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Optimization of Cellulase Enzyme Production by Co-cultures of Fungi Isolated from Lignocellulosic Waste

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ABSTRACT

Fruit wastes were incubated with the mixture of cellulolytic fungi Penicillium citrinum, Aspergillus oryzae, and Trichoderma viride to hydrolyze the cellulosic components and to increase the degree of degradation. . The batch experiments are statistically designed and performed using Box-Benhken method of Response Surface Methodology to investigate the influence of major parameters viz., incubation time, temperature, pH, moisture content and substrate concentration on cellulase enzyme production. Maximum cellulase production of 2.03 Units/ ml (U/ ml) was detected by the RSM method in a mixed culture containing fungi at a ratio of 1: 1: 1 under optimal conditions at an incubation time of 5.27 days, a temperature of 34.09 °C, pH 4.85, moisture content of 63.83% and a substrate concentration of 5.03%.

KEYWORDS: *Fruit waste, Mixed culture, Optimization, Cellulase production*

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I. INTRODUCTION

The worldwide supply of non-renewable form of energy is entering a phase of decline, while the requirement for energy is mounting. The current universal demand for energy is mainly met by utilizing fossil fuel resources such as coal, oil and petroleum. With the global increasing demand for energy, energy shortage will be a worldwide problem. There is also widespread prediction that the world population will likely increase by 50% in the next 50 years with increase in the world demand for petroleum energy (Igbinadalor,2012). The significance of alternate source of bioenergy has gained a lot of importance not only because of the continuous exhaustion of limited reserves of fossil fuels, but also for safer use of the surroundings and, as a result, a source of sustainable energy.

Lignocellulosic waste is produced daily all over the world in huge quantities through agricultural practices, mainly from different agricultural and forestry industries. These renewable materials accumulate a lot in the world and, if not exploited properly, they will cause environmental pollution. But if used correctly, it will benefit the economic development of our country.

"The hassle in waste utilization is to develop a process that is economically feasible and safe.. Bioconversion offers an inexpensive and safe method of disposing these wastes. It also has the

potential to convert the waste into usable forms such as substrate for production of cellulase enzymes and reducing sugars that can be used for fuel production".(Acharya et al., 2008).

Microorganisms are important in the bioconversion of cellulosic waste and in the production of enzymes from waste. Although bacteria and fungi can produce cellulases, fungal cellulases are generally preferred because they are extracellular, adaptive and generally secreted in large quantities (up to 2% by weight) during growth. "According to Maciel et al., 2008 cellulase enzymes are mainly produced from Trichoderma species, Aspergillus species and Penicillium species."

In this work, we focus at the relative potentials of fruit waste as microbial substrate for cellulase enzyme production by utilizing strains of A. oryzae, P.citrinum and T. viride as a mixed culture. These strains are isolated from lignocellulosic wastes. Also in a mixed culture a synergistic effect is present where the fungi co-operate and the effect on cellulase enzyme production is greater than the sum of the effects in the individual monocultures.

II. Materials & Methods

A. Cellulosic Materials

The rotten fruit waste which was used as substrate was collected in a sterile container and agitated on a rotary shaker at 250 rpm for 1 hour to disperse the sample. These samples were diluted and spread plated on a starch case in medium. The plates were incubated for 7 days at 28°C.

B. Microbial Stains

Fungi P.citrinum, A.oryzae and T.viride were isolated from decayed lignocellulosic Waste. The culture was maintained on potato dextrose agar slant at 4°C& sub-cultured on fresh sterile PDA slant & incubated for 72-120 hr.

C. Cellulase Assay

"Cellulase activity was measured as per the 3, 5-dinitrosalicylic acid (DNS) methods (Mandels, 1969) by determining the amount of reducing sugars released during 30 minutes in a reaction mixture. The cellulase activity was assayed by mixing 0.5 ml of culture supernatant with 0.5 ml of 1% carboxy methyl cellulose solution in 0.5 M acetate buffer pH 5 for fungal enzymes and incubated for 30 minutes at 50oC. One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1µmole reducing sugar in 1 minute reaction".

