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OPTIMIZATION OF PROCESS PARAMETERS FOR THE ENHANCED PRODUCTION OF A -AMYLASE USING *A.NIGER* IN SUBMERGED FERMENTATION

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ABSTRACT

Optimization of five process parameters - pH, temperature, fermentation time, inoculum size and substrate concentration - were attempted for the optimal production of alpha amylase using central composite design of response surface methodology by *A.niger* MTCC-282 under submerged culture conditions. The optimization process was carried out using the central composite design and response surface methodology. Maximum amylase activity of 25.0 U/ml was obtained at the fermentation time of 75.55 hr when an inoculum size and substrate concentration of 5.17 % and 21.2 g/L respectively were used at a pH of 4.84 and temperature of 29.93 ^{o}C.

INTRODUCTION

Large quantities of agricultural wastes generated from the various agricultural and industrial process, represents one of the most abundant source of carbon in the nature (Nigam *et al.*, 2001). Utilization of this biomass for the production of value added products can reduce the production costs to considerable extends. Wheat bran-the byproduct of wheat processing- is one such source which has been successfully utilized as substrate for many micro-organisms. Use of wheat bran as substrate for amylase production is also reported.

Alpha amylase is an extracellular enzyme, which catalyzes the hydrolysis of internal α-1,4-O-glycosidic bonds in starch and related polysaccharides liberating α-anomeric sugars and limit dextrins (Sivaramakrishnan *et al.*, 2006). It is an industrially important enzyme with a wide spectrum of applications in food, textile and paper industry along with its use in clinical, medical and analytical chemistry (Ramachandran *et al.*, 2004). Fungal amylases particularly from *Aspergillus* species have a high efficiency in saccharification compared to bacterial amylases and thus find various applications in antistaling, haze clarification in fruit juices and alcoholic beverages, glucose and maltose syrup production and other food products (Van der Maarel *et al.*, 2002; Aquino *et al.*, 2003). Though α-amylase can be produced from a variety of sources microbial amylases are of industrial importance. It is secreted as a primary metabolite and its production is growth associated (Spohr *et al.*, 1998; Sudo *et al.*, 1994). Production of α-amylase by submerged fermentation of microorganism has certain advantages such as: reliable scale up, simple process control, reasonably high yield and easy recovery by a series of simple fractionation and purification steps (Esfahanibolandbalaie *et al.*, 2008).

Amylase activity is strongly influenced by the various process parameters such as pH, temperature, fermentation time, inoculum size and substrate concentration. Thus the optimization of these parameters is crucial for the enhanced yield of amylase. The conventional 'one-factor-at-a-time' method is time consuming, laborious and assumes there is no interaction between the variables, that the response is a function of single parameter. The drawbacks of this method can be eliminated by central composite design using response surface methodology, which takes into account the variable interactions in generating the response. The current study thus aims at the optimization of the aforementioned process parameters by central composite design of RSM for maximizing the activity of amylase.

MATERIALS AND METHODS

Microorganism and maintenance

Aspergillus niger MTCC-282 obtained from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India was used in the present study. The culture was propagated on potato dextrose agar (PDA) and subcultured at an interval of three months. Slants were grown for 72 hrs and stored at 4^{o}C.

Inoculum preparation

The spore suspension was prepared from a sporulated (72 hr old) PDA slant culture using 10ml sterile water. The spores on the surface of the medium were dislodged using inoculation needle under aseptic conditions. The spore suspensions were filtered using sterile muslin cloth into a sterile 250 ml Erlenmeyer flask containing 100 ml of potato dextrose broth and incubated for three days at 25^{o}C. Appropriate volumes of inoculums (% v/v) were used to inoculate the production medium.

