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Orange Peel Waste For Amylase Production From *Bacillus* Sp. Using Response Surface Methodology

KEYWORDS: amylase, Bacillus, Plackett-Burman design

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ABSTRACT *Amylase production using orange peel as substrate by Bacillus subtilis. Bacillus sp. were isolated from soil contaminated with decaying material of fruit waste. Bacillus sp. EB-9 showed maximum halo zone on starch agar plate (27mm) which was considered as promising isolate for amylase production The nutritional components and operating conditions such as orange peel, glucose, yeast extract, MgSO4, KH2PO4, K2HPO4, NaCl, inoculum size and incubation period were varied according to the Plackett-Burman statistical designs. The variation of α- amylase production in the fifteen trials of Box-Behnken design was ranged from 102.27 to286.62 U /min /ml. The optimum value of the variables in actual unit was predicted as orange peel (4.27 %), yeast extract (0.44 %) and incubation period (43.39 h), with the predicted maximum α- amylase production of 287.92 U/min/ml of fermented media. A mean value of 290.42 ± 0.2462 U/min/ml of α- amylase was acquired from real experiments.*

1. INTRODUCTION

Amylases are the enzymes first to be commercially produced and marketed. The annual sale of alpha amylases in global market is estimated to be $11 million. In food industry, it is required for the production of glucose syrup, and crystalline glucose. Alpha amylase family can roughly be divided in to two groups such as the starch hydrolyzing enzymes and the starch modifying or transglycosylating enzymes. The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of generated products, lower energy requirements and elimination of neutralization steps due to the increasing demand, for these enzymes in various industries**.** *Bacillus* species are heterogeneous forms and gram positive bacteria commonly found in soil, known to be good producers of alpha amylases and these have been widely used for commercial production of the enzymes.

MATERIALS AND METHODS

Soil samples were collected from soil contaminated with decaying material of fruit waste in Namakkal. Microorganism isolated from the soil sample by serial dilution method was characterized morphologically and biochemically by Bergey's Manual of Systematic Bacteriology (Sneath, 1986). Medium optimization for amylase production was done in the submerged fermentation and maize flour as a carbon source. Crude enzyme extract was obtained by centrifuging the culture broth at 10,000 rpm for 20 minutes and supernatant was used for enzyme assay. Maltose was used as standard reference for enzyme activity. The activity of α-amylase was assayed and observed was measured in a UV/VIS spectrophotometer at 550nm.

The Plackett–Burman statistical experimental design is used for experiments confronted with two factor interaction. (Pedreschi *et al.*, 2008). The statistical software package Minitab version 15 (Minitab Ltd., Coventry CV3 2TE, UK) was used for analyzing the experimental data. PB experimental design is based on the first order model as given in equation 1.

$$Y = \beta_0 + \Sigma \beta i\, xi \ldots\ldots (1)$$

Where, Y is the response (enzyme activity), β_0 is the model intercept, β_i is variable estimates and X_i are independent variables.

Response surface methodology used for evaluation of relationship between cluster of controlled experimental factors and measured response. The Box-Behnken was proceeded to obtain a quadratic model to estimate the pure process variability with amylase production as response. The linear quadratic model with 3 variables expressed as:

$$Y=\beta_o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_{12} + \beta_{22}X_{22} + \beta_{33}X_{32} + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$$

Where y is the measured response, β0 is the intercept term, β1, β2, β3 are linear coefficient, β11, β22, β33 are quadratic coefficient, β12, β13, β23 are interaction coefficient and X1, X2 , X3 are coded independent variables.

The statistical software package Minitab version 15 (Minitab Ltd., Coventry CV3 2TE, UK) was used for regression and graphical analyses of the data obtained. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

RESULTS

Bacillus sp. was isolated from soil contaminated with decaying material of fruit waste. Among 15 isolates, the EB-9 showed maximum halo zone on starch agar plate (27mm). By Bergys Manual of classification EB-9 identified as *Bacillus* sp. The data on enzyme activity according to Plackett-Burman design in table 1. The results indicated that there was a variation of α- amylase production in the twelve trials in the range from 106.42 to261.28 U /min /ml.

On analysis of regression coefficients and t-value of 9 factors were presented in table-2. Orange peel, yeast extract and incubation periods were found to be highly significant factor for α- amylase production. Orange peel (confidence level- 99.10%), yeast extract (98.50%) and incubation time (98.40%) were found as most important factors, which influences the- amylase production.

