3.1. Overview

Chox is a bacterial flavoenzyme that catalyzes the oxidation of cholesterol to 4-cholesten-3-one with the simultaneous reduction of molecular oxygen to hydrogen peroxide (MacLachlan, 2000). Chox is a valuable biotechnological tool, due to its enormous applications in the field of clinical diagnostics, food and pharmaceutical industry. Chox is a widely used enzyme for the estimation of total cholesterol in human serum (Allain, 1974), for the estimation of cholesterol concentrations in food samples for the quality control assay, and the commercial production of precursors of the steroidal hormones from cholesterol and its derivatives (Mahato and Garai, 1997). An increasing need for specific estimation of cholesterol in clinical samples has enhanced the importance and demand of Chox in the pharmaceutical industry.

Chox is exclusively a microbial enzyme produced by a wide array of microorganisms including bacteria, actinomycetes, and fungi. Due to its growing applications in diverse fields, Chox has been produced and purified by various microorganisms such as *Nocardia rhodochrous* (Buchland, 1975), *Arthrobacter simplex* (Liu et al., 1988), *Pseudomonas sp.* (Noriyaku and Rikizo, 1998), *Actinomyces lavendulae* (Petrova et al., 1981; Shirshova et al., 1992), *Streptomyces fradiae* (Tabatabei et al., 2001), etc. Most microorganisms produce cell-bound Chox which are often difficult for isolation while some others are pathogenic. Apart from these, the microbial flora belonging to the group of *Actinomycetes* has been reported to produce high levels of extracellular Chox (Doukyu and Aono, 1998; Fukuda et al., 1973; Inoye et al., 1982; Lartillot and Kedziora, 1990; Varma and Nene, 2003) easier for both isolation and purification than the intracellular and cell-bound enzymes. *Streptomyces* is the largest genus of the phylum *Actinobacteria*; characterized by complex secondary metabolism, melanoid pigment (light

brown, brown-black or distinct brown) formation in the medium. They are gram-positive and spore-forming microorganisms (Sharma, 2014). *Streptomyces* are potent producers of valuable antibiotics and are also non-pathogenic. Chox from *Streptomyces* species for serum cholesterol assay has been reported to be superior to those from other microorganisms, due to lower cost of production and longer shelf life (Lolekha and Jantaveesirirat, 1992). At present time, the necessity of non-pathogenic and high-yielding strain which could meet the demand of relatively high production of Chox has led us to screen for Chox producer among various *Streptomyces species*.

Several assay methods have been reported for the estimation of Chox activity including colorometric enzymatic method (Allain, 1974), UV method using 4-cholestenone as a standard (Varma and Nene, 2003), and fluorometric enzymatic method (Huang, 1975; Amundson and Zhou, 1999). The UV method measures the concentration of 4-cholestenone in the reaction mixture (absorption maxima at 240nm). This method shows interference with the color imparted by the medium constituents (viz. yeast extract, peptone, etc.) in the crude enzyme extract. The fluorometric method makes use of expensive fluorescence dyes. Among the methods described above, the enzymatic assay method involving Chox coupled with H_2O_2 is extremely simple, specific, and highly sensitive. It indicates the relative concentration of cholesterol indirectly by the measurement of H_2O_2. Coupling of H_2O_2 with chromogen like 4-aminoantipyrine and o-dianisidine in the presence of peroxidase yields an adduct that exhibits highly absorbing chromophore Quiononeimine allowing more sensitive measurement of cholesterol than any other method. This approach makes it an effective assay method.

A general understanding of the particular features of enzymes produced by any new microbial source is required. Enzyme activity depends decisively on defined conditions

with respect to pH, temperature, ionic strength of the buffer, substrate concentration, enzyme concentration, reaction time, etc. Standardization of these parameters is desirable to attain optimum activity of the enzyme (Bisswanger, 2014). Enzymes display their highest activity at their respective optimum conditions, and deviations from the optimum cause a reduction in the activity, depending on the degree of deviation. Optimization of reaction conditions for an enzyme provides direct information about the optimal values of the parameter under study and combined effects of various enzyme assay parameters for the enhancement of activity (Singh and Banik, 2014; Liang, 2010, Burkert, 2006). Singh and Banik optimized the reaction conditions for L-Glutaminase using RSM and ANN models (Singh and Banik, 2014). Parameters optimization for an enzyme assay by conventional method (one variable at a time i.e., OVAT) is a complex process and lacks interaction of the factors on which they depend. This complexity can be minimized up to a certain extent with the advent of new tools and methodologies in mathematics and statistics. Therefore, a combination of mathematical and computational methods (RSM and ANN) capable of predicting enzyme activity would be helpful to enhance the Chox activity, thus optimizing the assay parameters for Chox activity. ANN mimics the neural functioning of the human brain and is a data-driven approach primarily based on input-output data (Shera et al., 2018). ANN is a model-independent technique capable of predicting highly variable and non-linear bio-catalytic reactions like enzymatic assays with high accuracy.

The objective of this study is to select a potent *Streptomyces* strain capable of producing high-yielding Chox and the optimization of assay parameters for the estimation of Chox produced by the species of *Streptomyces* i.e. *Streptomyces olivaceus* MTCC *6820 (S. olivaceus MTCC 6820)*. In order to achieve enhanced Chox activity (U/ml), the assay

conditions for each microbial source need to be optimized as the effective operating conditions for every new enzyme are different. The assay parameters were optimized with respect to substrate concentration (cholesterol and 4-aminoantipyrine), pH of reaction mixture, and enzyme concentrations (Chox and peroxidase). Further, these assay parameters were incorporated as inputs (for RSM and ANN) to enable the development of a robust ANN network for the prediction of the response, which further improve the Chox activity.

3.2. Experimental

3.2.1. Chemicals Used

Cholesterol was purchased from Sigma Aldrich Pvt. Ltd and Horseradish peroxidase was purchased from Sisco Research Laboratories, Mumbai, India. Yeast extract, Peptone, Malt extract, glucose, and agar were purchased from Himedia, Mumbai, India. KH_2PO_4, $CaCO_3$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, and Tween-80 were purchased from Qualigens, Mumbai, India. All the chemicals used were of analytical grade.