Enzyme production in submerged fermentation

The agricultural byproduct wheat bran collected from shops near Chidambaram, Tamilnadu, India was utilized as the substrate. The substrates were powdered in a laboratory grinder and sieved using a 40mm sieve after drying it at 80^{o}C for 12 hours in an oven. Adequate amount of the powdered substrate was mixed with 100ml of the mineral salt media in a

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250ml Erlenmeyer flask. The composition of mineral salt media was as follows: KH_2PO_4, 8.943 g/L; $MgSO_4$, 2.324 g/L; NaCl, 2.20 g/L; $MnSO_4$, 0.572 g/L and $NaNO_3$, 0.874 g/L (optimization data not shown). After adjusting the pH the contents of the flask were sterilized in an autoclave at 121°C and 15 psi pressure for 15 minutes. Appropriate volumes of inoculums were added to the flasks after cooling it down to room temperature.

Enzyme extraction

At the end of the fermentation period, contents of the flask were filtered using a Whatman No.44 filter paper followed by filtration through a muslin cloth. The filtrate was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was used as the source of enzyme for assay.

Enzyme assay

0.1ml of the enzyme extract was taken in a test tube along with 0.2 ml of buffered soluble starch (0.1 M acetate buffer pH 5.0) and incubated at 40°C for 10 minutes. After the incubation time, the reaction was stopped by adding 5 ml of iodine reagent which gives a blue colour due to the formation of starch-iodine complex. Iodine reagent was prepared freshly by diluting 1.0 ml of stock solution (0.5 % I2 in 5.0 % KI) in 500 ml of distilled water containing 5 ml of 5N HCL. A blank was also kept without adding the enzyme solution. The absorbance was then measured at 620 nm against the blank. One unit of amylase activity was defined as the amount of enzyme which produced 0.01% reduction in the intensity of the blue colour of the starch-iodine complex under assay conditions (Abou-Zeid, 1997) and the enzyme activity has been expressed as U/ml of fermentation broth.

Optimization of process parameters

In order to enhance the production of amylase, central composite design of response surface methodology (RSM) was employed to optimize the five variables selected for the study. A 2^5 factorial design was employed with 52 experimental runs. These 52 experiments were performed with different combinations of the five independent variables. The five independent variables were studied at five different levels −2.37, -1, 0, 1 and 2.37 and a set of 52 experiments was carried out. All variables were taken at a central coded value zero. All the experiments were carried out in triplicates and the average value was taken as the response.

RESULTS AND DISCUSSION

A central composite design was employed to analyze the interactive effects of the five process parameters for the production of amylase by *A.niger* MTCC-282 utilizing wheat bran, in order to arrive at an optimum value. Table 1 shows the summary of the variables and their variation levels. Submerged fermentation was carried out according to the design. The fermented samples were extracted and analyzed for amylase activity. The results obtained were analyzed using MINITAB15.

The 52 run design matrix along with the experimental and the predicted responses are given in Table 2. The results of the regression analysis of the second order polynomial model are given in Table 3. The second order polynomial equation fitted by the regression analysis for amylase production (Y) is given by:

$$Y = 24.9803 - 0.0683A - 0.1003B - 0.5651C + 0.1737D + 0.1542E - 0.5398A^2 - 0.9172B^2 - 0.9676C^2 - 0.5239D^2 - 0.9994E^2 - 0.4431AB + 0.7000AC - 0.0619AD + 0.3544AE + 0.2388BC - 0.0194BD - 0.0994BE + 0.0625CD - 0.0488CE - 0.1569DE \quad \text{Eqn.....(1)}$$

where A, pH; B, Temperature (°C); C, Fermentation time (hr); D, Inoculum size (%) and E, Substrate concentration (g/L).

Analysis of variance (ANOVA) was used to check the model adequacy and the significant parameters. From the results of ANOVA shown in Table 3, the model terms A, B, C, D, E, A^2, B^2, C^2, D^2, E^2, AB, AC, AD, AE, BC, BD, BE, CD and DE are found to be significant. The R^2 value of 0.9954 indicates good correlation between the experimental and predicted values. The predicted R^2 values 0.9835 is also in good agreement with the corresponding R^2 adjusted value of 0.9825. The parity plot between the experimental and predicted value is shown in Fig 1.

