4.1. Overview

Several microorganisms are reported to produce Chox with specific properties including bacteria, actinomycetes, and fungi (Buchland, 1975; Liu et al., 1988; Noriyaku and Rikizo, 1998; Petrova et al., 1981; Shirshova et al., 1992; Tabatabei et al., 2001). Some microorganisms viz. *Nocardia* and *Mycobacterium* species produce Chox as intrinsic membrane-bound enzymes whereas the members belonging to *Actinomycetes* include some species of *Streptomyces* viz. *S. parvus*, *S. violascens*, *Brevibacterium sterolicum*, *Streptoverticillium cholesterolicum*, *Rhodococcus equi*, *Rhodococcus erythropolis* secrete Chox in the medium in extracellular form (Salva et al., 1999). The production of intracellular or extra-cellular enzymes depends upon the physiology of the microorganism. Despite their widespread potential applications, the commercial production of Chox is still a challenging aspect, due to its low yield through the fermentation process (Yazdi et al., 2001; Chauhan et al., 2009). There are some underlying reasons behind this fact; firstly, the production and availability of Chox are confined only to the microbial fermentation process, on the other hand, no other sources (animal or plant) have been documented until now. Secondly, the production of Chox by several microorganisms mostly exhibits an inducible expression pattern, i.e., no constitutive expression of the Chox gene. Thirdly, the pathogenicity of producer organisms is also a problem in some cases, while some others are intracellular Chox producers. These reasons not only limit its production but also mark it as an expensive enzyme for industrial as well as clinical applications.

One of the primary strategies applied for maximizing yield in the fermentative production of enzymes and metabolites is the optimization of process parameters. The growth of microorganisms and production of enzymes is significantly affected by variation

in the culture conditions; hence it becomes necessary to optimize them for increasing the microbial production of the enzyme. The optimized cultural conditions viz. medium pH, incubation temperature, inoculum size, inoculum age, fermentation period, and shaking speed enhances the microbial production of enzymes and biochemicals under submerged fermentation. Optimization of process parameters in biological systems is yet a cumbersome task. The classical method of optimization involves varying one variable at a time (OVAT) while keeping others at a constant level (Chauhan et al., 2009). Statistical methods account for the interaction of the variables under study and reduce the number of experiments (Rathi et al., 2001). Response surface methodology (RSM) was used as a statistical tool for optimization of bioprocess where the predicted response was determined using quadratic function. RSM is based on the assumption of linear quadratic correlation for optimizing the response. The complexity of interactions increases with the increase in variables (more than 7), as the biological systems mostly represent complex non-linear relationships. RSM fails to explain the object function accurately in such cases; consequently, RSM could not explain complex interactions (Rafigh et al., 2014).

Artificial intelligence (AI) has emerged as an attractive tool for developing non-linear empirical models and optimizing the multifactor time-variant bioprocess (Rafigh et al., 2014; Desai et al., 2008; Liu et al., 2009; Patnaik, 2006; Jacob and Banerjee, 2016). ANN is a biologically inspired computational tool, which mimics the nervous system in the human body, where the neuron functions as fundamental processing units. ANN offers a sophisticated mathematical model which overcomes the shortcomings of regression models for noisy data and successfully accounts for the optimization and nonlinear modeling of complex biological processes. The learning algorithm of ANN enables it to

recognize and establish the cause-effect relationship through training for multiple input-output systems, and the performance evaluation is done on the unseen set of data, which makes it efficient for even more complex systems (Rafigh et al., 2014; Liu et al., 2009).

In the present study, we generated a central composite design (CCD) based experimental design. RSM coupled with ANN was employed to optimize the culture conditions viz. medium pH, incubation temperature, inoculum size, inoculum age, fermentation period, and shaking speed for augmenting the Chox production by *S. olivaceus MTCC 6820.* A comparative performance evaluation of RSM and ANN techniques was done.

4.2. Experimental

4.2.1. Chemicals used

Cholesterol was purchased from Sigma Aldrich Pvt. Ltd. and Horseradish peroxidase was purchased from Sisco Research Laboratories, Mumbai, India. Glucose, soluble starch, yeast extract, peptone, malt extract, and agar were purchased from Himedia, Mumbai, India. KH_2PO_4, $CaCl_2$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, and $FeSO_4$ were purchased from Thermo Fisher Scientific Pvt. Ltd., Mumbai, India. Tween-80 and Triton X - 100 were purchased from Qualigens, Mumbai, India. All other chemicals used were of analytical grade.

4.2.2. Microorganism and Culture Conditions

S. olivaceus MTCC 6820, used in this study was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India and was maintained in the *Streptomyces* growth medium containing (g/L): glucose - 4, yeast extract - 4, malt extract - 1, $CaCO_3$ - 2 and agar - 12 (pH 7.2, adjusted with KOH). The aseptically

inoculated slants were incubated at 30 ± 2˚C for 48 - 72 h for the growth of the organism; the cultures were preserved at 4˚C in the refrigerator and were routinely sub-cultured in every 30 days interval.

4.2.3. Fermentation Conditions

The inoculum was prepared by scraping the spores of *S. olivaceus* from the slants, transferring them into 3ml sterile distilled water, and the spore suspension was homogenized before transferring into 50 ml sterile seed medium in a 250ml Erlenmeyer flask. The flask was incubated at 30 ± 2ºC for 48 h in an orbital shaker (Orbitek, Scigenics Biotech Pvt. Ltd., Chennai, India) at 150 rpm. The production medium of 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, $CuSO_4$ - 0.002, $ZnSO_4$ - 0.002, $FeSO_4$ - 0.02, $CaCl_2$ - 0.0002 and Tween 80 - 10ml (Yazdi et al., 2001). Cholesterol was homogenized into the medium by ultra-sonication (Hielscher Ultrasound Technology, UP200S, Germany) for 15 min to avoid the deposition of undissolved cholesterol into the medium and the pH was adjusted to 7.5 before sterilization. The composition of the seed medium and the production medium remained the same. Fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of production medium. The production medium was inoculated with 10% (v/v) of inoculum of 48 h old *Streptomyces* culture. The inoculated flasks were incubated at 30 ± 2ºC for 120 h at 180 rpm in an orbital shaker. Samples were collected at every 12 h intervals and centrifuged at 12,000 rpm at 4ºC in an ultracentrifuge for 20 min. The supernatant was collected as a source of crude extract of extracellular Chox whereas the pellet was washed and the cells were recovered for the estimation of cell biomass.

4.2.4. Enzyme Assay

Chox activity assay was performed by the modified method of Allain et al. (Allain, 1974) as per the optimized assay conditions mentioned in the previous chapter. 50µL of 0.6% (w/v) cholesterol (dissolved in dimethyl formamide containing 5% (v/v) Triton X-100) was added to 1ml of reaction mixture containing 1.5mM 4- aminoantipyrine, 5 mM phenol, 10 U/ml horseradish peroxidase and sodium phosphate buffer (20mM, pH 8.0); pre-incubated for 3 min at 30˚C. The reaction was initiated by the addition of 100 µL of crude enzyme extract and incubated at 30˚C for 10 min. The reaction was terminated by placing the samples in a boiling water bath for 2 min followed by immediately placing them 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 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.

4.2.5 Estimation of cell biomass

The quantification of biomass was done by dry cell-weight analysis of the cell biomass in the fermentation broth. The fermented broth was centrifuged at 4ºC and 12,000rpm in an ultracentrifuge for 20 min. The pellet was dissolved in a minimum amount of sodium phosphate buffer (10 mM, pH 7.5). The collected cells were washed twice with the same buffer and transferred to pre-weighed cups prepared from aluminum foil. The cups containing fresh cell biomass were dried to a constant weight in a hot air oven at 80˚C overnight.

4.2.6 Preliminary studies for the production of Cholesterol oxidase

In order to investigate various parameters for Chox production, the preliminary studies were carried out in shake flask culture. The effect of initial pH on the production of Chox was investigated by varying the pH of the media in the pH range of 5 - 10 with the help of HCl (0.2 N) and NaOH (0.2 N). The effect of temperature on the production of Chox was studied in the temperature range 25˚C - 45˚C by carrying out the fermentation using orbital shakers adjusted at different temperatures. The effect of inoculum age on Chox production was studied by using the spore suspension (12 % v/v) of different ages ranging from 12 to 48 h. The optimum inoculum concentration was determined by the addition of 5 - 15% (v/v) inoculum volume (48 h old). The study of optimum fermentation period was carried out by varying the incubation time (12 - 120 h) of the inoculated media. The effect of shaking speed on the Chox production was studied by incubating the culture flasks at different shaking speeds (100 - 350 rpm). All the experiments were performed in triplicate.

