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RESPONSE SURFACE METHODOLOGICAL OPTIMIZATION OF PRODUCTION CONDITION OF HYPERTHERMOSTABLE β AMYLASE FROM *Bacillus subtilis* DJ5 UNDER SOLID STATE FERMENTATION USING BARLEY AS SUBSTRATE

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ABSTRACT

Eight different agro-residues were screened for production of hyperthermostable β amylase by *Bacillus subtilis* DJ5 using SSF. Barley showed highest production of 120.34 U/gdm after 41 hours of incubation. Three process parameters (initial moisture content, pH and incubation temperature) affecting enzyme production under SSF using barley as substrate were optimized with RSM. RSM experiments designed by 'Stat Ease' software determined optimum conditions for maximum enzyme production. The best preferred physical conditions were 63.40% initial moisture content, medium pH 6.947 and incubation temperature of 38.50 °C. Under such optimal condition, highest enzyme production of 130.62 U/gdm was recorded after 41 hours of incubation. Incubation temperature was found to play most crucial role for microbial growth and enzyme prodcution under SSF.

KEYWORDS

Response surface methodology, hyperthermostable β amylase, Solid State fermentation, barley, *Bacillus subtilis* DJ5.

INTRODUCTION

Thermostable amylolytic enzymes have attained immense academic and industrial interest[1-3]. Thermostability has been considered as most crucial factor for several industrial applications in order to survive the harsh condition in industrial setup[4]. Among the major group termed 'amylases'[5], microbial β amylases (E.C.3.2.1.2, α-1,4maltoglucan hydrolase) with its severe industrial applications is still suffering from scanty publications[6,7]. A major breakthrough in this path was achieved with the finding of novel hyperthermostable β amylase from mesophilic bacterium *Bacillus subtilis* DJ5[8]. In our several studies, we have reported some positive results regarding industrial application of the enzyme and the producer strain[8,9].

One of the major concerns of fermentation industry is to lower the production cost without compromising production volume. With such view researchers devote efforts in designing low cost fermentation medium and optimize process parameters[10,11]. In this respect SSF offers a big choice of selecting low cost agro residues that are left unutilized after human consumption. Even it offers numerous advantages over SmF in processing of agro-industrial residues as it has low capital investment, lower levels of catabolite repression[12] and end product inhibition, low waste water output, better product recovery, and high quality production[13] and are environmental-friendly.

Present day biotechnology has given response surface methodology (RSM) tremendous importance for bioprocess optimization[14,15]. In fermentation process, the process parameters interact and influence each other to a significant level. RSM can successfully combine the factors through a limited series of experimental strategies, mathematical methods and statistical inferences[16]. Several efforts have been given to find out suitable substrate and process condition for SSF for production of thermostable amylolytic enzymes to meet the growing demand of several industries[15,17].

This paper describes optimization of major process parameters for hyperthermostable β amylase production by *Bacillus subtilis* DJ5 using SSF with the help of full factorial composite design using RSM.

MATERIALS AND METHODS

(i) Microorganism:

Bacillus subtilis DJ5[8] (GenBank Accession Number GU357825) was used in this study. The organism was maintained on Starch Peptone agar medium with the following composition (gram per liter): Peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4,7H_2O$, 0.1; NaH_2PO_4, $2H_2O$, 0.5; soluble starch (Sigma, USA), 5; agar-agar, 15; pH 6.9.

(ii) Inoculum preparation:

Initial study indicated that *Bacillus subtilis* DJ5 is producing maximum enzyme only at six and half hour in starch peptone broth during SmF. For SSF study, such highly active cells were harvested by inoculating 6% of 24 hour broth culture in fresh sterile 100 mL starch peptone broth in a rotary shaker at 160 rpm at 37 °C. Active cells were prepared nutrient free by centrifuging the cells at 8000 rpm for 10 min and dissolving in same amount of sterile 0.1 M phosphate buffer (pH 6.9). It was used as inoculum for SSF. By serial dilution and plating

the number of viable colonies in the inoculum was found to be $3 \cdot 10^8$ CFU/mL.