D. Cellulase enzyme production using fruitwaste as substrate

"10 grams of fruit waste was moistened with Mandel and Reese medium to get initial moisture content of 50% and autoclaved at 121 0C for 15 minutes at 15 psi pressure. After cooling, sterilized flask were inoculated with mixed cultures. For mixed cultures (mixture of three fungi), 1ml of each culture was added. The final spore concentration was maintained as 3x106spores/ml. The content in the flask were mixed thoroughly to ensure uniform distribution of inoculums and flask was incubated at 300C for 12 days. The samples were withdrawn after each day till 10thday and estimated cellullase activity (Rahana et.al 2014)

E. Optimization of enzyme production

In order to find the influences of Incubation time, temperature, pH, substrate concentration and moisture content for optimization of cellulase enzyme production, mixed fungal strains were cultivated with incubation time of 1-10 days, varying temperatures of 15°C-45°C, pH range 2-8, moisture content of 40% - 80% and substrate concentration of 2% -7% by keeping all other parameters constant for 10 days.

F. Experimental design

Our work is based on determining the relationship between the amount of cellulase enzyme production and operating parameters such as Incubation time, temperature, pH, moisture content and substrate concentration, "Response surface methodology (RSM) is used as a statistical technique for the modeling and optimization of various variables, which determines the optimum process conditions" (Fang H 2010). **Table 1** gives the parameters and the operating ranges covered.

Table 1 The level and range of variables chosen for cellulase enzyme production.

Independent variable	Coded levels		
	-1	0	1
Incubation time(days)	1	5.5	10
Temperature (°C)	15	30	45
Initial pH	2	5	8
Moisture content (%)	40	60	80
Substrate Concentration	2	4.5	7

"The Substrate concentration, pH, and temperature are referred by uncoded variables as X1, X2 and X3 respectively. The variables in uncoded form are converted to coded form: x1, x2 and x3 using the following equation." (P.A Solomon 2009)

$$x = \frac{X - ((X_{max} + X_{min})/2)}{((X_{max} - X_{min})/2)} \quad \text{-- (1)}$$

"The Box–Behnken experimental design of RSM has been chosen to find the relationship between the response functions and variables using the statistical software tool MINITAB 16 (PA, USA). In the Box–Behnken method a total number of 46 experiments are carried out to estimate the amount of cellulose enzyme production. The interaction between the variables and the analysis of variance (ANOVA) has been studied by using RSM. The quality of the fit of this model is expressed by the coefficient of determination R2 (A.M Manilal and P.A Solomon 2020)

III. RESULTS AND DISCUSSIONS

A. Response surface methodology

The responses viz. cellulase enzyme production for different combinations of Incubation time, temperature, initial pH,
moisture content and substrate concentration for the 46 sets of experimental conditions as proposed by the RSM design are reported in Table 2. In order to make sure the consistency of results, every run was carried out thrice, average being reported.

Table 2 Design of experiment and experimental response for cellulase enzyme production

SI.No.	Time (Days) X1	Temperature (°C) X2	pH X3	Moisture content (%) X4	Substrate Concentration (%) X5	Cellulase enzyme production (U/ml)
1	1.0	15	5	60	7.0	1.4483
2	10.0	15	5	60	4.5	1.6338
3	1.0	30	8	60	7.0	1.6460
4	5.5	30	5	60	4.5	1.9900
5	5.5	30	2	40	4.5	1.6830
6	10	30	5	40	7	1.5603
7	5.5	45	2	80	4.5	1.7310
8	5.5	15	8	80	4.5	1.5778
9	5.5	15	5	60	2.0	1.4750
10	5.5	45	5	60	2.0	1.6247
11	5.5	15	5	60	7.0	1.6135
12	5.5	30	5	60	4.5	1.9960
13	1.0	45	2	60	4.5	1.5210
14	10.0	30	2	60	4.5	1.6828
15	1.0	45	8	60	4.5	1.4315
16	10.0	30	5	60	4.5	1.8280
17	5.5	45	2	40	2.0	1.3545
18	10.0	45	5	80	2.0	1.3433
19	5.5	30	5	40	7.0	1.7507
20	5.5	30	5	80	7.0	1.7530
21	5.5	15	2	60	4.5	1.4800
22	5.5	30	5	60	4.5	1.9990
23	5.5	15	8	60	4.5	1.6200
24	5.5	30	5	60	4.5	2.0210
25	1.0	45	5	40	7.0	1.4540
26	10.0	45	5	40	4.5	1.7017
27	1.0	45	5	80	7.0	1.7890
28	10.0	30	5	80	4.5	1.6575
29	1.0	45	2	60	2.0	1.2005
30	5.5	30	8	60	2.0	1.4525
31	5.5	30	2	60	7.0	1.6506
32	5.5	30	8	60	7.0	1.6700
33	1.0	45	5	60	2.0	1.2873

