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STATISTICAL OPTIMIZATION OF AMYLASE PRODUCTION FROM *ASPERGILLUS FLAVUS* USING POD BIOMASS OF *PITHECELLOBIUM DULCE* AS SUBSTRATE.

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ABSTRACT

Microorganisms are extremely good with an amazing array of valuable products. But they usually produce them only in amounts that they need for their own benefit; thus, they tend not to overproduce their metabolites. Therefore, attempts have been made to specify the conditions to produce higher amylase on commercial scale. Present study mainly focused on optimization of amylase production in submerged fermentation by *Aspergillus flavus*. Three optimization parameters as pH, incubation time and temperature were treated as independent variables for the maximum production of amylase. These parameters were studied for the amylase production by using response surface methodology (RSM) based central composite design (CCD). The model equation provide a suitable model for the response surface for amylase production and from the optimal concentrations of the medium components, a model was predicted. The maximum amylase activity was predicted 22.89 IU/ml, when incubation time was 96 hrs, pH 6 and temperature 30°C.

KEYWORDS: Optimization, central composite design (CCD), amylase activity, independent variables, substrate.

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INTRODUCTION

The biotechnological exploration due to the advancements in techniques has provided an insight into the enormous potential of microbial communities as bioreactors. Among these bioreactors, amylases have received great deal of attention. The spectrum of amylase has widened in global enzyme market due to application in many fields such as food, pharmaceuticals, textiles, detergents, etc.[1] Their enormous commercial potential is indicated by the fact that they alone constitute approximately 25-30% of the enzyme market.[2] However, one of the problems associated with commercialization of amylase production is feedstock availability and its cost. Hence critical stage in a biological process is optimization to improve a system and increase the efficiency of process without increasing the cost.[3] The cost of substrate contributes more than 40% of the total cost of enzyme production.[4] As an attempt to seek novel, economical industrially valuable substrate, the pod biomass of *Pithecellobium dulce* have been used as substrate for amylase production .Fungi are widely distributed in environment especially in soil in saprophytic mode because they produce variety of hydrolytic enzymes.[5] Hence , we used soil isolated fungal culture of *Aspergillus flavus* for the production of amylase .When developing an industrial fermentation, different parameters have critical importance because they significantly affect product concentration, yield, and productivity. Therefore, process optimization can be used for the overproduction of enzymes in large quantities to meet industrial demands and economical efficient fermentation .[6] The traditional pattern of optimize a production process by conducting the experiments by altering “one variable at a time” (OVAT), while keeping all others at a predetermined level is very inefficient in many cases, since it involves carrying out many experiments which are time-consuming and laborious . Moreover, these OVAT designs often overlook the interactions among the variables.[7] Some of these confusions may be avoided with multi-parameter optimized study. In this reference the response surface methodology (RSM) based on a central composite design (CCD) is widely applicable for optimization conditions.[8] Generally, RSM is a statistical and mathematical tool for designing experiments, building model, evaluating the effect of many variables, investigating the optimum conditions for desirable response, and reducing the number of required experiments. Recently it has been extensively used for the process and media optimization studies by microbes for enzyme production.[9] In the last few years, RSM has been applied to optimize and evaluate interactive effects of independent factors in numerous chemical and biochemical processes.[10] It determines the optimum process conditions through combining experimental designs with interpolation by first- or second-order polynomial equations in a sequential testing procedure.[11] It represents a set of statistical techniques for executing planning and evaluating the effect of independent variables on desired variable response.[12] Compared to classical method of optimization, central composite design (CCD) was more effective in bioprocess optimization.[13] In this context, this study was conducted for the amylase production by using response surface methodology (RSM) based central composite design (CCD). we also evaluate the potency of *Aspergillus flavus* by using waste biomass of *pithecellobium dulce* as substrate with optimum culture conditions and factors involved in maximum production of amylase.

MATERIALS AND METHODS

Chemicals

Soluble starch, used in the amylase assay, was purchased From Sigma aldrich. All other media components and analytical reagents used were of highest purity grade available commercially in India either from Hi Media, or Merk.

