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Optimization of amylase production from *Bacillus* sp. using statistics based experimental design

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Abstract: Production of amylase under submerged fermentation *Bacillus* sp. was investigated using wheat bran, soybean meal and $CaCO_3$ (WSC) medium. Response surface methodology (RSM) was used to evaluate the effect of the main variables, i.e., pH (11.35), temperature (35.16°C) and inoculum size (2.95%) on amylase production by applying a full factorial central composite design (CCD). The mutual interaction between these variables resulted into 4.64 fold increase in amylase activity as compared to the non-optimized environmental factors in the basal medium.

Key words: Amylase, *Bacillus* sp., central composite design, response surface methodology

تعظيم انتاج الأميليز من نوع الباسيلوس باستخدام تصميمات تجارب علي اسس احصائية

ف. بز زامبير

مركز ابحاث التصنيع الحيوي والتنمية،معهد التعدين والتكنولوجيا في جنوب داكوتا

501 جنوب سانت جوزيف ، مدينة رابد ، جنوب داكوتا ، الولايات المتحدة الامريكية

الملخص: تم دراسة انتاج الأميليز بواسطة التخمير بالغمر من نوع الباسيلوس باسخدام بيئة نخالة القمح، مسخلص فول الصويا و كربونات الكالسيوم(WSC). استخدمت تقنية الأستجابة السطحية (RSM) لتقييم تأثير العوامل الرئيسية، مثل درجة الحموضة (pH (11.35))، الحرارة (35.16oC) وحجم الالبادئ (2.95%) علي انتاج الأميليز بواسطة تطبيق تقنية المركب المركزي المتكامل (CCD). أدي التفاعل بين هذه المتغيرات إلى زيادة قدرها 4.64 مرات مثل في نشاط الأميليز بالمقارنة بحالة عدم عدم تعديل المتغيرات.

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Introduction

Amylases are the hydrolytic enzymes that cleave the α- 1-4 glucosidal linkage of complex polysaccharides (Pandey et al., 2000). Amylases are obtained from various origins like plant, animal, bacterial and fungal. Several researchers produced amylase enzyme using *Bacillus* sp. (Yuguo et al., 2000; Young et al., 2001; Dharani, 2004; Zambare, 2010a). Rumen bacteria also reported for amylase and protease production in sheep (Moharrery, 2003) Amylase has many applications in food, textile, paper and pulp, pharmaceuticals, baking and beverages, detergent and leather industries (Pandey et al., 2000; Reddy et al., 2003a; Kar et al., 2010). Industrially important enzymes including amylases have traditionally been obtained from submerged cultures because of easy handling, greater control of environmental and nutritional factors. The most frequently used operation in biotechnology is to improve the fermentation conditions for maximizing cell density, high level of desired metabolic product or enzyme levels in microbial system (Mukhopadhyay et al., 2008). This approach is time consuming and also ignores the combined interactions between physical as well as nutritional factors.

In contrast, RSM includes factorial design and regression analysis which helps in evaluating the effective factors and their interaction and to find out the optimum conditions of variables for a desirable response (Tunga et al., 1999; Coninck et al., 2000; Reddy et al., 2003b; Kunamneni et al., 2005; Gangadharan et al., 2008). Recently, a number of statistical experimental designs with response surface methodology have been employed for optimizing enzyme production from microorganisms (Koteswara et al., 2006; Thys et al., 2006; Zambare, 2010b; Mohandas et al., 2010). However, 3D and counter plots for response surfaces can provide a good way for visualizing the parameter interaction. Therefore, statistical technique is often used for predicting optimum process conditions for microbial enzyme production (Mullai et al., 2010). It is well known that extracellular enzyme production in microorganisms is greatly influenced by nutritional factors like carbon sources, nitrogen sources and mineral salts (Dey et al., 2001; Adinarayana and Elliaiah, 2002; Chauhan and Gupta, 2004). Enhancement in extracellular amylase production from *Bacillus* sp. by environmental factor optimization has not been attempted so far. Therefore, considering the many industrial applications of amylase, we report here the optimization of extracellular amylase production from *Bacillus* sp. as a result of the interactive effects of three variables (i.e. pH, temperature and inoculum size) using response surface methodology.

