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Utilization of Agricultural Waste as a Carbon Source for the Production of Cellulase Enzyme

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ABSTRACT:

In India large quantities of agricultural wastes are produced every year. The amount of residues in the form of pulp, straw, peel and seeds. These residues are disposed in the municipal bins, much of the agricultural wastes are disposed of by burning, which creates environmental pollution. It can be reduced by utilization of agricultural wastes as a carbon source for the production of cellulase. In this present work deals with the production of Cellulase by *Cellulomonas fimi* using rice straw in submerged fermentation(smF), was enhanced by Optimization of process parameters. The central composite design (CCD) of RSM was employed to optimize four process parameters namely temperature, initial pH, inoculum concentration and shaker speed for the production of cellulase. The optimum conditions for the maximum production of cellulase was determined by response surface analysis and also estimated by optimizer tool using statistical software package “Minitab 15”..The optimum conditions are temperature – 30 ^{o}C, initial pH – 6.7, inoculum concentration – 9.6 % v/v , and shaker speed – 192 rpm

KEYWORDS: Cellulase, Central Composite design (CCD), rice straw, Cellulomonas fimi

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INTROUCTION

Lignocellulose is a major renewable natural resource. but it 's chemical composition based on sugars other, they could be used for the production of biofuels, cheap energy sources for fermentation, enzymes, food additives, organic acids, improved animal feeds, human nutrients, and others[1]. One of the most important and difficult technological challenges is to overcome the recalcitrance of natural lignocellulosic materials, that can be hydrolyzed by the enzyme to produce fermentable sugars. The biodegradation of agricultural has led to extensive studies on cellulase enzymes produced by fungi and bacteria[2] which are capable of degrading and utilizing cellulose and hemicelluloses as carbon and energy sources. Cellulases are enzymes produced by microorganisms including both fungi and bacteria during their growth on cellulosic materials[3,4]. These microorganisms can be mesophilic or thermophilic, aerobic, anaerobic,. They belong to the genera of Aspergillus, Trichoderma, Clostridium, and Cellulomonas[5,6,7].

MATERIALS AND METHODS

Micro organisms used for fermentative production of cellulase in this study was *Cellulomonas fimi*. The above microorganism was purchased from the National Chemical Laboratory, Pune and was maintained on Nutrient Agar Slants and stored in test tubes at 4^{o}C and sub-cultured monthly.

Fermentative Production of Cellulase Using Batch Fermentation

Alkali pretreated rice straw was used as substrates for cellulase production. Fermentation was carried out in Erlenmeyer flasks (250 mL) with 10 g/L of alkali pretreated substrate in 100 ml of enzyme production medium, the composition of media varied according to the experimental design described in this work. pH of the medium was adjusted to 7.0 with 1 mol NaOH or 1 mol HCl. Each flask was covered with hydrophobic cotton and autoclaved at 121^{o}C for 20 min. After cooling, each flask was inoculated with 10% v/v of inoculum incubated at 28 ^{o}C for 72 hrs.

Recovery of Enzyme

After incubation, the samples were withdrawn at regular time intervals and the samples were filtered through GD-120 glass fiber filter disks (Whatman) to remove the residual insoluble substrate. Then, the liquid content obtained after filtration was centrifuged at 10,000×g for 10 min at 4◦Cto separate the cells. The cell-free supernatant was analyzed for enzyme activity by DNS method[8].

Filter Paper Assay for Cellulase Enzyme

The cellulase activity is measured as FPase Activity Units. A 1×6 cm strip of Whatman number 1 filter paper was added to a total volume of 1.5ml of culture filtrate in 50mMcitrate buffer (pH 4.5). The samples were incubated for 1 h at 60◦C. The hydrolysis was terminated by addition of 3 ml of DNS solution, followed by 5min boiling. After cooling, 20ml of distilled water was added and the absorbance was read at 540nm using glucose as standard. One FPA unit of cellulase activity is expressed as the amount of enzyme that releases one micromole of glucose unit/ml/min under the above experimental conditions[9].