3.2.2.Microorganisms and Growth Media

Streptomyces sp. including *S. niger* MTCC 4010, *S. fradiae* MTCC 4002, *S. olivaceus* MTCC 6820, *S. hygroscopicus* MTCC 4003, *S. annulatus* MTCC 6818, and *S. clavifer* MTCC 4150 were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. All the *Streptomyces* cultures were maintained in the *Streptomyces* growth medium containing (g/L): glucose - 4, yeast extract - 4, malt extract - 1, $CaCO_3$ - 2 and agar - 12; pH was adjusted to 7.2 with KOH. The slants were incubated at 30˚C ± 2˚C for 48 - 72 h

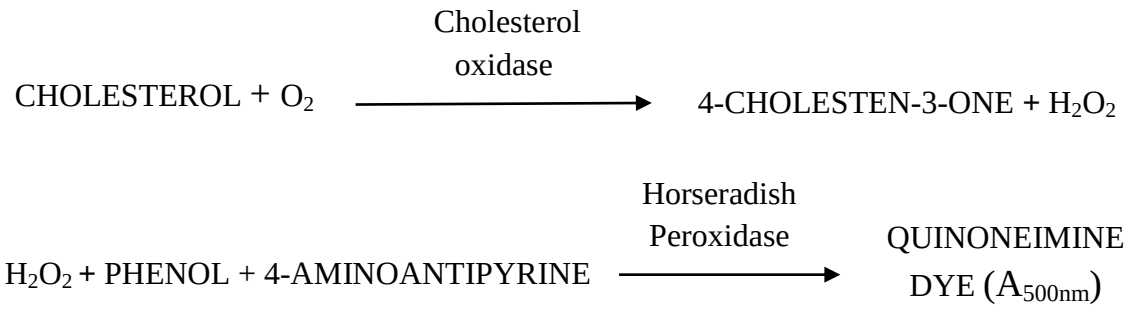
for the growth of the organism and preserved at 4˚C in the refrigerator. All the cultures were routinely sub-cultured every 30 days.

3.2.3. Medium Components and Culture Conditions

The production medium for Chox contained (g/L): cholesterol - 2, glucose - 12, starch - 9, yeast extract - 6, peptone - 4, $(NH_4)_2SO_4$ - 7.5, K_2HPO_4 - 1, $MgSO_4$ - 0.5, NaCl - 1, $MnSO_4$ - 0.008, $CaSO_4$ - 0.002, $ZnSO_4$ - 0.002, $FeSO_4$ - 0.02, $CaCl_2$ - 0.0002 and Tween 80 - 10 ml (Tabatabei, 2001). Chox production was carried out in 250 ml Erlenmeyer flask, incubated at 30˚C ± 2˚C for 72 h in an orbital shaker (Orbitek, Scigenics Biotech Pvt. Ltd., Chennai, India) at 180 rev/min.

3.2.4. Estimation of Cholesterol oxidase Activity

Chox activity assay was estimated by the method described by Allain et al. (Allain, 1974). 50 µL of 6g/L cholesterol (dissolved in dimethylformamide containing 5% (v/v) TritonX-100) was added to 1 ml of reaction mixture containing 1mM 4-aminoantipyrine, 5 mM phenol, 5 U/ml horseradish peroxidase and sodium phosphate buffer (20mM, pH 7.0); pre-incubated for 5 min at 30˚C. 100 µL of crude enzyme extract was added to the pre-incubated reaction mixture to start the reaction and the incubation continued for 10 min at 30˚C. The reaction was terminated by placing the samples in a boiling water bath for 2 min and then immediately placed in an ice bath for 2 min for color development. Absorbance was recorded at 500 nm (UV 1800 Spectrophotometer, Shimadzu, Japan). Blank was prepared by adding an inactivated enzyme to the reaction mixture. One unit of Chox activity was defined as the amount of enzyme that converts 1µmol of cholesterol into 4-cholesten-3-one per minute at 30˚C.



3.2.5. Protein Estimation by Bradford's Method

Protein concentration was determined by Bradford's method using Coomassie Brilliant Blue G-250 dye (Bradford, 1976). 0.2 g Coomassie Brilliant Blue (G-250) dye was dissolved in 95% ortho-phosphoric acid H_3PO_4(100 ml) containing 5% (v/v) absolute ethanol overnight using a magnetic stirrer under dark conditions. The next day volume was made up to 1 liter with ultrapure water (Merck Millipore) in a dark room and was kept on stirring for 1 hour. The prepared reagent was filtered using Wattmann filter paper and stored in an amber-brown colored bottle at 4˚C. The prepared reagent was deep reddish-brown in color. Precaution was taken for maintaining dark conditions while handling Bradford's reagent due to its sensitivity towards light.

A standard curve was prepared using bovine serum albumin(BSA) having concentrations of 0.002, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1mg/ml. BSA stock solution (1mg/ml) was prepared and pipetted into 11 different test tubes with 10-100µl volume respectively. Volume was made up to 1ml with distilled water in each test tube. Bradford's reagent (5ml) was added to each test tube and incubated at room temperature for 5 min. Absorbance was recorded at 595 nm against the blank containing distilled water in place of BSA. A calibration curve was prepared for concentration of BSA (mg/ml) versus Absorbance (595 nm) **(see Appendix 1)**. 100µl of test samples were

directly used without dilution for total protein estimation. Total protein content was calculated with the slope of the calibration curve.

3.2.6. Estimation of extracellular and cell-bound Cholesterol oxidase activity

Streptomyces culture supernatant obtained by the centrifugation of the fermented broth (12,000 rev/min 4ºC for 20 min.) was considered as the source of extracellular Chox. The organism so obtained was washed twice with 0.1M phosphate buffer (pH 7.2) and subjected to the detection of cell-bound Chox. When no activity was found in the buffer, the pellet was re-suspended in the same buffer containing 0.1% Triton X-100, Tween-20, and Tween-80 and incubated in an orbital shaker at 180 rev/min, 30ºC for 30 min. The suspension was recentrifuged (12,000 rev/min, 4ºC for 20 min) and the supernatant was tested for the activity of cell-bound Chox.