34	10.0	45	5	60	2.0	1.4707
35	1.0	45	8	60	7.0	1.4840
36	10.0	30	5	60	4.5	1.8669
37	5.5	15	5	40	4.5	1.6478
38	5.5	30	5	40	4.5	1.8700
39	5.5	15	5	80	4.5	1.6525
40	5.5	30	5	60	4.5	1.9980
41	5.5	30	5	60	4.5	2.0377
42	5.5	30	5	60	4.5	2.0195
43	5.5	30	5	60	4.5	2.0020
44	5.5	30	5	60	4.5	2.0559
45	5.5	30	5	60	4.5	1.9649
46	5.5	30	5	60	4.5	2.0013

"The experimental data obtained were fitted with a total quadratic model with regression coefficients. Other than the intercept, linear, and quadratic terms, the model also reveals the two-way interactions through the interaction terms incorporated. The model can be represented as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{55} x_5^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{45} x_4 x_5 \quad \text{-----(2)}$$

where y is the amount of cellulase enzyme production in U/ml. β_0 is a constant, β_1, β_2 and β_3 are the regression coefficients for linear effects, β_{11}, β_{22} and β_{33} are the quadratic coefficients and $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}$, and β_{34} are the interaction coefficients".(A.M. Manilal and P.A. Solomon 2020). The coefficients of the model are given in Table 3.

Table 3 Estimated regression coefficients and corresponding t- and p-value for cellulase production

Factor	Coefficient of the model in coded factors	t-value	p-value	Significance level (%)
β_0	2.01	276.31	0	significant
β_1	0.03	3.15	0.004	significant
β_2	0.08	8.99	0	significant
β_3	-0.02	-2.09	0.047	significant
β_4	0.04	4.80	0	significant
β_5	0.08	8.45	0	significant
β_{11}	-0.19	-17.46	0	significant
β_{22}	-0.17	-13.79	0	significant
β_{33}	-0.22	-23.58	0	significant
β_{44}	-0.11	-10.62	0	significant
β_{55}	-0.21	-21.46	0	significant
β_{12}	-0.03	-2.07	0.16	Not significant
β_{13}	-0.06	-3.86	0.001	significant
β_{14}	-0.14	-7.80	0	significant
β_{15}	-0.10	-5.61	0	significant
β_{23}	-0.10	-7.98	0	significant
β_{24}	0.03	2.53	0.018	significant
β_{25}	0.026	1.98	0.15	Not significant
β_{34}	0.04	2.98	0.006	significant
β_{35}	0.04	3.48	0.002	significant
B_{45}	-0.039	-2.54	0.018	significant

By means of above-mentioned cellulase production model proposed by RSM, any combination of these five parameters can be considered within the experimental influence range to predict its quantity. The p-test and t-test were used to analyze the significance of the regression coefficient of cellulase production. Tables 3 and 4 give the "p", "t" and effective levels, respectively. As can be seen from the table, all but two interaction terms have a significant effect on the response. The temperature-substrate concentration interaction term is the least affected term in the model.

The equation can also be written in uncoded form as

$$y. = -2.59 + 0.287x_1 + 0.055x_2 + 0.26x_3 + 0.041x_4 + 0.384x_5 + -0.009x_1^2 + -.00007x_2^2 + -0.025x_3^2 + -0..0003x_4^2 + -0.033x_5^2 + -0.0005x_1x_2 + -0.005x_1x_3 + -0.002x_1x_4 + -0.009x_1x_5 + -0.002x_2x_3 + -0.00009x_2x_4 + 0.0006x_2x_5 + 0.007x_3x_4 + 0.005x_3x_5 + -0.0008x_4x_5 \quad (3)$$