Analysis of variance (ANOVA) of established model was given in table-3. The *F*-value of α- amylase production equation is 28.62, and the value of 'Prob (*P*) >*F*' is less than 0.034, which indicates that the model is highly significant at 0.05 level; Moreover, determination coefficient (R^2 = 0.9923) is close to 1 for a good statistical simulation, which implies that the model can explain 99.23 % variation in the experiment.

Pareto chart plotted (Figure- 1) showed a vertical line indicating statistical significance (*p* = 0.05). Normal plot of standardized effects showed negative (low level of variable) impact of significant variables (Figure-2).

Based on Plackett- Burman design, orange peel, yeast extract and incubation periods were selected for further optimization using response surface methodology. The results indicated that there was a variation of α- amylase production in the fifteen trials in the range from 102.27 to 286.62 U /min /ml.

The results obtained from the Box-Behnken design were fitted to a second order polynomial equation to explain the dependence of α- amylase production on the medium components. The interaction effect of yeast extract *incubation periods (p= 0. 815) were not found to have influence on α- amylase production.

Based on the response from the design, the value for correlation coefficient was determined using regression analysis and was found to be (R^2) 99.69%. A higher value of the correlation coefficient signifies a good correlation between the independent variables and α- amylase production.

The equation resulted in an empirical model that relates the measured response to the independent variables of the experiment.

$$Y= 284.04 - 27.40*X_1 - 6.61*X_2 - 16.59*X_3 - 96.54*X_1^2 - 38.06*X_2^2 - 45.61*X_3^2 - 8.20*X_1*X_2 + 8.37*X_1*X_3 + 0.70*X_2*X_3$$

Where Y is the predicted response α- amylase production, X_1, X_2 and X_3 were the coded values of orange peel, yeast extract and incubation periods respectively.

Analysis of variance (ANOVA) was used to test the significance and adequacy of the second order polynomial model. 95% confidence level, which was used to evaluate the adequacy of the fitted model. In this study, the ANOVA of the regression model demonstrates that the model is highly significant, this is evident from the calculated F-value (F-model = 179.99) and probability value (P = 0.000). It is evident that the linear and quadratic effect (p = 0.000) and interaction effect (p= 0.045) of the variables had greater influence on α- amylase production.

The response surfaces and counter plot filled for the optimization are shown in Figures 3 - 5. Each figure presents the effect of two factors on α- amylase activity, while the third factor was held at the middle level.

These data revealed that the α- amylase activity would increase as orange peel and incubation period increase, but further increases in these two factors after the optimal point would reverse the trend (Figure 3a,b).

In figure- 4a,b, orange peel concentration of 3.9 to 4.6 % and incubation periods of 35 to 52 h exhibited the highest α- amylase activity.

The interaction effect of yeast extract and incubation period, the maximum α- amylase activity observe between 0.38 and 0.48 % of yeast extract and also 36 and 50 h of incubation period (Figure-5a, b)

The response optimizer in MINITAB 17.0 software was used to optimum value of the variables for maximum α-amylase production by indigenous isolate of *Bacillus subtilis* EB-9. The optimum value of the variables in actual unit was predicted as orange peel (4.27 %), yeast extract (0.44 %) and incubation period (43.39 h), with the predicted maximum α- amylase production of 287.92 U/min/ml of fermented media (Figure-6). A mean value of 290.42 ± 0.2462 U/min/ml of α- amylase was acquired from real experiments.

DISCUSSION

In the present investigation, ten isolates of *Bacillus* sp. were isolated from soil contaminated with decaying material of fruits waste in Erode district. All isolates were aerobic with straight rods, motile, endospore forming, gram positive, strongly catalase positive and indicative of *Bacillus* species, which designated as, EB1 to EB15.

In screening for amylase production, *Bacillus* sp. EB-9 showed maximum halo zone on starch agar. When starch was broken down into sugars, there were clear zones surrounding streaked lines, which indicate starch hydrolysis (Alfred, 2007).