In order to confirm the extracellular activity of the enzyme, the Chox indicator plates were prepared by adding 0.1% (w/v) cholesterol dissolved in 0.1% Triton X-100, 0.2% (w/v) 4-aminoantipyrine, and 100 U horseradish peroxidase to 100 ml of *Streptomyces* growth medium containing (g/L): Glucose – 4, Yeast Extract – 4, Malt Extract – 1, $CaCO_3$ – 2 and Agar – 12 and the pH was adjusted to 7.2 with KOH. *S. olivaceus* was cultured on these plates and incubated at 30 ± 2ºC for 48 -72 h for the growth of the organism.

3.2.7.Optimization of Cholesterol oxidase Assay Parameters using Response Surface Methodology

Enzyme activity is significantly influenced by substrate concentration, enzyme concentration, pH, and incubation temperature. Five assay parameters viz. pH of the reaction mixture (X_1), cholesterol concentration (X_2), 4-aminoantipyrine concentration (X_3), crude Chox volume (X_4), and horseradish peroxidase (X_5) were chosen as the

independent variables for optimization of Chox activity (U/ml) as response. The range and levels of the independent factors studied are given in **Table 3.1**. A five-level-five factor Central Composite Design (CCD) was employed using Minitab 17.0 statistical software to obtain an experimental design matrix consisting of 32 runs, displayed in **Table 3.2**. The experimental data were analyzed by surface response analysis on the basis of multiple regressions and ANOVA. The input range provided in the Minitab was based on the results of the preliminary studies. The second-order polynomial model equation fitted for the prediction of optimal levels is given in equation 3.1:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_{ij} \ldots\ldots\ldots \quad (3.1)$$

Where, Y = Predicted response, β_0 = Intercept coefficient, β_i = Linear coefficient, β_{ii} = Quadratic coefficient and β_{ij} = Interaction coefficient

The interaction effects of significant variables on Chox activity were studied using contour plots.

Table 3.1 Independent variables used for the experimental design along with their upper and lower levels

Factors	Name of factors	Levels				
		-α	**-1**	**0**	**+1**	**+α**
X_1	pH of the reaction mixture	6	7	8	9	10
X_2	Cholesterol concentration	0.2	0.4	0.6	0.8	1.0
X_3	4-Aminoantipyrine	-0.5	0.5	1.0	1.5	2.5
X_4	Crude Chox volume	0	50	100	150	200
X_5	Horseradish Peroxidase	0	5	10	15	20

3.2.8. Artificial Neural Network Modeling

The neural model was created in MATLAB 2017a (Mathworks, USA) using a neural network toolbox (command nftool) with five input variables (X_1, X_2, X_3, X_4, and X_5) and one output variable Y (Chox activity). The five assay parameters considered as independent variables in the experimental design were the determinants of Chox activity assay and served as network inputs in ANN modeling where Chox activity (U/ml) was the output. A multilayer feed-forward back propagation neural network was employed for the modeling and prediction of Chox activity. The network architecture consisted of one input layer, one hidden layer, and one output layer **(shown in Figure 3.1).** ANN simulated the same experimental data designed by CCD except the replicated data obtained at the center point **(Table 3.2)**. The replicates do not improve the prediction ability of the ANN network (Bas and Boyaci, 2007). The network was trained using the Levenberg-Marquardt learning algorithm till the preferred network accuracy was achieved by multiple trainings of the network concurrently adjusting the number of neurons in the hidden layer (Lou and Nakai, 2001). The performance of the network was evaluated in terms of Mean Squared Error (MSE) which indicates the difference between the output variable and pre-specified external desired signal. The input and output datasets were divided into three sets: Training (22), Validation (5), and Testing (5). Network performance was monitored through performance plot and regression analysis at each step consisting of training, validation, and testing.

The performance of ANN was statistically evaluated in terms of the regression coefficient (R^2) and the prediction efficiency Mean Absolute Percentage Error (MAPE). R^2, MAPE, and MSE were calculated according to the following Equations (3.2, 3.3, and 3.4) respectively.

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(y_i - y_{di})^2}{\sum_{i=1}^{n}(y_{di} - y_m)^2} \quad \text{..... (3.2)}$$

$$\text{MAPE} = \frac{100}{n}\sum_{i=1}^{n} |ydi - yi/ydi| \text{..... (3.3)}$$

$$\text{MSE} = \frac{1}{n}\sum_{i=1}^{n}(\theta i, p - \theta i, e)^2 \text{ ..... (3.4)}$$

Where, y_{di} is the experimental value; y_i is the predicted response; y_m is the average of actual values and n is the number of experiments. θ_i, p is the predicted value obtained from ANN model, θ_i, e is experimental value.

R^2 is the measure of the amount of variability in response obtained by using repressor variables in the model. R^2 value close to 1.0 and a minimum value of MSE and MAPE is indicative of good adequacy of the model (Ebrahimpour et al., 2011).

Table 3.2 CCD of five independent variables displaying Experimental, RSM and ANN predicted Cholesterol oxidase activity

Run Order	X_1	X_2	X_3	X_4	X_5	Chox Activity (U/ml)		
						Experimental	RSM Predicted	ANN Predicted
1	7	0.8	0.5	50	5	0.047	0.017	0.058
2	8	0.6	1.5	100	20	0.803	0.807	0.796
3	8	0.6	1.5	100	10	1.100	1.076	1.073
4	9	0.4	2.5	150	5	0.580	0.566	0.596
5	8	0.6	1.5	100	10	1.070	1.076	1.073
6	8	0.6	1.5	200	10	0.671	0.673	0.664
7	7	0.8	2.5	50	15	0.595	0.598	0.392
8	8	0.6	1.5	100	10	1.080	1.076	1.073
9	6	0.6	1.5	100	10	0.656	0.675	0.615
10	7	0.4	0.5	50	15	0.545	0.515	0.471
11	8	0.6	1.5	100	10	1.100	1.076	1.073
12	9	0.4	0.5	50	5	0.005	-0.0429	0.007
13	7	0.4	2.5	50	5	0.509	0.486	0.355
14	9	0.4	2.5	50	15	0.504	0.489	0.500
15	8	0.6	1.5	100	10	1.090	1.076	1.073
16	8	0.6	-0.5	100	10	0.322	0.394	0.413
17	8	0.6	1.5	100	0	0.268	0.325	0.256
18	8	1.0	1.5	100	10	0.919	0.923	0.891
19	9	0.8	2.5	150	15	0.882	0.894	0.873
20	7	0.8	2.5	150	5	0.705	0.710	0.769
21	9	0.8	2.5	50	5	0.444	0.429	0.501

