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Statistical Optimization of Cellulase and Xylanase Enzyme Production by *Penicillium Crustosum* Using Sugar Beet Peel Substrate by Response Surface Methodology.

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ABSTRACT

Response Surface Methodology (RSM) is a powerful and efficient mathematical approach widely applied in the optimization process of selected variables. Cellulase and xylanase enzyme production by *Penicillium crustosum* using agrowaste substrate (sugar beet peel) was investigated by optimizing various process parameters such as carbon and nitrogen source, pH and inoculum size. In the present study optimization was based on statistical design and employed to enhance the production of cellulase and xylanase enzyme production through submerged fermentation. A fractional factorial design (2^4) was applied to elucidate the process parameters that significantly affect cellulase and xylanase production. Carbon and nitrogen source, pH, inoculum size were identified as important process parameters effecting cellulase and xylanase enzyme production. The optimum yield of cellulase and xylanase activity was (5.56 U/ml), (36.14 U/ml) respectively.

Keywords: Cellulase, xylanase, sugar beet peel, Response surface methodology (RSM).

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INTRODUCTION

Sugar beet (*Beta vulgaris*) has its origin in early nineteenth century and was introduced in India in 1950s with the aim to supplement sugar production. It is a native of the temperate climate but its cultivation has extended to subtropical countries and is being successfully grown in India, Iran, Iraq, Algeria, Egypt and Afghanistan [1]. Sugar beet peel contains a high proportion of cellulosic matter which is easily decomposed by a combination of physical, chemical and biological processes. The amount of peel produced each day is increasing with increasing population worldwide, and India is no exception. India is an agricultural country and many agricultural wastes are discarded every day. The agro wastes referred to those are end products of production and consumption that have not been used, or recycled. In some industrialized countries such as USA, China, Germany, India etc these wastes have been converted into some useful products for the improvement of energy such as gas and oil [2]. There are several studies on the use of different agricultural wastes which include bagasse [3, 4] sawdust [5, 6] wheat bran [7] and wheat straw [8] as lignocellulosic substrate for cellulase hydrolysis. Different microorganisms can produce cellulase and xylanase enzymes, with the filamentous fungus *Trichoderma reesei* mostly used in industrial enzyme production [9]. Several studies focusing on the characterization of cellulase and xylanase in terms of the nitrogen and carbon source, pH and inoculum size parameters have been made because of a large interest in understanding the action of these enzymes under different conditions. Different variables, carbon and nitrogen, pH, inoculum size, have been used in statistical experimental design such as response surface methodology (RSM) to identify the optimal values of carbon and nitrogen pH, inoculum size to analyze the relationships between these variables. In the present work, the optimization of cellulolytic and xylanolytic enzymes produced by a selected fungi *Penicillium crustosum* under submerged fermentation using RSM was carried out.

MATERIALS AND METHODS

Freshly harvested matured sugar beet (*Beta vulgaris*) was sourced from a local market in Bangalore/ Karnataka, India. The tubers were washed thoroughly with tap water to remove of sand and dirt. Thereafter it was then peeled, sun dried about 4 to 7 days, milled and sieved using a mesh size 150µ in order to obtain very fine powder and stored in polyethylene bags at room temperature.

Sample collection

Soil samples were collected from an agricultural waste compost area in and around Bangalore.

Fungi isolation and identification

A fungus was isolated and identified by serial dilution and wet mount technique [10].

Inoculum preparation

The *Penicillium crustosum* obtained from soil was grown on PDA medium in Petri dishes. Spores were transferred into conical flask (250 ml) containing 100 ml of 0.9% of saline water and these flasks were shaken continuously in controlled incubator shaker (New Brunswick Scientific Co., USA) at 200 rpm and 30^{o}C for 1 h before it was used for the fermentation. The spore concentration in the suspension was determined by counting it in a Neubauer chamber. The volume of inoculum size [11] (number of spores present per ml) were 4.92 x 10^{9} were used to inoculate the fermentation medium.

Submerged fermentation

A submerged fermentation was carried out with 5 grams of finely powered[12] sugar beet peel was taken and similarly 1% (w/v) of manitol and ammonium sulphate as carbon and nitrogen source added separately. Production medium was inoculated and set in static condition for 7 days at 30°C. The fermented sugar beet peel was filtered with muslin cloth and centrifuged at 10000 × g for 10 min at 4°C [13]. The supernatant was used as crude enzyme and was stored at 4 °C until used.