4.2.7. Effect of nutrients on Cholesterol oxidase production

The effect of various nutrients on Chox production was studied by cultivating the *S. olivaceus* strain with different nutrients using one variable at a time (OVAT) method. All the experiments were performed in shake flask culture in duplicates.

4.2.7.1. Effect of different carbon and nitrogen sources

Supplementation experiments were performed to study the effect of nutrients on Chox production. Various sources of carbon (2% w/v) viz. glucose, sucrose, lactose, glycerol, oatmeal, D-mannitol, D-sorbitol, and soluble starch were supplemented in the production medium and their effect on enzyme production was studied. The effect of

different complex nitrogen sources was investigated using yeast extract, peptone, malt extract, meat peptone, soyabean meal, casein hydrolysate, beef extract, and urea. The control medium consisted of no carbon and nitrogen sources, instead, cholesterol and trace metal ions were added consistently in all the flasks.

4.2.7.2. Effect of surfactants

Surfactants act as stimulants for enzyme production, due to the fact that they affect the cell membrane permeability. Various non-ionic and ionic detergents viz. octylphenol (ethylene glycol) ether (Triton X-100), poly(oxyethylene)$_{20}$-sorbitan monolaureate (Tween - 20), poly(oxyethylene)$_{80}$-sorbitan monooleate (Tween 80), Extran MA 20 (commercial detergent: a mixture of anionic and non-ionic detergent) and Lauryl alcohol (1- Dodecanol) were used in order to solubilize cholesterol and to study the effect of surfactant supplementation on Chox production. Production media consisting of 1% (v/v) of different surfactants was prepared and autoclaved. The sterilized production media (with different surfactants) were inoculated with subsequent seed medium and the fermentation was carried out for 72h. The control medium consisted of no detergent.

4.2.7.3. Effect of cholesterol as inducer

The effect of different concentrations of cholesterol varying in the range of 0-0.5 %(w/v) was examined on Chox production. Culture grown without inducer was considered as control for the detection of constitutive expression of Chox.

4.2.8. Optimization of culture conditions using RSM and ANN methodology

4.2.8.1 Experimental design

A five-level-six factor CCD was employed using Minitab statistical software package, version 17.0 to generate the experimental design matrix consisting of 53

experimental trials. Six fermentation parameters viz. pH of media (X_1), inoculum age (X_2), inoculum size (X_3), fermentation period (X_4), incubation temperature (X_5), and shaking speed (X_6) were chosen as the independent variables, their coded and uncoded levels are displayed in **Table 4.1**. The design matrix comprised of nine replications at center points in order to evaluate the curvature and to simplify the pure error estimation, so that the significant lack of fit of the models could be predicted (Naggar et al., 2015). The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. The response surface is a multivariable polynomial model that was intended to determine optimum setpoints for the above-mentioned independent variables to optimize the dependent variable or response (Y) viz. Chox concentration (U/ml) in this study. The Chox activity for each experimental run was estimated in duplicate, and their average values were presented in **Table 4.2**. The experimental data were further analyzed using multiple regression and a second-order polynomial model fitted for predicting optimal levels was expressed in Eq. 4.1:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \text{(4.1)}$$

Where, Y is the predicted response, β_0 is the intercept coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient. The effect of the variables on the response and their interaction has been analyzed by conducting tests of significance and ANOVA to check the adequacy of the model. The optimized variables were chosen by using the response optimizer function of Minitab 17.0 software.

Table 4.1 Independent Variables Chosen for the CCD

Factor codes	Independent variables	Unit	Coded factor levels				
			-α	**-1**	**0**	**+1**	**+α**
X_1	pH of media	-	3.9324	6	7.5	9	11.0676
X_2	Inoculum age	hours (h)	5.1885	30	48	66	90.8115
X_3	Inoculum size	% (v/v)	2.3309	7.5	11.25	15	20.1691
X_4	Fermentation period	hours (h)	-13.623	36	72	108	157.623
X_5	Incubation Temperature	°C	6.2159	20	30	40	53.7841
X_6	Shaking speed	rev/min (rpm)	-3.381	100	175	250	353.381

The input range of the six independent variables (-1 to +1) were taken on the basis of results obtained from the preliminary experiments.

Table 4.2 Central Composite Design for Six Independent Variables and One Response (Cholesterol oxidase concentration) with RSM and ANN Predicted Activity for Chox (U/ml)

Run Order	pH of media	Inoculum age	Inoculum size	Fermentation Period	Temperature	Shaking speed	Chox Activity (U/ml)		
							Experimental	RSM Predicted	ANN Predicted
1	7.5	48	11.25	-13.623	30	175	1.500	1.7608	1.50
2	7.5	48	11.25	72	30	175	3.890	4.0511	4.0617
3	7.5	48	11.25	72	30	175	4.040	4.0511	4.0617
4	9	66	7.5	108	20	250	3.040	2.8916	2.9653
5	6	66	15	36	20	100	2.800	2.6103	2.8000
6	9	30	15	36	20	100	2.770	2.6230	2.7701
7	6	30	15	108	40	250	1.540	1.5855	1.5399
8	9	30	15	108	40	100	2.150	2.1821	2.1500
9	7.5	48	11.25	72	30	175	4.150	4.0511	4.2117
10	6	66	15	108	20	250	1.700	1.9578	1.6999
11	7.5	48	11.25	72	53.7841	175	1.400	1.2402	1.4000
12	9	30	7.5	36	20	250	2.990	3.0401	2.9898
13	9	66	7.5	36	20	100	1.940	1.8341	1.4381
14	7.5	48	11.25	72	30	175	4.110	4.0511	4.0617
15	6	30	15	108	20	100	3.850	3.9209	3.8498
16	6	30	7.5	108	40	100	2.880	3.0573	2.8800
17	9	30	7.5	36	40	100	2.530	2.3934	2.5299
18	3.9324	48	11.25	72	30	175	1.750	1.6293	2.0265
19	9	66	7.5	36	40	250	2.910	2.7063	2.9099
20	6	30	15	36	40	100	2.240	2.3921	2.2400
21	6	30	7.5	108	20	250	3.500	3.4707	3.4999
22	7.5	90.8115	11.25	72	30	175	1.730	2.2532	1.2827
23	7.5	48	11.25	72	30	353.381	2.992	3.1280	3.0113
24	9	30	7.5	108	20	100	3.810	3.8522	3.2103
25	7.5	48	11.25	72	30	175	4.122	4.0511	4.0617

26	9	30	7.5	108	40	250	3.650	3.2969	2.9887
27	6	66	15	108	40	100	2.510	2.3804	2.5099
28	9	66	15	108	40	250	2.490	1.9286	2.4899
29	7.5	5.1885	11.25	72	30	175	3.670	3.4789	3.6699
30	7.5	48	11.25	72	30	175	4.116	4.0511	4.0617
31	6	66	7.5	36	40	100	2.500	2.6671	2.4999
32	6	30	7.5	36	40	250	2.150	1.6253	2.1499
33	6	66	7.5	36	20	250	1.790	1.6530	1.7899
34	6	66	7.5	108	40	250	1.942	2.1207	1.6703
35	7.5	48	11.25	72	6.2159	175	1.770	2.2435	1.7699
36	7.5	48	11.25	157.623	30	175	2.950	2.7959	2.950
37	9	66	15	108	20	100	1.850	1.9673	1.8501
38	9	66	15	36	20	250	2.130	1.9094	1.6292
39	11.067	48	11.25	72	30	175	0.740	1.8174	0.7400
40	7.5	48	20.1691	72	30	175	3.050	3.0884	2.6465
41	9	66	15	36	40	100	2.840	2.0986	2.8401
42	6	66	7.5	108	20	100	2.970	2.7009	2.9699
43	7.5	48	2.3309	72	30	175	3.440	4.0123	3.4400
44	7.5	48	11.25	72	30	-3.381	3.894	3.8647	3.8940
45	6	66	15	36	40	250	1.760	1.7025	1.7599
46	7.5	48	11.25	72	30	175	4.083	4.0511	4.0617
47	9	30	15	108	20	250	3.620	3.4204	3.6197
48	7.5	48	11.25	72	30	175	3.989	4.0511	4.0617
49	7.5	48	11.25	72	30	175	4.092	4.0511	4.0617
50	6	30	15	36	20	250	2.890	2.8138	2.8898
51	9	30	15	36	40	250	1.640	1.7400	1.6399
52	6	30	7.5	36	20	100	2.875	3.0498	2.8748
53	9	66	7.5	108	40	100	3.210	3.0892	3.6338

