Statistical Optimization of Culture Conditions by Response Surface Methodology for Synthesis of A-Amylase with Indigenous Isolate of *Bacillus* KKC- 24

R.C. Senthamil Selvi[1], N. Thangaraj[2]and R. Rajendiran*[3]

[1]Dep of Biological Science, Kongunadu College of Education, Salem district, Tamiladu, India

[2]Principal, Kandaswami Kandar's College, Velur-638 182, Namakkal district, Tamilnadu, India

[3]Poultech Agro Research Centre, Namakkal district, Tamiladu, India-636 302

Abstract

A soil bacterium capable of degrading starch on starch agar plates was isolated and identified as *Bacillus* isolates which designated as *Bacillus* KKC-17 - 24. Of the 8 isolates, the maximum alpha amylase producing *Bacillus* KKC-24 was seen by observation in Petridish of the clear zone of starch hydrolysis. A two stage method was used to optimise the conditions of cultivation for the production of α-amylase by this bacterium. The results were first analysed using the Plackett-Burman design on a variety of medium components. NaCl and $CaCl_2$ have had a positive impact on the development of enzymes by different components tested while other variables have negative effects. However, due to a powerful curvature effect, the linear model was not sufficient to specify the optimal levels for these components. In the second step, to evaluate the optimum values, Box-Behnken response surface methodology was used. It was noted that the experimental data the matched correctly with a quadratic polynomial equation. The optimal concentrations for corn flour (g/L), peptone (g/L) and incubation periods (hrs) were found to be 12.83, 3.01 and 34.67 respectively, with an estimated value of alpha-amylase of 258.26 U/mL/ min. Practical alpha-amylase development hit 257.97 U/mL/min using this statistically optimised medium.

Key words: *Bacillus isolates, α-amylase, media optimization, response surface methodology.*

Introduction

Industrial uses of microbial enzymes are growing and among them amylases have attained a great deal of attention due to their use in many sectors and economic benefits. Alpha amylase (EC 3.2.1.1, alpha-1,4-glucan-4-glucanohydrolase) spontaneously cleaves alpha-1,4 glycoside bonds of endo-attacking starch, amylose, amylopectin and associated polysaccharides and creates various sizes of alpha-anomeric oligosaccharides (Kubrak et al., 2010). Alpha-amylase is a primary metabolite produced for growth by microorganisms, and its formation is a mechanism correlated with growth (Sudo et al., 1994; Spohr et al., 1998). There have been records of alpha-amylase from numerous sources, including bacterial and fungal sources. *Bacillu*s spp. are commonly used in the production of thermostable alpha-amylase to satisfy industrial needs such as food, textile, pharmaceutical and detergent industries, starch-processing, brewing, alcohol production etc (Prescott and Dunn, 1987).

In order to satisfy the growing demands of economically significant alpha-amylase, highly efficient enzymes need to be discovered to increase the yield and decrease the production costs (Haq et al., 2003). Media optimization is considered a promising technique in order to maximise enzyme yield. Medium component and culture condition greatly influences the growth and enzyme production of the organism, so optimizing media components could increase product efficiency (Djekrif et al., 2006). Schmidell et al. (1988) stated that variables such as composition of culture medium, initial polysaccharide concentration, conditions of cultivation and the microorganism itself may have an effect on the development of enzymes.

Using statistical experimental design techniques, modifications of nutrients and culture conditions are valuable resources for screening nutrients with a substantial growth rate effect, as they can provide statistical models that help explain the relationships between process parameters at different levels. The interactions of variables between the medium components are not accounted for by a well-known traditional process, one variable at a time (OVAT), commonly used but time consuming (Korbhati et al., 2007). For instance, Plackett-Burman factorial experimental design and response surface methodology (RSM) may therefore be used to solve these problems and to evaluate the relationships between the variables (Sharma et al., 2009). RSM is a set of mathematical and computational techniques that are useful for modelling problems in which response is influenced by a variety of variables and the aim is to maximise response. This optimization process involves three major steps: Performing the statistically designed experiments, fitting experimentally defined response data into a quadratic model and estimating the coefficients in a mathematical model, and predicting the response and testing the adequacy of the model (Vohra and Satyanarayana, 2002). The key benefit of RSM is the reduced number of laboratory experiments needed to define and measure relevant correlations between the variables (Karacan et al., 2007).

In this study, Plackett-Burman factorial design screened important nutrients and culture conditions affecting alpha-amylase production, and then Box-Behnken design was used to identify the optimum levels of the important variables to produce the highest yield of alpha-amylase.