Microorganism and inoculum preparation

The fungus *Aspergillus flavus* used for the productionof amylase was isolated from soil samples of Haryana. For inoculum preparation, sterilized normal saline (20-40 mL) containing tween-80 (0.1% v/v) was added to five days old slants of fungal culture grown on PDA medium. The spores were scratched by sterile wire loop and shaken vigorously for preparing a homogenous spore suspension. A standard spore count was done using a hemocytometer. One milliliter of spore suspension containing $1x10^7$ spores was used as inoculum for submerged fermentation.

Substrate Preparation

The pod biomass of *Pithecellobium dulce* was collected from Rohtak city which is located at a latitude of 30°1`N and longitude of 75°17`E and its nearby places. The pod biomass washed with tap water and then oven-dried at 70°C till constant weight. Then the biomass grinded and sieved to obtain mean particle size (≤ 425um) .It stored in sealed plastic bags at room temperature for carrying out further experiments of amylase production in submerged fermentation.

Medium Preparation And Culture Conditions For Amylase Production

For submerged fermentation, 50 mL Mendel & Reese media[12] containing (g/l) protease peptone, 1; $(NH_4)_2So_4$, 1.4; KH_2Po_4, 2.0; urea, 0.3; $CaCl_2$, 0.3; $MgSo_4.7H_2O$, 0.3; $FeSo_4.7H_2O$, 0.005; Tween-80, 0.1 % (v/v), supplemented with 1 % (w/v) pod biomass of *pithecellobum dulce* as substrate was added to 250 mL Erlenmeyer flasks and autoclaved at 121°C for 20 minutes. After sterilization, the media was allowed to cool and inoculated with 1.0 mL spore suspension of the fungus containing approximately $1x10^6$ spores. The inoculated flasks were incubated at relevent temerature for six days in an orbital incubator shaker with agitation speed of 180 rpm. At predicted intervals of fermentation period, 1.0 mL of the culture filtrate was withdrawn under aseptic conditions and centrifuged at 10,000 rpm for 10 minutes at 4°C to remove unwanted particles and spores. The cell free supernatants obtained after centrifugation were used as the crude extracellular enzyme source and assayed amylase activity .

Amylase Assay

The dialysed sample will be assayed for amylase activity. The enzyme sample will be incubated with soluble starch at desired temperature and pH. After 10 mintues, the liberated reducing sugars will be estimated using 3,5- dinitrosalicyclic acid (DNS) method .[15] The colour developed was read by measuring its optical density using a spectrophotometer at 540 nm. One unit of enzymatic activity (IU) was defined as the amount of enzyme releasing 1 µmol of sugar in 1 minute under standard assay conditions.

Optimization Of Amylase Production By Response Surface Methodology Based Central Composite Design (CCD).

In order to obtain maximum enzyme production while keeping in view the interactions between different factors of the 'one-at-a-time' optimized medium, a statistical design approach, CCD, was used. The effect of three variables, viz., incubation time, incubation temperature, pH was studied by this method. A 2^3 factorial design, with fourteen axial points and six replicates at the centre point with a total number of 20 experiments as listed in Table 2, was employed. The minimum and maximum ranges selected for the three variables are listed in Table 1.

Table 1

Range of variables used in central composite design

Variables	Name of Factor	Range studied		Coded Values		Mean
		Minimum	Maximum			value
A	Incubation Time	24	152	-1.000=24	+1.000=120	72
B	Temperature	20	45	-1.000=30	+1.000=40	35
C	pH of media	2	8	-1.000=3	+1.000=8	5

Table 2

Experimental design of RSM showing experimental and predicted values of amylase production

	Factor 1	Factor 2	Factor 3	Responses	
Run	A:Incubation time (Hours)	B:Temperature (°C)	C:pH of media	Experimental (U/ml)	Predicted (U/ml)
1	72	32.5	5.5	16.6	15.99
2	120	40	8	9.98	10.07
3	72	45	5.5	8.87	8.86
4	72	20	5.5	9.87	8.89
5	120	25	3	10.56	10.67
6	96	30	6	22.89	21.09
7	72	32.5	5.5	16.23	16.2
8	72	32.5	1.5	6.67	5.99
9	24	40	3	5.67	5.34
10	120	40	3	4.89	3.89
11	120	25	8	10.45	11.67
12	72	32.5	5.5	18.67	19.03
13	120	32.5	6	21.28	21.98
14	24	25	3	6.34	6.43
15	152	32.5	5.5	12.45	13.02
16	24	40	8	2.54	2.96
17	72	32.5	8	14.01	15.03
18	24	25	8	3.1	3.3
19	72	32.5	5.5	18.67	19.03
20	48	32.5	5.5	13.99	14.07