(iii) Solid state fermentation:

(a) Selection of suitable substrate:

Eight different starchy agricultural products namely Barley (B), Sattu (S), Wheat Bran (WB), Corn Flour (CF), Arrowroot (A), Yellow Peas Split (YPS), Rice Husk (RH), Rice Powder (RP) were screened individually under fixed process parameters i.e. moisture content 60%, pH 7, incubation temperature 37 °C. The solid substrate that induced highest enzyme activity was used in further experiments.

(b) Preparation of substrate and enzyme extraction:

5 gm of substrate was taken in 250 mL Erlenmeyer flask and was autoclaved at 121 °C for 20 min. It was then dried in an oven at 80 °C for 24 hour. To maintain desired moisture level (% by mass per volume), sterile 0.1 M phosphate buffer (pH 6.9) was added aseptically and mixed thoroughly. Culture equivalent to 6% inoculum load of SmF were added to it, mixed thoroughly and incubated at 37 °C for 41 hours.

Unless it is specified otherwise, 0.5 gm of fermented mass was mixed with 2.5 mL of extraction buffer (0.1 M phosphate buffer pH 6.9 mixed with 0.1% Tween 80), vortexed well and centrifuged at 10,000 rpm at 15 min at 4 °C. Supernatant was used as a source of crude enzyme for enzyme assay.

(iv) Statistical optimization procedure:

The optimization of process parameters for β-amylase production by *Bacillus subtilis* DJ5 under SSF using barley as substrate was carried out by central composite design and response surface methodology.

A 2^3 factorial central composite experimental design with 14 noncentral points and 6 replicates at the central point, resulting in 20 experiments generated by Design Expert (Version 8.0.6.1 Stat-Ease Inc., Minneapolis, MN) statistical software. Each set of experiment was performed in triplicates and their values were averaged. The experimental design is shown in Table 1 and the coded variables are reflected in Table 2. A regression model containing 3 linear (β_1, β_2, β_3), 3 quadratic (β_{11}, β_{22}, β_{33}), 3 interactions (β_{12}, β_{23}, β_{13}) and β_0 intercept term was used. The overall second order polynomial mathematical relationship of the response Y (U/gdm) and three variables i.e. initial moisture content (A, %), medium pH (B) and incubation temperature (C, °C) could be approximated by following quadratic equation 1:

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{23}BC+\beta_{13}AC \quad \text{Eqn 1}$$

Experimental data was analyzed to plot response surface. ANOVA was used to estimate statistical parameters.

Table 1

Central composite design consisting of 20 experiments for the study of three experimental factors in coded units

Run	A	B	C
1	-1.000	1.000	1.000
2	0.000	-1.000	0.000
3	0.000	0.000	-1.000
4	1.000	-1.000	-1.000
5	1.000	0.000	0.000
6	1.000	-1.000	1.000
7	0.000	0.000	0.000
8	0.000	0.000	1.000
9	1.000	1.000	1.000
10	0.000	0.000	0.000

11	0.000	0.000	0.000
12	-1.000	1.000	-1.000
13	-1.000	-1.000	1.000
14	0.000	0.000	0.000
15	0.000	0.000	0.000
16	0.000	0.000	0.000
17	1.000	1.000	-1.000
18	0.000	1.000	0.000
19	-1.000	-1.000	-1.000
20	-1.000	0.000	0.000

Table 2
Experimental range and levels of the independent variables

Experimental variables	Coded symbol	Range and levels		
		-1	0	+1
Initial Moisture Content (%)	A	50	60	70
pH	B	5	7	9
Incubation temperature (°C)	C	37	41	45

(v) Analytical method:

β amylolytic activity was measured by the method described by Bernfeld (1955)[18]. Assay mixture contained 0.5 mL of 0.1M phosphate buffer (pH 6.9), 1 mL soluble starch (0.5%, Sigma, USA) and 0.1 mL of enzyme. Control was prepared as same without adding substrate. The reaction mixture was incubated at 100 °C for 15 min. Enzyme-substrate reaction was then stopped by addition of 1 mL 2M NaOH. Both the assay mixture and control were then allowed to boil in boiling water bath for 10 min after addition of 0.5 mL of 3, 5-dinitrosalisylic acid reagent (Merck, Germany). After cooling the assay mixture at room temperature, absorbance were measured spectrophotometrically (Elico, India) at 540 nm. Amount of maltose released (mg) was measured from standard curve of maltose. One unit (U) of β amylolytic activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute per mL from soluble starch (Sigma) under the standard assay conditions. The enzymatic activities used for representations are the average values of three independent experiments. Maximum enzyme titer was expressed as units per gram of dry mass of substrate (U/gdm).

RESULT AND DISCUSSION

1. Selection of suitable substrate

Selection of a proper substrate is a key aspect of SSF. In SSF, solid material is non-soluble that acts both as physical support and source of nutrients. Solid material could be a naturally occurring solid substrate such as agricultural crops, agro-industrial residues or inert support[19,20]. Result of this study indicated (Table 3), all substrates supported microbial growth and enzyme production with varying extent. Barley and Wheat Bran showed highest enzyme production of 120.34 U/gdm and 119.79 U/gdm respectively after 41 hours of incubation. For optimization purpose barley was selected as solid substrate.

Table 3
Effect of different substrates on β amylase producing under SSF

Substrate	β amylase activity (U/gdm)
Barley (B)	120.34
Sattu (S)	113.4
Wheat Bran (WB)	119.79
Corn Flour (CF)	84.29
Arrowroot (A)	24.58
Yellow Peas Split (YPS)	26.59
Rice Husk (RH)	35.19
Rice Powder (RP)	96.39

2. Optimization of process parameter

To detect the effect of 3 major key factors (initial moisture content, pH, and incubation temperature) responsible for hyperthermostable β amylase production, each factor was studied at 3 different levels (-1, 0, +1) by central composite design. The experimental and predicted response of β-amylase production after 41 hours of cultivation by *Bacillus subtilis* DJ5 in barley under SSF are shown in Table 4. The predicted versus actual value curve (Figure 1) is the graphical representation of Table 4 and indicates that there is little variation between predicted and actual enzymatic activity. On the basis of quadratic polynomial equation of response surface model of Eqn 1, the present model successfully defined optimum fermentation conditions as well as illustrated combined effect of independent variables on enzymatic activity.

The coefficients of regression equation were calculated using Design Expert and the following equation were obtained:

$$Y = +87.24 + 12.46A + 0.94B - 17.94C + 6.89AB - 7.11AC - 6.25BC - 8.42\ A^2 - 46.71B^2 + 10.96C^2$$ ..... Eqn 2

Where Y is the response (β amylase activity in U/gdm) and A, B, C are coded values of the test variables, initial moisture contnet (%), medium pH, incubation temperature (°C) respectively

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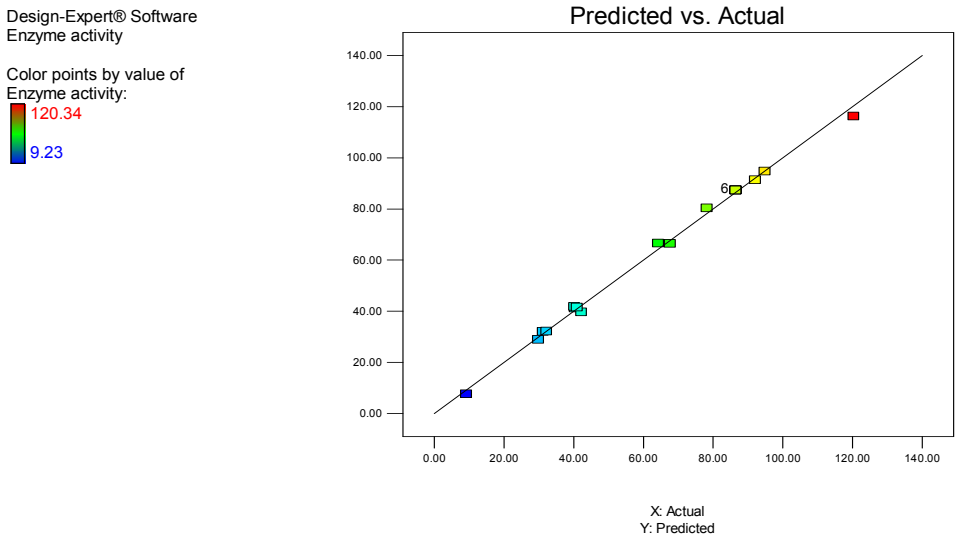
Table 4
Observed responses and predicted values