Materials and Methods

Microorganism

Bacillus sp. isolated from soil showed true potential in extracellular amylase secretion. Amylase secretion was tested on starch agar plates. After 24h of incubation the plate was flooded with iodine solution. The amylase activity was measured in terms of clear zone diameter with dark blue background. It was maintained on 2 % Nutrient agar slants at 4°C and also as a glycerol stocks at -20°C. The isolate was identified on the basis of various morphological characteristics (colony size, shape, margin elevation, color, opacity, Gram's nature, spore staining and motility), physiological test (growth at 25 and 42°C, growth at pH 4 and 10, growth in NaCl at 1 and 7% concentration) and biochemical tests (glucose utilization, indole production, methyl red, Voges Prokaurer, citrate utilization, catalase, oxidase, arginine dehydrolase, lysine decarboxylase, hydrolysis of gelatin, starch, casein, tributyrine and acid from other carbohydrates etc. Above all tests were performed according to Bergey's manual of systematic bacteriology (Sneath et al., 1986).

Chemicals and media

Chemicals and media were all of analytical grade and purchased from Sigma (St Louis, USA).

Production medium

Production medium containing 1 %-wheat bran (local market), 1%-soybean meal (Sigma) and 0.3%-$CaCO_3$ (Sigma), was used for the growth and amylase production by *Bacillus* sp.

Inoculum preparation

Seed inoculum was prepared by growing the isolate on Nutrient agar (Sigma) in Roux bottle at 30°C for 24 h. The cells were suspended in saline and cell density was measured with spectrophotometer (Shimatzu UV-2501 PC, Japan) at 600 nm.

Experimental design and optimization by RSM

In the RSM the interactive effects of three variables, i.e. pH, temperature and inoculum size was studied for amylase production. Each factor in the CCD was studied at three different levels (-1, 0, +1). The minimum and maximum ranges of variables were investigated with respect to their values in actual and coded form (Table 1). To optimize the conditions for amylase production, Design-Expert 8.0 CCD-RSM software (Minneapolis, U.S.A.) was used. A 2^3 factorial CCD proposed by Box et al. (1978) with three factors leading to a total of 20 sets per experiment was formulated to optimize the process parameters. This experiment included 8 factorial design, 6 star and 6 central points. All the variables i.e. pH, temperature and inoculum size were taken at a central coded value and considered as zero. The conditions of these environmental factors for the production medium were varied according to the experimental design (Table 2). All the experiments were carried out in duplicates.

Table 1. Experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors.

Variable	Range of levels					
	Actual	Coded	Actual	Coded	Actual	Coded
X_1 - pH	10	-1	11	0	12	+1
X_2 – Temperature (^{0}C)	30	-1	35	0	40	+1
X_3 – % Inoculum	1	-1	3	0	5	+1

Table 2. Central composite design in coded units.

Run No.	X_1	X_2	X_3	Coefficients assessed by
1	-1	-1	-1	Fractional 2^3 factorial design
2	+1	-1	-1	
3	-1	+1	-1	
4	+1	+1	-1	
5	-1	-1	+1	
6	+1	-1	+1	
7	-1	+1	+1	
8	+1	+1	+1	
9	0	0	-1	Star points (6 points)
10	0	-1	0	
11	-1	0	0	
12	+1	0	0	
13	0	0	+1	
14	0	+1	0	
15	0	0	0	Central points
16	0	0	0	
17	0	0	0	
18	0	0	0	
19	0	0	0	
20	0	0	0	

The experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml of sterilized WSC medium of different pH 10-12, inoculated with the freshly prepared 1-5% (2 x 10^8 cells/ml) inoculum (as discussed earlier) and incubated for 12 hrs at 30-40°C under shaking culture condition (150 rpm). After fermentation, the cell-free supernatant was obtained by centrifugation at 10,000 rpm and used for amylase activity.

Using RSM, the relationship among the variables, i.e. pH, temperature and inoculum size were expressed mathematically in the form of a polynomial model, which gave the response as a function of relevant variables. The present work was based on the CCD to obtain the experimental data, which would fit in an empirical, full second-order polynomial model representing the response surfaces over a relatively broad range of parameters as shown in Eq. (1).

$$y = a_0 + \sum_{i-1}^{3} a_i x_i + \sum_{i=1}^{3} \sum_{j=i}^{3} aijx_i x_j \ldots\ldots\ldots\ldots\ldots (1)$$

where, *y* was the predicted response (amylase production) used as a dependent variable; *xi* (*i* = 1, 2 and 3) were the input predictors or controlling variables; and a_0, *ai* (*i* = 1, 2, 3) and *aij* (*i* = 1, 2, 3; *j* = *i*, . . . , 3) were the model coefficient parameters. The coefficient parameters were estimated by multiple linear regression analysis using the least-squares method. A second-order polynomial equation was then fitted to the data by least-squares optimization technique. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