Materials and Methods

Strain and medium: *Bacillus* spp. isolated from the different maize rhizosphere soil at Namakkal district, Tamilnadu, India using thermal selection process (Travers et al., 1987) and identified on the basis of standard morphological and biochemical tests. The isolates were maintained at 4°C, on plates of nutrient agar slants for further study.

Screening of amylolytic *Bacillus* spp.:The isolates were tested for amylase activity by employing zone clearing technique (Gomes *et al.,* 2001) using starch agar medium. The 24hrs cultures of isolates were spot inoculated on starch agar plates which were incubated at 37°C for 3 days. The region of starch hydrolysis was observed after incubation by flooding the plates with iodine solution. The blue colour development revealed the presence of starch when the areas around the hydrolytic bacteria tended to be transparent. The diameter was estimated in terms of millimetres for the regions around the colonies.

Production of α-amylase: The production media is prepared in a 250 ml flask of Erlenmeyer comprising 100 ml of broth prepared on the basis of a statistical design. The medium was sterilised for 20min. by autoclaving at 121ºC and then inoculated with bacterial suspension. In the submerged fermentation, medium optimization for alpha-amylase production was carried out using corn meal, yeast extract, peptone, NaCl, KH_2PO_4, $MgSO_4$, $CaCl_2$, inoculum size and incubation periods as nutritional ingredients and processing variables.

Extraction of enzyme: The enzyme was removed at the end of 4 days with a 4-fold v / v of 0.05 M phosphate buffer at pH 7.0. The buffer was applied to the culture flasks and agitated at 150 rpm for 1 hour. The mixture was purified using a muslin cloth and the filtrate was centrifuged at 5000 rpm for 30 minutes. The supernatant was extracted and used as a raw source of the enzyme.

Enzyme activity assay: Amylase activity was measured using the Miller (1959) technique with slight modifications. Amylase activity was determined by incubation of a mixture of 1 ml of enzyme source and 1 percent soluble starch at 40°C for 30 min. The reaction was halted by adding 2 ml of DNS (3, 5-dinitrosalicylic acid) followed by boiling for 10 minutes. The final volume was raised to 20 ml with sterilised water and the sugar was determined by the absorbance calculation at 540 nm wavelength. Values have been obtained and compared to the standard maltose graph. One unit of enzyme activity is the amount of enzyme that releases 1 micromol glucose per minute and is expressed in U/mL/min.

Identification of significant nutrients and culture conditions by Plackett-Burman design: Plackett-Burman design was used to determine important factors for production of amylase (Majumder and Goyal, 2008) (Table-1). A total of nine factors were chosen for the analysis, each of which was expressed at two stages, high

(+) and low (−) and two dummy variables in 12 trials (Table 2). Each row represents a test and each column represents an independent (assigned) or dummy (unassigned) variable used to quantify experimental data interpretation errors (Soliman et al., 2005). Plackett - Burman design is based on the first-order polynomial equation

$$Y = ß_o + \Sigma ß_i X_i \quad (1)$$

Where, Y, is the α-amylase yield; $ß_o$, is the model intercept; $ß_i$, is the linear coefficient; X_i, is the level of the independent variable.

The Plackett-Burman design was only used to screen and evaluate the significant variables that could affect yield, as this model does not explain the interaction between the different variables (Purama and Goyal, 2008). Following the choosing of significant variables, a Box-Behnken design (BBD) was conducted to examine the relationship between variables and a surface response equation was derived. The BBD approach was used to evaluate the optimum level of essential variables (identified by Plackett-Burman) for the enhancement of enzyme production. The effect of each variable on enzyme production was examined at three different concentrations. The enzymatic activity was calculated in triplicate in 15 separate experimental series (Table 3). The development of amylase was evaluated using a second-order polynomial equation and the data were fitted into the multiple-regression equation (Acikalin et al., 2005; Aslan, 2007):

$$Y=b_o+b_1x_1+b_2x_2+b_3x_3+b_{11}x_1^2+b_{22}x_2^2+b_{33}x_3^2+b_{12}x_1x_2+b_{13}x_1x_3+b_{23}x_{23} \quad (2)$$

Where Y is the percentage reaction conversion, b_0 is the value of fitted response at the center point of design, $b_{1...3}$, $b_{11...33}$ and $b_{12,13,23}$ are the linear, quadratic and interaction terms respectively, and $x_{1...3}$ the dimensionless coded variables. The second-order model includes all the terms in the first-order model, plus all quadratic terms like $b_{11}x_1^2$ and all cross product terms like $B_{13}x_1x_3$ (Tanyildizi et al., 2005).

