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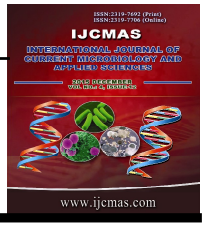

Original Research Article

Optimization of Some Culture Conditions to Enhance α-Amylase Production using Response Surface Methodology

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Keywords

Optimization, RSM, α-Amylase and Culture conditions

A B S T R A C T

The main objective of this research was the optimization of some culture conditions to enhance Alpha amylase production. Screening, isolation and identification of α-amylase producing bacteria from different locations in Khartoum was determined. Twenty five soil samples were collected from different locations. The bacterial isolates for amylase production reflected by a large zone using iodine method. These isolates were identified according to microscopical and biochemical test. 28%were found to be *Bacillus subtilis* while 24% was found to be *Bacillus licheniformis* Statistical experimental design was used to optimize the physical conditions such as temperature and incubation time to enhance alpha amylase production from *Bacillus licheniformis.* Response surface methodology with appropriate statistical experimental design was employed for this purpose. The maximal level of extracellular alpha amylase was achieved at 0.445 mg⁄ml/hr when the temperature and time of incubation were 47°C and 46.3 hr. respectively.

Introduction

Amylases (E.C.3.2.1.1) are enzymes that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such glucose, maltose and maltotriose units (Gupta et al.,2003 ; Rajagopalan and Krishnan 2008). Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (Reddy et al., 2003). They can be obtained from several sources, such as plants, animals and microorganisms. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal amylases (Tanyildizi et al., 2005). The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy

to manipulate to obtain enzymes of desired characteristics. Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. Fungal and bacterial amylases could be potentially useful in the pharmaceutical and fine-chemical industries. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries (Kandra 2003; Pandey et al., 2000). Starch is an important constituent of the human diet and is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. Starch converting enzymes are used in the production of maltodextrin, modified starches, or glucose and fructose syrups. A large number of microbial α-amylases has applications in different industrial sectors such as food, textile, paper and detergent industries. The production of α-amylases has generally been carried out using submerged fermentation, but solid state fermentation systems appear as a promising technology. The properties of each α-amylases such as thermostability, pH profile, pH stability, and Ca-independency are important in the development of fermentation process. (Paula and Pérola, 2010) The production of α-amylases by submerged fermentation (SmF) and solid state fermentation (SSF) has been investigated and depend on a variety of physicochemical factors. SmF has been traditionally used for the production of industrially important enzymes because of the ease of control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture (Gangadharan et al., 2008; Couto and Sanromán, 2006). α-amylases are one of the most popular and important form of industrial amylases and the present review point out the microorganisms that produce this enzymes. The optimization of fermentation conditions, particularly physical and chemical parameters are important in the development of fermentation processes due to their impact on the economy and practicability of the process (Francis et al., 2003). The role of various factors, including pH, temperature, metal ions, carbon and nitrogen source, surface acting agents, phosphate and agitation have been studied for α-amylase production. The properties of each α-amylase such as thermostability, pH profile, pH stability, and Ca-independency must be matched to its application. For example, α-amylases used in starch industry must be active and stable at low pH, but at high pH values in the detergent industry.

The statistical experimental design constitutes an efficient tool and well adopted for treating problems with large number of variables, and allows simultaneous, systemic and efficient analysis of all variables (Ibrahim et al., 2003). The conventional methods for multifactor experimental design are time-consuming and incapable of detecting the true optimum, due especially to the interactions among the factors; this can be eliminated using response surface methodology (RSM). RSM is a statistical technique for the modeling and optimization of multiple variables, which determines the optimum process conditions through combining experimental designs with interpolation by first- or second-order polynomial equations in a sequential testing procedure (Myers et al., 2009).

Materials and Method

Sampling

Twenty five soil samples were collected

during March to April 2015 from different locations in Khartoum state into sterilized plastic bags. Samples were taken from 15-20 cm depth after removing approximately 3 cm of earth surface and given number from 1-25.