Amylases enzymes are divided into endoamylases and exoamylases. Initially, endoamylases randomly catalyze hydrolysis of starch molecule, causing the formation of linear and branched oligosaccharides; subsequently exoamylases hydrolyze the intermediate substrate to glucose molecules (Gupta *et al*., 2003). The variation of α-amylase production by *B. subtilis* EB-9 in the twelve trials of Plackett Burman design was ranged from 106.42 to 261.28 U /min /ml. On analysis of regression coefficients and t-value of 9 factors such as $MgSO_4$, K_2HPO_4, and NaCl showed a positive sign (high level of coded value) on α-amylase production, the remaining all factors shown a negative sign (low level of coded value) on α-amylase production. When the sign of the concentration effect of the tested variable was positive, the influence of the variable upon the amylase production was greater at a high concentration, and when it was negative, the influence of the variable was greater at a low concentration (Dhanya *et al*., 2008).

In the present work, variables with confidence levels great-

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

er than 95% were considered as significant. Orange peel (confidence level- 99.10%), yeast extract (98.50%) and incubation time (98.40%) were found as most important factors which influences the α- amylase production. The composition and concentration of media greatly affect the growth and production of extracellular amylase production in bacteria (Srivastava and Baruah, 1986).

The three significant factors (orange peel, yeast extract and incubation periods) found in by PB design were selected for optimization using Box-Behnken design for response surface methodology. The results indicated that there was a variation of α- amylase production in the fifteen trials in the range from 102.27 to 286.62 U /min /ml. The coefficients t and p values for linear, quadratic and combined effects are given at 95% significance level.

The response optimizer in MINITAB 17.0 software was used to optimum value of the variables for maximum α-amylase production by indigenous isolate of *Bacillus subtilis* EB-9. The optimum value of the variables in actual unit was predicted as orange peel (4.27 %), yeast extract (0.44 %) and incubation period (43.39 h) with the predicted maximum α- amylase production of 287.92 U/min/ml of fermented media. A mean value of 290.42 ± 0.2462 U/min/ml of α- amylase was acquired from real experiments.

The statistical design of experiment offer efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for α-amylase production by *B. subtilis* EB-9

CONCLUSION

The use of an experimental design was to reveal the influence of various nutrient component and process variables on α- amylase production allowed the rapid screening of large experimental domain in search of the best culture conditions for optimization of α- amylase production by *Bacillus subtilis* EB-9. The optimization of the medium resulted in a reduced cost of medium constituent. The chosen method of optimization of medium composition was efficient, relatively simple, and time and material saving.

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Table-1. Plackett-Burman design of 12 runs for 9 variables along with observed concentration of Alpha amylase production in fermentation broth

Run	Orange Peel (%)	Glucose (%)	Yeast Extract (%)	$MgSO_4$ (%)	K_2HPO_4 (%)	KH_2PO_4 (%)	NaCl (%)	Inoculum size (%)	Incubation Periods (Days)	Experimental Alpha amylase activity (U /min /ml)	Predicted Alpha amylase activity (U/min /ml)
1	6	1	0.9	0.2	0.2	0.2	5	3	72	106.42	105.4683
2	6	3	0.3	0.6	0.2	0.2	1	3	72	141.24	142.1917
3	3	3	0.9	0.2	0.4	0.2	1	1	72	147.14	152.285
4	6	1	0.9	0.6	0.2	0.4	1	1	24	169.85	174.995
5	6	3	0.3	0.6	0.4	0.2	5	1	24	195.92	194.9683
6	6	3	0.9	0.2	0.4	0.4	1	3	24	132.08	126.935
7	3	3	0.9	0.6	0.2	0.4	5	1	72	186.34	181.195
8	3	1	0.9	0.6	0.4	0.2	5	3	24	199.48	200.4317
9	3	1	0.3	0.6	0.4	0.4	1	3	72	205.62	204.6683
10	6	1	0.3	0.2	0.4	0.4	5	1	72	161.53	162.4817
11	3	3	0.3	0.2	0.2	0.4	5	3	24	231.84	236.985
12	3	1	0.3	0.2	0.2	0.2	1	1	24	261.28	256.135

Sign '+1' is for high concentration of variables and '-1' is for low concentration of variables

Table -2. Statistical analysis of Plackett-Burman design on α- amylase activity by Bacillus subtilis-EB 9