Enzyme assay

Cellulase activity was measured using an assay based on methodology given by Ghose [14.] A volume of the appropriately diluted enzyme extract was incubated at 100ºC for 10 min with 1% Carboxy Methyl Cellulose CMC (Sigma, USA) solution prepared in 0.05 mMol/L citrate buffer at pH 4.8 as a substrate. One unit of cellulase activity corresponds to 1 mmol of glucose released per minute at pH4.8 and 100^0C.

Xylanase activity was measured according to Ghose [14] by incubating a volume of the appropriately diluted enzyme extract at 50^0C for 30 min with a 1% oat spelt xylan (Sigma, USA) solution prepared in 0.5 citrate buffer pH 5.0 as a substrate. One unit of xylanase activity corresponds to 1 µmol of xylose released per minute at pH 5.0 and 50^0C. The quantification of the reducing sugar released from both the assays was performed according to the DNS method [15].

Experimental Design

The present experiment consisted of four independent variables which were manitol (X_1), potassium nitrate (X_2), pH (X_3) and inoculum size (X_4) and their possible interaction in the enzymatic activity. 2^4 factorial designs was used for this experiment. Approximately 30 experiments were designed with the combination of four factors as listed in Table 2. The design consisted of low and high level values shown in Table 1. The variable responses were cellulase and xylanase activities. The statistical software package MATLAB software version 7.5.0.342 was used to analyse the experimental data, analysis of variance (ANOVA) data and the plotting surface [16] all variables were taken at a central coded value of zero.

Table 1: Codes and actual levels of the independent variables for design of experiment.

Variables	Code	Level				
		-2	-1	0	1	2
Manitol (g)	X_1	0.25	0.5	0.75	1	1.25
Potassium nitrate (g)	X_2	0.25	0.5	0.75	1	1.25
pH	X_3	4.5	5.5	6.5	7.5	8.5
Inoculum size (ml)	X_4	1	2	3	4	5

Validation of the experimental model

To validate the model equation, experiments were conducted in triplicates for cellulase and xylanase enzyme production under optimum conditions predicted by the model.

RESULTS AND DISCUSSION

The fungal colony was identified as *Penicillium crustosum* with the following characteristics.

Penicillium crustosum is a species of *Penicillium,* within the phylum Ascomycota. The end of each conidiophore has clusters of 2 or more branches, each supporting a cluster of conideogenous cells called phialides[17].

The selected variables for optimization

The manitol, potassium nitrate, pH, and inoculum size have effect on cellulase and xylanase activities produced by the filamentous fungus *Penicillium crustosum* grown on sugar beet peel under SmF were evaluated by using the statistical design of experiments and response surface methodology analysis.
Influence of factors to target the levels of the relation of the four variables for enzymes yield was described according to equation below:

$$\mathbf{Y} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_1X_2 + b_6X_1X_3 + b_7X_1X_4 + b_8X_2X_3 + b_9X_2X_4 + b_{10}X_3X_4 + b_{11}X^2_1 + b_{12}X^2_2 + b_{13}X^2_3 + b_{14}X^2_4.$$

Where - manitol (X_1), potassium nitrate (X_2), pH (X_3) and inoculum size (X_4)

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Cellulase activity (IU/ml)

$$Y_1 = -3.9881 + 0.4303X^1 + 1.8869X^2 + 0.8701X^3 + 0.9259X^4 - 1.4600X^1X^2 - 0.1950X^1X^3 + 0.3350X^1X^4 + 0.0783X^2X^3 - 0.0850X^2X^4 - 0.0987X^3X^4 + 0.7700X^21 - 0.6967X^22 - 0.0285X^23 - 0.0935X^24.$$

Xylanase activity (IU/ml)

$$Y_2 = 12.6790 - 2.4778X_1 - 17.0667X_2 + 4.2738X_3 - 4.1609X_4 - 0.5805X_1X_2 - 2.9629X_1X_3 + 1.5908X_1X_4 - 1.8441X_2X_3 + 1.3609X_2X_4 - 0.0209X_3X_4 + 12.9139X^2_1 + 16.2632X^2_2 + 0.0496X^2_3 + 0.4713X^2_4..$$

Figure 1a depicts the effect of the interaction of manitol with potassium nitrate with the fixed coded value of incubation time (7days). An increasing manitol with increase in potassium nitrate let to an initial increase in cellulase activity until they reached the optimal cellulase production. Figure. 1b depicts that the plots illustrating the effect of the inoculum size and manitol. The plot predicted that the increasing of inoculum size cellulase production was low and increasing manitol resulted in an increasing cellulase production. Figure: 1c depicts increasing pH resulted in increasing cellulase activity whereas increasing inoculums size resulted in less production of cellulase activity. Figure 2a and 2b showed that an initial increase in pH with simultaneous increase in manitol concentration resulted in an increase xylanase production. However on increase in potassium nitrate concentration beyond this limit has affected xylanase production. The optimum conditions for the maximum production of cellulase and xylanase were determined by response surface analysis and also estimated by optimizer tool using statistical.