Variable code	Variable	Unit	Value	
			-1	+1
X_1	Corn flour	g/L	10	20
X_2	Yeast extract	g/L	1	3
X_3	Peptone	g/L	2	4
X_4	NaCl	g/L	1	2
X_5	KH_2PO_4	g/L	0.5	1
X_6	$MgSO_4$	g/L	0.5	1
X_7	$CaCl_2$	g/L	0.1	0.5
X_8	Inoculum size	%	1	2
X_9	Incubation periods	hrs	24	72

Table (1) Variables and their levels employed in Plackett-Burman design for screening of culture conditions affecting α-amylase production by *Bacillus* KKC-24.

Statistical analysis: The statistical software package Minitab version 17 (Minitab Ltd., Coventry CV3 2TE, UK) was used for regression and graphical analyses of the data obtained. The optimal concentrations of the critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

Results and Discussion

A total of 8 starch degrading *Bacillus* isolates were obtained from starch agar plate and designated *Bacillus* KKC-17-24 (Fig.1). Identification of selected *Bacillus* strain was identified on the basis of standard morphological and biochemical tests according to Bergey's Manual of determinative Bacteriology (Buchana and Gibbons, 1974). Among eight isolates, the most efficient *Bacillus* KKC-24 isolate was selected α-amylase

production on the basis of size of the halo zone around the streaked colony and the isolates showing the largest halo zones were selected for further studies.

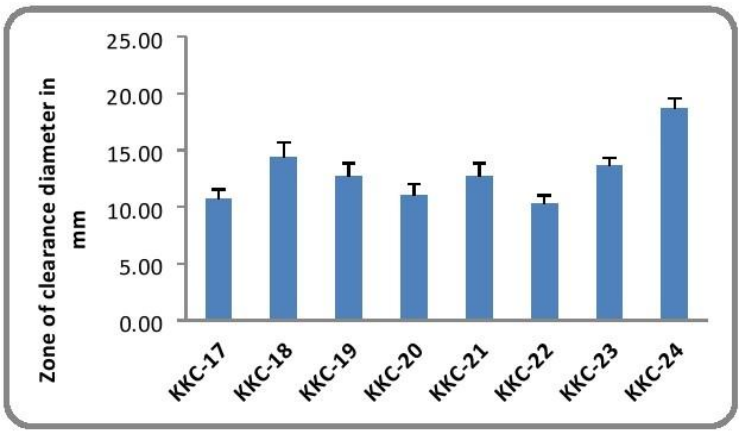

Fig (1) α-amylase activities of different *Bacillus* isolates

As seen in Table 2, the maximum and minimum values of the variables selected for the Plackett-Burman experimental design trials reflect a wide difference in nutritional sources. The findings showed that there was a difference in the development of alpha-amylase between 127.45 and 232.45 U / ml / min in the twelve trials. The findings of the statistical study shown in Table 3 revealed that X_1 (Corn flour), X_3 (Peptone) and X_9 (incubation periods) confidence levels were 98.90%, 98.10% and 97.30%, respectively, which are known to have a major impact on the production of alpha-amylase. Other independent variables were usually deemed negligible, with confidence levels below 95%. The coefficient of determination (R^2) values provides a measure of how much uncertainty can be interpreted by the experimental variables and their interactions in the observed response values.

The value of the coefficient of determination (R^2) explains how much uncertainty can be shown by experimental variables in the observed response values. R^2 value always varies from 0, 1. The closer the R^2 value to 1, the model is stronger and the better predicted response (Kaushik et al., 2006). In this case , the value of the determination coefficient (R2 = 0.9898) reveals that the independent variables were related to 98.98% of the uncertainty in the response and only 1.02% of the overall differences were not clarified by the independent variables. Moreover, the value of the adjusted coefficient of determination (Adj. R2 = 0.9441) is also very high, suggesting the model's high significance (Akhnazarova and Kafarov, 1982).