Isolation of Microorganism

Isolation of microorganism was performed by the soil dilution plate technique (Waksman, 1961). 1gm of each soil sample was taken in 9 ml of sterilized distilled water in a presterilized test tube. Dilution process was continuing until 10^{-4} obtain. Different aqueous dilutions (10^{-3}, 10^{-5}, and 10^{-7}) of the soil suspension were applied separately into sterilized Petri-dishes with nutrient agar. After gently rotating the plates they incubated at 37˚C for 24 hours.

Colonies, with *Bacillus* morphological characteristic that appeared in the incubated plates, were repeatedly sub-cultured for further studies.

Starch Hydrolysis

Starch medium was sterilized at 121˚C for 15 minutes and then poured into petri dishes to cool. Starch agar media were inoculated with isolated bacteria at 37˚C for 48 hr. The Petri dishes were flooded with 5.0 ml of iodine solution. The clear zone surrounding the colony was measured in (mm) from the edge of the colony to the limit of clearing (inhibition zone).

The identification of purified isolates were carried out according to (Cowan and Steel, 1974).

Enzyme Extraction

Preparation of Inoculums

The isolates that showed large inhibition zone on starch agar media were used for inoculums preparation and experimental design (isolate 7). A volume of 200 ml of nutrient broth taken in a 250-ml Erlenmeyer flask was inoculated with a loop full of cells from a 24-hr. old slant and kept at 37 ºC in a rotary shaker . After 24 hour of incubation, 10 ml of this nutrient broth culture was used as the inoculums.

Media Preparation

Starch liquid media was prepared by dissolving (g/liter) Soluble starch 1%(w/v), Peptone 0.5%(w/v),, Yeast extract 0.5%(w/v), $MgSO_4.7H_2O$ 0.02%(w/v), K_2HPO_4 0.1%(w/v), and $Na_2CO_3$1% in distiller water , the final pH value of the medium was adjusted to 7.0. The medium was portioned into 100 ml in 250ml Erlenmeyer flasks. The flasks were sterilized by autoclaving at121ºC for 15 minutes, Na_2CO_3 was autoclaved separately and then added under flame to the media (Horikoshi, 1971). 1ml of inoculums which prepared as in 3.5.1 was inoculated into 100 ml of sterilized starch liquid media in 250 ml cotton plugged Erlenmeyer flasks. The flasks were incubated at 37 ºC on a rotary shaker operating at 140 rpm for 3 days. The culture broth was then centrifuged (Hettich Mikro 20) at 40.000 rpm for 30 min. at 4°C, the free-cell supernatant was used as an extracellular crude enzyme (Ibrahim et al., 2004)

Enzyme Assay

Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate 6. 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. The reaction was stopped by adding 400 µl of 3,5-dinitrosalicylic acid

solution followed by heating in a boiling water bath for 5 min and cooling at room temperature and then 8 ml of deionized water was added. Absorbance of each solutions containing the brown reduction product was measured at 472 nm in a UV-visible spectrophotometer (Akcan et al., 2011). The enzyme activity was measured according to DNS method based on the standard curve of sugar

Experimental Design

Response surface design was used for the optimization of variables. The variables determined were temperature and incubation period. the variables (coded ,uncoded) of each constituent at level -1 , 0 , +1 are give in Table (1).

A 2^3 factorial design and 5 replicate at the center point, with a total number of 13 experiments were employed as in table 2. Results were analyzed statistically by Minitab program (version 16) and optimum conditions were computed and graphically presented.

The optimization of the temperature and incubation time for α-amylas activity, the surface response for enzyme production as a function of selected key variable has to be predetermined. Therefore, according to the literature review, the temperature range tested in the final optimization step was (26-54°C) and incubation time of (24- 72). The minimum and maximum ranges of variables were investigated the tables above

Results and Discussion

Isolation and Identification of Bacterial Isolates

The twenty five soils samples (25) were collected from different locations in Khartoum. 52% of the isolates were found to be of *Bacillus*. 7 out of 25 were found to be *Bacillus subtilis* while 6 out of 25 was found to be *Bacillus licheniformis*. The isolate no (2, 5, 7 and 12) showed larger zone upon treated with iodine solution and a disappearance of the blue color around colony, were selected for further characterization and for α- amylase production.