Run	β amylase activity (U/gdm)	
	Observed response (Y_{obs})	Predicted response (Y_{pred})
1	9.23	7.586
2	42.23	39.583
3	120.34	116.141
4	64.32	66.504
5	92.12	91.281
6	29.87	28.891
7	86.52	87.239
8	78.22	80.263
9	32.23	32.069
10	86.52	87.239
11	86.52	87.239
12	40.21	41.729
13	31.23	31.973
14	86.52	87.239
15	86.52	87.239
16	86.52	87.239
17	94.87	94.667
18	40.98	41.471
19	40.43	41.131
20	67.67	66.353

Figure 1
The predicted vs actual response



The result of the second order response surface model fitting in the form of ANOVA are giben in Table 5. The Model F-value of 394.23 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. This model presented a high determination coefficient (R^2 = 0.9972) explaining 97.5% of the variability in the response. Moreover "Pred R-Squared" of 0.9756 is in reasonable agreement with the "Adj R-Squared" of 0.9947 indicating a high significance of this model. "Adeq Precision" of 71.240 indicates an adequate signal for the signal noise ratio. A very small value of coefficient of variation (C.V.) 3.31% clearly indicate a very high degree of precision and a good reliability of the experimental values.

Table 5
ANOVA for quadratic model

Source	Sum of suqares	df	Mean square	F value	p-value Prob > F	Remarks*
Model	16476.70	9	1830.74	394.23	< 0.0001	s
A-Moisture content	1553.51	1	1553.51	334.53	< 0.0001	
B-pH	8.91	1	8.91	1.92	0.1961	ns
C- Temp	3218.08	1	3218.08	692.97	< 0.0001	s
AB	379.91	1	379.91	81.81	< 0.0001	s
AC	404.84	1	404.84	87.18	< 0.0001	s
BC	312.13	1	312.13	67.21	< 0.0001	s
A^2	195.05	1	195.05	42.00	< 0.0001	s
B^2	6000.48	1	6000.48	1292.12	< 0.0001	s
C^2	330.53	1	330.53	71.17	< 0.0001	s

Residual	46.44	10	4.64
Lack of fit	46.44	5	9.29
Pure error	0.000	5	0.000
Cor Total	16523.14	19	

R^2 = 0.9972, Adj R^2 = 0.9947, Pred R^2 = 0.9756, C.V. = 3.31%, Adeq Precision = 71.240