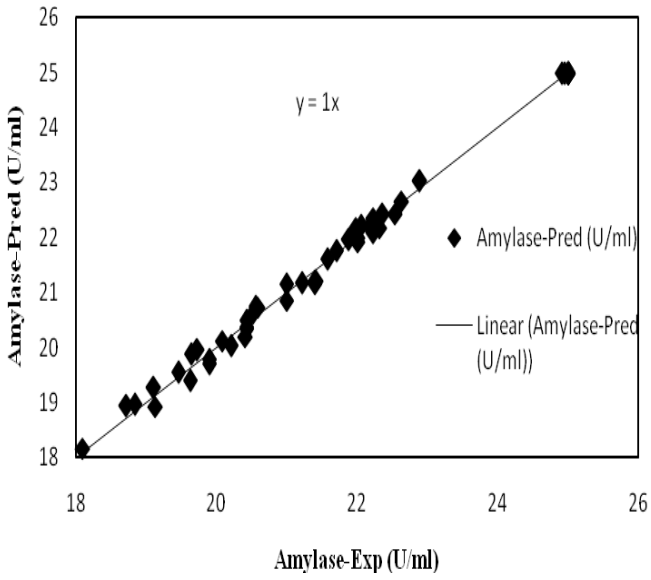


Fig 1 Parity plot between the experimental and predicted values of process parameters

The response surface curves for determining the interaction effects and optimal levels of the variables are represented in Fig.2 (a-j).

Figure 23D plots showing the effects of process parameters on amylase activity

Fig.2(a) Response surface plot for amylase production showing the interactive effect of temperature and pH.

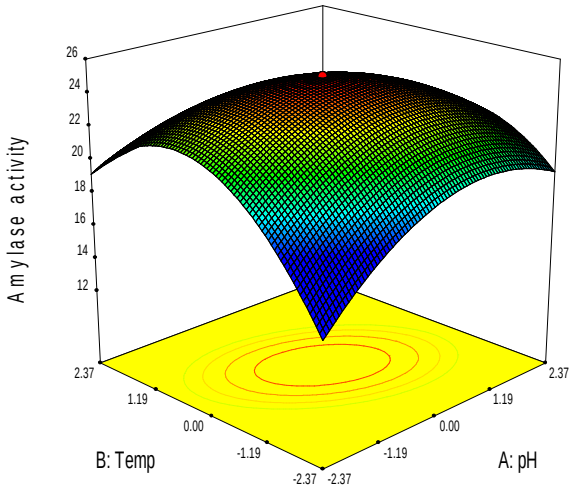


Fig.2(b) Response surface plot for amylase production showing the interactive effect of time and pH.

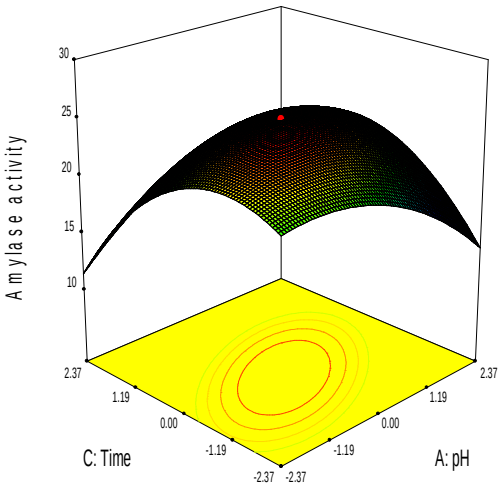


Fig.2(c) Response surface plot for amylase production showing the interactive effect of inoculum size and pH.

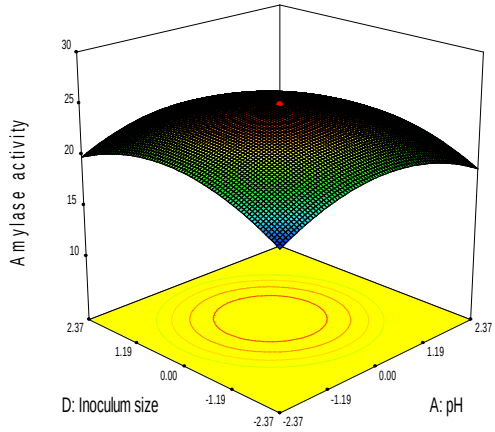


Fig.2(d) Response surface plot for amylase production showing the interactive effect of substrate concentrationand pH.