Factorial Desigen and Analysis of the Results

The overall effect of experimental operation factors; temperature and incubation time on alpha amylase production, was found that those factors exerted certain effect on response and the percent variability explained (R^2) was 78.13%.This indicates that the variation in those seleceted factors could explain the variation in alpha amylase activity up to 78.13%. These make the fitted second-order polynomial as acceptable. Regression coefficients obtained from MINITAB pakege (release 16) analysis are shown in Table (3). All coefficients in the second order polynomial in terms of linearity and quardratic were significant at 1%. The interaction coefficients showed significant result at 5%. The lack of fit was not significant . The analysis was done using uncoded units.

The significance of each coefficient was determined by t-values and P-values which are listed in Table 3. The larger the magnitude of t-test and value and smaller the P-value indicates the high significance of the corresponding coefficient (Karthikeyan et al., 1996).

Local Solution gave by MINITAB Package was

Temp = 46.9996
Incubation time = 46.2858

Table.1 Process Variables and their Levels in the Two Variables-Two Levels Response Surface Design

Independent variables	**symbol**		**levels**	
	coded	uncoded	coded	uncoded
Temperature (°C)	X_1	°C	1	26
			0	40
			-1	54
Incubation time (hr.)	X_2	Hrs	1	24
			0	48
			-1	72

Table.2 Expermintal Design of α- amylase Production

Experiment No	StdOrder	RunOrder	PtType	Blocks	Temp. °C	Incubation Time (hr.)
1	2	1	1	1	54.0000	24.0000
2	5	2	-1	1	20.2010	48.0000
3	12	3	0	1	40.0000	48.0000
4	9	4	0	1	40.0000	48.0000
5	1	5	1	1	26.0000	24.0000
6	3	6	1	1	26.0000	72.0000
7	10	7	0	1	40.0000	48.0000
8	7	8	-1	1	40.0000	14.0589
9	4	9	1	1	54.0000	72.0000
10	13	10	0	1	40.0000	48.0000
11	6	11	-1	1	59.7990	48.0000
12	11	12	0	1	40.0000	48.0000
13	8	13	-1	1	40.0000	81.9411

Table.3 Regression Result of a Polynomial Model for Alpha Amylase Production

Term	Coef	SE Coef	T	P
Constant	-0.483510	0.238581	-2.027	0.082
temp	0.023959	0.009067	2.642	0.033
time	0.015779	0.004634	3.405	0.011
temp*temp	-0.000226	0.000102	-2.219	0.062
time*time	-0.000140	0.000035	-4.036	0.005
temp*time	-0.000059	0.000078	-0.759	0.473

S = 0.0526234 PRESS = 0.115440, R-Sq = 78.13% R-Sq(pred) = 0.00% R-Sq(adj) = 62.50%

Table.4.Analysis of Variance for Alpha Amylase Production

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.069232	0.069232	0.013846	5.00	0.029
Linear	2	0.014468	0.038387	0.019193	6.93	0.022
temp	1	0.014466	0.019335	0.019335	6.98	0.033
time	1	0.000001	0.032113	0.032113	11.60	0.011
Square	2	0.053170	0.053170	0.026585	9.60	0.010
temp*temp	1	0.008070	0.013634	0.013634	4.92	0.062
time*time	1	0.045100	0.045100	0.045100	16.29	0.005
Interaction	1	0.001594	0.001594	0.001594	0.58	0.473
temp*time	1	0.001594	0.001594	0.001594	0.58	0.473
Residual Error	7	0.019385	0.019385	0.002769		
Lack-of-Fit	3	0.015346	0.015346	0.005115	5.07	0.075
Pure Error	4	0.004038	0.004038	0.001010		
Total	12	0.088616				