4.2.8.2 ANN modeling

A multi-layer perceptron (MLP) feed forward back propagation type neural network (FFBP-NN) was employed using MATLAB 2012b (Math Works Inc., USA). The six determinants of Chox production (X_1, X_2, X_3, X_4, X_5, and X_6; **Table 4.1**) served as network inputs. The output (Chox concentration U/ml) was predicted by training the FFBP-NN with Levenberg-Marquardt training algorithm using MATLAB *trainlm* function. The selection of optimal neural network architecture and topology augments the predictability of the output. The MLP architecture of ANN essentially comprises an input, a hidden, and an output layer. Different architectures of FFBP-NN were designed and trained using neural network tool-box of MATLAB 2012b (Math Works Inc., USA) and the network topology of 6-25-1 was found to be optimum, illustrated in **Fig 4.1**. The *'Tansig'* and *'Purelin'* transfer functions were used in layer 1 and 2 respectively as input and hidden layers with biases at each layer. The neural network was trained and simulated on experimental values of Chox concentration as the target, the same used for RSM, (**Table 4.2**), and the entire experimental data (53 runs) from CCD were divided into 70%, 15%, and 15% for training, validation, and testing respectively. The splitting of experimental data enables to measure the performance of the neural network to predict the unseen data (not used for training) and to assess the generalization capability of ANN. Training was done until the network Mean Square Error (MSE) reached the lowest value and correlation coefficient (R) close to 1. The trained network models were validated using the validation data set (experimental data excluding the training data) for precision.

The performance of the network was evaluated in terms of MSE; the minimum MSE value imitates the optimum number of neurons in the hidden layer. Each input data

(X_i) passed through the input layer to the hidden layer hold some weights. The interconnection between neurons in the MLP network is defined by *synaptic weight* (W_{ij}), which corresponds to the extent of influence one neuron has on another, while the onset for the activation of these neurons is introduced in terms of *bias* (θ_j). The summation of the weighted outputs ($XiWij$) is added to the bias term (θ_j) and regulates the neuron input (I_j) in the outer layer, given in Eq. 4.2:

$$I_j = \sum XiWij + \theta_j \quad \text{(4.2)}$$

This input neuron has to further pass through an activation function $f(I_j)$ and transformed to output neuron by using sigmoid transform function, described in Eq. 4.3:

$$f(I_j) = 1/\ 1 + e^{-Ij} \quad \text{(4.3)}$$

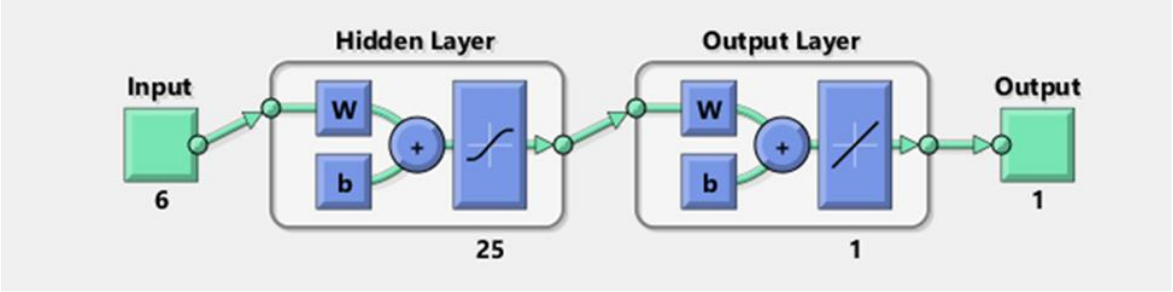
4.2.8.3 Evaluation of model predictability

The adequacy of the developed ANN models was assessed by using MSE and mean absolute percentage error (MAPE), given in Eq.s 4.4 and 4.5:

$$\text{MSE} = \frac{1}{n}\sum_{i=1}^{n}(\theta i, p - \theta i, e)^2 \quad \text{(4.4)}$$

$$\text{MAPE} = \frac{100}{n}\sum_{i=1}^{n}|ydi - yi/ydi| \quad \text{(4.5)}$$

where, n is the number of data points/experiments, $\theta i, p$ is predicted value obtained from ANN model, $\theta i, e$ is experimental value, *ydi* is the actual response and *yi* is the predicted response. The efficiency of the ANN model was evaluated based on the MSE, MAPE, and regression values obtained. The network performance was evaluated by the Performance plot.



Network architecture of 6-25-1 representing the input, hidden and output layers; optimum topology for the prediction of desired response

Fig 4.1Architecture of optimized feed forward back propagation neural network

4.2.8.4 Performance evaluation of RSM and ANN models

The capability of prediction efficiency of RSM and ANN were examined by comparing the predicted responses with the experimental values. The performance of the predicted response of Chox concentration obtained from RSM and ANN were assessed in terms of coefficient of determination (R^2), the Pearson's correlation coefficient (r), and average absolute deviation (AAD). The R^2 and AAD were calculated by Eqs. 4.6 and 4.7 respectively:

$$R^2 = 1 - \sum_{i=1}^{n} \left(\frac{(yi,cal - yi,exp)^2}{yiavg,exp - yi,exp^2} \right) \quad \text{..... (4.6)}$$

$$\text{AAD } \% = \sum \left| \frac{\frac{yi,exp - y,cal}{y,exp}}{n} \right| \times 100 \quad \text{..... (4.7)}$$

Where, n is the number of experimental data, yi,cal is the calculated values, yi,exp is the experimental values, yi,avg,exp is the average experimental values. R^2 is a measure of the reduction in the amount of variability of the response by using the repressor variables in the model while AAD is a direct method to measure the dispersion or variability in the data (Betiku et al., 2015). AAD explains the deviation of predicted data from observed data.

The value of R^2 must be close to unity while the AAD between predicted and experimental data must be as small as possible (Ebrahimpour et al., 2008). Pearson's correlation coefficient (r) is a statistical measure of the linear correlation between two variables and its value lies between +1 and -1.

4.3 Results and Discussion

4.3.1. Fermentation profile

The fermentation profile of Chox produced by *S. olivaceus MTCC 6820* is presented in **Fig 4.2**. It is evident from **Fig 4.2**, the exponential phase of the cells starts from around 12 h and lasts up to 36 h, where it enters into a stationary phase which was up to 96 h, afterwards the cells enter the decline phase. Although, the production of Chox started in small quantities with the initiation of the exponential phase but maximum enzyme was produced during the stationary phase of the cells viz. 1.07 U/ml in 72 h fermentation time period. It was interesting to scrutinize here that, though there was a sharp increase in the Chox activity during the fermentation time period 48 - 72 h, at the same time the biomass slightly dropped during 36 - 48 h at the beginning of the stationary phase. Simultaneously, a pH drop in the fermentation medium was observed from 7.5 to 5.0 with the increasing cell biomass during exponential phase up to 48 h which again increased gradually in the stationary phase and remained steady afterwards. As these results were reproduced, they could not be attributed to experimental error. One possible explanation for this observation could be the utilization of carbon and nitrogen sources for the purpose of enzyme synthesis and the cells being acclimatizing to utilize the secondary carbon source (oligosaccharides present in the medium) for longer sustainability in the stationary phase.

