Biological Forum – An International Journal **15(5): 988-993(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Optimization of α-amylase Production from a Novel Bacterial Strain, *Geobacillus kaustophilus* Strain AN11, Employing Response Surface Methodology

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(Received: 08 March 2023; Revised: 17 April 2023; Accepted: 22 April 2023; Published: 20 May 2023)
(Published by Research Trend)

ABSTRACT: To overcome numerous industrial barriers, it is critical to investigate thermostable α-amylase-producing bacteria in demanding conditions. This study aimed to identify and characterize α-amylase-producing bacteria isolated from hot springs. The isolates were identified as *Geobacillus kaustophilus* strain AN11 based on their molecular characterization. An optimization technique based on statistical experimental design is applied to increase thermostable α-amylase production by a thermotolerant *Geobacillus kaustophilus* strain AN11 isolate. The pH, temperature, salt (NaCl) level, and nitrogen supply (tryptophan) were found to have a substantial influence on enzyme production using the one variant at a time approach. The response surface methodology (RSM) was used to determine the optimal process conditions among the variables picked. A Box-Behnken design was utilized to develop a polynomial quadratic model that correlated the link between the four variables and α-amylase activity. The optimum culture variables for α-amylase synthesis were pH 5, temperature 55°C, tryptophan 0.4 gm, and NaCl 2.52 gm. Temperature studies demonstrated that the crude enzyme had the highest activity and stability at 60, 65 and 70°C. The temperature and α-amylase production had a linear relationship. Thus, molecular identification of this novel strain *Geobacillus kaustophilus* strain AN11 isolates, purification, and production of amylases are proposed for effective application in various industries. Industrial detergent's effectiveness to wash clothes might be enhanced by adding enzymes to the detergent. This study has shown that these thermostable bacteria are a great supply of amylase that may be employed commercially to sustain economic activity.

Keywords: α-amylase, Response surface methodology, Industries, Enzymes, Box-Behnken.

INTRODUCTION

Amylases are prominent enzymes with a crucial purpose in biotechnology. Plants, animals, and microbes make them. The baking and bread industry, fermentations, textiles, alcohols, medicines, and detergents use amylases extensively (Mageswari *et al.,* 2012). Additionally, they are utilized in the manufacture of malt, low-calorie beer, apple and pear juice purification, corn, and chocolate syrup, as well as in the removal of adhesive in the paper industry (Gupta *et al*., 2003). Because they can be made quickly, cheaply, and simply, microbial amylases have traditionally been preferred to other plant and animal amylases, and unlike other microbes, bacterial amylase manufacturing is less expensive and quicker. Numerous industrial enzymatic processes benefit from high temperatures by reducing the risk of contamination, speeding up diffusion, and resisting denaturing chemicals and proteolytic enzymes (Saxena *et al.,* 2007).

Distinct *Bacillus* species produce different amylases with varying pH ranges and temperatures (Vaikundamoorthy *et al.*, 2018). A source of thermophilic bacteria that produce thermostable amylase, which is needed in many industry sectors, is the hot springs (Fooladi & Sajjadian 2010). The need for thermostable amylases is growing in both biotechnology and commercial applications. However, few reports on bacterial strains could synthesize thermostable amylase (Indriati *et al.,* 2018). Thermophilic fermentation is also considered beneficial for environmental and technical uses. The benefits include cheaper cooling costs, improved substrate solubility, quicker mixing and pumping, the lesser possibility of potential contaminants, and lower viscosity.

However, operating systems to produce α-amylase at higher temperatures will necessitate innovative process design and enhanced comprehension of thermophilic bacteria (Leveque *et al.*, 2000). Optimizing the media constituents and the cultural parameters is crucial for standardizing a bioprocess since the proportion of the medium has a significant impact on growth and enzyme production. Response surface methodology (RSM), a statistical and mathematical tool for forecasting the behavior of process variables and elucidating their relationships, has been utilized to improve α-amylase production (Ojha *et al.*, 2020).

In the current investigation, we identified and characterized the thermostable α-amylase-producing

bacteria from hot springs. Through the application of response surface methodology (RSM) design, we also investigated the optimization of four major parameters influencing the production of thermostable amylase from *Geobacillus kaustophilus* strain AN11isolate. The thermostability of the enzyme was also investigated after medium optimization.

MATERIAL AND METHOD

A. Sampling and Isolation

A water sample was obtained from Rajwadi Hot Spring in Rajwadi, Maharashtra, and placed in a sterile container to isolate thermophilic bacteria. The sample was diluted in sterile 0.9% saline solution ten times before being cultured on nutrient agar plates with 100 µl of each dilution. The plates were incubated for 48 h at 55°C. After incubation, bacterial colonies with distinctive morphologies were selected and purified on sterile nutrient agar plates.

B. Screening and Selection

Through sub-culturing on agar plates supplied with 2% soluble starch, 1% peptone, 0.3% meat extract, and 0.5% NaCl at pH 7, the isolates were evaluated for their ability to produce α-amylase. The plates were incubated for 48 h at 55°C. Using the iodine test, the clearance zone was used to select and identify the isolate that produced the α-amylase (Fazal *et al.,* 2022).