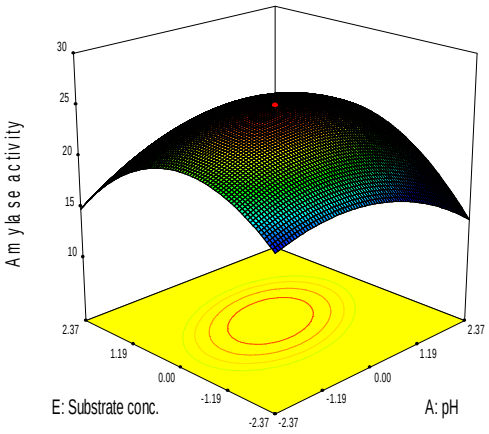


Fig.2(e) Response surface plot for amylase production showing the interactive effect of time andtemperature.

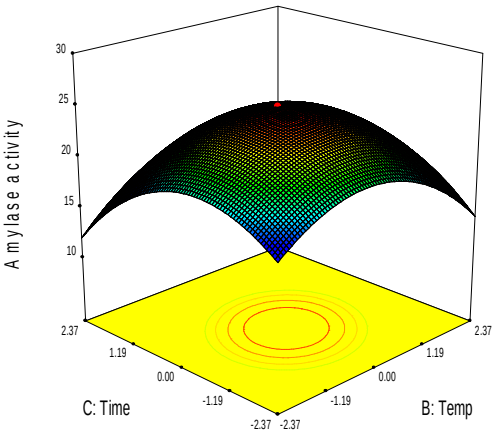


Fig.2(f) Response surface plot for amylase production showing the interactive effect of inoculum size and temperature

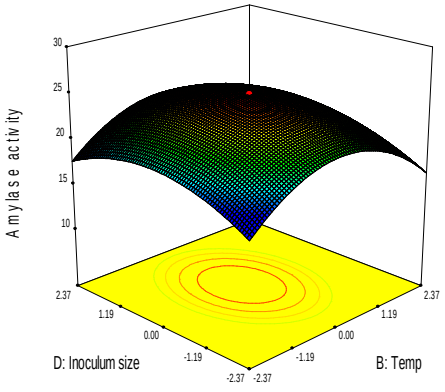


Table 1 Summary of variables and their variation levels employed in CCD

Variables	Symbol	Coded levels				
		-2	**-1**	**0**	**+1**	**+2**
pH	A	4	4.5	5	5.5	6
Temperature (^{0}C)	B	24	27	30	33	36
Time (hr)	C	66	72	78	84	90
Inoculum size (%)	D	3	4	5	6	7
Substrate (g/L)	E	10	15	20	25	30

Table 2 The Central Composite experimental design with five independent variables