22	7	0.4	2.5	150	15	0.532	0.537	0.764
23	8	0.6	3.5	100	10	0.788	0.778	0.809
24	8	0.6	1.5	0	10	0.105	0.165	0.097
25	7	0.4	0.5	150	5	0.387	0.358	0.364
26	8	0.2	1.5	100	10	0.667	0.724	0.643
27	9	0.8	0.5	150	5	0.473	0.452	0.265
28	9	0.4	0.5	150	15	0.657	0.635	0.730
29	8	0.6	1.5	100	10	1.080	1.076	1.073
30	7	0.8	0.5	150	15	0.809	0.807	0.757
31	10	0.6	1.5	100	10	0.588	0.631	0.601
32	9	0.8	0.5	50	15	0.455	0.432	0.247

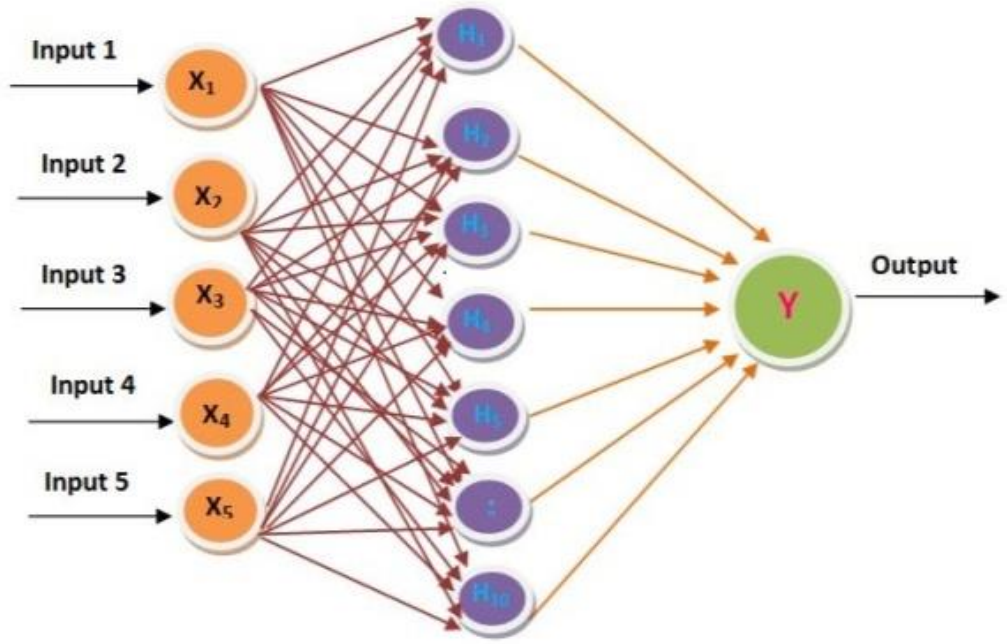
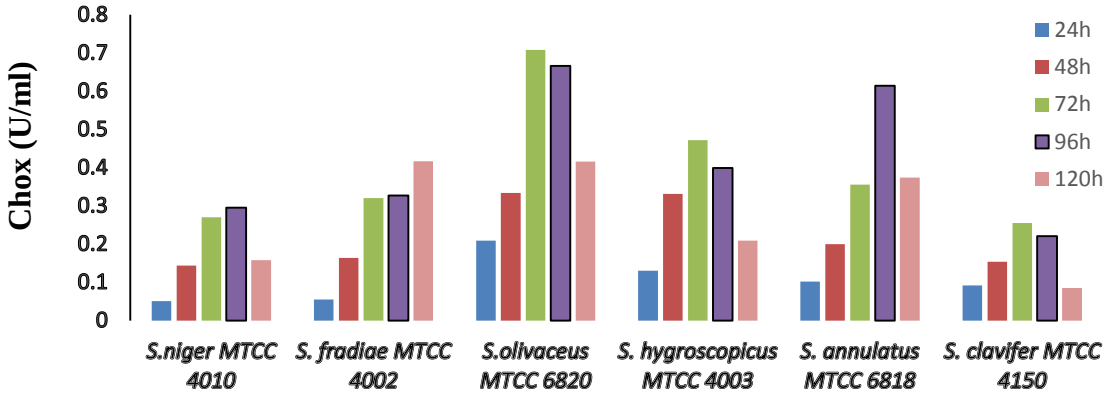


Fig 3.1 ANN architecture showing five input parameters at Input Layer (X_1, X_2, X_3, X_4 and X_5), neurons in the Hidden Layer (H_1, H_2, H_3,H_4,….,H_{10}) and Output Layer (Y)

3.3. Results and Discussion

3.3.1. Selection of potent microorganism for Cholesterol oxidase production

Chox from various microbial sources including *Streptomyces sp., Nocardia sp., Pseudomonas sp.,* are commercially available, but the Chox obtained from *Streptomyces* has been found to be superior. *Streptomyces* being the producers of extracellular Chox are preferred for their potential in-vitro applications such as clinical pathology (Allain, 1974); (Lolekha and Jantaveesirirat, 1992), pharmaceutical and food industry (Mahato and Garai, 1997); (Ahmad and Goswami, 2014), biosensors (Aggarwal et al., 2016); (Gholivand and Khodadadian, 2014), etc. A number of *Streptomyces sp.* have been reported to produce extracellular Chox, based on which six *Streptomyces* strains were used in this study to select a potent strain with high Chox yielding capacity, listed in **Table 3.3.** All the six *Streptomyces* strains showed positive results for extracellular Chox production (**Fig 3.2**). All the microorganisms gave maximum production at 72h except *S. annulatus MTCC 6818* which showed maximum production at 96h. Amongst the six *Streptomyces sp.* used, *Streptomyces olivaceus MTCC 6820* was found to produce a significantly high level of Chox under unoptimized assay conditions, therefore, it was further selected for the optimization study of Chox enzyme production and assay conditions.