Table: 2 Full Factorial Central Composite Design (CCD) of four variables in coded and natural units along with the observed responses.

Run	X_1	X_2	X_3	X_4	Cellulase: IU/ml		Xylanase: IU/ml	
					Experimental	Predicted	Experimental	Predicted
1.	0.5	0.5	6.5	2.4	1.1400	1.0664	18.7460	18.1285
2.	1	0.5	6.5	2.4	1.2733	1.2928	19.7340	19.8727
3.	0.5	1	6.5	2.4	1.2400	1.2528	17.1585	17.9370
4.	1	1	6.5	2.4	1.1933	1.1142	20.2640	19.5361
5.	0.5	0.5	8.5	2.4	1.4933	1.5528	22.2640	23.0755
6.	1	0.5	8.5	2.4	1.6133	1.5842	22.6013	21.8569
7.	0.5	1	8.5	2.4	1.7333	1.8175	20.9147	21.0398
8.	1	1	8.5	2.4	1.4200	1.4839	20.0713	19.6761
9.	0.5	0.5	6.5	4.8	1.0533	0.9594	17.7100	18.1841
10.	1	0.5	6.5	4.8	1.5667	1.5208	22.2633	21.5191
11.	0.5	1	6.5	4.8	0.9933	1.0608	19.2280	19.3534
12.	1	1	6.5	4.8	1.3467	1.2572	23.2760	22.5433
13.	0.5	0.5	8.5	4.8	0.9333	1.0508	22.9387	23.0475
14.	1	0.5	8.5	4.8	1.4600	1.4172	24.1193	23.4197
15.	0.5	1	8.5	4.8	1.2800	1.2306	22.4327	22.3727
16.	1	1	8.5	4.8	1.1200	1.2319	22.6013	22.5998
17.	0.25	0.75	7.5	1.2	1.5600	1.5019	21.9267	20.7835
18.	1.25	0.75	7.5	1.2	1.6800	1.7297	21.0713	22.7548
19.	0.75	0.25	7.5	1.2	1.2000	1.2486	22.7460	23.1122
20.	0.75	1.25	7.5	1.2	1.3067	1.2497	21.9267	22.1007
21.	0.75	0.75	5.5	1.2	0.9333	1.0786	15.8547	16.2374
22.	0.75	0.75	9.5	1.2	1.6933	1.5397	21.0833	21.2409
23.	0.75	0.75	7.5	6	1.2533	1.2286	18.8907	18.9362
24.	0.75	0.75	7.5	6	0.8533	0.8697	21.4207	21.9154
25.	0.75	0.75	7.5	3.6	1.4400	1.4233	18.5867	18.5407

All the experiments were carried out in triplicates

The volume of cellulase and xylanase were determined by actual response value. The data reported represented its mean. Statistical significance was evaluated using the Analysis of Variance (ANOVA), $p<0.05$ and $p < 0.01$ were considered as significant [18]. Second-order polynomial regressed equations were

established on the basis of the experimental data. Optimum parameters were defined by the MATLAB software version 7.5.0.342 (R2007b).

Table 3: Model coefficients estimated by multiple linear regressions of Cellulase

Serial No.	Parameters	Co-efficient	Standard error	t-value	p-value
1	Constant	-3.9881	1.2793	-3.1174	0.00706
2	X_1	0.4303	0.8826	0.4875	0.63293
3	X_2	1.8869	0.8826	2.1381	0.04938
4	X_3	0.8701	0.2739	3.1765	0.00626 *
5	X_4	0.9259	0.2206	4.1965	0.00078 *
6	X_1X_2	-1.4600	0.3900	-3.7432	0.00196 *
7	$X_1 X_3$	-0.1950	0.0975	-1.9998	0.06397
8	$X_1 X_4$	0.3350	0.0975	3.4355	0.00368 *
9	$X_2 X_3$	0.0783	0.0975	0.8033	0.43433
10	$X_2 X_4$	-0.0850	0.0975	-0.8717	0.39711
11	$X_3 X_4$	-0.0987	0.0244	-4.0508	0.00105 **
12	X^2_1	0.7700	0.2979	2.5847	0.02072
13	X^2_2	-0.6967	0.2979	-2.3386	0.03362
14	X^2_3	-0.0285	0.0186	-1.5330	0.14611
15	X^2_4	-0.0935	0.0186	-5.0240	0.00015**

