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A STATISTICAL STRATEGY FOR THE PRODUCTION OF CELLULASE, XYLANASE AND α-AMYLASE BY CLADOSPORIUM *CLADOSPORIOIDES*

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ABSTRACT

In the present study Response surface methodology (RSM) was used to investigate the combined effect of relevant process variables to enhance the production of cellulase, xylanase and α-amylase under submerged fermentation by Cladosporium *cladosporioides*. The process variables included maltose, ammonium sulphate, pH and inoculum size. A 25 factorial central composite design (CCD) using response surface methodology (RSM) was employed to obtain interaction between the process variables and optimizing cellulase, xylanase and α-amylase titers. It was observed that the fungi displayed high production of cellulase, xylanase and α-amylase using economical substrate such as carrot peel. Cellulase increased up to 1.74U/mL with an approximate of 2.8 fold improvement over the previous production of 0.62 U/mL and xylanase increased up to 19.2 U/mL with an approximate of 2.2 fold over the previous production of 8.43 U/mL. The optimization of media led to2.5 fold increased in α-amylase activity up to 0.639U/mL over the previous production 0.24 U/mL.

Keywords: Cellulase, xylanase, α-amylase, carrot peel, Response SurfaceMethodology (RSM)

INTRODUCTION

The carrot (Daucus carota) is a root vegetable, which grows in tropical and subtropical regions. The worldwide agricultural industries produce large quantity of carrot peels annually of 24 million tons **(FAO.org, 2011)** causing serious problem to the environment.The crisis occurs in the countries where the agriculture is large and intensive is negligence of agro wastes. Inadequate methods of disposal of solid waste can cause various hazards, such as air pollution, deterioration of water quality and negative impacts on public health. Microorganisms are the most important sources for enzyme production.The production of enzymes for industrial, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper **(Alariya *et al.*,2013).**

The cellulase has three major hydrolases: the endo-β-1,4-glucanases (EG I, EG II, EG III, EG IV, and EG V; EC 3.2.1.4), which hydrolyze the glucosidic bonds randomly in cellulose fiber; the exo- β-1,4-glucanases or cellobiohydrolases (CBH I and CBH II; EC 3.2.1.91), which act on the reducing and no reducing ends of polymers, releasing cellobiose; and the β-1,4-glucosidases (BG I and BG II; EC 3.2.1.21), which hydrolyze oligosaccharides and cellobiose into glucose **(Chandra, *et al.*, 2010).**

Xylanases are group of enzymes mainly consisting of endo-xylanase (EC 3.2.1.8) which primarily cleaves β -1, 4 linked xylan backbone and β-xylosidase (EC 3.2.1.37) which converts xylooligomers to monomeric xylose sub unit **(Ritter *et al.*, 2013).**

The α-amylase comprises a group of enzymes with a variety of different specificities that all act on one type of substrate being glucose residues linked through α- 1-1, α-1-4, α-1-6, glycosidic bonds **(Shruti *et al.*, *2016*)**. The α-amylase is used in the first step of enzymatic degradation yielding a mixture of glucose and fructose with high fructose content. The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands because it is economical when produced in large quantities **(Alariya *et al.*, 2013).**The microbial production of α-amylase is greatly influenced by the components of the culture medium, especially the carbon and nitrogen sources and physical conditions, such as the pH, temperature, agitation, level of dissolved oxygen and the inoculum concentration **(Rezaei *et al.*, 2010).**Submerged fermentation is the cultivation of the microorganism in a liquid medium containing soluble carbon source and nutrients maintained under agitation. The use of the submerged culture is advantageous because of the ease of sterilization and process control rendering it easier to engineer in these systems **(Shruti *et al., 2016*).**In the present study optimization of various process parameters were done under submerged
fermentation by response surface methodology for enhanced cellulase, xylanase and α-amylase production.

MATERIALS AND METHOD

Microorganism and inoculum preparation

The fungi Cladosporium *cladosporioides* was isolated from soil and was maintained on Potato Dextrose Agar (PDA). The spore suspension was prepared by using sterile distilled water and thenumber of spores present per ml were 4.87×10^7**(Sharma,1989)**.