***Streptomyces sp.* producing Cholesterol oxidase**

Fig 3.2 Different species of *Streptomyces* producing Cholesterol oxidase under unoptimized assay conditions

Table 3.3 Estimation of Cholesterol oxidase activity at 72h fermentation time among different *Streptomyces sp.*

Streptomyces sp.	Chox activity (U/ml)
S. niger MTCC 4010	0.27 ± 0.01
S. fradiae MTCC 4002	0.32 ± 0.15
S. olivaceus MTCC 6820	0.675 ± 0.02
S. hygrroscopicus MTCC 4003	0.472 ± 0.22
S. annulatus MTCC 6818	0.355 ± 0.45
S. clavifer MTCC 4150	0.254 ± 0.48

All the experiments for the estimation of cholesterol oxidase activity were performed in triplicates. Results are displayed in terms of the mean and standard deviation of three independent experiments.

3.3.2. Release of extracellular Cholesterol oxidase

The culture supernatant of *S. olivaceus* showed positive results for the Chox activity which proved the production of extracellular Chox in the medium. While the pellets washed with 0.1M phosphate buffer (pH 7.0) and the pellet re-suspended in the same buffer containing different non-ionic surfactants i.e., Triton X-100, Tween-20 and Tween-80 did not show the presence of Chox activity in it. This proved that there was no intracellular or cell-bound Chox present in *S. olivaceus*.

The release of extracellular Chox by *S. olivaceus* was also confirmed by the Chox indicator plates. The enzyme Chox converts cholesterol into 4-cholesten-3-one and hydrogen peroxide (H_2O_2). As the cholesterol present in the medium penetrates into *Streptomyces* cells it gets converted into hydrogen peroxide by the action of Chox produced by the microorganism. The H_2O_2 causes the reduction of 4-aminoantipyrine present in the medium catalyzed by peroxidase leading to the formation of azo-dye which gives intense brown color to the medium. The change in color of the medium was noticeable after 48 h of incubation and it turned to intense brown after 72h which proved the production of extracellular Chox, presented in **Fig 3.3**.

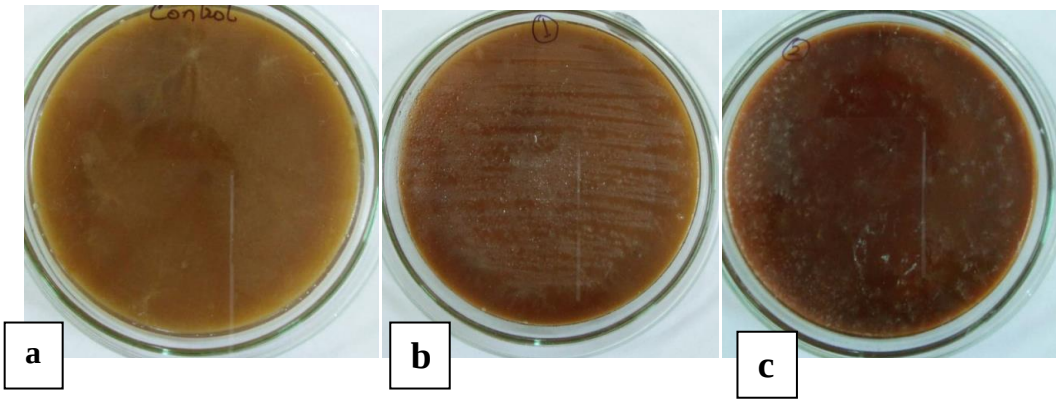


Fig 3.3 Plate Assay (a) Control (Bare Cholesterol oxidase indicator plates) (b) *S. olivaceus* cultured by streak plate method and (c) *S. olivaceus* cultured by spread plate method

3.3.3.Optimization of Assay Parameters using Response Surface Methodology

Chox activity was estimated by modified method of Allain et al. (1974). Response Surface Methodology (RSM) was employed to thirty two experiments designed by CCD in order to optimize the five assay parameters of Chox activity and the RSM predicted were tabulated in **Table 3.2.** The experimental values of Chox activity were fitted to the quadratic equation (Eq. 3.1), and the following second-order polynomial regression equation (Eq. 3.5) in coded units was obtained:

$$Y = -7.087 + 1.522 X_1 + 0.530 X_2 + 0.510 X_3 + 0.01054 X_4 + 0.1310 X_5 - 0.10593 X_1^2 - 1.580 X_2^2 - 0.12267 X_3^2 - 0.000066 X_4^2 - 0.005100 X_5^2 + 0.1013 X_1*X_2 + 0.0166 X_1*X_3 + 0.000558 X_1*X_4 + 0.00203 X_1*X_5 + 0.0975 X_2*X_3 + 0.00460 X_2* X_4 + 0.0197 X_2* X_5 - 0.000783 X_3* X_4 - 0.01598 X_3* X_5 - 0.00009 X_4* X_5 \ldots\ldots\ldots\ldots\ldots (3.5)$$

Where Y is the predicted response variable viz., Chox activity (U/ml);

X_1, X_2, X_3, X_4, and X_5 represent the independent variables - pH of the reaction mixture, Cholesterol concentration,4-Aminoantipyrine, Crude Chox volume, and Horseradish Peroxidase. The significance of each individual factor and their interaction effects on Chox activity was explained by their corresponding *P*-values (displayed in **Table 3.4**). All the individual variables have a positive significant effect on Chox activity whereas the high coefficient value of Crude Chox volume and horseradish peroxidase shows their high degree of significant effect on Chox activity. The individual terms X_2, X_3, X_4, andX_5 were significant terms, and the terms X_1*X_4, X_2*X_4, X_3*X_4, X_3*X_5, and X_4*X_5 were found to be highly significant interaction terms with $P<0.05$ (95% confidence level, a = 0.05).