Table 4 ANOVA report for the RSM model of Cellulase enzyme production

Source	dF	Sum of squares	Mean squares	F-value	p-value	Remark
Regression	20	2.340	0.1170	179.96	0	Significant
Linear	5	0.4404	0.0214	32.94	0	Significant
X_1- Incubation Time	1	0.1516	0.0064	9.72	0.005	Significant
X_2- Temperature	1	0.063	0.5200	78.05	0	Significant
X_3 – pH	1	0.00126	0.0027	4.42	0.046	Significant
X_4-Moisture concentration	1	0.00007	0.0149	24.87	0	Significant
X_5-Substrate Concentration	1	0.2257	0.0461	69.92	0	Significant
Square	5	1.735	0.282	433.5	0	Significant
X_1^2	1	0.513	0.194	296.92	0	Significant
X_2^2	1	0.4420	0.122	189.41	0	Significant
X_3^2	1	0.318	0.357	550.56	0	Significant
X_4^2	1	0.064	0.072	107.23	0	Significant
X_5^2	1	0.399	0.296	456.95	0	Significant
Interaction	10	0.1618	0.0164	24.90	0	Significant
X_1X_2	1	0.0014	0.0026	4.16	0.14	Not significant
X_1X_3	1	0.0029	0.0094	14.44	0.001	Significant
X_1X_4	1	0.0467	0.038	57.96	0	Significant
X_1X_5	1	0.046	0.0198	30.53	0	Significant
X_2X_3	1	0.046	0.0407	61.21	0	Significant
X_2X_4	1	0.0006	0.0041	5.51	0.018	Significant
X_2X_5	1	0.0017	0.0025	3.89	0.06	Not significant
X_3X_4	1	0.0066	0.0057	8.27	0.007	Significant
X_3X_5	1	0.006	0.0077	12.28	0.002	Significant
X_4X_5	1	0.0039	0.0039	6.00	0.022	Significant
Residual Error	25	0.0163	0.0006			
Lack-of-Fit	14	0.00943	0.0009	1.09	0.453	
Pure Error	11	0.0068	0.0007			
Total	45	2.356				

"Finally, the graphical analyses of the data was done using Analysis of Variance (ANOVA) through the statistical analysis software (MINITAB 16 (PA, USA)). The model terms were evaluated by the p-value (probability) with a 95% confidence level and the statistical significance was checked by the Fisher F-test. The quality of the fit was expressed by the coefficient of determination R^2 and adjusted R^2. (A.M Manilal and P.A Solomon 2020)

The importance of the regression coefficients of the parameters on cellulase enzyme production was tested with the Fisher F-test. The regression coefficient values, F value, and p-value are obtained in Tables 2 and 3. It can be seen that incubation time, temperature, pH, moisture content and substrate concentration all are most important variables for the cellulase enzyme production which can be linked with the significance of p-values which is less than 0.05.

"Further, the model competency was checked by computing the coefficient of determination R^2. The R^2 value can reveal how well the model in reproducing the observed outcomes of the experiments. A high R^2 value for the coefficient guaranteed satisfactory representation of the proposed model to the experimental observations" (A.M Manilal and P.A Solomon 2020)

B. Combined Effect of Parameters

The influence of five parameters on quantity of cellulase enzyme production is carried out. Figure 1 shows the contour plots the joint effect of these variables on cellulase production.