Submerged fermentation

A submerged fermentation was carried out with the concentration of 5.8 % (w/v) of substrates (carrot peel),and 0.8% (w/v) of maltose and ammonium sulphate as carbon and nitrogen source.Production medium was inoculated and set in static condition for 7 days at 30°C. The fermented biomass were filtered with Whatman filter papers and centrifuged at 10000×g for 10 min at 4°C, and the clear cell-free supernatant was used as a source of enzymes.

Cellulase and α-amylase assay

Cellulase and α-amylase activity were determined by carboxymethyl cellulose assay (CMC) (Ghose, 1987). Initially 0.5 ml of enzyme was addedto 0.5 ml of 0.05M citrate buffer (pH 5.0)and transferred into test tubes containing 0.5 ml of 1% (w/v)CMC and incubated at 50º C for 30 minutes in water bath. After 30 minutes, DNS (3ml) was added. Cellulase activity was determined in calorimeter at 540nm **(Miller, 1959)**.

Xylanase assay

A volume of 500µl of diluted enzyme was added to 1.5 ml of 1% (w/v) birch wood xylan (sigma). The xylanase obtained was vortexed vigorously and incubated at 50º C for 5 minutes, and DNS was added to test tubes, mixed and kept in water bath for 5 min and absorbance was measured at 540nm. D-xylose (sigma) was used as standard solution of this reaction. Xylanase activity was expressed in international units, IU, where 1IU=µmole reducing sugar (xylose) liberated per minute **(Bailey *et al.*,1992).**

Experimental design and statistical analysis

To determine which variable affects cellulase, xylanase and α-amylase production the different variables have been studied. Maltose (X_1), ammonium sulphate (X_2), pH (X_3) and inoculum size (X_4) were considered for Cladosporium *cladosporioides* (Table 1).A fractional factorial design (2^4) was applied to elucidate the process parameters for cellulase, xylanase and α-amylase production by Cladosporium *cladosporioides* inoculated on carrot peel as substrate (Table4).The package MATLAB software version 7.5.0.342 was used to analyze the experimental, the predicted data and the plotting response surface plots **(Rashmi *et al*.,2014).**The design also suggested that parameters for optimizing enzymes with expected value of enzyme at the certain desirability were considered. The central composite design (CCD) was used to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the process variability with cellulase, xylanase and α-amylase production.

Table 1 Experimental codes & levels of the variables chosen for the model

Variables	Code	Level				
		-2	-1	0	1	2
Maltose(g)	X_1	0.25	0.5	0.75	1	1.25
Ammonium Sulphate (g)	X_2	0.25	0.5	0.75	1	1.25
pH	X_3	5.5	6.5	7.5	8.5	9.5
Inoculum size (ml)	X_4	1.2	2.4	3.6	4.8	6

RESULTS AND DISCUSSION

Optimization of the selected variables

The plot figures obtained by the analysis of the experimental data of Central Composite Design (CCD) exhibited a relationship between two variables at time while maintaining third variables at constant level. These figures were helpful in understanding both linear and interaction effect of two variables. Yield of any microbial product can be improved by optimization of medium components that are required in fermentation processes. Application of statistical methodology in fermentation process development can result in improved yields of the product, reduced process variability, closer confirmation of the output response to normal and target requirements, reduced development time with overall costs **(Mrudula *et al*.,2011a)**.

Leading the completion of experiments, the maximum cellulase, xylanase and α-amylase activity were taken as the response (Y). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the responsemeasured to the independent variables. The second-order regression equations provided the levels of the relation of the four variables for enzymes yield is:

$$Y= c_0 + c_1X_1 + b_2X_2 + c_3X_3 + c_4X_4 + c_5X_1X_2 + c_6X_1X_3 + c_7X_1X_4 + c_8X_2X_3 + c_9X_2X_4 + c_{10}X_3X_4 + c_{11}X^2_1 + c_{12}X^2_2 + c_{13}X^2_3 + c_{14}X^2_4$$

Where Yis the predicted yield of cellulase, xylanase and α-amylase activity U/mL respectively for process parameters.Response surface methodology (RSM) using CCD was applied to determine the optimum level of thefour selected variables that affected enzymes production, and the mean predicted and observed responses are presented (Table 2). The regression equations obtained after the analysis of variance (ANOVA) provided the levels of enzymes produced as a function of the values of X_1, X_2, X_3 and X_4 size. The production of cellulase, xylanase and α-amylase were calculated by the following equation:

Cellulase activity (U/ml)

$$Y_1= -1.0221+0.9468X_1 + 0.8391X_2 - 0.0997X_3 + 0.6771X_4 - 0.9769X_1X_2 + 0.0725X_1X_3 + 0.0725X_1X_4 + 0.0358X_2X_3 - 0.0444X^2_2X_4 - 0.0476X_3X_4 - 0.2038X^2_1 - 0.1890X^2_2 + 0.0215X^2_3 - 0.0288X^2_4.$$

Xylanase activity (U/ml)

$$Y_2= -33.7585 - 1.8621X_1 - 17.4836X_2 + 15.4470X_3 - 2.6039X_4 + 0.7514X_1X_2 - 0.0609X_1X_3 + 2.9411X_1X_4 + 2.7157X_2X_3 + 1.4785X_2X_4 + 0.2417X_3X_4 - 4.2782X^2_1 - 7.3936X^2_2 - 1.1554X^2_3 - 0.2988X^2_4.$$

α-amylase activity (U/ml)

$$Y_3= -1.2755 + 0.1428X_1 + 0.1899X_2 + 0.4212X_3 - 0.0511X_4 - 0.2033X_1X_2 - 0.0417X_1X_3 + 0.0465X_1X_4 - 0.625X_2X_3 + 0.0403X_2X_4 + 0.0036X_3X_4 - 0.928X^2_1 - 0.0728X^2_2 - 0.0213X^2_3 - 0.0078X^2_4.$$

Analysis of Variance presents thevalidity of the model and could explain whether this model adequately fits the variation observed in enzyme produced at the designed level. A p value lower than 0.05 indicated that the model is considered to be statistically significant **(Myers, 2002).** From the ANOVA, the interactions between X_1, X_1X_2, X_3X_4; interactions between X_3, X_1X_4, X_2X_3, X_2X_4, N^2; and interactions between X_3, X_2X_3 are significantly contributed to cellulase, xylanase and α-amylase production from carrot peel (Tables 3, 5, 7).The p-value suggested that the coefficient for the linear effect ($p< 0.05$) and ($p< 0.01$) were statistically analyzed for cellulase, xylanase and α-amylase production. The R^2 values were close to 1that confirms that the model was better for variabilityof experimental values to the predicted values. For a fit model R^2 should be more than 0.80 (**Joglekar*et al*.,1987).**

Table 2 Full factorial central composite design of four variables in coded and natural units along with the observed responses

Serial numbers	X_1	X_2	X_3	X_4	Cellulase: U/ml		Xylanase: U/ml		α-amylase: U/mL	
					Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1.	0.5	0.5	6.5	2.4	0.69	0.68	14.88	15.16	0.39	0.37
2.	1	0.5	6.5	2.4	0.83	0.87	14.21	14.54	0.38	0.38
3.	0.5	1	6.5	2.4	0.74	0.77	11.34	11.66	0.31	0.31
4.	1	1	6.5	2.4	0.73	0.72	11.68	11.22	0.28	0.27
5.	0.5	0.5	8.5	2.4	1.04	1.00	14.90	15.20	0.51	0.49
6.	1	0.5	8.5	2.4	1.30	1.27	14.42	14.52	0.46	0.46
7.	0.5	1	8.5	2.4	1.10	1.14	13.87	14.42	0.35	0.37
8.	1	1	8.5	2.4	1.21	1.16	13.54	13.93	0.32	0.29
9.	0.5	0.5	6.5	4.8	0.89	0.89	12.25	12.82	0.26	0.27
10.	1	0.5	6.5	4.8	0.97	0.96	16.76	15.73	0.37	0.34
11.	0.5	1	6.5	4.8	0.88	0.93	11.68	11.09	0.27	0.27
12.	1	1	6.5	4.8	0.78	0.76	13.54	14.19	0.28	0.28
13.	0.5	0.5	8.5	4.8	0.96	0.99	14.06	14.02	0.42	0.41
14.	1	0.5	8.5	4.8	1.22	1.13	16.23	16.87	0.46	0.43
15.	0.5	1	8.5	4.8	1.16	1.07	14.39	15.02	0.36	0.34
16.	1	1	8.5	4.8	0.93	0.96	18.82	18.05	0.30	0.31
17.	0.25	0.75	7.5	1.2	0.93	0.91	15.10	14.31	0.40	0.40
18.	1.25	0.75	7.5	1.2	0.94	0.99	16.42	16.73	0.37	0.39
19.	0.75	0.25	7.5	1.2	0.96	0.99	16.25	15.90	0.45	0.48
20.	0.75	1.25	7.5	1.2	0.92	0.92	13.71	13.58	0.31	0.30
21.	0.75	0.75	5.5	1.2	0.88	0.82	9.82	10.02	0.22	0.21
22.	0.75	0.75	9.5	1.2	1.27	1.35	14.60	13.93	0.34	0.36
23.	0.75	0.75	7.5	6	0.82	0.82	14.60	13.98	0.35	0.36
24.	0.75	0.75	7.5	6	0.88	0.95	16.10	16.53	0.31	0.34
25.	0.75	0.75	7.5	3.6	1.05	1.00	16.88	16.59	0.39	0.37