3.3.2.1 Statistical Analysis by ANOVA

RSM data was analyzed by multiple regression analysis. Analysis of variance (ANOVA) of assay conditions for the estimation of Chox activity is given in **Table 3.5**; it explains whether a more complex model is required for a better fit. If the *F*- test for the model is significant at 5% level ($P< 0.05$), then the model is supposed to be fit and can effectively interpret for the variation observed. The goodness of fit of the model was described by the coefficient of determination R^2, obtained as 0.9909 in this study. The obtained R^2 value represents 99.09% of sample variation attributed to the testing variables and only 0.91% of the total variance could not be explained by the model. This indicates a very good fit between the observed and the predicted responses inferring that the model is reliable for predicting Chox activity in this study. The extremely low *P*-value of lack of fit for the model (0.001) in **Table 3.5** represents that the model adequacy well establishing the relationship between assay parameter factors and the response variable viz., Chox activity.

The test of significance and model adequacy presented by the ANOVA of quadratic regression model **(Table 3.5**) suggests that the model is highly significant as evident from the high *F*-value (59.99) and a very low *P*-value (0.000) obtained from Fischer's F-Test. This implies that the combinatorial influence of all the independent variables considerably contributed to maximizing the response i.e., Chox concentration (U/ml).

Table 3.4 Regression of CCD showing coefficients of RSM model and significant terms for Cholesterol oxidase activity

Term	Coef	SE Coef	*T*	*P*
Constant	1.0764	0.0196	54.90	0.000
X_1	0.0110	0.0100	-1.10	0.296
X_2	0.0498	0.0100	4.96	0.000
X_3	0.0960	0.0100	9.57	0.000
X_4	0.1271	0.0100	12.67	0.000
X_5	0.1207	0.0100	12.03	0.000
X_1^2	-0.10593	0.00908	-11.67	0.000
X_2^2	-0.06318	0.00908	-6.96	0.000
X_3^2	-0.12267	0.00908	-13.52	0.000
X_4^2	-0.16438	0.00908	-18.11	0.000
X_5^2	-0.12751	0.00908	-14.05	0.000
$X_1 * X_2$	0.0203	0.0123	1.65	0.128
$X_1 * X_3$	0.0166	0.0123	1.35	0.203
$X_1 * X_4$	0.0279	0.0123	2.27	0.044
$X_1 * X_5$	0.0101	0.0123	0.82	0.427
$X_2 * X_3$	0.0195	0.0123	1.59	0.141
$X_2 * X_4$	0.0460	0.0123	3.74	0.003
$X_1 * X_5$	0.0197	0.0123	1.61	0.136
$X_3 * X_4$	-0.0391	0.0123	-3.18	0.009
$X_3 * X_5$	-0.0799	0.0123	-6.50	0.000
$X_4 * X_5$	-0.0224	0.0123	-1.82	0.046

Where, X_1 = pH of the reaction mixture, X_2 = Cholesterol concentration, X_3 = 4-Aminoantipyrine, X_4 = Crude Chox volume, and X_5 = Horeseradish Peroxidase

Table 3.5 ANOVA table analyzing model fitness for Cholesterol oxidase activity

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	20	2.89895	2.89895	0.144948	**59.99**	**0.000**
Linear	5	1.02118	1.02118	0.204236	84.52	0.000
Square	5	1.67193	1.67193	0.334385	138.38	0.000
Interaction	10	0.20585	0.20585	0.020585	8.52	0.001
Residual Error	11	0.02658	0.02658	0.002416		
Lack of Fit	6	0.02585	0.02585	0.004308	29.37	**0.001**
Pure Error	5	0.00073	0.00073	0.000147		
Total	31	2.92553	2.92553			

3.3.2.2 Contour Plots

The interaction effects of the assay parameter variables on Chox activity along with the prediction of their optimum activity were explained with the help of contour plots. The contour plots of the interacting variables showing positive significant effects are presented in **Fig 3.4 (a-e)**. The contour plots showed an increase in Chox activity as the values of the selected assay parameters increases until they attain the optimum Chox activity value, on further increase in assay parameter values the Chox activity decreases gradually. **Fig 3.5** shows the optimization plot of Chox activity which represents the optimum value obtained for each assay parameter. The optimal levels of assay parameters were obtained as pH of the reaction mixture (8.0), Cholesterol concentration (0.6% w/v), 4-Aminoantipyrine (1.5mM), Crude Chox volume (100µl), and Horseradish Peroxidase (10 U). The optimum values found for pH of the reaction mixture (8.0), 4-Aminoantipyrine (1.5mM), and Crude Chox volume (100µl) obtained through the experimental design are different from those reported for Chox activity from other sources of Chox (Yang and Zhang, 2012; Lin et al., 2010). Also the enhanced Chox activity obtained at the optimum values of these assay parameters shows the significance of assay optimization from a new microbial source of cholesterol oxidase. Maximum Chox activity predicted by RSM was 1.0764 U/ml) at optimum levels of the parameters.