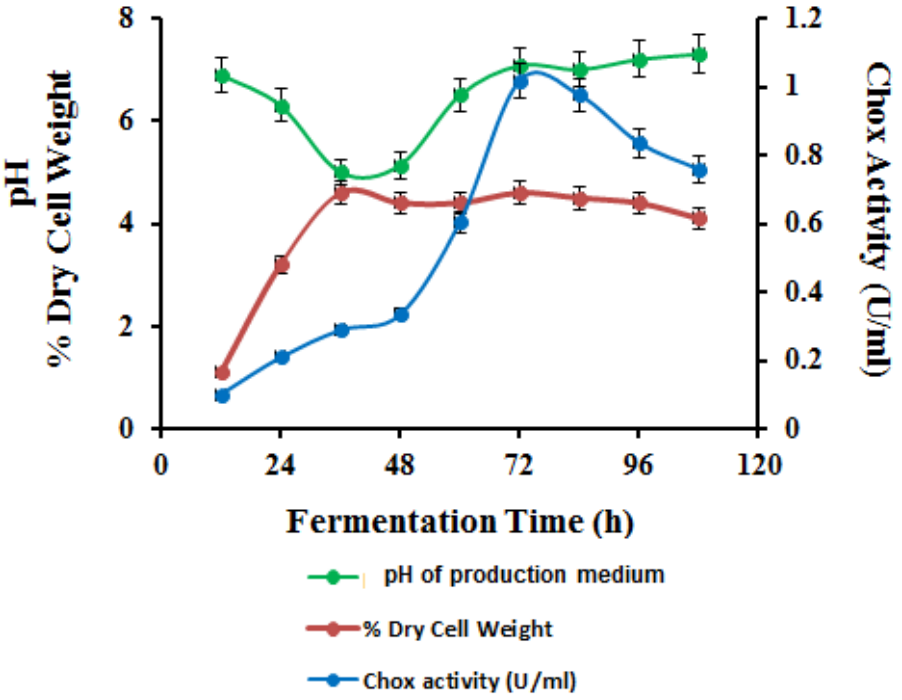


Fig 4.2 Fermentation profile of Cholesterol oxidase produced in shake flask cultures at 30˚C and 180 rpm

Since the pH drop was observed during the growth phase, which may be due to the acidic environment generated due to the accumulation of metabolic intermediates by the increased number of bacterial cells. Impermeability of bacterial cells towards highly charged chemical species allows them to accumulate charged nutrients and intermediate metabolic compounds, thus maintaining a significant difference between the internal and external concentrations of H^+ (Willem H. Kampen). The difference in H^+ ion concentration brings about the pH change in the medium. In the stationary phase (72 - 96 h), the pH was retained to 7.5 again with the maximum secretion of extracellular Chox in the medium, which shows that the pH drop has no adverse effect on the growth and Chox production. It

shows that the pH of the media plays an influential role in Chox production through the submerged fermentation process.

4.3.2 Effect of nutrients on Cholesterol oxidase production

4.3.2.1 Effect of different carbon and nitrogen sources

The effect of nutrients on enzyme production was carried out by performing supplementation experiments. Various sources of carbon and nitrogen were supplemented (1% w/v) in the production medium. The results of supplementation effect on Chox production were measured in terms of Chox concentration (U/ml). All the carbon sources used in the study enhanced the Chox production as compared to the control. Supplementation of D-mannitol, glycerol, and starch imparted approximately three folds increase in the Chox production. The maximum Chox production was observed in the D-sorbitol and oatmeal supplemented medium with 3.7 and 3.68 folds increment in Chox production respectively **(Fig. 4.3)**. However, the use of oatmeal increased the viscosity of the medium which was problematic for the recovery of supernatant of fermentation broth for enzyme activity estimation. D-sorbitol was sparingly soluble in the medium and was easily available for microbial metabolism thereby increasing the rate of cell growth and enzyme production; therefore it was chosen for further experiments. It was observed that dextrose did not enhance the Chox activity substantially; while a significant increase in Chox production was observed with other carbon sources. One possible explanation for this may be that the maximum enzyme was secreted in the stationary phase **(Fig. 4.2)**, where Chox was produced as a secondary metabolite by *S. olivaceus*. It may be noted that genus *Streptomyces* are well characterized as the producers of valuable antibiotics; most of which are secondary metabolites. A large amount of energy reservoir made available by the

disaccharides supports primary cell growth and the cell viability sustains till the stationary phase to exhibit the secondary metabolism. While on the other hand, dextrose being a monosaccharide is quickly consumed by the organism in cell growth and does not sustain till the stationary phase to support enzyme secretion as well. Yazdi et al. (1999) used 1.2% glucose and 0.9% starch to enhance Chox production. Nene and Varma used 1.5 %(w/v) potato starch to enhance the Chox production (Nene and Varma, 2003).

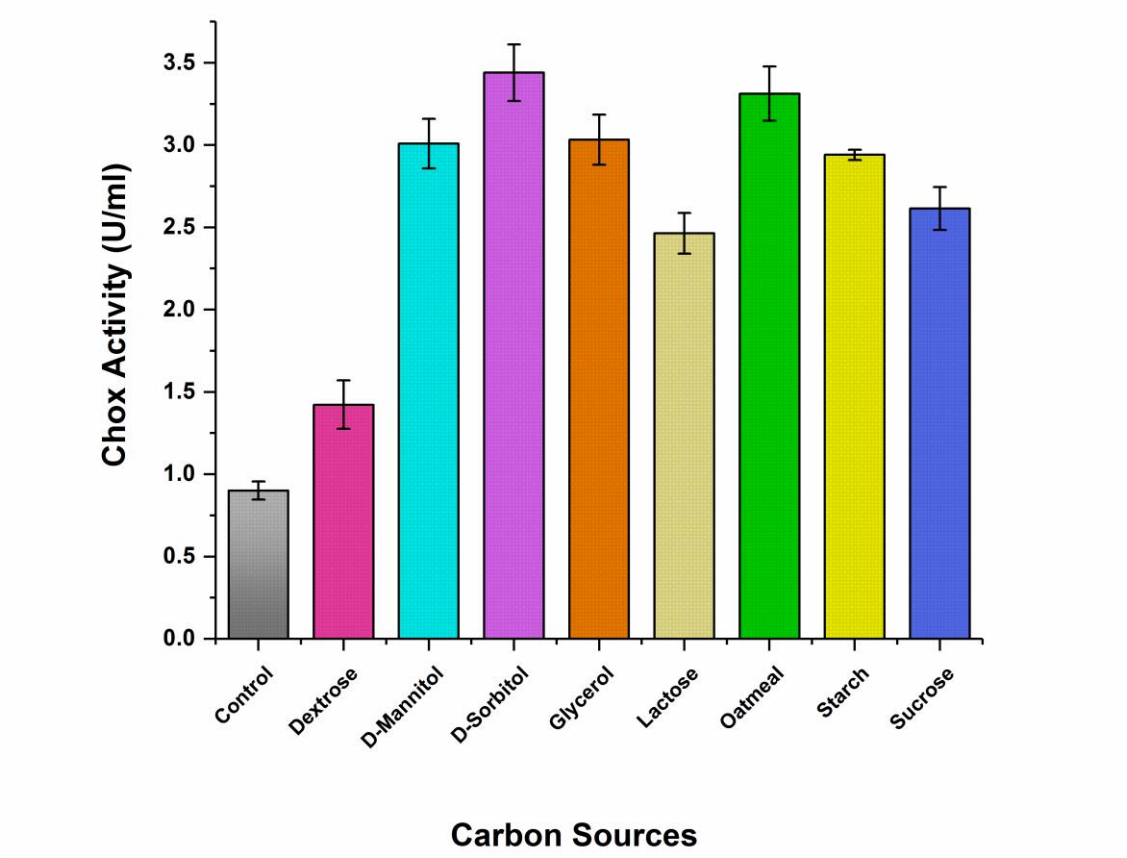


Fig. 4.3 Effect of different carbon sources on Cholesterol oxidase production

The effect of different nitrogen sources on Chox production is displayed in **Fig. 4.4**. Supplementation of peptone in the production medium showed nearly two folds increase in Chox production. Casein hydrolysate and yeast extract showed 1.83 and 1.7

folds increase in enzyme production while the combination of yeast extract and peptone significantly enhanced the enzyme production showing three folds increment. The maximum enzyme production was achieved with the supplementation of soyabean meal showing 3.2 fold increase in Chox production as compared to the control; it was selected for further studies. Complex nitrogen sources consist of essential amino acids necessary for protein synthesis. Similar results were obtained by Yazdi et al. where the cell growth and enzyme production was enhanced by using the combination of yeast extract and peptone (Yazdi et al., 1999).