****s = significant; ns = not significant.***

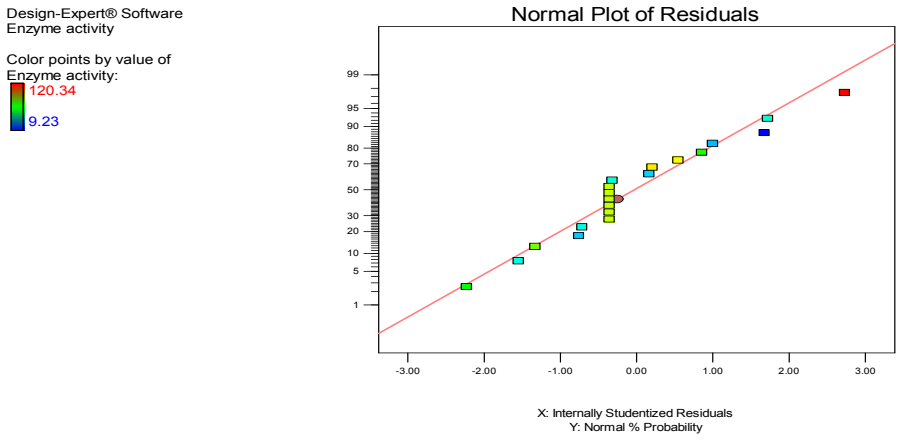
The significane of each coefficient was determined by confidence interval indicated in Table 6. The smaller the confidence length the more significant the facor is. Result indicate that temperature alone affects enzyme production more significantly. Even in interaction, AC and BC have influence on enzyme production as evidenced from lower values of confidence length.

Table 6
Confidence interval (CI) of model factors

Factor	Coefficient Estimate	Standard Error	95%CI Low	95% CI High
Intercept	87.24	0.74	85.59	88.89
A-Moisture content	12.46	0.68	10.95	13.98
B-pH	0.94	0.68	-0.57	2.46
C-Temp	-17.94	0.68	-19.46	-16.42
AB	6.89	0.76	5.19	8.59
AC	-7.11	0.76	-8.81	-5.42
BC	-6.25	0.76	-7.94	-4.55
A^2	-8.42	1.30	-11.32	-5.53
B^2	-46.71	1.30	-49.61	-43.82
C^2	10.96	1.30	8.07	13.86

The resuiduals from the least squares also play an important role in judging model accuracy[21]. A normal probability plot of the residuals (Figure 2) showed a satisfactory straight line that conclude the emperical model is adequate to describe the β amylolytic activity by response surface.

Figure 2
Normal plot of residuals



The 3D response surface and the 2D contour plots are the graphical representation of the regression equation (Figure 3, 4, 5). The main goal of plot is hunt for the optimum values of the variables such that response is maximized. Each contour curve represnts an infinite number of combination of two test variables with the other maintained at their zero level. Elliptical contour is considered as a measure of perfect interactions among independent variables[22] and the maximum predicted value is present in the smallest eclipse in the contour diagram[17]. Figure 2 indicates the response surface is nearly elliptical. Higher production of β amylase was recorded at pH ~ 7 and moisture content of ~ 70%.

Similarly when interaction of temperature and pH was considered, higher production was recorded at pH ~7 and temperature higher thant 37 °C (Figure 3). Contour diagram is not truly elliptical that indicates the particular optimum point in this interaction is present beyond the experimental setup. Similar pattern of highest enzymatic production was also recoded from Figure 4. To determine optimum values of test variables (A, B, and C) regression equation was solved using equation functions of Microsoft Excel 2007. The optimum values of test variables in coded units were A=0.340127, B= -0.02649 and C= 0.921210. At these values, the actual optimum moistuture content, pH and incubation temperature were 63.40%, 6.947 and 38.50 °C respectively.

Figure 3

3D response surface plot showing effects of pH and moisture content on hyperthermostable β amylase production with other variable constant at middle point

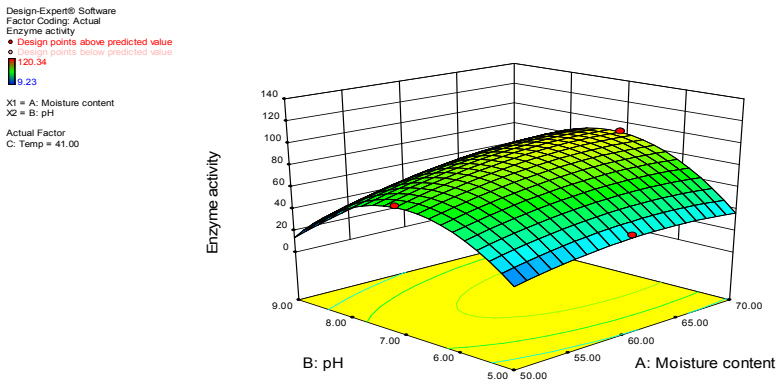


Figure 4

3D response surface plot showing effect of temperature and pH on hyperthermostable β amylase production with other variable constant at middle point

