in temperature up to 40ºC and the maximum activity was observed at 40ºC. However the increase of temperature from 50-60ºC resulted in nearly 80% of activity. Thermal stability was also studied at 40ºC with respect to the incubation time. Tobis and Tiller [35] reported that the effect of temperature on the lipase activity showed that the optimum temperature for the enzyme was 37ºC and about 90% of its maximum activity occurred at 50ºC.

Immobilization

The crude, partially purified and standard enzyme has been immobilized and their enzyme activity has been observed [35,36].

Comparing the enzyme activity

The crude enzyme, partially purified enzyme and the standard enzyme has been compared with each other according to its enzyme activity (Table 5) [37,38].

Table 5: Characterization of lipases activity after immobilization in standard, crude and partially purified enzyme

Concentration (µg)	Enzyme activity(units/ml)		
	Standard	Crude	Partially purified
0.5	14.92	7.46	11.19
1.0	33.58	11.19	29.85
1.5	48.5	18.65	44.77
2.0	65.97	26.11	58.5

Abd-Elhakeem and Elsayed [39] used Tris buffer which showed highest lipolytic activity and obtained the value as 61.5 U/ml that was similar to the present study. Stuer et al. [40] has found that the partial purification using ammonium sulphate precipitation recovered at 30-70 saturation and precipitation was obtained in the concentration between 70-90% was obtained.

According to Dong et al. [41] an extracellular lipase from *Pseudomonas* sp. was purified by extraction, Bio-gel P-10 chromatography and Superose 12B chromatography, and a 37-fold purification was accomplished. The purified enzyme showed a single band when it was imperilled to SDS/PAGE and isoelectric focusing. The SDS/PAGE electrophoresis indicated a molecular mass of 52 kDa for this lipase. Its isoelectric point was 4.5. The optimum pH and temperature for hydrolysis were 7.0-9.0 and 45-60ºC, respectively.

CONCLUSION

The main objective of the study is to isolate the microorganism from soil that can produce lipase enzyme. *Bacillus subterraneus* TNUS15 was identified by 16S rRNA sequencing. The organism was grown in submerged fermentation followed by enzyme extraction. The crude enzyme was evaluated for enzyme's activity the protein estimation. This paper also described the use of design of experiments (DOE) for conducting experiments. A model was developed for predicting lipase enzyme production using Response Surface Methodology (RSM) and Artificial Neural Network (ANN). The predictive ANN model is found to be capable of better predictions of response within the range that they had been trained. The results of the ANN model indicate it is much more robust and accurate in estimating the values of response–enzyme production when compared with the response surface model. In conjunction with this *Bacillus subterraneus* TNUS15 was screened for maximum enzyme production against three different strains and optimized for the production of utmost production of by adjusting the growth and culture parameters. The enzyme was further purified and stability analysis was also done. Further it was purified by ammonium sulfate precipitation, dialysis and column chromatography using silica gel. The enzyme activity and the protein estimation have been done for all the three purification process respectively. SDS PAGE was performed to determine the molecular weight (52 kDa) of the enzyme. After purification the partially purified enzyme has been obtained. The process of immobilization was carried out for the crude, partially purified and standard enzyme and it has been done using sodium alginate. The activity of the immobilized enzyme has been performed and its activity is compared with the standard enzyme.