Term	Effect	Coef	T-Value	P-Value	Confidence interval (95%)
Constant		178.23	68.13	0.000*	100*
Orange Peel (%)	-54.11	-27.06	-10.34	0.009*	99.1*
Glucose (%)	-11.60	-5.80	-2.22	0.157	84.3
Yeast Extract (%)	-42.69	-21.34	-8.16	0.015*	98.5*
$MgSO_4$ (%)	9.69	4.85	1.85	0.205	79.5
K_2HPO_4 (%)	-9.20	-4.60	-1.76	0.221	77.9
KH_2PO_4 (%)	5.96	2.98	1.14	0.373	62.7
NaCl (%)	4.05	2.03	0.77	0.520	48.0
Inoculum size (%)	-17.56	-8.78	-3.36	0.078	92.2
Incubation Periods (Days)	-40.36	-20.18	-7.71	0.016*	98.4*
R-sq : 99.23% R-sq(adj):95.76%					

SE- Standard error, t – student's test, p – corresponding level of significance, *-significant

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

Table -3. Analysis of variance (ANOVA) for linear model on effect of independent variables on α- amylase production by Bacillus subtilis-EB-9

Source	Degree of freedom (DF)	Adj sum of squares (ASS)	Adjusted sum of squares (ASS)	F	P
Main Effects	9	21158.0	2350.89	28.62	0.034*
Residual Error	2	164.3	82.13		
Total	11	21322.3			

F – Fishers's function, p – corresponding level of significance, *-significant.

Figure-1. Pareto chart of effects of process variables for α- amylase production by Bacillus subtilis-EB 9

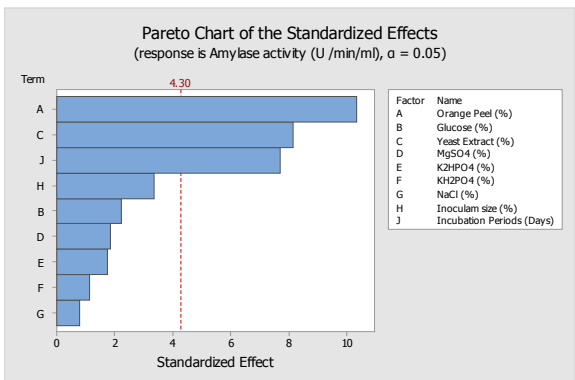


Figure-2. Normal plot chart of the standardized effects of process variables for α- amylase production by Bacillus subtilis-EB-9

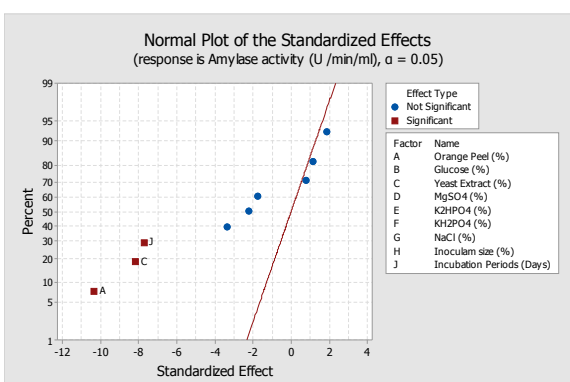


Figure-3a. Contour plot of interaction effect of orange peel and yeast extract on amylase activity

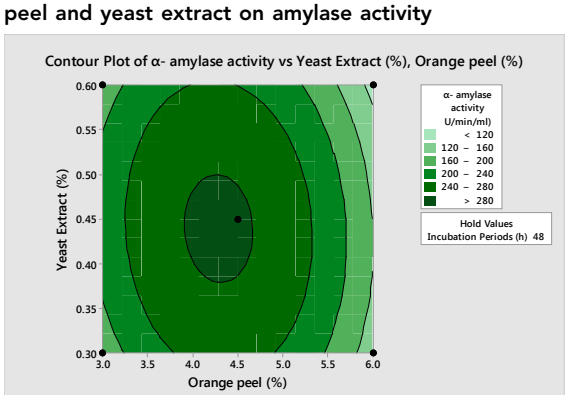


Figure-3b. Surface plot of interaction effect of orange peel and yeast extract on amylase activity

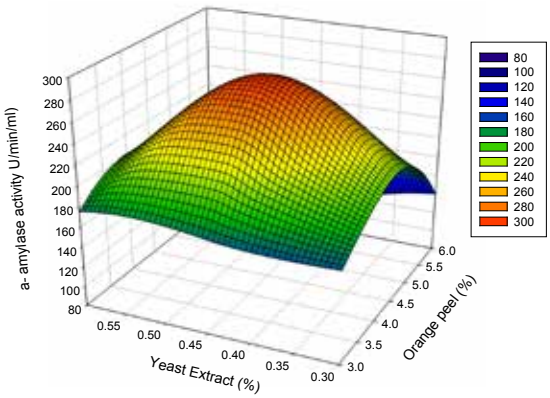


Figure-4a- Contour plot of interaction effect of orange peel and incubation periods on amylase activity