Fermentation experiments **were** carried out separately for each with replicates. Upon completion of experiments, the average amylase activity was taken as a dependant variable or response (Y). Regression analysis was then performed on the data obtained. The results of CCD were then used to fit the following second-order polynomial equation:

$$Y= \beta 0 + \beta 1\ A + \beta 2\ B + \beta 3\ C + \beta 11\ A^2 + \beta 22\ B^2 + \beta 33\ C^2 + \beta 12\ AB + \beta 23\ BC + \beta 13\ AC$$

where Y = predicted response; β 0=regression coefficient; β 1, β 2, β 3 = linear effect; β 11, β 22, β 33 = squared effect; β 12, β 23, β 13 =interaction effect.

Statistical Analysis Software

Experimental designs and the polynomial coefficients were calculated and analyzed using a trial version of Design-Expert software (version 9, Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Validation of Model

The statistical model was validated with respect to all three variables within the design space. A verification assay under the optimized conditions was used for validation of the statistical model.

RESULTS AND DISCUSSION

Optimization of parameters by CCD

Three optimization parameters pH, incubation time and temperature were treated as independent variables had to be varied was determined by using CCD. The data recorded for amylase production were fed into Design-Expert software and analysed. The data were analysed using analysis of variance (ANOVA) as appropriate to the experimental design used. The calculated regression equation for the optimization of medium components showed the amylase production (Y) as a function of these variables. By applying multiple regression analysis on the experimental data, the following equation was found to explain amylase production:

$$Y=-72.27697-0.26409A+3.90861B+6.03305C+1.35577A^2+0.061993\ B^2+0.74121\ C^2\ 2.71814AB + 0.014534AC - 0.038362BC$$

where Y represents amylase production (U /mL) and A, B, C, A^2, B^2, C^2, AB, BC and AC are the independent and interacting variables. The experimental levels of amylase production are given Table 2 along with the predicted data. The quality of the model can be checked using various criteria. The coefficient of determination (R^2) was calculated to be 0.9874, which ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that 98.74 % of the variability in the response could be explained by the model. The closer the R^2 value is to 1.0, the stronger the model and the better it predicts the response. The values of correlation coefficients (adj. R^2 and pred. R^2) for amylase production were 0.9407 and 0.8904, suggesting a strong agreement between the experimental and predicted values of amylase production. The Model F-value of 14.23 implies the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant.The three-dimensional response surface curves were then plotted to understand the interaction of the given factors and the optimum concentration of each component required for optimum amylase production. The response surface curves (Figs. 1–3) show the relative effects of the two variables when the concentration of the third variable is maintained at a constant level.

Figure 1

Response surface plot of amylase production from Aspergillus flavus as a function of incubation temperature and time.

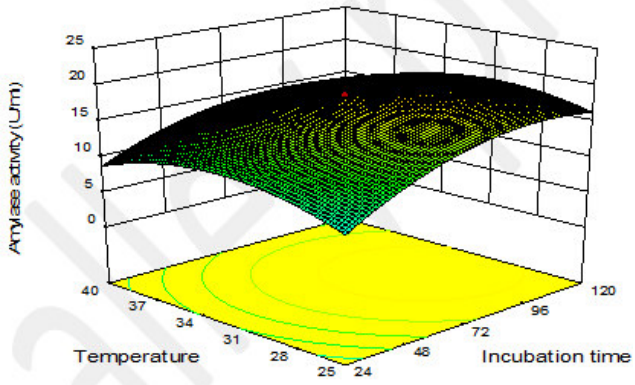

Figure 2

Response surface plot of amylase production from Aspergillus flavus as a function of incubation temperature and pH of media.