Central Composite Design Optimization Using Response Surface Methodology

An orthogonal 2^4 factorial central composite experimental design with resulting in a total of 30 experiments were used to optimize the chosen key variables for the production of cellulase in batch process from rice straw,.

The experiments with five different temperature 24°C, 28°C, 32°C, 36°C& 40°C five different pH, 5.0, 6.0,7.0, 8.0&9.0 and five different inoculum concentration 3, 5, 7, 9,&11 (v/v) and five different shaker speed 90,120, 150, 180&210 rpm were employed simultaneously covering the spectrum of variables for the production of cellulase in the central composite design. Table 1 indicates the range and levels of independent variables selected for the production of cellulase enzymes.

Table No.1: "The Range and Levels of the Independent Variables"

Independent variable		Range and levels of independent variables				
	Code	-2	-1	0	1	2
Temperature (°C)	A	24	28	32	36	40
pH	B	5	6	7	8	9
Inoculum Concentration(V/ V)	C	3	5	7	9	11
Shakers speed (rpm)	D	90	120	150	180	210

RESULTS AND DISCUSSION

The central composite design (CCD) of RSM was employed to optimize four process parameters namelytemperature, initial pH, inoculum concentration and shaker speedfor the production of cellulase. The four independent variables were studied at five different levels (Table

No.1) and a set of 30 experiments were carried out (Table No. 2) and the results were analyzed by ANOVA.

Table No.2: "CCD Design Matrix with Experimental and Predicted Values for Cellulase Production"

Run	A	B	C	D	Cellulase IU/mL	
					Observed	Predicted
1	0	0	0	0	0.86	0.87
2	1	1	-1	-1	0.48	0.53
3	-1	1	-1	-1	0.51	0.51
4	-1	1	1	1	0.86	0.84
5	-1	1	-1	1	0.47	0.48
6	1	-1	-1	-1	0.49	0.48
7	1	-1	-1	1	0.59	0.63
8	0	0	0	0	0.85	0.87
9	-1	1	1	-1	0.76	0.75
10	2	0	0	0	0.53	0.49
11	0	0	0	0	0.87	0.87
12	0	0	2	0	0.78	0.84
13	0	0	0	0	0.87	0.87
14	-1	-1	-1	1	0.59	0.55
15	1	-1	1	-1	0.52	0.54
16	-1	-1	1	1	0.92	0.9
17	1	-1	1	1	0.82	0.8
18	1	1	1	1	0.67	0.64
19	0	0	-2	0	0.48	0.42
20	0	-2	0	0	0.54	0.56
21	1	1	1	-1	0.58	0.6
22	-1	-1	1	-1	0.65	0.6
23	0	0	0	-2	0.61	0.58
24	1	1	-1	1	0.43	0.46
25	0	0	0	0	0.87	0.87
26	-1	-1	-1	-1	0.31	0.36
27	0	2	0	0	0.57	0.55
28	0	0	0	0	0.87	0.87
29	0	0	0	2	0.79	0.82
30	-2	0	0	0	0.52	0.56

The relationship and interrelationship of the variables were determined by fitting the second-order polynomial equation to data obtained from 30 experiments. The response values (Y_1) used in each trial was the average of the duplicates. The second-order regression equations provided the levels of cellulase as a function of temperature, initial pH, inoculum concentration and shaker speedwhich can be represented in terms of coded factors as in the following equations (1)

$$Y_1 = 0.87 - 0.02 * A - 2.917E-003 * B + 0.10 * C + 0.059 * D - 0.024 * A * B - 0.044 * A * C - 0.011 * A * D + 3.125E-003 * B * C - 0.053 * B * D + 0.029 * C * D - 0.086 * 0.078 * B^2 - 0.059 * C^2 - - 0.042 * D^2 \quad \ldots(1)$$

Where, Y_1 cellulase activity. *A*, *B*, *C* and *D*are the coded values of temperature, initial pH, inoculum concentration and shaker speedrespectively. ANOVA for the response surface is shown in Table No.3.