REFERENCE

[1] R. Gupta, N. Gupta, P. Rathi, *Appli. Microbiol. Biotechnol*., **2004**, 64, 763-781.
[2] Kishore Patil, Manojkumar Chopda, Raghunath Mahajan, *Indian J. Sci. Technol.,* **2011**, 4, 971-982.
[3] D. Kumar, R. Parshad, V.K. Gupta, *Int. J. Biol. Macromol.,* **2014**, 66, 97-107.
[4] R. Sharma, Y. Chisti, U. Chand Banerjee, *Biotechnol. Adv.,* **2001**, 19, 627-662.
[5] F. Valero, F. Ayats, J. Lopez-Santin, M. Poch, *Biotechnol. Lett*., **1988**, 10, 741-744.
[6] G. Narendrakumar, S.K.R. Namasivayam, R.A.L. Singh, *Res. J. Pharm. Technol.*, **2015**, 8, 280.
[7] K. Shabbiri, A. Adnan, B. Noor, S. Jamil, *Ann. Microbiol.,* **2012,** 62, 523-532.
[8] L. Gao, J.H. Xu, X.J. Li, Z.Z. Liu, *J. Ind. Microbiol. Biotechnol.,* **2004**, 31, 525-530.
[9] Y. Chisti, M.C. Flickinger, S.W. Drew, NY, USA, Wiley, **2002**, 2446-2462.
[10] M. Chaturvedi, M.R. Singh, C. Man, S. Pandey, *Res. J. Microbiol.,* **2010**, 5, 725-730.
[11] C. Hemachander, N. Bose, R. Puvanakrishnan, *Proc. Biochem.,* **2001**, 36, 629-633.
[12] S.E. Petrovic, M. Skrinjar, I.F. Becarevic, L. Banka, *Biotechnol. Lett.,* **1990**, 12, 299-304.
[13] P. Anbu, G. Annadurai, J.F. Lee, B.K. Hur, *J. Chem. Technol. Biotechnol.,* **2011**, 84, 54-62.
[14] G. Kouker, K.E. Jaeger, *Appl. Environ. Microbiol.,* **1987**, 53, 211-213.
[15] S. Fadıloglu, Z. Soylemez, *Food Res. Intern.,* 1997, 30, 171-175.
[16] F. Beisson, A. Tiss, C. Riviera, R. Verger, *Eur. J. Lipid Sci. Technol.,* **2000**, 102, 133-115.
[17] S. Bae, M. Shoda, *Biotechnol. Bioeng*., **2005**, 90, 20-28.
[18] R. Thyagarajan, G. Narendrakumar, V.R. Kumar, S.K.R. Namasivayam, *Res. J. Pharm. Technol.,* **2016**, 9, 430.
[19] K. Levenberg, *Quart. Appl. Math.,* **1944**, 2, 164-168.
[20] E.J. Gilbert, A. Cornish, C.W. Jones, *J. Gen. Microbiol.,* **1991**, 137, 2223-2229.
[21] O. Lowry, N. Rosebrough, A.L. Farr, R. Randall, *J. Biol. Chem.,* **1951**, 193, 265-275.
[22] N. Nawani, R. Singh, J. Kaur, *Electronic J. Biotechnol.,* **2006**, 9, 559-565.
[23] N. Bourguet, J.P. Torreton, O. Galy, V. Arondel, M. Goutx, *Appl. Environ. Microbiol.,* **2003**, 69, 7395-7400.
[24] Y. Kojima, M. Yokoe, T. Mase, *Biosci. Biotechnol. Biochem.,* **1994**, 58, 1564-1568.
[25] D. Clans, W. Berkley, N.S. Mair, H.E. Sharpe, Williams and Wilkins, Baltimore, **1986**, 1105-1139.
[26] S. Kanso, A. Greene, B. Pate, *Int. J. Syst. Evol. Microbiol.,* **2002**, 52, 869-874.
[27] M. Essamri, D. Valerie, C. Louis, *J. Biotechnol.,* **1998**, 60, 97-103.
[29] K. Shabbiri, A. Adnan, *World Appl. Sci. J.*, **2011**, 13, 1059-1066.
[30] A. Khoramnia, O. Ming Lai, A. Ebrahimpour, C. Josue Tanduba, T. Siow Voon, S. Mukhlis, *Elect. J. Biotechnol.,* **2010**, 13, 5-10.
[31] A. Rajendran, V. Thangavelu, *J. Bioproc. Biotech.,* **2012**, 2, 1-9.
[32] E. Lesuisse, K. Schanck, C. Colson, *Eur. J. Biochem.,* **1993**, 216, 155-160.
[33] T. Izumi, K. Nakamura, T. Fukase, *Agr. Boil. Chem.,* **1990**, 54, 1253-1258.
[34] W.S. Xia, D.X. Lee, *Carbohyd. Polym.,* **2008**, 74, 544-551.
[35] J. Tobis, J.C. Tiller, *Biotechnol. Lett*., **2014**, 36, 1661-1667.
[36] M.A. Longo, I.S. Novella, L.A. Garcia, M. Diaz, *Enzyme Microb. Technol.,* **1992**, 14, 586-590.
[37] T.S. Brush, R. Chapman, R. Kurzman, D.P. Williams, *Bioorg. Med. Chem.,* **1999**, 7, 2131-2138.
[38] Y. Chen, J. Liu, C. Xia, C. Zhao, Z. Ren, W. Zhang, *Biotechnol. Appl. Biochem.,* **2014**, 62, 101-106.
[39] M. Abd-Elhakeem, A. Elsayed, T. Alkhulaqi, *Am. J. Analyt. Chem.,* **2013**, 4, 442-444.
[40] W. Stuer, K.E. Jaeger, U.K. Winkler, *J. Bacteriol*., **1986**, 168, 1070-1074.
[41] H. Dong, S. Gao, S.P. Han, S.G. Cao, *Biotechnol. Appl. Biochem*., **1999**, 30, 251-256.