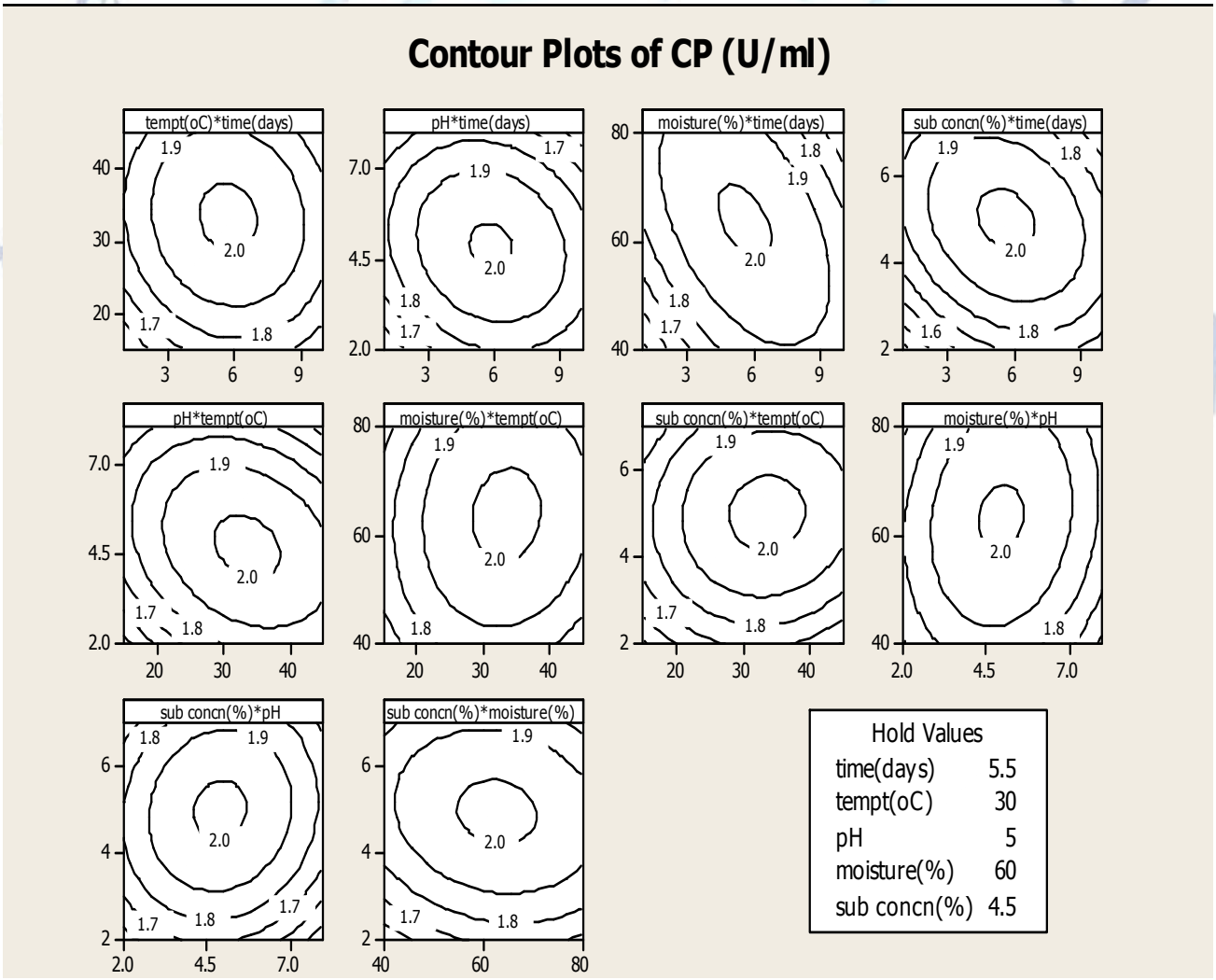

Fig. 1 Contour plot of variables

In each contour plot, two parameters are varied, while the others are unchanged. The plots are obtained from the second order models of Eq. 2. It was obvious that the incubation time, Temperature,

pH, Substrate concentration and moisture content exhibited the same tendency on cellulase production. All the ten plots evidently indicated that the response surfaces for cellulase production are showing a clear peak, signifying that optimum settings for maximum cellulase enzyme production are within the boundary.

The optimum operating conditions as predicted by the response optimizer tool of MINITAB 16 were 2.0303U/ml for the parameter values of 5.27 days for incubation time, 34.090C for temperature, 4.85 for initial pH `63.84% for moisture content and 5.03% for substrate concentration. The optimization plot obtained is shown in Figure 2. This suggests that the proposed quadratic regression models reasonably optimize the operating conditions.