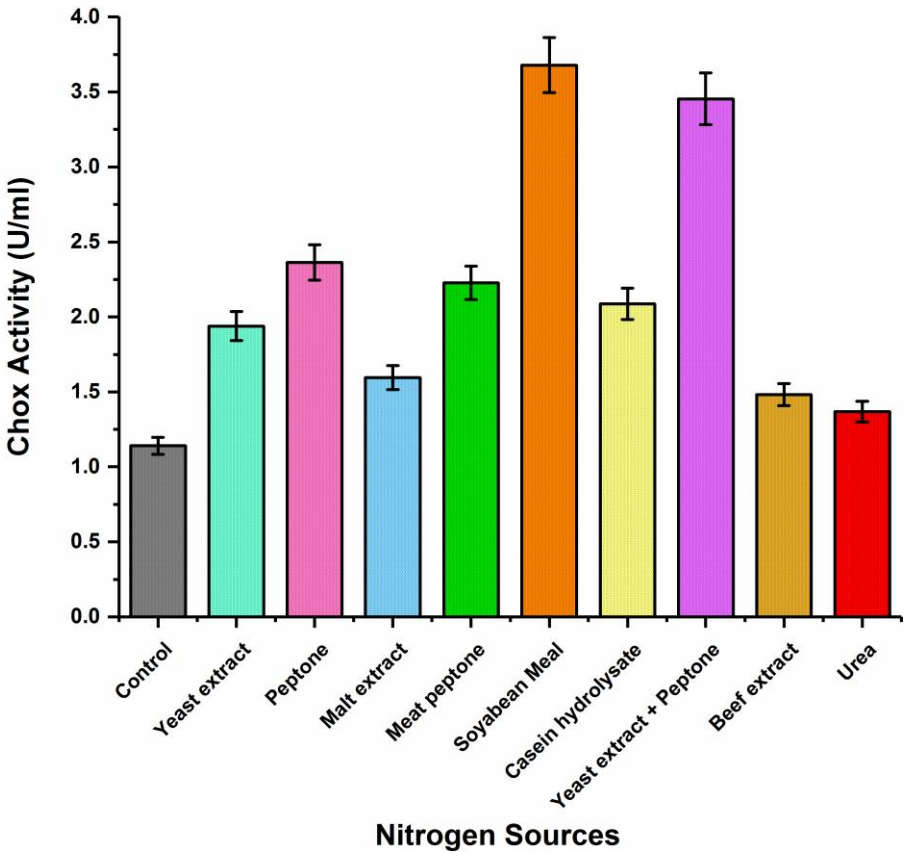


Fig. 4.4 Effect of different complex nitrogen sources on Cholesterol oxidase production

4.3.2.2 Effect of surfactants

The effect of various surfactants on Chox production was studied **(Fig. 4.5**). Cholesterol dispersed in the production medium without detergent was used as a control. Cholesterol is a water-insoluble molecule. Non-ionic detergents have been used by researchers for the solubilization of cholesterol. The efficiency of solubility of cholesterol depends upon the type of surfactant used in the medium. All the surfactants used in the production medium increased the Chox production except Dodecanol. Tween - 20 showed maximum Chox production (3.905 U/ml) with 3.45 fold increase, so it was selected for further studies. It shows that Tween - 20 was able to solubilize cholesterol efficiently which makes them available to the microorganism in easily metabolizable form thereby augmenting the Chox production.

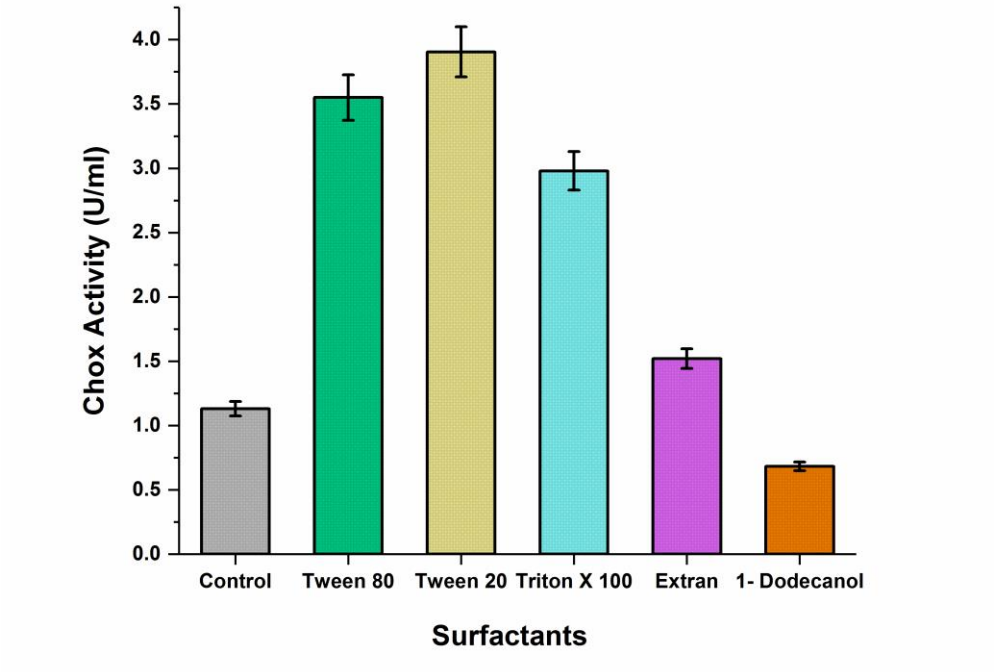


Fig. 4.5 Effect of different surfactants on Cholesterol oxidase production

4.3.2.3 Effect of inducer concentration

The study for constitutive expression of Chox was carried out using a production medium without cholesterol. It was found that no Chox production was detected in the medium without cholesterol; which means there was no constitutive expression of Chox. As Chox production was induced only in the presence of cholesterol (inducer), so cholesterol was used as an inducer in the production medium. Production medium without cholesterol was used as a control for the optimization study of inducer concentration. The suitable inducer concentration was investigated using cholesterol (solubilized with Tween - 20) at different concentrations ranging from 0 - 0.5 % (w/v) in the production medium, as shown in **Fig. 4.6**. Chox production was found to be higher at cholesterol concentrations 0.2 – 0.3 (%w/v) while the medium containing 0.25 (%w/v) cholesterol resulted in maximum enzyme production. Niwas et al. (2013) obtained 0.05 (% w/v) cholesterol concentrations as an optimum to produce maximum Chox production.

It is also evident in **Fig. 4.6.** that, initially with an increase in inducer concentration from 0.5 – 0.2(% w/v), the Chox production increased gradually; reached its maximum (3.7 U/ml) at 0.25 (%w/v) inducer concentration while on further increase beyond 0.3 (%w/v) inducer concentration the Chox production was reduced. The underlying reason for this may be attributed to the phenomena of substrate - inhibition. A greater possibility here is that, during the production of Chox, cholesterol primarily acts as an inducer while after the utilization of the preferred carbon source by the microbial population; it acts as a secondary carbon source. At higher cholesterol concentrations, cholesterol acts as an inhibitor of cell growth, leading to the reduction in Chox producing microbial cells; subsequently reducing the Chox production.