Assay of amylase

The amylase activity in the cell free supernatant (CFS) was measured by incubating 0.5 ml of CFS with 0.5 ml of 2% (w/v) starch at 37°C in 2 ml phosphate buffer (0.1 M, pH 6.0). The reducing sugars released were measured by 3,5-dinitrosalicylic acid method (Miller, 1959). A separate blank was set for each sample to correct the non-enzymatic release of sugars. One unit of amylase was defined as the amount of enzyme that released 1 µg of reducing sugar as maltose per minute under the standard assay conditions.

Results and Discussion

In present work, the isolate used is a soil isolate and showed potential amylase production on starch agar plate with zone of hydrolysis (50 mm diameter). The clear zone of hydrolysis around the bacterial colony was due to the hydrolysis of starch by amylolytic enzyme (Figure 1).

Figure 1. Amylase production on starch agar plate after iodine flooding (grown at 37 ^{0}C).

Morphological, physiological and biochemical tests were carried out for this isolate as described in Table 3, according to Bergey's manual of systematic bacteriology (Sneath et al., 1986). It was a Gram-positive, motile, rod shaped alkaliphilic bacterium. From Table 3, it was identified as *Bacillus* sp. Due to strange results in some of biochemical tests it was difficult to identify up to species level. Likewise, on the basis of various morphological, physiological and biochemical basis the protease producing bacterium Tap 5 was identified as *Bacillus firmus* (Joshi, 2010).

Table 3. Morphological and biochemical characteristics of the soil isolate.

Characteristics	Soil isolate	Characteristics	Soil isolate
Morphological Tests		***Biochemical Tests***	
Colony size	Small	Glucose utilization	±
Colony shape	Circular	Indole production	-
Margin	Rhizoid	Methyl red test	+
Elevation	Slightly raised	Voges Prokaurer test	-
Color	Off white	Citrate utilization test	-
Opacity	Opaque	Catalase	+
Gram's nature	Gram positive rods	Oxidase	+
Spore	Central	Arginine dehydrolase	+
Motility	+	Lysin decarboxylase	-
Physiological Tests		Nitrate reductase	+
Growth at 25 ^{0}C	+	Hydrolysis of :	
Growth at 42 ^{0}C	+	Gelatin	+
Growth at 4 pH	-	Starch	+
Growth at 10 pH	+	Casein	+
Growth in NaCl at 1 %	+	Tributyrine	-
Growth in NaCl at 7 %	-	Acid from other carbohydrates	-

+ = Positive; - = Negative; ± = not predictable

RSM had not only been used for optimization of medium components in the fermentation process (Puri et al., 2002) but also for studying the combined effects of culture parameters (Dutta et al., 2004; Nawani and Kapadnis, 2005). A submerged culture was used for the production of extracellular amylase from *Bacillus* sp. Preliminary experiments on amylase production from the above strain indicated that the most important environmental factors were pH, temperature and inoculum size. Hence these three factors were considered as the independent variables and their effect on amylase production was studied using a CCD of RSM. The results of CCD experiments for studying the effects of three independent variables, viz., pH, temperature and inoculum size, on amylase production are presented in Table 4 along with the predicted and observed responses. The standard deviations on the observed responses are also presented in Table 4. Maximum predicted activity of 511.77 U/ml was observed for central points while experimental results showed maximum activity of 604.17 U/ml for run number 10. The difference between few runs of experimental and predicted activities were due to the determination coefficients where, few percentages of the total variations are not explained by the model. The coefficients of the model were determined of least-squares optimization by the Gauss–Newton technique (Table 5). The overall second order polynomial equation for amylase production is given in Eq. (2).

Table 4. Observed responses and predicted values.

Run No	Amylase activity (U/ml)		Residual standard deviation
	Observed response	Predicted response	
1	110.83	129.97	-19.14
2	113.33	142.18	-28.85
3	10.42	71.35	-60.93
4	49.17	168.56	-119.39
5	173.33	69.39	103.94
6	78.33	32.85	45.48
7	25.42	12.02	13.40
8	64.17	60.48	3.69
9	437.50	474.77	-37.27
10	604.17	505.10	99.07
11	233.75	335.18	-101.43
12	482.92	319.69	163.23
13	562.50	334.18	228.32
14	83.33	249.85	-166.52
15	491.67	511.77	-20.10
16	490.67	511.77	-21.10
17	491.67	511.77	-20.10
18	490.67	511.77	-21.10
19	491.67	511.77	-20.10
20	490.67	511.77	-21.10

Table 5. Model coefficients estimated by multiple linear regressions.