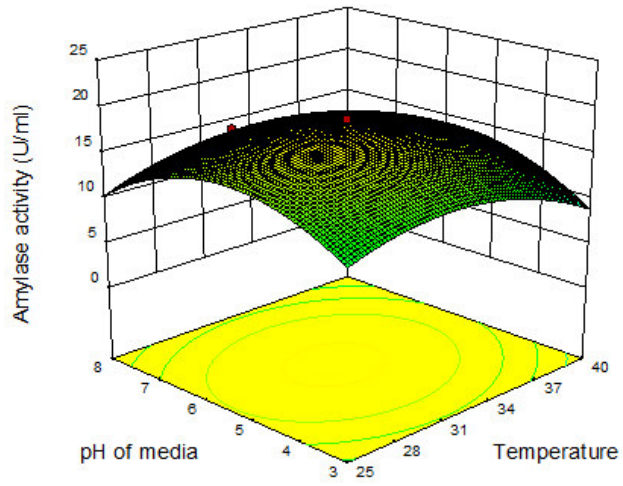

Figure *3*
Response surface plot of amylase production from Aspergillus flavus as a function of incubation time and pH of media.

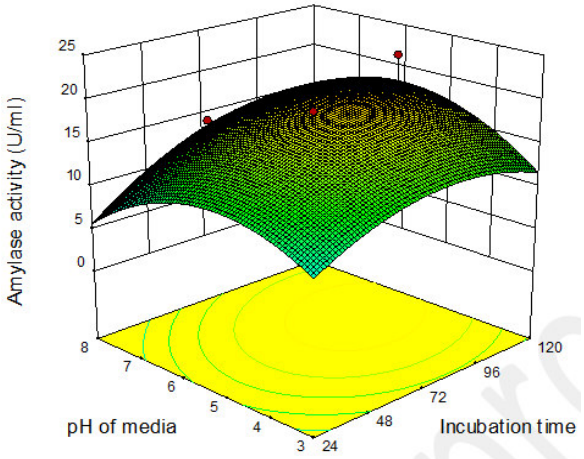

Figure 1 shows the response of relative effect of amylase production from *Aspergillus flavus* as a function of incubation temperature and time at constant pH of media. Here, a linear effect was observed with increasing incubation time from 96 hrs. Any further increase in the incubation period leads to decreased the amylase production. On the other hand, the highest contour levels were obtained within temperature range of 30 °C. Figure shows that the amylase production increased with the increase in the concentrations of both incubation time and pH of media at certain extent. The coordinates of this contour level in this case lie between72- 96 hrs on axis of incubation time, and 6 -7 on pH axis. Therefore, the optimum concentration of these two components are expected to fall around these values. At a constant incubation time , again a linear effect was observed between amylase production & incubation temperature. Results depicted that maximum amylase production was obtained at incubation time of 96 hrs and pH of 6 with temperature range of 30 °C. The response surface methodology, a smaller and less time consuming experimental design, could generally satisfy the optimization of many microbial processes.[16]After optimization of the medium components, the amylase production was enhanced to a significant level. The above findings led to the reasoning that the production of amylase in this case is highly influenced by the incubation time and pH of media and to some extent by the incubation temperature also.

Validation of the model

In order to validate the adequacy of the model, a verification experiment was conducted under optimal operation conditions obtained by the regression model, temperature –30°C, incubation time –96 hrs, spore and pH - 6. The experiments were performed in triplicates and the results are compared. The amylase activity (22.89U/mL) obtained from experiments was close to the predicted response (21.09 U/mL) predicted by the regression model, which proved the validity of the model.

CONCLUSIONS

Although, considerable research has been done in production of amylase, not much attention has been paid to readily available, high volume low cost by-product of industries and a potentially valuable biomass resource for industrial exploitation. Therefore, the study was undertaken with the aim of optimizing a fermentation process for the production of amylase from a non-conventional starch source of pod biomass of *pithecellobium dulce* as substrate. Because the need to develop and improve sustainable substrate is eminent due to the finite nature of economically important food crops. In the present work, we have demonstrated the use of a central composite design by determining the conditions leading to higher amylase production. Thus, smaller and less time consuming experimental designs could generally suffice for the optimization of fermentation parameters. The study ensure that waste pod biomass of *pithecellobium dulce* have its own potential for amylase production and can be used as a cheaper substrate for fermentation .It offers a potential pathway to low cost amylase production. The use of such wastes besides providing alternative substrates helps to solve environmental problems, which are otherwise caused by their disposal. The huge availability of this biomass may reduce the cost of importation and encourage self-reliance. This study becomes important to the industrial chemistry and likewise contributes to environmental management and economic stability.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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