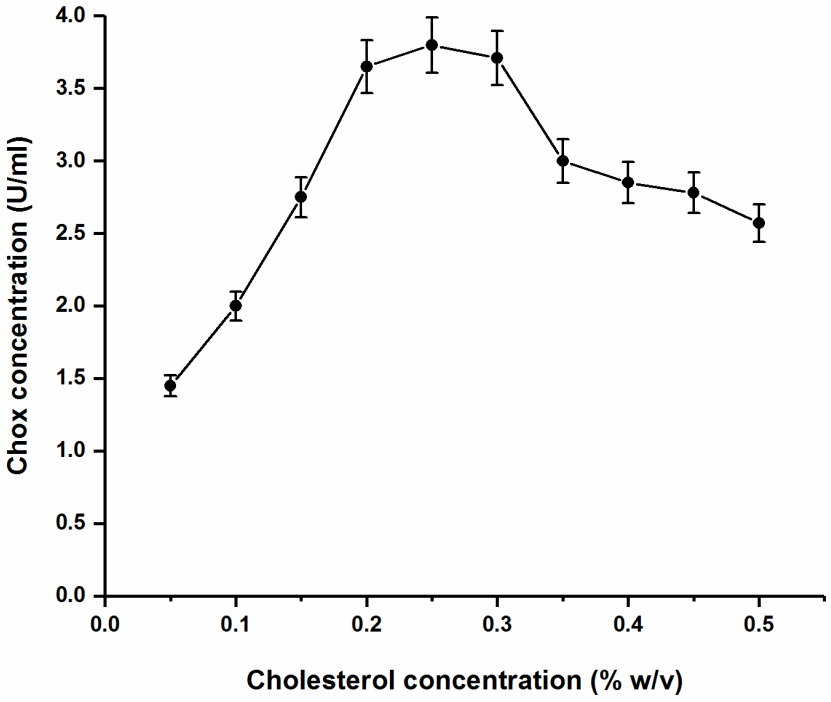


Fig. 4.6 Effect of cholesterol concentration on Cholesterol oxidase production

4.3.3 Optimization of culture conditions

4.3.3.1 Response surface regression model for Cholesterol oxidase production by RSM

RSM was performed to define the interactive effects of the culture conditions on Chox activity as well as to maximize its production. The experimental values of Chox were fitted to the quadratic equation (Eq. 4.1), and the following second-order polynomial regression equation (Eq.4.8) in coded units was obtained:

$$Y = -11.55 + 2.494\,X_1 + 0.0123\,X_2 + 0.4544\,X_3 + 0.05829\,X_4 + 0.1743\,X_5 - 0.00561\,X_6 - 0.1837\,X_1^2 - 0.000652\,X_2^2 - 0.00643\,X_3^2 - 0.000243\,X_4^2 - 0.004101\,X_5^2 - 0.000018\,X_6^2 - 0.02363\,X_1*X_3 + 0.000928\,X_1*X_4 + 0.00530\,X_1*X_5 + 0.001873\,X_1*X_6 - 0.000157\,X_2*X_4 + 0.001578\,X_2*X_5 -$$

$$0.000939\ X_3*X_4 - 0.003064\ X_3*X_5 - 0.000142\ X_3*X_6 - 0.000203\ X_4*X_5 - 0.000090\ X_5*X_6 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4.8)$$

where, Y is the response (Chox concentration, U/ml), X_1, X_2, X_3, X_4, X_5, and X_6 are the coded values of the independent variables viz. pH of media, inoculum age, inoculum size, fermentation period, incubation temperature, and shaking speed respectively. Four interaction terms X_1X_2, X_2X_3, X_2X_6, and X_4X_6 were not found to support model hierarchy and are highly insignificant (*P >0.1)*, therefore these terms were eliminated from the RSM model for better curve fitting.

The significance of the regression coefficient was tested by *t-test*. The *P*-values explain the significance of the interaction effects, which indicate the patterns of the interactions among the variables (Ebrahimpour et al., 2008; Montgomery, 1991). The significance of each individual factor and their interaction effects on Chox production were described by their corresponding *P*-values, (**Table 4.3**). The individual terms in the model as X_2, X_3, X_4, X_5, and X_6 were significant terms, the quadratic terms as X_1^2, X_2^2, X_3^2, X_4^2, X_5^2, X_6^2, and the terms X_1X_3, X_1X_5, X_1X_6, X_2X_4, X_2X_5, X_3X_4, X_3X_5, X_4X_5 were found to be the significant interaction terms with *P-values <0.05* (95 % confidence level, a = 0.05). The individual term X_1 was found to be insignificant (P >0.05), but the corresponding interactions terms X_1X_3, X_1X_5, X_1X_6 showed significant interaction. The interaction terms X_1X_4, X_3X_6, and X_5X_6 were found to be insignificant with *P-values >0.05*.

4.3.3.2 Statistical analysis by ANOVA

Multiple regression analysis was done to analyze the RSM data. The goodness of fit of the model was described by the coefficient of determination (R^2), found to be 0.9709 in this case, representing 97.09 % of the sample variation attributed to the testing variables and only 2.91 % of the total variance could not be explained by the model. The R-sq (adj) and R-sq (pred) were found to be 94.79 % and 87.22 % respectively, which reflected a very good fit between the observed and the predicted responses, inferring that

the model is reliable for Chox production in the present study. The *P*-value for lack of fit of the model (0.002) in **Table 4.4**, was very low which means that the model adequately describes the relationship between the factors and the response variable.

The test of significance and the adequacy of the model were presented by ANOVA, (**Table 4.4**). The ANOVA of the quadratic regression model shows that the model ishighly significant as is evident from the high *F* value (42.14) and very low valueof *P* (0.000) obtained from Fisher's F test. This implies that the combinatorial influence of all the independent variables substantially contributed to maximizing the response, i.e., Chox production.

Table 4.3 Regression Analysis of CCD Showing Model Coefficients and Significance of Regression Coefficient for Cholesterol oxidase Activity

Term	Coef	SE Coef	T-Value	P-Value
Constant	4.0617	0.0677	60.01	0.000
X_1	0.0395	0.0309	1.28	0.211
X_2	-0.2577	0.0309	-8.34	0.000
X_3	-0.1942	0.0309	-6.28	0.000
X_4	0.2176	0.0309	7.04	0.000
X_5	-0.2109	0.0309	-6.82	0.000
X_6	-0.1549	0.0309	-5.01	0.000
X_1^2	-0.4134	0.0264	-15.66	0.000
X_2^2	-0.2114	0.0264	-8.01	0.000
X_3^2	-0.0904	0.0264	-3.42	0.002
X_4^2	-0.3152	0.0264	-11.94	0.000
X_5^2	-0.4101	0.0264	-15.54	0.000
X_6^2	-0.0999	0.0264	-3.79	0.001
X_1*X_3	-0.1329	0.0360	-3.70	0.001
X_1*X_4	0.0501	0.0360	1.39	**0.174**
X_1*X_5	0.0795	0.0360	2.21	0.035
X_1*X_6	0.2107	0.0360	5.86	0.000
X_2*X_4	-0.1017	0.0360	-2.83	0.008
X_2*X_5	0.2840	0.0360	7.90	0.000
X_3*X_4	-0.1268	0.0360	-3.53	0.001
X_3*X_5	-0.1149	0.0360	-3.20	0.003
X_3*X_6	-0.0399	0.0360	-1.11	**0.276**
X_4*X_5	-0.0729	0.0360	-2.03	0.052
X_5*X_6	-0.0673	0.0360	-1.87	**0.071**
S = 0.2034 R-sq = 97.09 % R-sq (adj) = 94.79 % R-sq (pred) = 87.22 %				

Table 4.4 ANOVA for Quadratic model of Cholesterol oxidase Activity

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	23	40.0950	1.7433	42.14	0.000
Linear	6	9.5948	1.5991	38.65	0.000
Square	6	24.0178	4.0030	96.76	0.000
2-Way Interaction	11	6.4824	0.5893	14.24	0.000
Residual Error	29	1.1998	0.0414	-	-
Lack-of-Fit	21	1.1466	0.0546	8.21	0.002
Pure Error	8	0.0532	0.0066	-	-
Total	52	41.2948	-	-	-