Run No.	Coded values					Amylase Activity (U/ml)	
	A	B	C	D	E	Exp.	Pred.
1	0	0	0	0	0	24.91	24.980
2	-1	1	-1	-1	1	22.19	22.222
3	0	0	0	0	0	25.00	24.980
4	1	-1	-1	1	-1	21.22	21.180
5	0	0	-2.38	0	0	21.00	20.851
6	1	1	1	-1	-1	20.08	20.122
7	0	0	0	0	0	25.00	24.980
8	0	0	0	0	0	25.00	24.980
9	-1	1	1	-1	1	19.73	19.947
10	-1	1	1	1	1	20.41	20.191
11	1	-1	-1	-1	1	22.23	22.356
12	2.38	0	0	0	0	21.71	21.764
13	-1	-1	1	1	-1	19.64	19.878
14	0	0	0	-2.38	0	21.58	21.604
15	1	1	1	1	-1	20.56	20.746
16	0	0	0	0	0	25.00	24.980
17	-1	-1	1	-1	-1	19.13	18.929
18	0	0	2.38	0	0	18.10	18.163
19	-2.38	0	0	0	0	22.23	22.089
20	0	0	0	0	0	25.00	24.980
21	1	1	1	1	1	21.00	21.153
22	0	0	0	2.38	0	22.54	22.430
23	0	-2.38	0	0	0	20.21	20.030
24	-1	-1	-1	1	1	22.23	22.245
25	1	-1	-1	1	1	21.98	22.180
26	0	0	0	0	0	24.95	24.980
27	0	0	0	0	0	25.00	24.980
28	-1	-1	1	1	1	19.11	19.265
29	-1	-1	-1	-1	1	22.32	22.174
30	1	1	-1	1	-1	19.90	19.776
31	0	0	0	0	0	25.00	24.980
32	-1	1	-1	-1	-1	22.36	22.410
33	-1	-1	-1	-1	-1	21.88	21.964
34	-1	-1	1	-1	1	18.72	18.944
35	0	2.38	0	0	0	19.46	19.553
36	1	1	-1	1	1	20.43	20.378
37	1	-1	1	1	1	22.01	22.000
38	1	-1	1	-1	1	22.01	21.926
39	1	-1	1	-1	-1	20.43	20.494
40	0	0	0	0	-2.38	18.84	18.960
41	1	1	-1	-1	1	20.52	20.632
42	0	0	0	0	0	25.00	24.980
43	-1	1	1	-1	-1	20.43	20.330
44	1	1	-1	-1	-1	19.63	19.402
45	-1	-1	-1	1	-1	22.63	22.663
46	0	0	0	0	2.38	19.90	19.693
47	-1	1	-1	1	-1	22.89	23.031
48	-1	1	1	1	-1	21.40	21.201
49	1	1	1	-1	1	21.40	21.157
50	1	-1	-1	-1	-1	20.59	20.729
51	1	-1	1	1	-1	21.42	21.195
52	-1	1	-1	1	1	22.06	22.216

Table 3 Results of the regression analysis of second order polynomial model

Term constant	Regression coefficient	T-statistics	P-value
Intercept	24.9803	465.941	0.000
A	-0.0683	-2.634	0.013
B	-0.1003	-3.869	0.001
C	-0.5651	-21.803	0.000
D	0.1737	6.701	0.000
E	0.1542	5.951	0.000
A^2	-0.5398	-24.210	0.000
B^2	-0.9172	-41.137	0.000
C^2	-0.9676	-43.397	0.000
D^2	-0.5239	-23.497	0.000
E^2	-0.9994	-44.824	0.000
AB	-0.4431	-14.695	0.000
AC	0.7000	23.214	0.000
AD	-0.0619	-2.052	0.049
AE	0.3544	11.752	0.000
BC	0.2388	7.918	0.000
BD	-0.0194	-0.643	0.525
BE	-0.0994	-3.296	0.002
CD	0.0625	2.073	0.047
CE	-0.0488	-1.617	0.116
DE	-0.1569	-5.202	0.000

R-Sq = 99.54% R-Sq(pred) = 98.35% R-Sq(adj) = 99.25%

Table 4 ANOVA for the fitted polynomial model for parameter optimization

Sources of variation	Sum of squares	Degrees of freedom (DF)	Mean square (MS)	F- value	P-value
Regression	196.398	20	9.8199	337.48	0.000
Linear	16.807	5	3.3615	115.53	0.000
Square	150.345	5	30.0690	1033.39	0.000
Interaction	29.245	10	2.9245	100.51	0.000
Residual Error	0.902	31	0.0291		-
Lack-of-Fit	0.893	22	0.0406	42.30	0.000
Pure Error	0.009	9	0.0010	-	-
Total	197.300	51	-	-	-

Fig.2(g) Response surface plot for amylase production showing the interactive effect of substrate concentration and temperature.