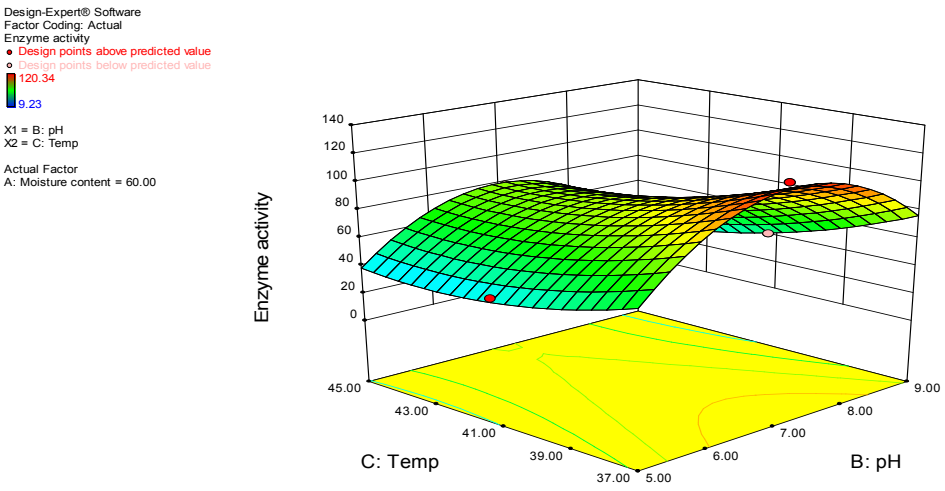
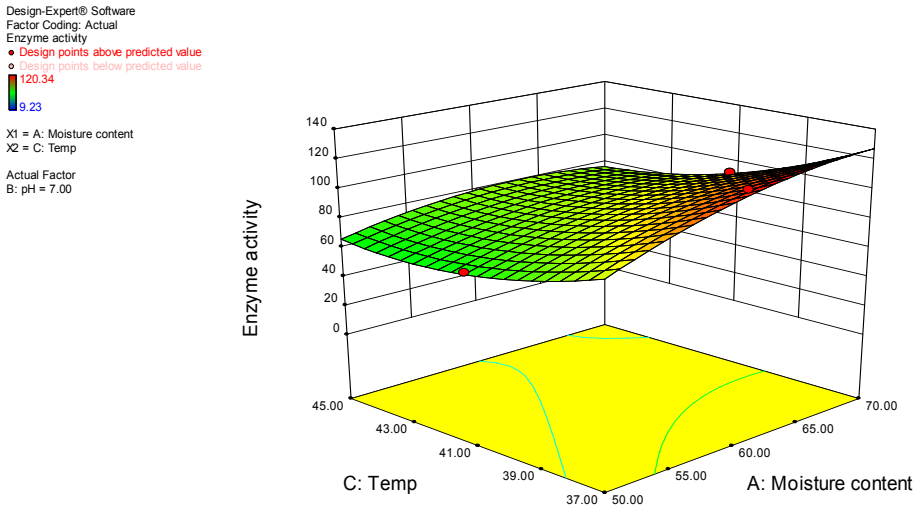


Figure 5

3D response surface plot showing effect of temperature and moisture content on hyperthermostable β amylase production with other variable constant at middle point



CONCLUSION

Among the several published report on response surface methodological optimization of process parameters for α amylase production, there was no report on optimization β amylase production condition. This study successfully established correlation among several process parameters and indicate that incubation temperature is the major contributing factor in SSF for β amylase production.

Abbreviation used:
RSM: Response surface methodology; SSF: Solid State Fermentation; SmF: Submerged Fermentation

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