Table 3 Regression coefficient results from the data of Central Composite Design Experiments for Cellulase activity

Serial numbers	Parameters	Co-efficient	Standard error	t-value	p-value
1	Constant	-1.02	1.57	-0.65	0.53
2	X_1	0.94	0.78	1.20	0.25
3	X_2	0.83	0.78	1.07	0.30
4	X_3	-0.09	0.30	-0.32	0.74
5	X_4	0.67	0.17	3.83	0.003*
6	X_1X_2	-0.97	0.29	-3.34	0.0074*
7	$X_1 X_3$	0.07	0.07	0.99	0.34
8	$X_1 X_4$	0.07	0.06	-1.66	0.12
9	$X_2 X_3$	0.03	0.07	0.49	0.63
10	N_2N_4	-0.04	0.06	-0.73	0.48
11	X_3X_4	-0.04	0.01	-3.12	0.01*
12	X^2_1	-0.20	0.30	-0.66	0.52
13	X^2_2	-0.18	0.30	-0.61	0.55
14	X^2_3	0.02	0.01	1.11	0.28
15	X^2_4	-0.02	0.01	-1.69	0.12

Table 4 Analysis of variance for response surface quadratic model for Cellulase activity

Sum of squares	degree of freedom	f-value	p-value	mean square error	R^2	Adjr^2
0.05	14.00	8.44	0.0009	0.0053	0.92	0.81

Table 5 Regression coefficient results from the data of Central Composite Design Experiments for Xylanase activity

Serial numbers	Parameters	Coefficient	Standard error	*t*-value	*p*-value
1	Constant	-33.75	17.45	-1.93	0.08
2	X_1	-1.86	8.69	-0.21	0.83
3	X_2	-17.48	8.69	-2.01	0.07
4	X_3	15.44	3.37	4.57	0.0010*
5	X_4	-2.60	1.96	-1.32	0.2138
6	X_1X_2	0.75	3.24	0.23	0.82
7	$X_1 X_3$	-0.06	0.81	-0.07	0.94
8	$X_1 X_4$	2.94	0.67	4.35	0.0014*
9	X_2X_3	2.71	0.81	3.34	0.0074*
10	X_2X_4	1.47	0.67	2.18	0.05*
11	X_3X_4	0.24	0.16	1.43	0.18
12	X^2_1	-4.27	3.41	-1.25	0.23
13	X^2_2	-7.39	3.41	-2.16	0.05
14	X^2_3	-1.15	0.21	-5.40	0.0003*
15	X^2_4	-0.29	0.18	-1.58	0.14

Table 6 Analysis of variance for response surface quadratic model for Xylanase activity

Sum of squares	Degree of freedom	f-value	p-value	Mean square error	R^2	Adj. R^2
6.58	14.00	9.94	0.0004	0.65	0.93	0.83

Table 7 Regression coefficient results from the data of Central Composite Design Experiments for α- amylase activity