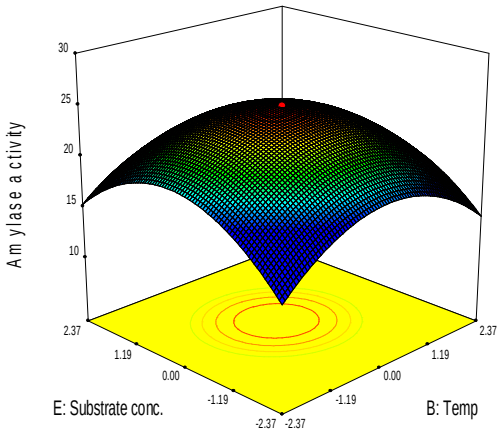


Fig.2(h) Response surface plot for amylase production showing the interactive effect of inoculum size and time

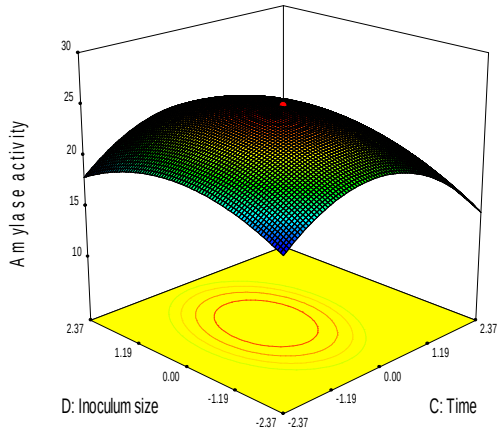


Fig.2(i) Response surface plot for amylase production showing the interactive effect of substrate cooncentration and time.

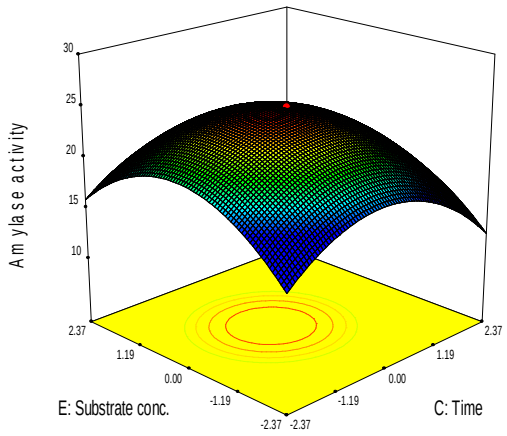
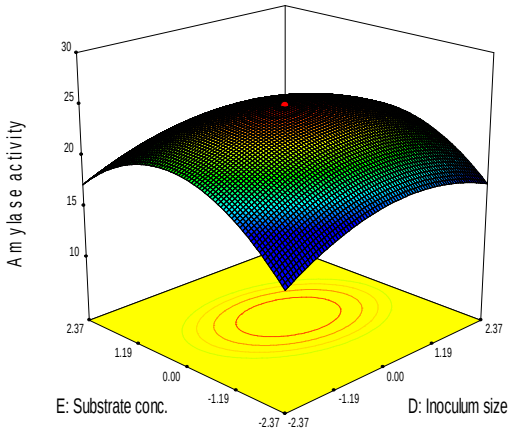


Fig 2(j) Response surface plot for amylase production showing the interactive effect of substrate concentration and inoculum size.



The optimum values obtained were pH, 4.84; temperature, 29.93; time, 75.55hr; inoculums size, 5.17% and substrate, 21.2 g/L. The pH and temperature optimum obtained are in good agreement with the works reported previously using *A.niger* (Hayashida and Teramoto, 1986; Carlsen *et al.*, 1996; Irfan *et al.*, 2012; Mong *et al.*, 2011). The inoculums size of 5% was also reported previously (Irfan *et al.*, 2012).

CONCLUSION

In the present work wheat bran was used as the substrate for the production of α-amylase by *A.niger* MTCC-282 under submerged fermentation conditions. Statistical analysis based on a central composite design was used to find the interaction effects of pH, temperature, fermentation time, inoculum size and substrate concentration for a better α-amylase activity. The results showed that inoculum size and pH are among the most important factors affecting α-amylase activity.

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