* $p < 0.05$, ** $p < 0.01$

Table 4: Model coefficients estimated by multiple linear regressions of Xylanase Activity

Serial No	Parameters	Coefficient	Standard error	*t*-value	*p*-value
1	Constant	12.6790	10.4788	1.2100	0.24502
2	X_1	-2.4778	7.2291	-0.3428	0.73654
3	X_2	-17.0667	7.2291	-2.3608	0.03219 *
4	X_3	4.2738	2.2436	1.9048	0.07616
5	X_4	-4.1609	1.8073	-2.3023	0.03606 *
6	X_1X_2	-0.5805	3.1949	-0.1817	0.85825
7	$X_1 X_3$	-2.9629	0.7987	-3.7095	0.00210 *
8	$X_1 X_4$	1.5908	0.7987	1.9917	0.06494
9	$X_2 X_3$	-1.8441	0.7987	-2.3088	0.03561*
10	$X_2 X_4$	1.3609	0.7987	1.7038	0.10904
11	$X_3 X_4$	-0.0209	0.1997	-0.1046	0.91809
12	X^2_1	12.9139	2.4402	5.2922	0.00009*
13	X^2_2	16.2632	2.4402	6.6648	0.00001 **
14	X^2_3	0.0496	0.1525	0.3253	0.74943
15	X^2_4	0.4713	0.1525	3.0902	0.00747*

* $p < 0.05$, ** $p < 0.01$

All the linear effects of X_1 , X_3 , X_3 ,X_4, X_1X_2, X_1X_4, X_3X_4 square effects of X^2_4 are significant model terms for cellulase production and where as the linear effects of X_4, X_1X_3, X_2X_3, and square effects of X^2_1, X^2_2, X^2_4 are significant model terms for xylanase production.

After all the experiments were conducted, the analysis of the enzymes response in the factorial design was diagnosed where the actual value of enzymes production were compared to predicted value designed as in enzymes response in Table 2. Evaluated by ANOVA analysis using 4-level factorial design is showed in Table 5 and 6. The p-value suggested that the coefficient for the linear effect ($p< 0.05$) and ($p< 0.01$) were statically for cellulase and xylanase production. The different concentrations of inoculums size, manitol, potassium nitrate and different pH have effect of cellulase and xylanase production by *Penicillium crustosum*.

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Table 5 Analysis of Variance (ANOVA) for Response Surface Quadratic Model: Cellulase activity

Sum of squares	degree of freedom	f-value	p-value	mean square error	R^2	$Adjr^2$
0.1426	14	10.8137	0.00002	0.0095	0.9099	0.8257

Table 6: Analysis of Variance (ANOVA) for Response Surface Quadratic Model: Xylanase Activity

Sum of squares	Degree of freedom	f-value	p-value	Mean square error	R^2	Adj. R^2
9.5695	14	13.2479	0.00001	0.6380	0.9252	0.8553

Although the coefficient of determination R^2 of the model was 0.9099 for cellulase activity and 0.9252 for xylanase activity indicating that the sample variation of 90.99% of cellulase activity and 92.52 % of xylanase was attributed to a given independent variables Fig 5, 6. They are still in a reasonable agreement with the $Adjr^2$ of 0.8257 for cellulase and $Adjr^2$ of 0.8553 for xylanase. To test the fit of the model equation, the regression based determination coefficient R^2 was evaluated. The nearer the values of R^2 close to 1, the model would explain better for variability of experimental values to the predicted values. For a fit model, R^2 should be more than 0.80 [19]. The regression coefficient in the response surface model for the linear, quadratic and interaction effects of the variables were shown along with *p*-value in Table 3, 4. The response surface curves were plotted to understand the interaction of the variables and to determine the optimum level of each variable for maximum response (Figure. 1a, 1b,1c) and 2b).The RSM model showed a satisfactory performance and offered a stable response in predicting the combined interactions of the four independent variables (manitol, potassium nitate, pH and innoculum size) with respect to the levels of cellulase and xylanase production.