Table No.3: "Analysis of Variance (ANOVA) for Cellulase"

Source	Coefficient Factor	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	0.87	0.83188	14	0.05942	32.0131	< 0.0001
A	-0.02	0.0092	1	0.0092	4.95884	0.0417
B	-2.92E-03	0.0002	1	0.0002	0.11	0.7447
C	0.1	0.2625	1	0.2625	141.427	< 0.0001
D	0.059	0.08284	1	0.08284	44.6296	< 0.0001
AB	-0.024	0.00951	1	0.00951	5.1216	0.0389
AC	-0.044	0.03151	1	0.03151	16.9743	0.0009
AD	-0.011	0.00181	1	0.00181	0.97314	0.3395
BC	3.13E-03	0.00016	1	0.00016	0.08418	0.7757
BD	-0.053	0.04516	1	0.04516	24.3284	0.0002
CD	0.029	0.01381	1	0.01381	7.43827	0.0156
A^2	-0.086	0.20159	1	0.20159	108.607	< 0.0001
B^2	-0.078	0.16786	1	0.16786	90.435	< 0.0001
C^2	-0.059	0.09704	1	0.09704	52.2792	< 0.0001
D^2	-0.042	0.04834	1	0.04834	26.0416	0.0001
Residual		0.02784	15	0.00186		
Lack of Fit		0.02776	10	0.00278	166.55	< 0.0001
Pure Error		8.3E-05	5	1.7E-05		
Cor Total		0.85972	29			

Std. Dev.-0.043, R-Squared -0.9676, Mean-0.66, Adj R-Squared-0.9374, C.V. %-6.57, Pred R-Squared-0.8139, PRESS-0.16, Adeq Precision-17.616

The independent variables were fitted to the second order model equation and examined for the goodness of fit. Several indicators were used to evaluate the adequacy of the fitted model and the results are shown in Table 2 The determination coefficient R^2 value, coefficients of variation and model significance (*F*-value) were used to judge the adequacy of the model. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model.

The Model F-value of 32.01 for cellulase implies the model is significant. There is only 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > *F*" less than 0.05 indicates that model terms are significant. The coefficient of variation indicates the degree of precision with which the treatments were compared. Usually, the higher the value of CV, lower is the reliability of experiment. Here, a lower value of CV for cellulase (6.57%) indicated a better precision and reliability of the experiments[10,11].

To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated, which is the proportion of variation in the response attributed to the model rather than to random error. The closer the values of R^2 to 1, the better the model would explain the variability

between the experimental and the model predicted values. The coefficient of determination (R^2) for cellulase activity was calculated as 0.9676 which explain the variability of the response. The predicted R^2 value of cellulase activity was 0.8139 and has a reasonable agreement with the adjusted R^2 value of cellulase activity of 0.9374."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Adeq Precision of 17.61 for cellulase indicates an adequate signal. These models can be used to navigate the design space.

The statistical significance of Eq. 1 was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 2 and Fig.2. The results demonstrated that the model is highly significant and is evident from Fischer's *F*-test with a low probability value (*P* model >*F* less than 0.05). Model coefficients estimated by regression analysis for each variable is shown in Table.2 The significance of each coefficient was determined by *t*-values and *P*-values. The larger the magnitude of *t*-test value and smaller the *P*-value indicates the high significance of the corresponding coefficient.

In the present work, the linear effects of *A*, *C* and *D* the interactive effects of *AB*, *AC*, *BD* and *CD* squared effects of A^2, B^2, C^2andD^2 are significant model terms for cellulase production. To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The nearer the values of R^2 to 1, the model would explain better for variability of experimental values to the predicted values. The above models can be used to predict the cellulase productions within the limits of the experimental factors. Fig.1 shows that the actual response values agree well with the predicted response values of cellulase. The interaction effect of the variables on cellulase production was investigated by plotting the 3D response surfaces with the vertical (Z) axis representing enzyme activity(response) yield and two horizontal axes representing the coded levels of two explanatory factors, while maintaining other variables at their median levels are shown in Fig.2(1)-Fig.2(6).