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	α-amylase activity (U/ml/min)
1	1	-1	1	-1	-1	-1	1	1	1	127.45
2	1	1	-1	1	-1	-1	-1	1	1	139.89
3	-1	1	1	-1	1	-1	-1	-1	1	145.55
4	1	-1	1	1	-1	1	-1	-1	-1	158.29
5	1	1	-1	1	1	-1	1	-1	-1	192.7
6	1	1	1	-1	1	1	-1	1	-1	128.94
7	-1	1	1	1	-1	1	1	-1	1	183.29
8	-1	-1	1	1	1	-1	1	1	-1	196.52
9	-1	-1	-1	1	1	1	-1	1	1	202.74
10	1	-1	-1	-1	1	1	1	-1	1	158.74
11	-1	1	-1	-1	-1	1	1	1	-1	229.14
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	232.45

Table (2) Plackett-Burman experimental design matrix for screening of factors affecting α-amylase production by *Bacillus* KKC-24

Term	Effect	Coef	T-value	P-value	Confidence level (%)
Constant		174.64	69.61	0.000*	100*
X_1	-47.28	-23.64	-9.42	0.011*	98.9*
X_2	-9.45	-4.72	-1.88	0.200	80
X_3	-35.94	-17.97	-7.16	0.019*	98.1*
X_4	8.53	4.26	1.70	0.231	76.9
X_5	-7.55	-3.78	-1.51	0.271	72.9
X_6	4.43	2.22	0.88	0.470	53
X_7	13.33	6.66	2.66	0.117	88.3
X_8	-7.72	-3.86	-1.54	0.264	73.6
X_9	-30.06	-15.03	-5.99	0.027*	97.3*

Coef- coefficient; T-T test; P-Probability,* Signifcant values

Table (3) Effect estimates for α-amylase production from the results of Placket-Burman design.

The media component and culture conditions were reduced to three major variables after the first optimization, showing that the design of Plackett-Burman is a powerful tool for screening variables. Hymavathi et al. (2010) stated that the Pareto effects chart was plotted to classify the factors that are important for the production of enzymes and indicates the main effect estimates of the factors on the horizontal axis seen in Fig.2. The specific optimal values of the individual factors were still uncertain, but the subsequent design of Box-Behnken could determine them.

The growth pattern and use of corn starch as a cheap carbon substrate by the amylolytic species *Bacillus* compared to soluble starch and nutrient broth medium was determined (Nortemann, 1992) Media containing starch-rich flours such as corn is suitable for the production of ш-amylase. It is well known that higher yields of amylase can be produced in complex raw materials containing starch from maize, barley, wheat and malt (Aiyer, 2004). As a result, these starch-rich flours can prove useful as cheaper alternative sources of carbon and energy for bacterial amylase production (Prakash et al., 2009).

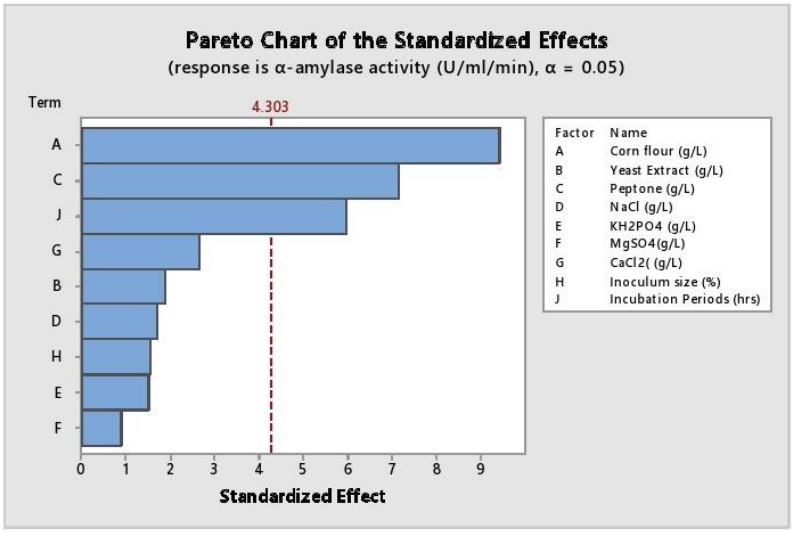

Fig (2) Standardized main effect Pareto chart for the Plackett–Burman design.
Vertical line in the chart defines the critical value associated with 95% confidence level

Organic sources such as yeast extract and peptone typically promote the production of α-amylase (Hewitt and Solomons, 1996; Hamilton et al., 1999). Oziengbe and Onilude (2012) observed that when applied to the development medium, peptone had the greatest α-amylase activity. Fooladi and Sajjadian (2010) observed that growth and α-amylase activity were increased by adding peptone (1%) to the mineral medium. Similar findings were observed by El-Banna et al. (2007), who reported that peptone from *B.subtilis* was the best source of nitrogen for amylase production ($2.7\mu ml^{-1}$).