Fig 1: 1a, 1b &1c: 3D Response surface plot showing interactive effect of Potassium nitrate and manitol (a), innoculum size and monitol (b), innoculum size and pH (c) on cellulase activity.

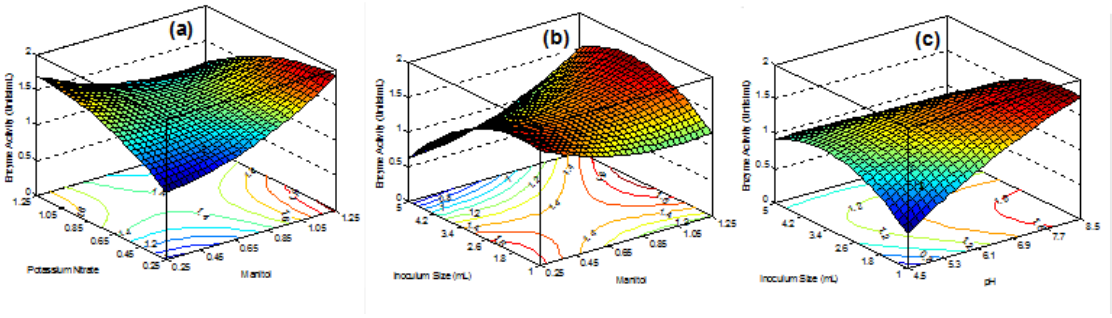
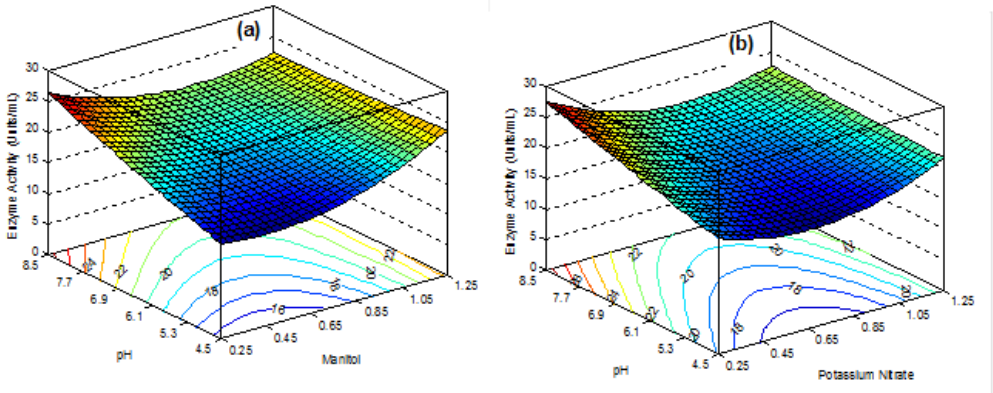


Fig 2: 2a & 2b: 3D Response surface plot showing interactive effect of pH and manitol (a), pH and Potassium nitrate (b) on xylanase activity.



Validation of the experimental

Validation of the predicted results was done under optimized conditions in four independent experiments. In this model, the experimental cellulase and xylanase activity of 5.56U/ml and 36.14. Respectively were obtained which correlated to the predicted activity of 5.02 U/ml and 32.52U/ml confirming the rationality of the model. The cellulase and xylanase activity obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model.

DISCUSSION

To date there are many reports in literature on optimization of cellulase and xyalanase production using RSM. Although numerous fungal species producing cellulase activity have been described in literature. The cellulase and xylanase production have been influenced by carbon and nitrogen source which are important in growth of microbial reported Mudrula [20]. Carbon and nitrogen sources are important variables that affect the growth and products of microbes [21, 22] their concentrations are important for optimum production of enzymes. It has been noted that microorganisms are dependent on the pH for their cell growth and enzyme production Kumar [23]. It was found that the increase in inoculum size resulted in a rapid increase in enzyme production [24]. Zambare, [25] reported the production extracellular amylase from *Bacillus spp.* in submerged fermentation and the most important factors employed were pH, temperature and inoculum size.

CONLUSION

Response surface methodology was successfully employed to optimize the process variables for cellulase and xylanase production. Sugar beet peel compared to the other agro-waste material studied is a very promising substrate for cellulase and xylanase production. Application of RSM for optimization studies is an effective method for improving the enzyme production and also understanding the interaction effects between the variables with less number of experiments. The successful use of sugar beet peel as renewable agro wastes is dependent on the development of economically feasible processes for cellulase and xylanase enzyme production.

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