4.3.3.3 Contour plots

The analysis of the interaction amongst the significant variables and prediction of their optimum conditions for Chox production was represented with the help of contour plots (**Fig 4.7 a-h**). Interactions of pH with inoculum size, incubation temperature, and shaking speed are shown in **Fig 4.7(a-c**), respectively, the change in color of the contour indicates that the production of Chox was affected mainly by the change in pH of the medium as compared to other parameters studied. With the increase in medium pH, the production of Chox increased until it reaches the optimum pH (7.5), whereas a further increase in pH decreased its production. A pH drop from 7.5 to 5.0 in the fermentation broth was observed (Fig 4.1) after 24 h fermentation period, while the pH again increased to 7.5 after 60 h fermentation period. Since this pH drop was observed during the growth phase, it may be attributed to the acidic environment generated due to the accumulation of metabolic intermediates by the increased number of bacterial cells. Bacterial cells are impermeable to highly charged chemical species present in the medium. This allows the cell to contain a reservoir of charged nutrients and intermediate metabolic compounds, thus

maintaining a significant difference between the internal and external concentrations of small cations (ex. H^+, K^+, Na^+) (Willem H. Kampen). The difference in H^+ ion concentration brings about the pH change in the medium. In the stationary phase (72 - 96 h), the pH was retained to 7.5 again with the maximum secretion of extracellular Chox in the medium, which shows that the pH drop in the growth phase has no adverse effect on the Chox production. This phenomenon gives an idea that the pH of the media plays an influential role in Chox production through the submerged fermentation process. The interaction of shaking speed with pH (**Fig 4.7 (c**) shows maximum Chox production at 175 rpm and pH 7.5. The change in the color of contour in **Fig 4.7 (c)** indicates that the Chox production decreased on further increasing the shaking speed. *Streptomyces* is a shear-sensitive microorganism belonging to the group Actinobacteria. An increase in shaking speed more than 200 rpm causes damage to the cell structure due to unbearable shear force, thereby increasing the cell mortality rate in the fermentation medium, leading to a reduction in the Chox production.

The interaction of inoculum age with fermentation period and incubation temperature respectively was significant (**Table 4.3**) and showed a positive impact on Chox production, presented in **Fig 4.7 (d - e)**. With the increase in inoculum age up to 48 h, the Chox production increased to its maximum while subsequently decreased on further increase in inoculum age. Inoculum age of 48 h was found to be optimum for *S. olivaceus* MTCC 6820, ascertaining that it is a slow-growing microorganism as compared to other bacteria. The response was also influenced by incubation temperature; increasing the incubation temperature above 30°C led to a decrease in Chox production, **Fig 4.7 (d**), while it remained less influenced with the fermentation period **Fig 4.7 (e**). As shown in **Fig**

4.7 (f), the fermentation period has a positive impact on Chox production, and maximum production was obtained in the stationary phase which started around 72 h and lasted up to 120 h. The simultaneous increase in the fermentation period and inoculum size resulted in the enhanced Chox production; it decreased sharply on a further increase of inoculum size beyond the optimum 11.25 % (v/v). The interactive effect of inoculum size and incubation temperature has significant positive effects, as shown in **Fig 4.7(g**), the production of Chox improves with the increase in both the culture parameters until its optimum is reached, further increase in both the parameters causes a decline in the production of Chox. **Fig 4.7(h),** explains an equal effect of both the culture parameters on Chox production, as the rapid change in the color of contour indicates improvement in response with the simultaneous increase in fermentation period and incubation temperature till it reaches its optimum and decreases sharply on further increase. The optimal levels of fermentation conditions are media pH (7.5), inoculum age (48 h), inoculum size (11.25%), fermentation period (72 h), incubation temperature (30°C), and shaking speed (175 rpm). Graphical analysis was combined with the numerical optimization and the production of Chox obtained was 4.05 U/ml under these optimum culture conditions.

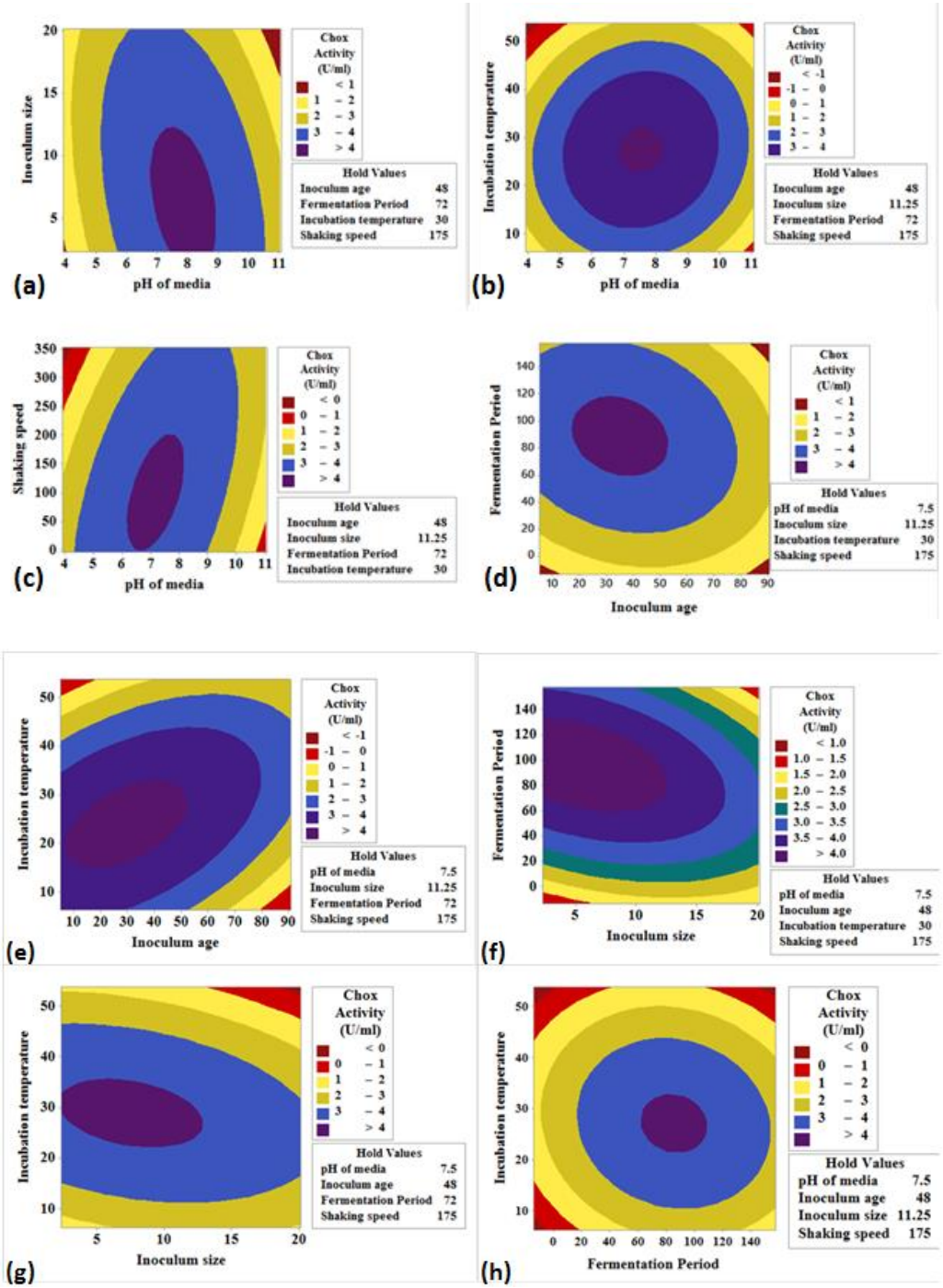


Fig.4.7 Contour plots showing the interactive effect of selected independent variables on Cholesterol oxidase activity **(a)** pH and inoculum size **(b)** pH and incubation temperature **(c)** pH and shaking speed **(d)** inoculum age and fermentation period **(e)** inoculum age and incubation temperature **(f)** inoculum size and fermentation period **(g)** inoculum size and incubation temperature **(h)** fermentation period and incubation temperature

4.3.3.4 ANN Modeling

Artificial neural network provides a non-linear mapping between the input and output variables based on the training directly from the raw data, which enables it to minimize the error between the target data and the simulated output (Masoumietal., 2011). The network architecture of 6-25-1 was found to be optimum for the prediction of desired response (Fig 2). The adequacy of the ANN model was evaluated by the MSE and MAPE values. The MSE value was 0.039 and the MAPE value was 3.46%. A minimum MSE value and MAPE ≤ 10% indicates good prediction accuracy (Yadav et al., 2014; Shera et al., 2018). The regression coefficient for training, validation, and testing (0.99) were close to 1, indicating that the non-linearity in response was better captured by the ANN model. This proves the capability of ANN to be highly competent in representing the relationship between culture condition parameters (i.e., pH of media, inoculum age, inoculum size, fermentation period, incubation temperature, and shaking speed) and Choxproduction. The ANN simulated predicted values of the response (Chox concentration, U/ml) for six different culture parameters have been given in **Table 4.2**. The maximum amount of Chox produced was 4.2 U/ml under optimized culture conditions using the ANN methodology. The Chox produced by *S. olivaceus* was in a significantly good amount as compared to the maximum Chox obtained by other researchers in case of *S. lavendulae* (2.21 U/ml) (Chauhan et al., 2009), *S. badius* (1.4 U/ml) (Moradpour et al., 2013), *Brevibacterium sp.* (1.469 U/ml) (Zhang and Yang, 2012).