Figure.1 Contour Plot of Alpha Amylase Activity

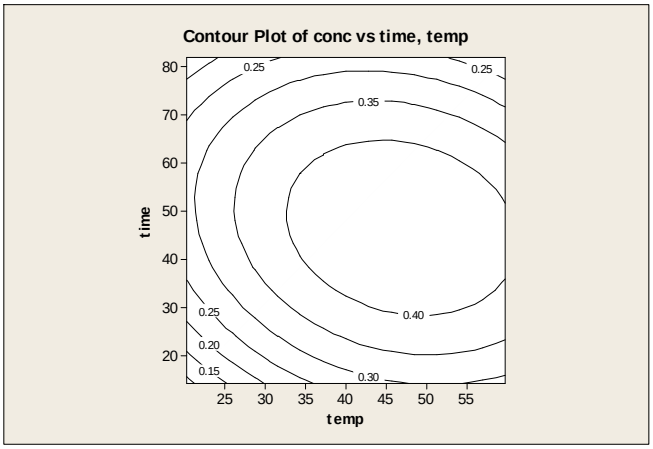


Figure.2 Fitted Surface of Temperature –Time- Enzyme Activity

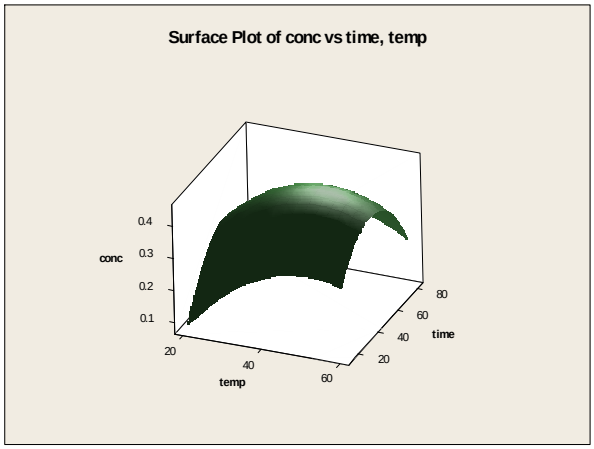
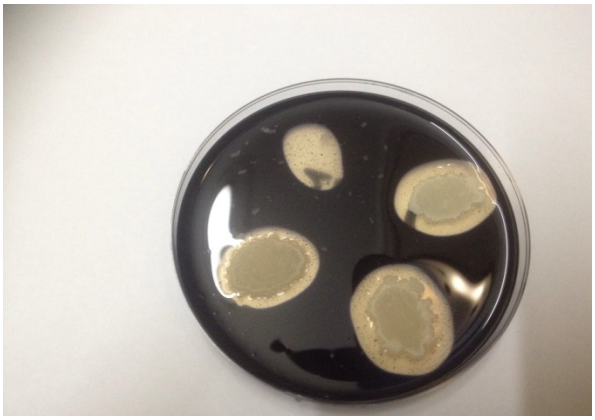


Plate.1 Starch Hydrolysis Test (Iodine Method)



Predicted Responses

Composite Desirability = 0.034871
Enzyme activity = 0.445222,
Desirability = 0.034871

To obtain the maximum optimum activity, the factor levels was set at the values given by MINITAB's multiple response optimizer under global solution of desirability equal to 0.034871. That is temperature would be set at 46.9996 and time at 46.2858. That indicated as temperature increase the enzyme activity increase also time has significant effect on the activity of enzyme.

Figure 1 and 2 represent the isoresponse contour plots for the optimization of temperature and time on alpha amylase production. Elliptical contours are obtained when there is a perfect interaction between the independent variables. The derived optimum levels of the physical environment were temperature of 46.9996 °C and time 46.2858 hr. which maximum yield was 0.445222 mg⁄ml. Previously, 37°C were reported as optimum temperature for alpha amylase production by several authors (Anto et al.,2006; Baysal et al.,2003). In other research the optimum temperatures for growth and α-Amylase production were found to be 45°C to 46 °C and 50 °C (Kenneth et al.,1993) which nearly to my result. Other author found that the maximum yield of enzyme produced after 48hr. these result nearly similar to my result (Rameshkumar and Sivasudha, 2011). The production of enzyme decreased after 72hr. A similar result was reported by (Gangadharan et al., 2006). Enzyme production is related to the growth of the microorganism. Growth of the organisms would have reached a stage (due to insufficient nutrients) that indirectly stimulated production of secondary metabolites (Febe et al., 2002).

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