Effect of Temperature

The Fig.2(1), Fig.2(2), and Fig.2(3) shows the effect of temperature on cellulase production. Incubation temperature plays an important role in the metabolic activities of microorganism. A higher temperature alters the cell membrane composition and stimulates protein catabolism, thus, causes the cell death. Optimum temperature recorded for maximum cellulase productivity was at 30°C for *Cellulomonas spp.* and *Bacillus pumilus.*

Effect of Initial Ph

It was observed in Fig.2.9(1), Fig.2(4) and Fig.2(5) which shows the effect of initial pH on cellulase production. The cellulase activity increases with increase in initial pH up to 6.7 and

thereafter cellulase activity decreases with further increase in initial pH. Cellulolytic enzyme, endogluconase obtained from *Cellulomonas*, *Bacillus*, and *Micrococcus* spp.

Effect of Inoculum Concentration

The Fig.2.9(2), Fig.2(4), and Fig.2(6), shows the effect of inoculum concentration on cellulase production. The cellulase activity increases with increase in inoculum concentration up to 9.6 %v/vand thereafter cellulase decreases with further increase in inoculum concentration.

Effect of Shaker Speed

The effect of shaker speed on cellulase production was observed from the Fig.2.9(3), Fig.2(5), and Fig.2(6). The cellulase activity increases with increase in shaker speed up to 192 rpm and thereafter cellulase activity decreases with further increase in shaker speed. Increase in the agitation speed beyond 200 rpm resulted in low xylanase yields.

Validation of the Experimental Model for Production of Cellulase

The optimum conditions for the maximum production of cellulase was determined by response surface analysis and also estimated by optimizer tool using statistical software package "Minitab 15". The optimum conditions are temperature – 30^{o}C, initial pH –6.7, inoculum concentration– 9.6 %v/v, and shaker speed–192 rpm.

Validation of the experimental model was done by carrying out the batch experiment under optimal operating conditions. The experiments were done in triplicate and the results were compared. The cellulase activity obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model. The percentage of error between experimental and model predicted values of cellulase activitiy is 1.722. At these optimized conditions, the maximum cellulase activities was found to be 1.01 IU/mL.

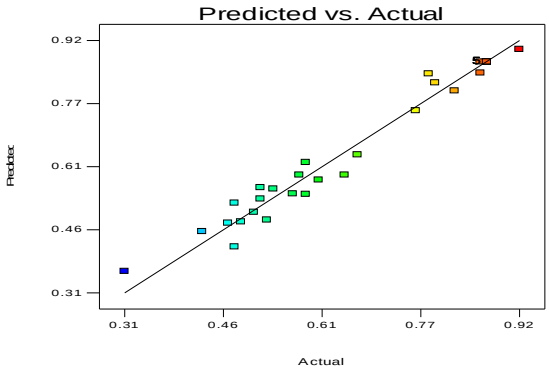
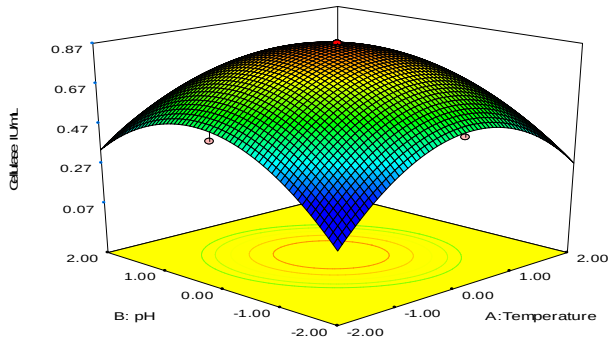
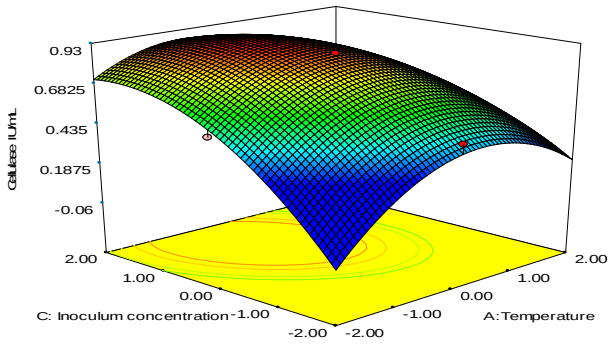