Serial numbers	Parameters	Co-efficient	standard error	*t*-value	*p*-value
1	Constant	-1.27	0.58	-2.16	0.05
2	X_1	0.14	0.29	0.48	0.63
3	X_2	0.18	0.29	0.64	0.53
4	X_3	0.42	0.11	3.69	0.0041*
5	X_4	-0.05	0.06	-0.77	0.45
6	X_1X_2	-0.20	0.10	-1.85	0.09
7	X_1X_3	-0.04	0.02	-1.52	0.15
8	X_1X_4	0.04	0.02	2.04	0.06
9	$X_2 X_3$	-0.06	0.02	-2.28	0.0455*
10	X_2X_4	0.04	0.02	1.76	0.10
11	$X_3 X_4$	0.0036	0.0057	0.63	0.53
12	X^2_1	-0.09	0.11	-0.80	0.44
13	X^2_2	-0.07	0.11	0.63	0.54
14	X^2_3	-0.02	0.0072	-2.95	0.14
15	X^2_4	-0.0078	0.0064	-1.22	0.24

Table 8 Analysis of variance for response surface quadratic model for α- amylase activity

Sum of squares	Degree of freedom	f-value	p-value	Mean square error	R^2	Adj. R^2
0.0075	14.00	10.98	0.0003	0.0007	0.93	0.85

The statistical significance of the developed quadratic model was determined by t-test; the proportion of variance obtained by the model was provided by the multiple coefficients of determination, R^2.The nearer the values of R^2 close to 1, the model would explain better forvariability of experimental values to the predicted values.The coefficient of determination R^2 for cellulase, xylanase and α-amylaseproduction was calculated as $R^2 = 0.92$ for cellulase, 0.93 for xylanase and 0.93 for α- amylase which are nearly equal to 1; indicating a reasonable fit of the model to the experimental data (Tables 4, 6, 8). The multiple correlation coefficient R^2 values for cellulase and xylanase and α- amylase, varied between 92% and 93% which represent appreciable fitness of the model as well as significant effects of maltose, ammonium sulphate, pH, and inoculum size on cellulase, xylanase and α-amylase production.

Analysis of variance (ANOVA) was employed to signify the variables and their interaction effects on enzyme production. **Li *et al.*,(2005)** reported that when the R^2 value was higher than 0.9 considered as very high correlation. The three dimensional response surfaces were plotted to study the interaction among the various factors selected and to determine the optimum concentration for attaining maximum cellulase, xylanase and α-amylase production. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components. The interaction effects of variables on enzymes activity (U/ml) were studied by plotting three-dimensional (3D) surface curves against two independent variables and keeping other variables at their central (0) level. The 3D curves and contour plots from the interaction between variables were depicted:

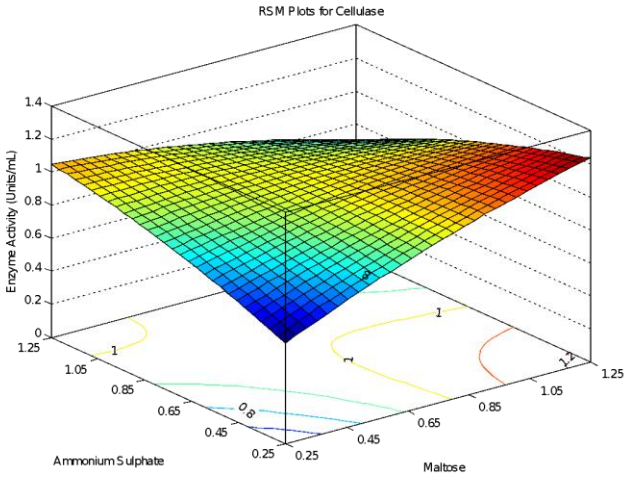

Fig. 1- 3D Response surface and contour subplots showing the relative effect of tested variables on Cellulase activity.

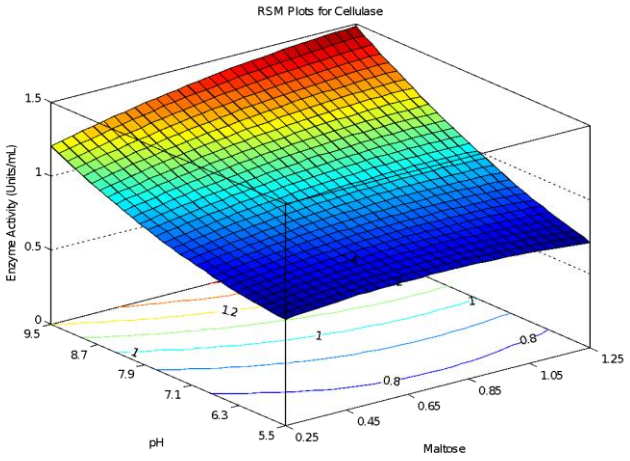

Fig. 2- 3D Response surface and contour subplots showing the relative effect of tested variables on Cellulase activity.