4.3.3.5 Performance evaluation of RSM and ANN models

The values of Chox activity (U/ml) predicted by ANN are closer to the actual experimental values as compared to the RSM predicted Chox concentration (**Fig 4.8a**). The regression coefficient (R^2) between RSM predicted Chox activity and the actual experimental production of Chox was 0.90 whereas (R^2) between the ANN predicted and experimental Chox activity was 0.96 (**Fig 4.8b**), it means the ANN predicted Chox activity is more close to the experimental Chox activity. It shows that ANN is a better predictor than RSM, so the ANN model is superior to the RSM model. Pearson's correlation coefficient (r) is a very good statistical method indicating how strong a relationship is between two variables. The value of 'r' ($ANN_{0.98} > RSM_{0.95}$) shows that ANN predicted values are closer to actual experimental values as compared to the RSM predicted values. The value of 'r' confirms that ANN is a better predictor than RSM. The absolute average deviation (AAD) for ANN (3.46 %) and RSM (9.87 %) reflects a higher deviation in RSM data than ANN. Singh et al.(Singh, 2013) obtained 18.47 % and 1.17 % AAD values for RSM and ANN respectively. By the above three statistical measures, i.e., regression coefficient (R^2), Pearson correlation coefficient (r), and AAD, it was proved that ANN methodology was superior to RSM for the prediction of experimental data.

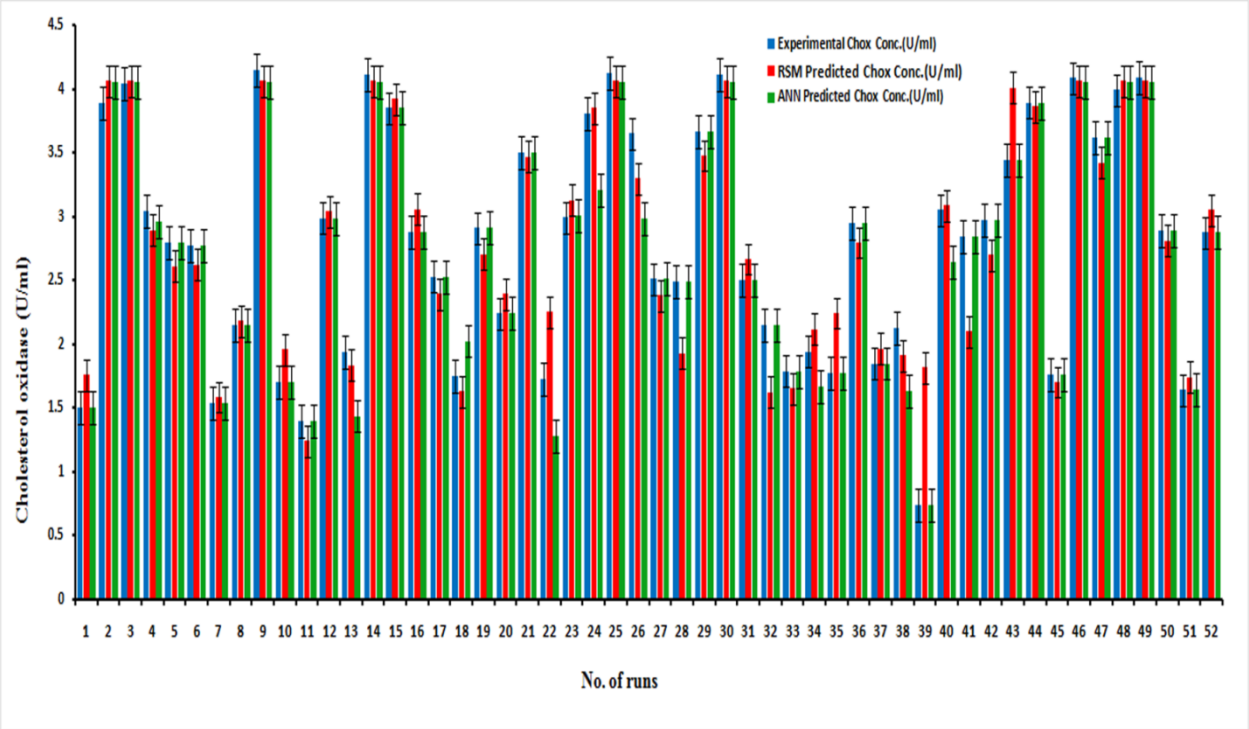


Fig. 4.8(a) Comparison of observed and predicted Cholesterol oxidase concentration (U/ml) for RSM and ANN models

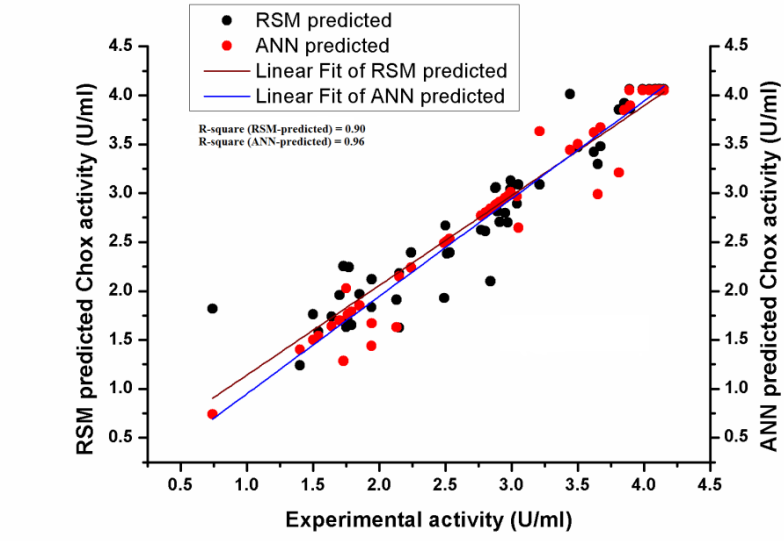


Fig. 4.8(b) Regression coefficients (R^2) for the RSM and ANN predicted Cholesterol oxidase concentration (U/ml). R^2 for RSM predicted Chox concentration is 0.90 while R^2 for ANN predicted Chox concentration is 0.96

4.3.3.6 Experimental validation of the model

The verification of the optimization results and accuracy of the model was accomplished by performing the experiments thrice under optimized culture conditions i.e., pH of the media (7.5), inoculum age (48 h), inoculum size (11.25 %), fermentation period (72 h), incubation temperature (30°C) and shaking speed (175 rpm). Under these culture conditions, the maximum Chox produced was 4.2 ± 0.51 U/ml, which corresponds very well to the value predicted by the ANN model.

4.4. Conclusion

The optimization of physical parameters for Chox production by *S. olivaceus* MTCC 6820 through submerged fermentation in shake flask culture was investigated. The carbon and nitrogen sources showing increase in Chox production were D-Sorbitol (1% w/v) and Soyabean meal (1% w/v). Medium containing inducer (cholesterol) concentration 0.25%(w/v) dissolved with Tween-20 was found to enhance Chox production. Both the RSM and ANN were employed to model the Chox production (U/ml) as a function of six independent variables and their optimum conditions were found. ANN optimized and established the crucial culture parameters and their interactions affecting Chox production. The present study signifies that ANN can be considered as an effective tool to model and predict optimum parameters for Chox production. The ANN model provided more accurate predictions than RSM with higher regression coefficient (R^2), greater Pearson correlation coefficient (r), and lower AAD values. The Chox production was enhanced by 2.2 fold after optimization of the culture conditions as compared to the unoptimized culture conditions (1.9 U/ml) with the maximum Chox activity reaching up to 4.2 U/ml.