Factor	Coefficient	F-value	p- value
Intercept	511.77	5.02	0.009*
X_1 - pH	15.77	0.14	0.719
X_2 – Temperature	-7.75	0.037	0.851
X_3 – Inoculum size	-42.17	1.09	0.320
X_{12}	21.25	0.22	0.647
X_{23}	-12.19	0.073	0.792
X_{13}	0.31	4.800E-005	0.994
X_{11}	-21.83	0.081	0.782
X_{22}	-184.33	5.74	0.037*
X_{33}	-219.75	8.16	0.017*

*Significant variables

Amylase activity $(y) = 511.77 + 35.77X_1 – 7.75X_2 – 42.17X_3 + 21.25X_{12} – 12.19X_{23} + 0.31X_{13} – 21.83X_{11} –184.33X_{22} – 219.75X_{33}$……..... (2)

where, X_1-pH, X_2-temperature in ^{0}C and X_3-inoculum size *in* %. The larger the magnitudes of F- value, the smaller the p-value, the more significant value is the corresponding coefficient (Akhnazarova and Kafarov, 1982; Rubinder et al., 2002). The results of the second order response surface model fitting in the form of analysis of variance (ANOVA) are in Table. 6. The fisher F-test with a very low probability value demonstrated a very high significance for the regression model (Olivera et al., 2004;

Zambare, 2010b). The fitting of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2= 0.818) indicates that only 18.2% of the total variations are not explained by the model. The value of the adjusted determination coefficient (Adj. R^2= 0.655) is also high, which indicates a higher significance (p value < 0.01) of the model (Adinarayana and Elliaiah, 2002; Olivera et al., 2004). Adequate precision measures the signal to noise ratio. An adequate precision value (5.74) was greater than 4 which indicates adequate signal. At the same time a relatively lower value of the coefficient of variation (CV=42.70) indicates improved precision and reliability of the conducted experiments (Adinarayana and Elliaiah, 2002).

Table 6. Analysis of variance (ANOVA) for the three factorial design.

Source of variation	Sum of squares	Degree of freedom	Mean square	F-value	P- value
Regression	7.352E+005	9	81688.75	5.02	0.009
Residual	1.628E+005	10	16277.28		
Total		19			

* Significant at < 0.01.R2= 0.818, Adjusted R2= 0.655, Adeq. Precision= 5.54, % C.V. = 42.70

The 3-D counter plots for response surfaces corresponding to the combined effects of pH-temperature (Figure 2), pH-inoculum (Figure 3) and temperature-inoculum (Figure 4) were plotted. The response *surfaces* obtained were suggesting that *Bacillus* sp. secreted amylase in more alkaline condition at moderate temperature and inoculum size. Thus the optimum operating conditions obtained from the RSM model were pH (11.35), temperature (35.16°C) and inoculum (2.95%) with predicted amylase activity of 515.30 U/ml. After optimization, 4.64 fold amylase activity (515.30 U/ml) was enhanced when compared with non-optimized environmental factors (pH 10, temperature 30°C, inoculum size 1 %) in basal medium (110.83 U/ml). Thus, RSM could be a very powerful and flexible tool for modeling the fermentation process due to corrective action arising from methodology and the associated estimation procedure.

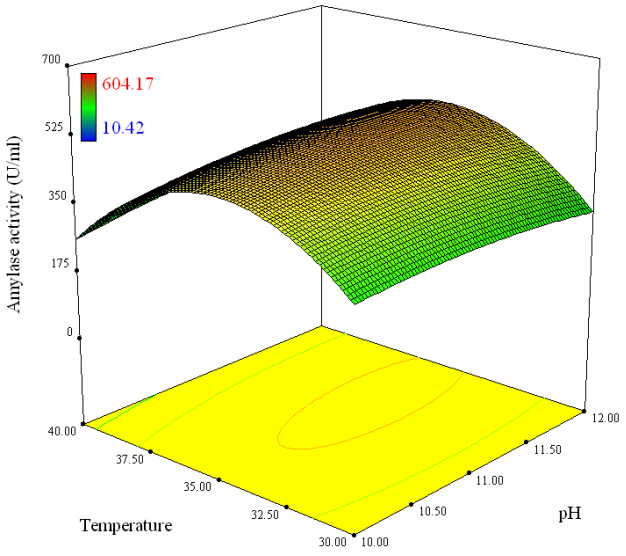

Figure 2. Three-D counter plot showing the mutual effect of pH and temperature on amylase activity with 3 % inoculums.

