RESEARCH ARTICLE

Statistical optimization of an amylase from *Bacillus* sp. by using response surface methodology

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Manuscript details:

Received: 20 August, 2014
Revised : 25 September, 2014
Revised received: 10 October, 2014
Accepted: 01 December, 2014
Published :30 December, 2014

Editor: Dr. Arvind Chavhan

Citation this article as:
Kadam Omprakash A (2014) Statistical optimization of an amylase from *Bacillus* sp. by using response surface methodology. *Int.J. of Life Sciences*, 2(4):378-381.

ABSTRACT

There are four *Bacillus sp*. were tentatively identified by using culture dependent method. Among them one of the Bacillus sp. having potential to produce maximum extracellular amylase on skimmed milk agar plate. The production of an extracellular amylase by *Bacillus sp was* statistically optimized by using response surface methodology **(**RSM). The maximum production of amylase from Bacillus sp. was achieved in presence of glucose and peptone as a carbon and nitrogen source respectively. The obtained ANOVA from central composite design showed a significant p value hence the designed model can be useful for to produce amylase by *Bacillus sp.*

Keywords: *Bacillus sp.,* Amylase, RSM.

INTRODUCTION

Amylases are hydrolyzing enzyme in function which causes hydrolysis of molecules. In biotechnology amylases are of the most important enzymes used. The main use of enzymes includes hydrolysis of starch to yield glucose syrup, amylase-rich flour and in the formation of dextrin during baking in food industries (Rubinder *et al.,* 2002). Furthermore, in the textile industry, amylases are used for removal of starch sizing and as additives in detergents. However, the cost of producing this enzyme is high and the cost of procurement by developing countries can be even higher as a result of importation. Cheap and readily available agricultural waste such as Potato peels, which presently constitutes a menace to solid waste management, may be a rich source of amylolytic bacteria (Reddy *et al.,* 2003a; 2003b). *Bacillus licheniformis, Bacillus amyloliquifaciens* and *Aspergillus nige are commonly used for production of amylase* . Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries (Akhnazarova and Kafarov, 1982).

In the present investigation, the *Bacillus sp.* used in this work could be used for the production of α-amylase, the most commercially interes-ting enzymes, in one fermentation process using inexpensive nutrients.

MATERIALS AND METHODS

Microorganism

α-Amlyase producing *Bacillus sp.* which was grown on nutrient agar at 37°C for 24 h for inoculum preparation. A loopful of the growth was transferred to Laura broth (LB) liquid medium (1% yeast extract, 0.5% peptone, 0.5% NaCI, (w/v), pH 7.0) (Agrawal *et al*., 2005).

Enzyme production:

The organism was grown at 37°C for 1 days in 25 ml of medium with shaking on a shaker (150 rpm). Samples were taken from 12 to 120 h. The supernatant of the culture after centrifugation (10.000 rpm, 10 min) at 4°C was used to determine extracellular amylase activity (Agrawal *et al*., 2005).

Enzyme assay: (Agrawal *et al*., 2005).

α-Amylase activity was measured by using Bernfeld method. The reaction mixture containing 200 µl of 1% substrate (w/v) in 0.1 M phosphate buffer (pH: 7.0) and 150 µl of enzyme solution was incubated for 30 min at 37°C. stop the reaction by using 400 µl of 3,5-dinitrosalicylic acid solution and heat in boiling water for 5 min and add 8 ml of distilled water. The OD was measured at 489 nm by using spectrophotometer.

Assay of protein concentration:

The total protein contents of the samples were determined according to the method described by Lowry. The protein standard used was BSA (Merck). Protein standard solution, in the range of 0.5 to 5 mg/ml was prepared in triplicate to obtain a standard curve. Samples (cell-free supernatant) were diluted to 1 ml with distilled water so that the protein content would be within the range of the standards. Alkaline copper sulphate reagent (5 ml) was added to each tube and mixed well. The solutions were kept at room temperature for 10 minutes followed by the addition of 0.5 ml Folin & Ciocalteu's Phenol reagent (Merck) working solution. Each tube was rapidly mixed, and incubated in dark for 30 minutes. Absorbance of the samples was measured spectrophotometrically at 570 nm using UV/Vis spectrophotometer (Adinarayana & Ellaiah, 2002).

Experimental design and optimization by RSM:

The statistical software package Design-Expert 8.0 (Stat-Ease Inc., Minneapolis, USA) was used to analyze the experimental design, data analysis, linear, quadratic model building. Response surface and contour plots were generated to understand the interaction of different variables (Dey *et al*., 2001).

Table 1: Selected parameters for the production of amylase with its lower and higher values.

Sr. no	Parameters	Lower	Higher
1.	Temperature	20	50
2.	pH	5	9
3.	Incubation time	20	72
4.	Carbon source	0.1	1
5.	Nitrogen source	1	3

RESULTS AND DISCUSSION

Microorganisms like fungi and bacteria have been extensively screened for α-amylase production. In the bacteria, *Bacillus* species such as *B. subtilis, B. licheniformis* and *B. stearothermophilus* can be used for the better production of α-amylase in shake flask.

Morphological, physiological and biochemical tests were carried out for selected isolate and tentatively confirmed according to Bergey's manual of systematic bacteriology (Agrawal *et al*., 2005).

In the present investigation RSM was used to optimize the medium components in the fermentation process. Amylase production from *Bacillus* sp. Was carried by using pH, temperature, incubation time, carbon source and nitrogen source. Hence these five factors were considered as the independent variables and their effect on amylase production was studied using a CCD of RSM.