The time of incubation showed that the concentration of amylase was most likely decreased due to nutritional declines, waste product accumulation, cell death, and catabolite repression over a prolonged incubation period (Abd-Elhalem et al., 2015). The effect of incubation time on the production of amylase showed that the optimal period for maximal amylase activity was 24hrs. This may be because the cells could have entered the decline period after 24 hrs and have shown low amylase synthesis (Sivakumar et al., 2012).

Optimization by response surface methodology: The interactions between the important factors in α-amylase production were analysed in the Box-Behnken design and their optimal levels were also determined. The other variables in the analysis were maintained in the Plackett-Burman studies at a median level. A total of 15 experiments have been conducted with various combinations of corn flour (g/L), peptone (g/L) and incubation periods (hrs), and the findings of the experiments are shown in Table 4 which revealed significant differences in the production of α-amylase.

The regression model equations (second order polynomial) for BBD relating the α-amylase production and process parameters were developed using the experimental results (Peng et al., 2020) and the corresponding equations (3) are:

$$Y=243.78-37.45x_1-6.02x_2-21.08x_3-57.73x_1^2-13.39x_2^2-28.65x_3^2-6.96x_1x_2+24.64x_1x_3-5.81x_{23} \quad (3)$$

Multiple regression analysis was used to analyze the data, the goodness of fit of the model was checked by the coefficient of determination (R^2) which was found to be 0.9959, indicating that the sample variation of 99.59% was attributed to the variables and only 0.49% of the total variance could not be explained by the model. The present R^2-value reflected a very good fit between the observed and predicted responses and implied that the model is reliable for α-amylase production in the present study. The p-values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. It can be seen from the degree of significance that the linear coefficients of X_1 (corn flour), X_2 (peptone) X_3 (incubation periods), quadratic effect of all variables and interaction effect of corn flour and peptone, corn flour and incubation periods are observed significantly difference in the model.

Runs	Corn clour (x_1) (g/L)	Peptone (x_2) (g/L)	Incubation periods (x_3) (h)	α-amylase activity (U/ml/min)	Predicted α-amylase activity (U/ml/min)
1	-1	-1	0	212.46	209.168
2	1	-1	0	146.27	148.193
3	-1	1	0	212.97	211.048
4	1	1	0	118.93	122.223
5	-1	0	-1	240.68	240.565
6	1	0	-1	121.71	116.380
7	-1	0	1	143.79	149.120
8	1	0	1	123.39	123.505
9	0	-1	-1	219.62	223.027
10	0	1	-1	220.57	222.607
11	0	-1	1	194.53	192.493
12	0	1	1	172.23	168.823
13	0	0	0	243.25	243.777
14	0	0	0	246.78	243.777
15	0	0	0	241.30	243.777

Table (4) Box-Behnken experimental design matrix with experimental and predicted values of α-amylase production by *Bacillus* KKC-24

Term	Effect	Coef	SE Coef	T-value	P-value
Intercept		243.78	2.98	81.91	0.000*
x_1	-74.90	-37.45	1.82	-20.55	0.000*
x_2	-12.05	-6.02	1.82	-3.30	0.021*
x_3	-42.16	-21.08	1.82	-11.57	0.000*
$x_1 x_1$	-115.46	-57.73	2.68	-21.52	0.000*
$x_2 x_2$	-26.77	-13.39	2.68	-4.99	0.004*
$x_3 x_3$	-57.30	-28.65	2.68	-10.68	0.000*
$x_1 x_2$	-13.93	-6.96	2.58	-2.70	0.043*
$x_1 x_3$	49.29	24.64	2.58	9.56	0.000*
$x_2 x_3$	-11.62	-5.81	2.58	-2.26	0.074

Coef- coefficient; SE coef- standard coefficient; T-T test; P-Probability, *Signifcant values

Table (5) Estimated Regression Coefficients for α-amylase production by *Bacillus* KKC-24

Analysis of variance (ANOVA) which is required to test the significance and adequacy of the model is presented in table 6. The analysis of variance (ANOVA) of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test (135.89) and a very low probability value (0.00). Optimal concentrations of the variable, obtained from the optimization experiment were verified experimentally and compared with the predicted data.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	32501.3	3611.3	135.89	0.000*
Linear	3	15065.1	5021.7	188.97	0.000*
Square	3	14678.1	4892.7	184.11	0.000*
2-Way Interaction	3	2758.1	919.4	34.60	0.001*
Error	5	132.9	26.6		
Lack-of-Fit	3	117.4	39.1	5.07	0.169
Pure Error	2	15.4	7.7		
Total	14	32634.2			

DF- Degree of freedom; Adj SS- Adjusted sum of squares; Adj MS- Adjusted mean sum of squares; F – Fishers's function; P-Probability, *Signifcant values

Table (6) Lack of fit for Box-Behnken experimental design showing model summary.