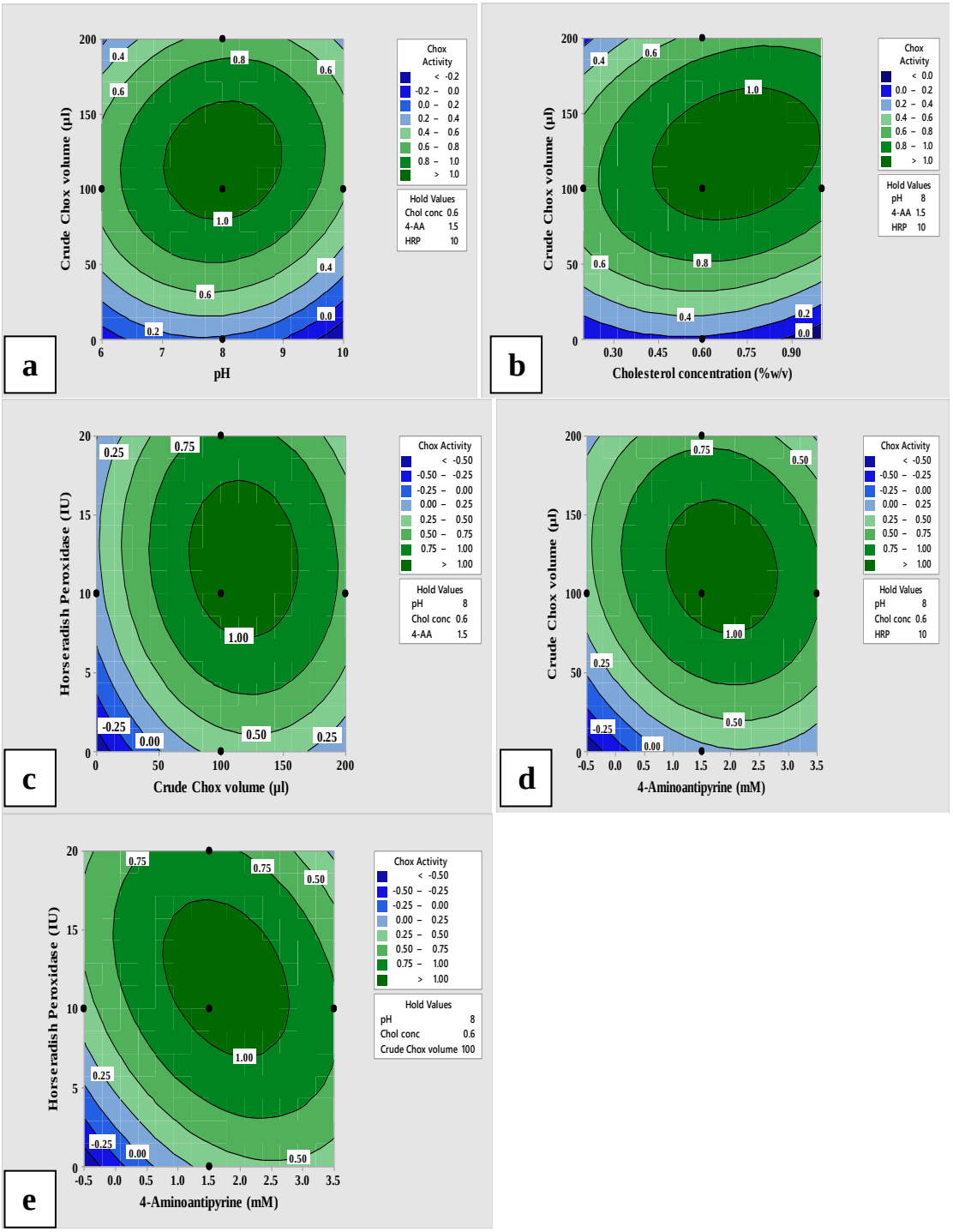
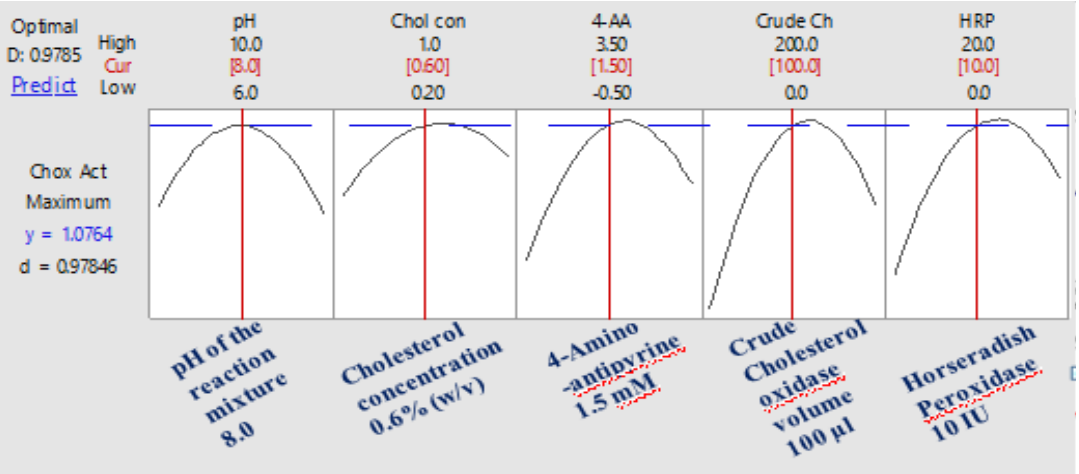


Fig 3.4 Contour plots showing interaction effects of the selected assay parameters on Cholesterol oxidase activity (a) pH and Crude Cholesterol oxidase volume (b) Cholesterol concentration and Crude Cholesterol oxidase volume (c) 4- aminoantipyrine and Crude Cholesterol oxidase volume (d) 4-aminoantipyrine and Horseradish Peroxidase(e) Crude Cholesterol oxidase volume andHorseradish Peroxidase



The values written in red color at the top of each assay parameter represent their optimum level for Cholesterol oxidase activity obtained after optimization

Fig 3.5 Optimization Plot: Prediction for cholesterol oxidase activity

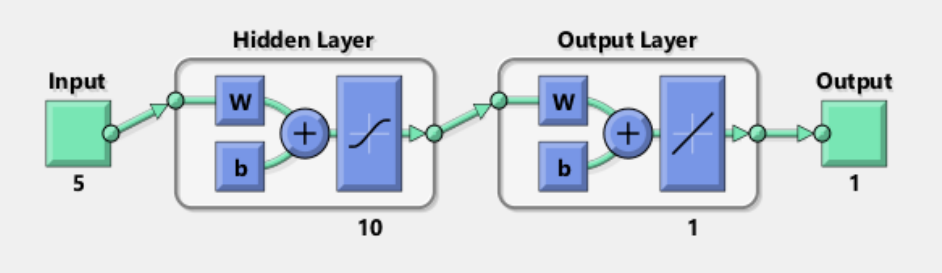
3.3.3 ANN Modeling

ANN model was developed with pH, cholesterol concentration, 4-aminoantipyrine, crude Chox volume, and horseradish peroxidase as five model inputs, and the experimental design matrix using CCD consisting of 32 experiments were prepared, as shown in **Table 3.2**. Chox activity (U/ml) served as an output of the ANN, which gives the quantitative prediction of the increase in enzyme activity when all the five independent variables are optimized. ANN captures the non-linear behavior of the input parameters thus, affecting the output of the system. The enzyme activity is largely affected by the physicochemical parameters of the reaction conditions provided. The above-mentioned reaction conditions needed to be optimized to predict the optimum activity of Chox produced by a new microorganism *S. olivaceus* MTCC 6820. Maximum experimental Chox activity observed was 1.1 U/ml which was very close to the ANN predicted Chox activity - 1.073 U/ml) at optimum levels of the parameters. The optimized enzyme activity is the result of a combined effect of these assay parameters as a whole,