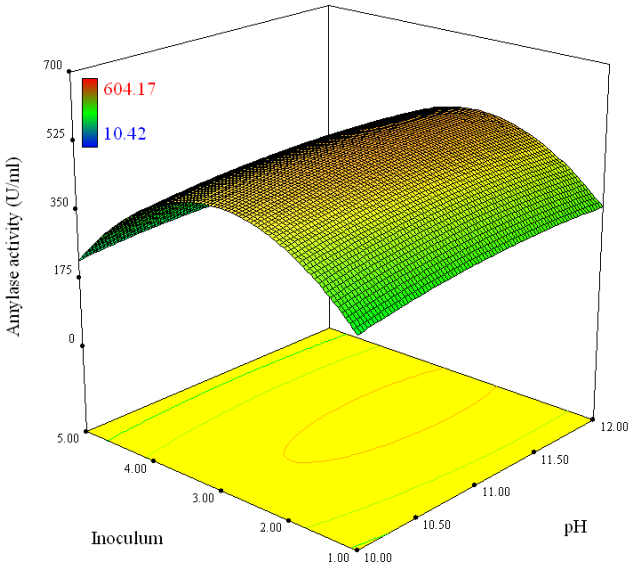

Figure 3. Three -D counter plot showing the mutual effect of pH and inoculum on amylase activity at 35°C.

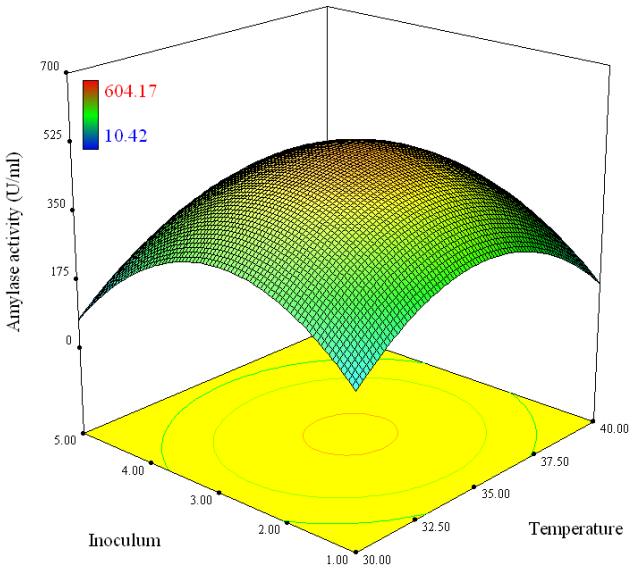

Figure 4. Three-D counter plot showing the mutual effect of temperature and inoculum on amylase activity at pH 11.

Among the physico-chemical parameters, the pH of the growth plays important role on the production of alpha amylase (Panday et al., 2000). In this study, for amylase production optimum at pH 11.35 was found; above and below this pH there was probably due poor microbial growth in acidic and alkaline medium (Kar and Ray, 2008). Also, similarly various research on optimization of physical parameters for amylase studies were reported

in literature (Agrawal et al., 2005; Kar and ray, 2008; Tamilarasan et al., 2010). The application of properly designed models with multi-factor analysis allow process and biochemical engineers to design scale up strategies for increasing enzyme production. Also, amylases have numbers of commercial applications in baking, brewing and alcohol industries because of their inherent unique properties (Kar and Ray, 2008). Hence the amylase produced from *Bacillus* sp. will be useful in the starch bioprocessing sectors, particularly for starch liquefaction for bioethanol production. Further study is in progress in our laboratory on the application of *Bacillus* amylase in saccharification of cassava starch and related substrates for production of ethanol and in wine making.

Conclusion

The result obtained in the present study indicated that *Bacillus* sp. could be a potential strain for amylase production in submerged fermentation using wheat bran like easily available carbon substrates. The RSM allowed the optimization of process parameters such as pH (11.35), temperature (35.16°C) and inoculum size (2.95%) for attaining higher yield of amylase.

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