Table 2: Selected parameters for the production of amylase with its lower and higher alpha values obtained from Design expert 8.

Sr. no	Parameters	Lower	Higher	-Alpha	+ Alpha
1	Temperature	20	50	-0.676213	70.6762
2	pH	5	9	2.24317	11.7568
3	Incubation time	20	72	-15.8388	107.839
4	Carbon source	0.1	1	-0.520286	1.62029
5	Nitrogen source	1	3	-0.378414	4.37841

Table 3. The Central composite design (CCD) matrix for screening of medium components for amylase production by *Bacillus sp.*

Run	Temperature (°C)	pH	Incubation time (Hrs)	Carbon source (%)	Nitrogen source (%)	Amylase production (U/mg)
1.	50	9	20	0.10	3.0	0.01
2.	35	10.64	46	0.55	2.0	0.01
3.	35	7	46	0.55	2.0	0.21
4.	20	9	72	1	1	0.1
5.	50	9	20	1	1	0.13
6.	35	7	46	1.37	2.0	0.34
7.	20	9	72	0.10	3	0.23
8.	50	5	72	0.10	3.0	0.31
9.	50	5	72	1	1	0.12
10.	20	5	20	0.10	1	0.15
11.	50	9	72	0.1	1.0	0.43
12.	35	3.36	46	0.55	2.0	0.21
13.	20	5	72	1	3	0.12
14.	35	7	24	0.55	1	1.4
15.	35	7	93.35	0.55	2	0.98
16.	50	5	20	1	3	0.32
17.	35	7	46	0.55	2.0	0.89
18.	7.68	7.0	46	0.55	2	0.89
19.	35	7	46	0.55	0.18	0.94
20.	35	7	46	0.55	2	0.81
21.	62.32	7	46	0.55	2	0.21
22.	20	9	20	1	3	0.3
23.	35	7	-1.35	0.55	2	00
24.	35	7	46	-0.27	2	0
25.	35	7	46	0.55	3.82	0.32
26.	35	7	46	0.55	2	0.34

Table 4 : ANOVA for Response Surface Cubic Model (Aliased).

Source	Sum of Squares df	Square	Mean Value	f Prob >F	p-value
Model2.94	22	0.13	1.17	0.0203	
A-Temperature	0.020	1	0.020	0.18	0.7036
B-pH	0.020	1	0.020	0.18	0.0036
C-Incubation time	0.48	1	0.48	4.21	0.1326
D-Carbon source	0.058	1	0.059	0.51	0.280
E-Nitrogen source	0.19	1	0.19	1.68	0.0851

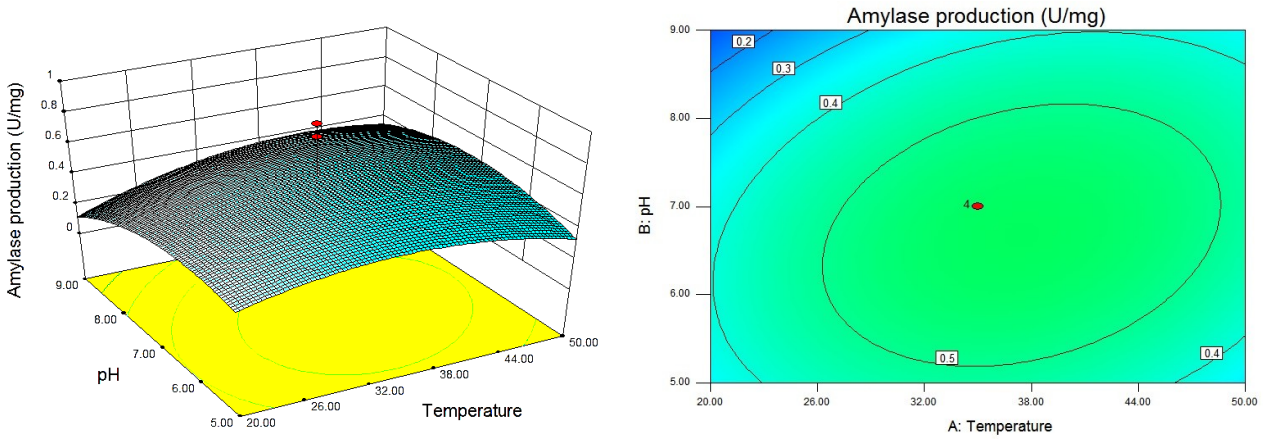

Fig. 1: (A) Response surface plot; (B) contour plot of amylase production showing the interaction between temperature (°C) and pH at the constant values of all other parameters.

In order to search for the optimum formulation of the medium, Central Composite design was used with five factors, which indicated that 26 experiments were required (Chauhan and Gupta, 2004; Box *et al*., 1978). The experimental design and corresponding amylase yields are shown in Table 3.

The results were analyzed by using ANOVA i.e. analysis of variance suitable for the experimental design used and cited in Table 4. The ANOVA of the cubic regression model indicates the model is significant. Statistical analysis of RSM demonstrates that the Model F-value of 1.17 implies that model is significant. There is only a 2.08% 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 pH, temperature, carbon source and nitrogen source are significant model terms. The obtained ANOVA indicated in table 4.

The "Model F-value" of 1.17 implies the model is significant relative to the noise. The response surface and counter plot is shown in fig 1.

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