which shows non-linearity. Hence, ANN modeling combined with the surface response analysis was the preferable method. The hit and trial method was employed to determine the optimal number of neurons in the hidden layer to train the network. The network topology of 5-10-1 was found to be optimum and is presented in **Figure 3.6.** The ANN predicted values of the output (Chox activity U/ml) for different ranges of 5 input parameters (pH, cholesterol concentration, 4-aminoantipyrine concentration, crude Chox volume, and horseradish peroxidase concentration) were presented in **Table 3.2**. The performance of the neural network and prediction efficiency of ANN was measured in terms of the regression coefficient (R^2), MSE, and MAPE, which were 0.9792%, 0.0075% and 0.12% respectively. The regression analysis of the training, validation and testing phase of the ANN is shown in **Figure 3.7.** The obtained R^2 value is close to unity which confirms the excellent network performance and indicates that 97.92% variability of the data can be explained by the ANN model while only 2.08% of the total variation is not explained by the model (Lin et. al., 2010). The MSE and MAPE values determine the scale for prediction accuracy. MAPE ≤ 10% indicates high prediction efficiency, 10% ≤ MAPE ≤ 20%depicts good prediction, and 20% ≤MAPE≤50% suggests reasonable prediction while MAPE ≥ 50% indicates inaccurate prediction (Yadav et al., 2014; Shera et al., 2018). The small MSE and MAPE values so obtained prove the excellent prediction efficiency of the model. The values of R^2, MSE, and MAPE altogether substantiated an efficient neural model for the prediction of responses.

The predicted condition was experimentally verified in triplicate by conducting the experiments at predicted optimum assay conditions viz., pH of the reaction mixture (8.0), Cholesterol concentration (0.6% w/v), 4-Aminoantipyrine (1.5mM), Crude Chox volume (100µl), and Horseradish Peroxidase (10 U). Chox activity obtained after conducting the experiments was 1.1 U/ml (ANN predicted - 1.073 U/ml) at optimum levels of parameters,

which was very close to the predicted response and is 1.71 times higher than the Chox activity obtained under unoptimized assay conditions.



ANN topology represents five input variables, ten neurons at hidden layer with tansigmoid activation function and one output layer with Purelin transfer function. *'w'* and *'b'* in the hidden and output layer represents the weight and bias respectively; their values were adjusted automatically by the ANN function.

Fig 3.6 Optimized ANN Topology

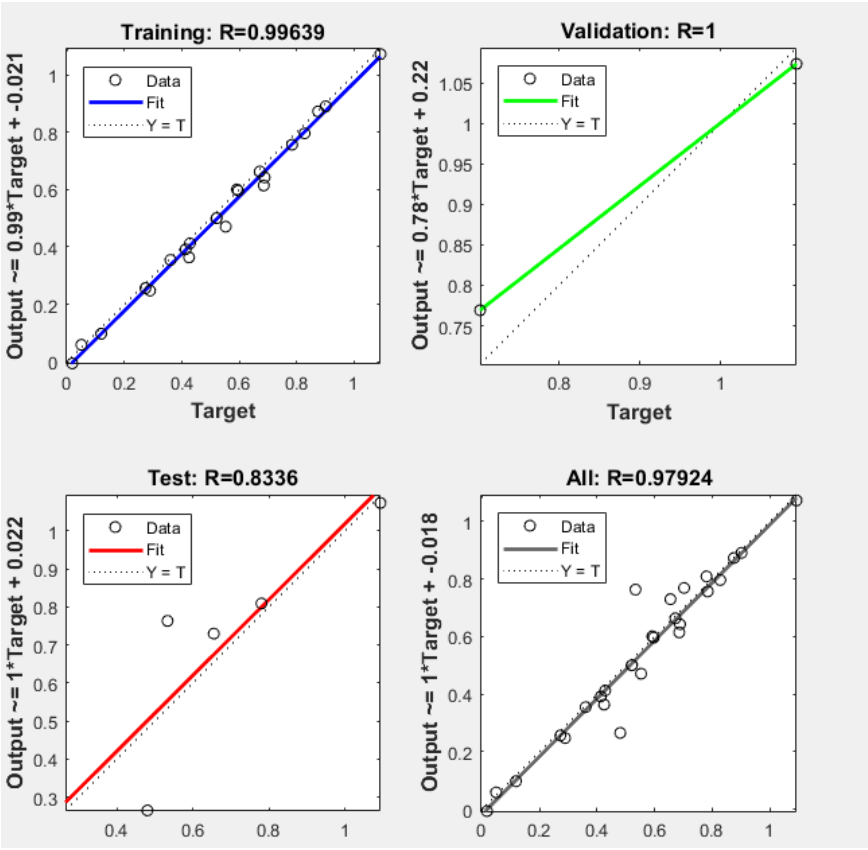


Fig 3.7 Regression analysis of ANN model generated by the ANN toolbox during training, validation and testing phase

3.4. Conclusion

S. olivaceus MTCC 6820 was selected as the most potent Chox producing strain among the different *Streptomyces sp.* studied, so it was selected for further studies. The Chox activity assay conditions for the Chox produced by the new strain *S. olivaceus MTCC 6820* were optimized using a combined Response Surface Methodology and Artificial Neural Network modeling based approach, resulting in enhanced Chox activity attaining unbiased optimum values for each assay parameter optimized. The developed ANN model was successful in predicting the Chox activity of *S. olivaceus MTCC 6820*. In addition, the excellent prediction accuracy of the ANN model illustrates its robustness in predicting the Chox activity in a similar type of system within the range of the dataset. ANN technique was based on the machine-learning approach, considering input-output data. This method helped minimize the labor, cost and enhanced the Chox activity to a greater extent. The activity of Chox was enhanced by 1.71 folds after optimization of reaction conditions viz. pH of reaction mixture (8.0), cholesterol concentration (0.6 %w/v), 4-aminoantipyrine (1.5 mM), crude Chox volume (100 µl) and horseradish peroxidase (10.0 U/ml). The generated ANN model will work as a template for the prediction, modeling, and estimation of Chox from other *Streptomyces sp.* or microorganisms using similar reaction conditions studied in this work.