The shapes of the contour plots, elliptical or circular, indicated whether or not the interactions between the corresponding variables were significant. An elliptical contour plot meant that the interactions between the variables were significant, while a circular contour plot meant the opposite (Luo, 2008; Lv et al., 2012). Fig. 3-5 showed contours plots for α-amylase production which were made from two selected independent variables keeping the value of third variable persistent at its central value to get optimum conditions for maximum α-amylase production. These plots were represented by different colors which indicated different levels of α-amylase production between two independent parameters and keeping third parameter at constant value. These graphs indicated that each parameter had significant impact on α-amylase production by *Bacillus* KKC -24 in submerged fermentation.

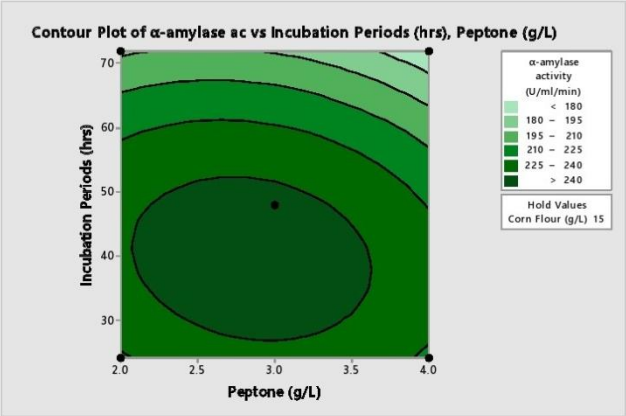

Fig (3) Response surface contour plots of α-amylase production by *Bacillus* KKC-24 showing the effect of peptone and incubation periods

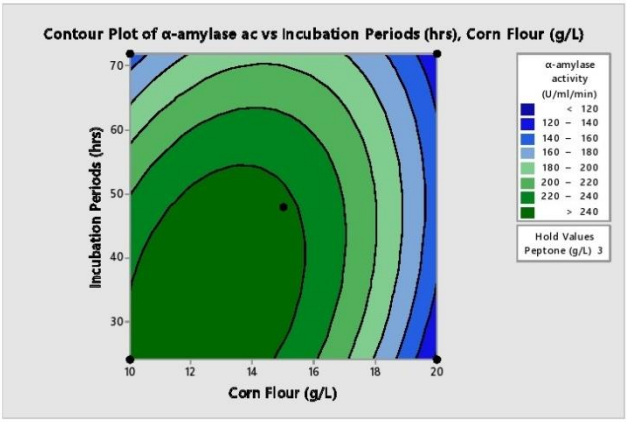

Fig (4) Response surface contour plots of α-amylase production by *Bacillus* KKC-24 showing the effect of corn flour and incubation periods

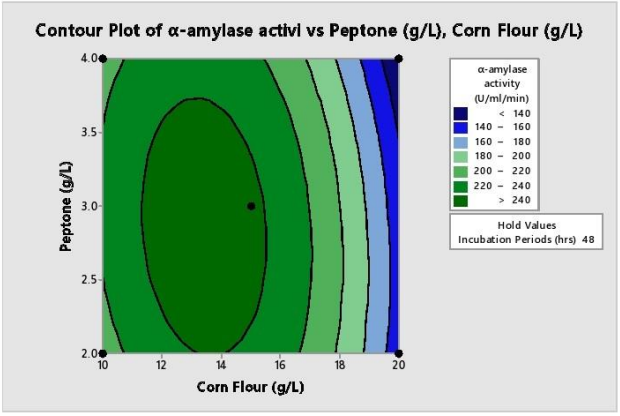

Fig (5) Response surface contour plots of α-amylase production by *Bacillus* KKC-24 showing the effect of corn flour and peptone

Validation of the statistical design model: The measured α-amylase production was 258.26 U/mL/min, where the predicted value from the polynomial model was 257.97 U/mL/min. The verification revealed a high degree of accuracy of the model indicating the model validation under the tested conditions. The optimal levels of the process variables for α-amylase production by *Bacillus* KKC - 24 were corn flour (12.83g/L), peptone (3.01g /L) and incubation periods (34.67hrs).