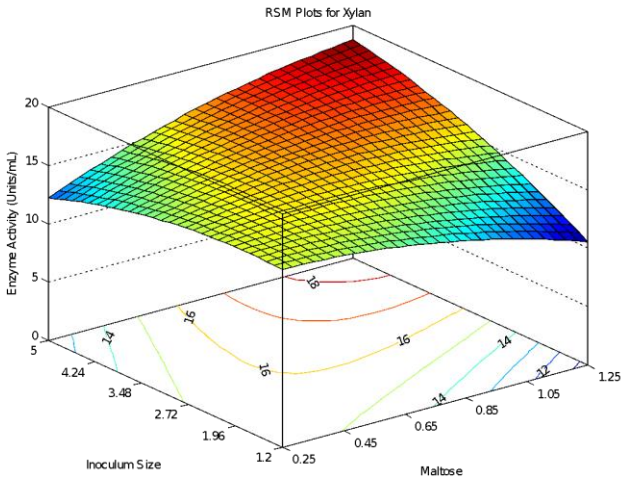

Fig. 3- 3D Response surface and contour subplots showing the relative effect of tested variables on Cellulase activity.

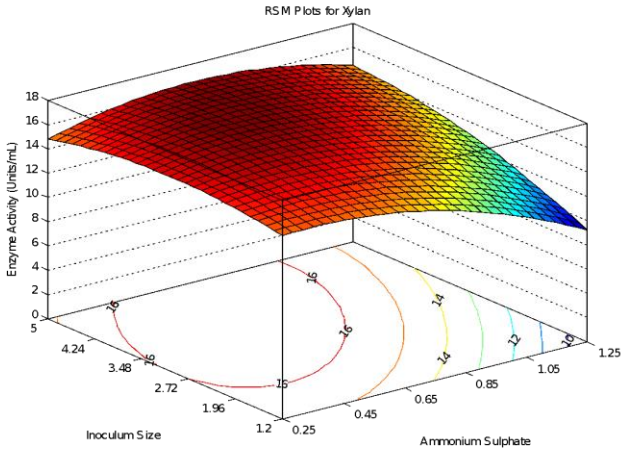

Fig. 4- 3D Response surface and contour subplots showing the relative effect of tested variables on Xylanase activity.

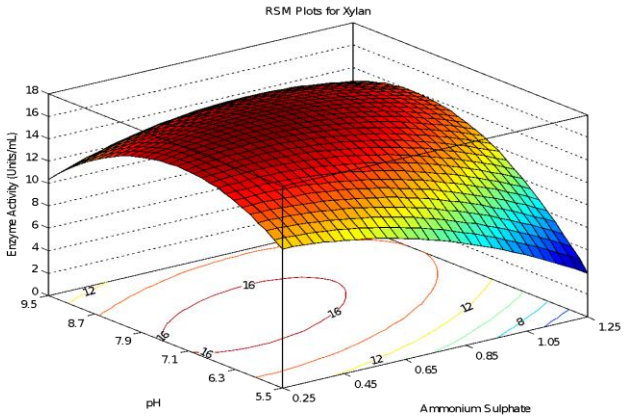

Fig. 5- 3D Response surface and contour subplots showing the relative effect of tested variables on Xylanase activity.