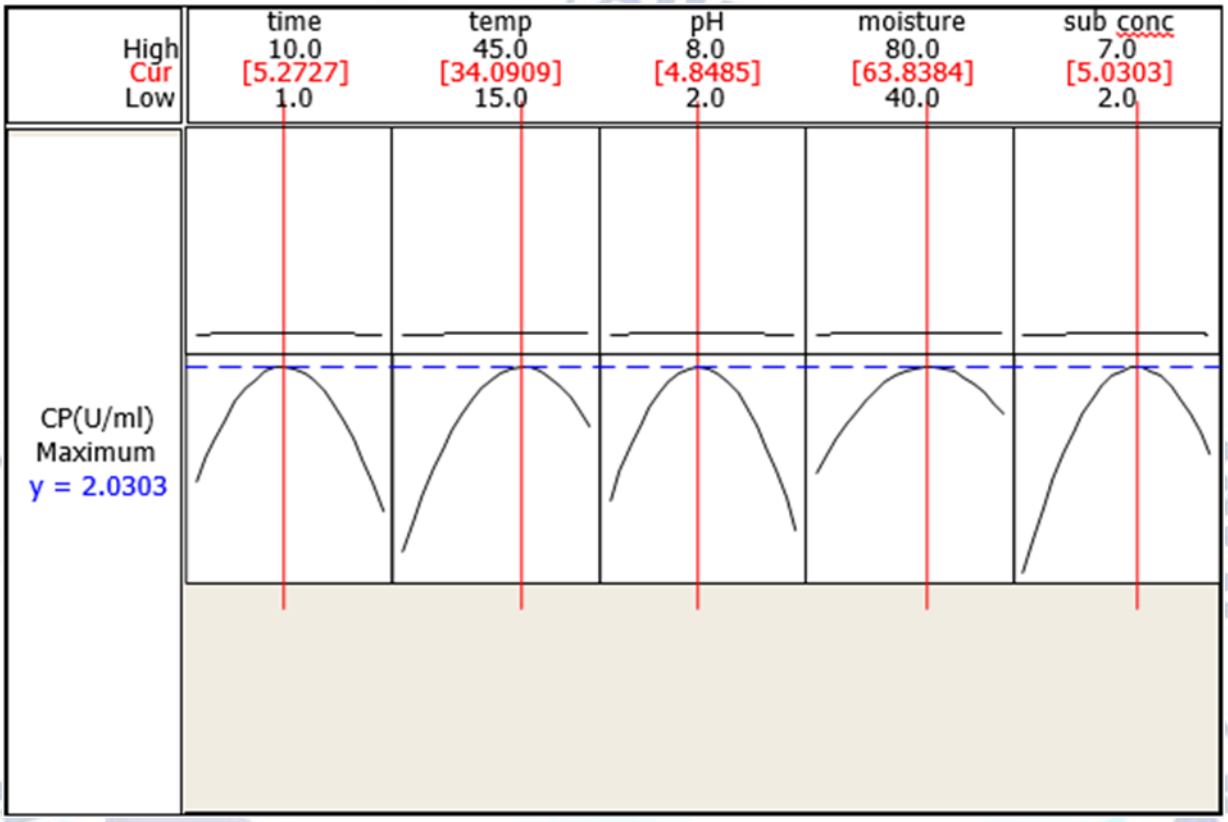

Fig. 2 Optimization plot for cellulase enzyme production

Incubation time is one of the important factors affecting the cellulase enzyme production. It was found that as the incubation time increased, cellulase activity also increased till 5 days and after that there is a decrease in cellulase activity. The optimum incubation time was found to be 5.27 days. "The optimization of the time course is of prime importance for saccharification by fungi" (Khud& Sing, 1993). The decrease in the cellulase activity after 5 days of incubation period may be because of the reduction of the nutrients or catabolic repression of cellulase enzyme by released glucose.

It was found that when there is an increase in temperature from 15 to 35°C, cellulase enzyme activity also increased. The optimum temperature was found to be 34.09°C at which maximum enzyme activity (2.03 U/ml) was achieved with mixed culture."As the temperature was further increased, there was a gradual reduction in the cellulase enzyme activity. This may be due to the fact that higher temperature denatures the enzymes mainly cellulase."(SolomonB.O.1999). "Results observed are in line with Mekala et al., (2008) who showed that cellulases production was maximum in flasks when incubated at 33°C and decreased with high temperature"

The optimum pH was found to be 4.85 at which cellulase enzyme activity was maximum and hence optimized for cellulase production from mixed fungal strains. "After that the production of cellulases decreased which might be due to the fact that cellulase are acidic proteins and are greatly affected by the neutral pH values" (Chandra et al., 2009).

The moisture content was varied between 40 to 80%. The optimum moisture content was found to be 63.84%.The moisture content has a significant effect on the growth of microorganism "Efficiency of mass transfer in solid- phase particles depends on the moisture and substrate characteristics; however, an excessive increase in moisture content inversely effects the enzyme production. It is because of the fact that more than its optimum amount of water leads to the reduction in the contact surface of the particles" (Dutt& Kumar 2014)

The cellulase activity increases up to 5 % substrate concentration and then it decreases. "A decrease in enzyme activity beyond maximum (5%) substrate concentration may be due to inhibitors.

The decrease may also be due to depletion of the nutrients other than the energy source or due to the specific binding of the enzymes with the substrate". (Milala et al ; 2005) conformity of the second-order regression model with the experimental data.

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