Conclusion

This study proved that statistical experimental design showed significant results for optimizing the process parameters for maximum α-amylase production by *Bacillus* KKC - 24 under submerged fermentation and allowed rapid screening of a large number of variables. The α-amylase production was found to be significantly influenced by corn flour, peptone and incubation periods. A maximum α-amylase production of 258.26 U/mL/min was achieved with the following optimized factors: corn flour (12.83g/L), yeast Extract (2g/L), peptone (3.01g/L), NaCl (1.5g/L), KH_2PO_4(0.75g/L), $MgSO_4$(0.75g/L), $CaCl_2$ (0.3g/L), inoculum size (1.5%), incubation periods (34.67hrs). Validation experiments were also carried out to verify the adequacy and the accuracy of the model and results showed that the predicted value agreed with the experimental values well and about 2-fold increase compared to the original medium was obtained.

Reference

Abd-Elhalem, B.T., M. El-Sawy, R.F. Gamal, K.A. Abou-Taleb (2015). Production of amylases from Bacillus amyloliquefaciens under submerged fermentation using some agro-industrial by-products. Ann. Agric. Sci. 60(2):193–202.

Acikalin, K., F. Karaca, E. Bolat (2005). Central composite rotatable design for liquefaction of pine barks. Fuel Process. Technol. 87: 17-24.

Aiyer, P.V.D (2004). Effect of C:N ratio on alpha amylase production by Bacillus licheniformis SPT 27. Afr. J. Biotechnol. 3(10): 519-522.

Akhnazarova, S. and V. Kafarov (1982). Experiment optimization in chemistry and chemical engineering. Mir Publication, Moscow.

Aslan, N (2007). Application of response surface methodology and central composite rotatable design for modeling the influence of some operating variables of a Multi-Gravity Separator for coal cleaning. Fuel 86: 769-776.

Buchana, R.E., and N.E. Gibbons (1974). Bergey's manual of determinative bacteriology, The Williams and Wilkins Co., Baltimore.

Djekrif, D.S., A.B. Gheribi, Z. Meraihi, and L. Bennamoun (2006). Application of a statistical design to the optimization of culture medium for alpha-amylase production by Aspergillus niger ATCC 16404 grown on orange waste powder. J. Food Eng. 73(2): 190−197.

El-Banna, T.E., A.A. Abd-Aziz, M.I. Abou-Dobara and R.I Ibrahim (2007). Production and immobilization of alpha-amylase from Bacillus subtilis. Pak. J. Biol. Sci. 10(12):2039–2047.

Fooladi, J., and A. Sajjaduan (2010). Screening the thermophilic and hyperthermophilic bacterial population of three Iranian hot-springs to detect the thermostable alpha-amylase producing strain. Iranian J. Microbiol. 2(1):49–53.

Gomes, I., M. Sultana, K. Uddin, J. Gomes, W. Steiner, and D.J. Gomes (2001). Nutrient composition and fermentation conditions for α-amylase production by Bacillus amyloliquefaciens. Bangladesh J. Microbiol. 18(2): 141-150.

Hamilton, L. M., C.T. Kelly and W.M. Fogarty (1999). Production and properties of the raw starch-digesting α-amylase of Bacillus sp. IMD 435. Process Biochem.35(1-2):27–31.

Haq, I., H. Ashraf and J. Iqbal (2003). Production of alpha amylase by Bacillus licheniformis using an economical medium. Bioresour. Technol. 87(5): 57−61.

Hewitt C. J., and G.L. Solomons (1996). The production of α-amylase (E.C.3.2.1.1.) by Bacillus amyloliquefaciens, in a complex and a totally defined synthetic culture medium. J. Ind. Microbiol. Biotechnol. 17(2):96–99.

Hymavathi, M., T. Sathish, P. Brahmaiah, and R.S. Prakasham ( ). Impact of carbon and nitrogen sources on L-asparaginase production by isolated Bacillus circulans (MTCC 8574): Application of saturated PlackettBurman design. Chem. Biochem. Eng. Q. 24(4): 473–480.

Karacan, F., U. Ozden, and S. Karacan (2007). Optimization of manufacturing conditions for activated carbon from Turkish lignite by chemical activation using response surface methodology. Appl. Therm. Eng. 27: 1212-1218.

Kaushik, R., S. Saran, J. Isar and R.K. Saxena (2006). Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by Aspergillus carneus. J. Mol. Catal. B: Enzym. 40: 121-126.

Korbhati, B.K., N. Aktas, A. Tanyolac (2007). Optimization of electrochemical treatment of industrial paint wastewater with response surface methodology. J. Hazard. Mater. 148: 83-90.