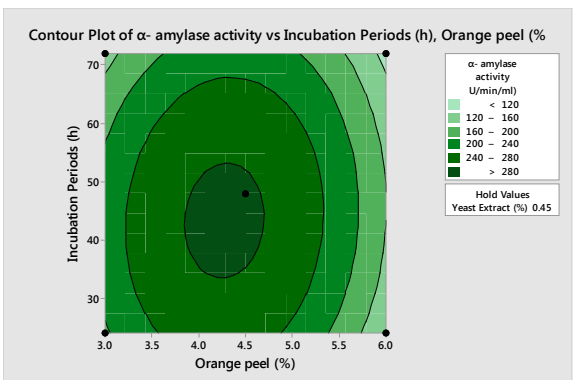


Figure-4b-Surface plot of interaction effect of orange peel and incubation periods on amylase activity

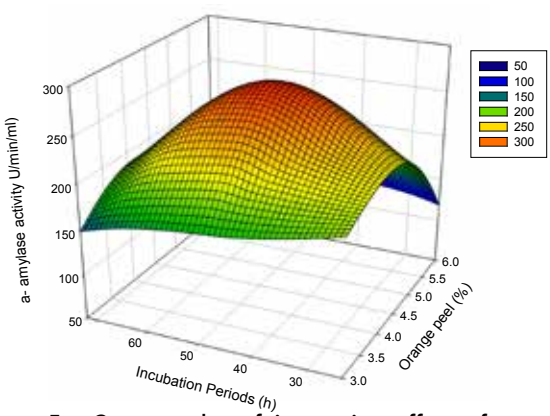
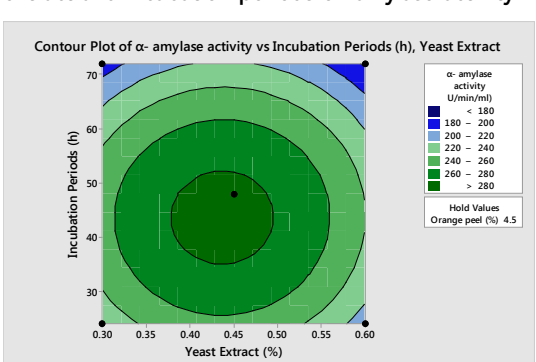


Figure-5a- Contour plot of interaction effect of yeast extract and incubation periods on amylase activity



Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

Figure-5b. Surface plot of interaction effect of yeast extract and incubation periods on amylase activity

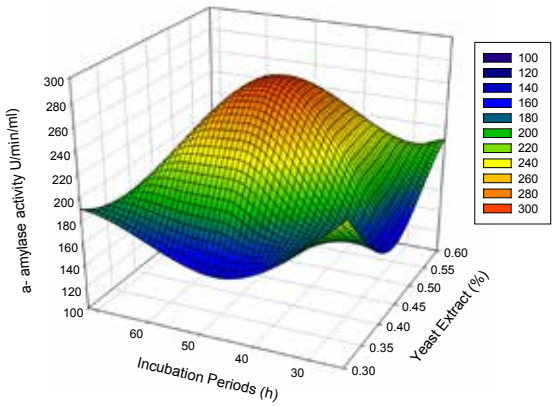
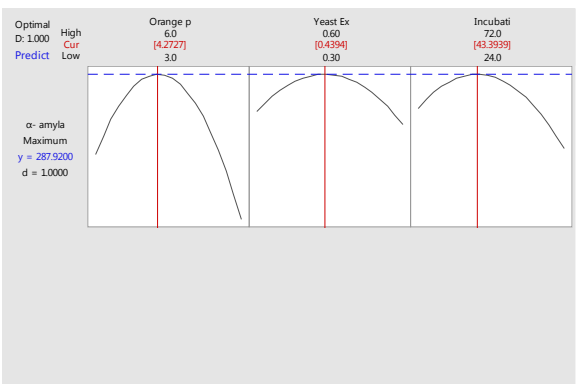


Figure –6. Optimization of parameters on predicted maximum α- amylase production



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