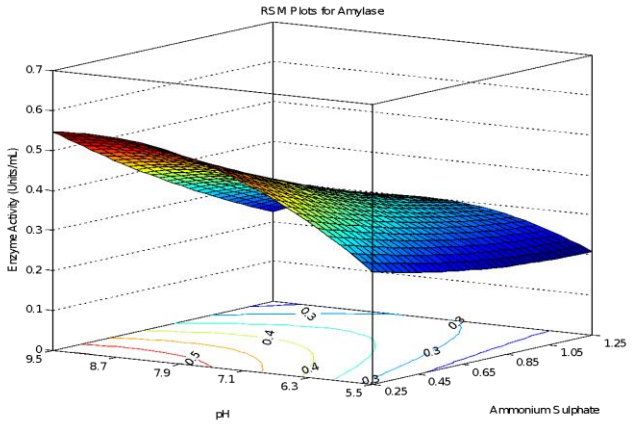

Fig. 6- 3D Response surface and contour subplots showing the relative effect of tested variables on α- amylase activity.

Fig:1 the ammonium sulphate and maltose on enzyme activity was depicted. Increasing maltose and ammonium sulphate concentration, maltose exhibited maximum cellulase activity more than ammonium concentration. Fig:2 the enzyme activity increased steadily with the increase pH and maltose concentration. RSM has been recently used for modeling and optimization of fermentation media **(Ruchi *et al.*,2008)**, conditions process **(Gao, 2005)**conditions of enzyme reaction **(Ferella *et al.*,2010)** and biohydrogen production (**Hallenbeck *et al.*,2010)**. Using RSM, the cellulolytic enzyme production from numerous microorganisms such as Scytalidium *thermophilum* **(Jatinder *et al.*,2006)**,Trichoderma *reesei* **(Hao *et al.*,2006)**, Aspergillus *heteromorphus* **(Singh *et al.*,2009)**, Debaryomyces *pseudopolymorphus* .**Barbosa *et al.*,(2010)** have been reported.Fig:3 depicts inoculum size and maltose concentration on enzyme activity. Increasing maltose concentration exhibited maximum enzyme activity with increasing inoculum size. Fig: 4 there is none effect of inoculum size on the enzyme activity was observed at low concentration of ammonium sulphate but the increase in enzyme activity was higher with inoculum size.Fig: 5 the effect of pH and ammonium sulphate on xylanase production was represented.Wide variation of enzyme activity was observed due to pH and ammonium concentration. Optimization of xylanase using RSM by different fungi and bacteria such as Penicilium *citrinum* MTCC 2553in submerged fermentation was reported **(Li, 2007** and **(Ghoshal, 2011)**. **Porsuk *et al.*, (2013)** isolated 5 groups of Streptomyces *sp* BA1, Streptomyces *sp* BA2, Streptomyces *sp* BS44, Streptomyces *sp* CA24, and Streptomyces *sp* CAH33 demonstrated approximately 50 U/ml xylanase activity. The orientation of xylanase by xylan has been found to increase number of xylanase producing by streptomycetes was reported **(Ninawe *et al.*,2008)**. Fig:6 depicts maximaα-amylase production with increase pH and less ammonium sulphate, then pH increase is very important factor for α-amylase production.The most important factors were pH, temperature and inoculum size for production of amylase from Bacillus sp was reported **(Zambare *et al.*,2011)**. **Pandey *et al.*, (2000)** reported that the physico-chemical parameters, the pH of the growth plays important role on the production of α- amylase.

Validation of the optimized condition

The optimized conditions generated during response surface methodology implementation were validated by conducting experiment on given optimal setting conditions. The experiments were performed using the predicted medium composition and the results were carried out in triplicate to confirm the results. The cellulase, xylanase and α-amylase activity obtained from the experiments were very close to the actual response predicted by the regression model, which proved the validity. At these optimized conditions xylanase had the maximum enzyme activity found to 19.20 U/ml and the fold increased to 2.2. The results obtained were in a good agreement with the predicted values as represented in linear fit model.

CONCLUSION

The present study using central composite design (CCD) of RSM is a collection of statistical techniques for designing experiments building models, evaluating the effect of factors and obtaining optimum conditions for desirable responses. Cellulse, xylanase and α- Amylase activity obtained by optimizing the medium contents was found to be significantly affected by the interaction of Cladosporium *cladosporioides* with the designed medium. Final, optimized conditions are obtained by solving inverse matrix from the equation and through statistical analysis of the constraints. The statistical design of experiment offers efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments. The maximum cellulase, xylanase and α-amylase production observed by the model were 49.23 U/mL, 135.66 U/mL, 1805.66 U/mL, 49.23 U/mL respectively.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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