Kubrak, O.I., J.M. Storey, K.B. Storey, and V.I. Lushchak (2010). Production and properties of alpha-amylase from Bacillus sp. BKL20. Can. J. Microbiol. 56: 279–88.

Luo, D (2008). Identification of structure and antioxidant activity of a fraction of polysaccharide purified from Dioscorea nipponica Makino. Carbohyd. Polym. 71:544–549.

Lv, H.Q., C. Hu, H.P. Zhong, H.B. Zheng, C.Wen. (2012). Optimization of technology for dietary fiber extraction from maixiansan by response surface methodology. Chin Med. 7(1):28.

Majumder, A., and A. Goyal (2008). Enhanced production of exocellular glucansucrase from Leuconostoc dextranicum NRRL B-1146 using response surface method. Bioresour. Technol. 99: 3685-3691.

Miller, G.L. (1959). Use of dinitrosalisylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426–428.

Nortemann, B. (1992). Total Degradation of EDTA by mixed cultures and a Bacterial isolate. Appl. Environ Microbiol. 58(2): 671-676.

Oziengbe, E.O., and A.A. Onilude (2012). Production of a thermostable A-amylase and its assay using Bacillus Licheniformis isolated from excavated land sites in Ibadan Nigeria. Bayero J. Pure Appl. Sci. 5(1):132–138.

peng, X., G. Yang, Y. Shi, Y. Zhou, M. Zhang and S. Li (2020). Box–Behnken design based statistical modeling for the extraction and physicochemical properties of pectin from sunflower heads and the comparison with commercial low-methoxyl pectin. Sci Rep ***10:*** *3595.*

Prakash, B., M. Vidyasagar, M.S. Madhukumar, G. Muralikrishna, K. Sreeramulu (2009). Production, purification, and characterization of two extremely halotolerant, thermostable, and alkali-stable α-amylases from Chromohalobacter sp. TVSP 101. Process Biochem. 44: 210-215.

Prescott, S., and A. Dunn (1987). Industrial microbiology 4th ed. CBS Publishers and Distributors, New Delhi, India, pp. 550-565.

Purama, R.K., and A. Goyal (2008). Screening and optimization of nutritional factors for higher dextransucrase production by Leuconostoc mesenteroides NRRL B-640 using statistical approach. Bioresour. Technol. 99: 7108-7114.

Schmidell, W., M.C.R. Facciotti, B.V. Kilikian, H. Aboutboul, and J.M.Z. Aguero (1988). Influence of pH oscillations in amyloglucosidase production by Aspergillus awamori. Revista de Microbiologia. 19: 71−77.

Sharma, S., A. Malik, S. Satya (2009). Application of response surface methodology (RSM) for optimization of nutrient supplementation for Cr (VI) removal by Aspergillus lentulus AML05. J. Hazard. Mater. 164: 1198-1204.

Sivakumar, T., T. Shankar, P. Vijayabaskar, J. Muthukumar, and E. Nagendrakannan (2012). Amylase production using Bacillus cereus isolated from a vermicompost site. Int. J. Microbiol. Res. 3(2): 117-123.

Soliman, N.A., M.B. Mahmoud, and Y.R. Abdel-Fattah (2005). Polyglutamic acid production by Bacillus sp. SAB-26: Application of PlackettBurman experimental design to evaluate culture requirements. Appl. Microbiol. Biotechnol. 69:259-267.

Spohr, A., M. Carlsen, J. Nielsen, and J. Villadsen (1998). α-Amylase production in recombinant Aspergillus oryzae during fed-batch and continuous cultivation. J. Ferment. Bioeng. 86: 49–56.

Sudo, S., T. Ishikawa, K. Sato, and T. Oba. (1994). Comparison of acid stable α-amylase production by Aspergillus kawachii in solid-state and submerged cultures. J. Ferment. Bioeng. 77: 483-489.

Tanyildizi, M.S., D. Ozer, and M. Elibol (2005). Optimization of -amylase production by Bacillus sp. using response surface methodology. Process Biochem. 40: 2291-2296.

Travers, R.S., P.A.W. Martin, and C.F. Reichelderfer (1987). Selective process for efficient isolation of soil Bacillus spp. Appl. Env. Microbiol. 53 (6): 1263 – 1266.

Vohra, A., and T. Satyanarayana (2002). Statistical optimization of the medium components by response surface methodology to enhance phytase production by Pichia anomala. Process Biochem. 37: 999-1004.