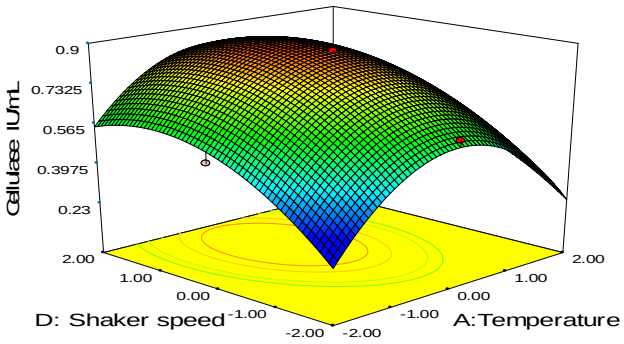
Fig.1 Predicted Response Vs Actual Value for Cellulase Production



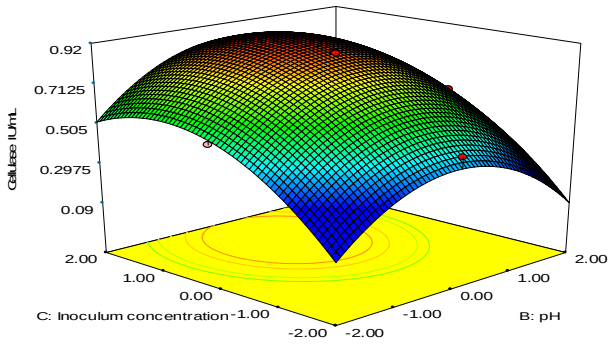
(1):Temperature and pH



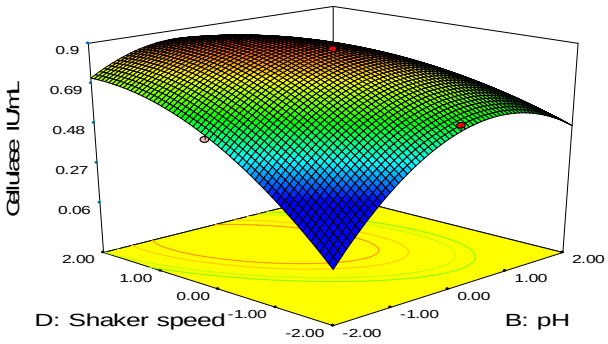
(2): Temperature and Inoculum Concentration



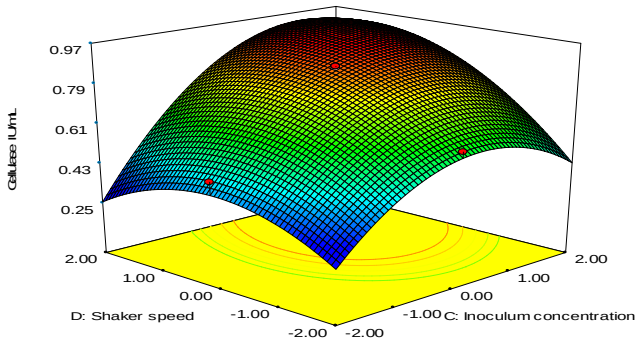
(3): Temperature and Shaker Speed



(4):pH and Inoculum Concentration



(5): pH and Shaker Speed



(6): Inoculum Concentration and Shaker Speed

Fig.2: 3D Response Surface Plot for Cellulase Production Showing the Interactive Effects of Process Parameters

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