C. Molecular identification

Using a Gene Elute Genomic DNA isolation kit, total genomic DNA was extracted (Sigma, USA). Almost the entire 16S rRNA gene was amplified using the primers 27F and 1492R. The Eppendorf Gradient Mastercycler system was used to conduct the PCR, which included a cycle of 94°C for 3 min, 32 cycles of 94°C for 45 sec, 51°C for 1 min, and 72°C for 1.30 min, and a final extension at 72°C for 10 mins, with the mixture being kept at 4°C. The Magnetic bead-based technique was used to clean the PCR product. The PCR result was sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). Following the manufacturer's instructions, the sequencing process and template preparation were completed and purified (Applied Biosystems). An ABI PRISM 3100 Genetic Analyzer was used to analyze the samples (Applied Biosystems). The sequencing results were examined using the DNA Analyzer computer program (Applied Biosystems). To check the sequence's identity, it was compared and submitted to NCBI. Phylogenetic trees of strains were created using MEGA6 to figure out the evolutionary connections between bacteria in the community (Kumar *et al.*, 2018).

D. Optimization of α-amylase enzyme activity employing RSM

The Box-Behnken design (BBD) was implemented to scrutinize the synergistic impacts of the parameters pH, temperature, salt concentration, and nitrogen source (tryptophan) on α-amylase enzyme activity. These variables were chosen to be examined in the BBD experiment's 29 runs. They have given the designations A, B, C, and D and boundary conditions for each factor with the actual design. Table 1 shows the range and three distinct levels of the aforementioned factors optimized to produce the highest possible α-amylase activity. Each independent variable's lowest and highest levels were denoted by the symbols -1 and +1, correspondingly, while the center level was designated 0. The interaction between the dependent and independent variables was demonstrated by employing a second-order polynomial equation as follows:

$$Y = \alpha_0 + \sum_{i=1}^{n} \alpha_i A_i + \sum_{i=1}^{n} \alpha_{ii} A_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \alpha_{ij} A_i A_j$$

Where Y denotes the predicted response; α_0 denotes the model intercept; A_i and $A_j$denote independent variables; and α_i, α_{ii}, and α_{ij} denote the regression coefficients for the linear, square, and interaction effects, correspondingly. The letter "n" denotes the study's n independent variables (4). The experimental models were created through Design-Expert (Version 11, Stat-Ease Inc., USA) statistical software.

Table 1: Actual levels for the four variables in the Box-Behnken design.

Independent Variables	Symbols	Coded and Actual levels		
		-1	0	+1
pH	A	5	7	10
Temperature	B	40	55	70
Salt concentration	C	0.05	2.525	5
Nitrogen source (Tryptophan)	D	0.2	0.4	0.6

E. Detection of α-amylase

The amylase activity was measured by utilizing the 3,5-dinitrosalicylic acid (DNSA) method, which involved the incubation of 0.8 ml of 1% starch in 50 mM Tris-HCl buffer (pH 7.5) with 2 ml of cell-free supernatant for 30 minutes at temperatures of 55°C. The reaction was ceased by adding 1 ml of DNSA reagent and boiling it in a water bath for 15 minutes. The amount of glucose released was calculated by measuring the absorbance at 540 nm using a Unico 7200 Spectrophotometer after cooling at room temperature. Using a calibration curve for glucose, amylase activity was calculated (Soy *et al.,* 2021).

F. α-amylase thermostability

The thermal stability of the crude enzyme was assessed using the DNSA method by incubating it at various temperatures of 60, 65, and 70°C for 48 h.

RESULT AND DISCUSSION

A. Isolation, screening, selection, and identification of potent bacteria

In order to identify the isolated culture on a nutrient agar plate and incubated at 55°C, a zone of clearing around the colonies that produced amylase employing the iodine test was observed. Additionally, to create the pure colonies, the separated colonies were sub-cultured. By performing a partial amplification and sequencing of the 16S rRNA gene, the isolated organism was identified as *Geobacillus kaustophilus* ML-1 (100 percent sequence identity). When submitted, a GenBank accession number (OP897632) was assigned to the nucleotide sequence. Fig. 1 shows a phylogenetic analysis with the alignment of the acquired gene sequence.

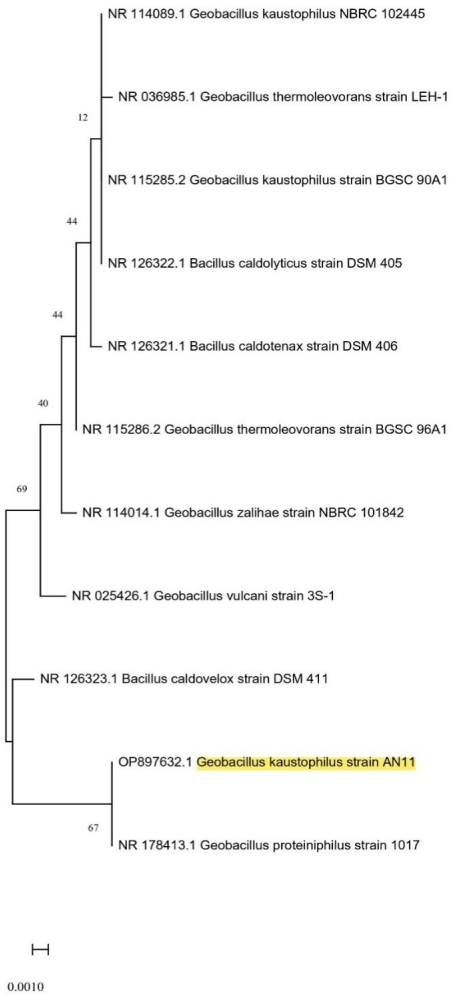

Fig. 1. Phylogenetic analysis with the alignment of the acquired gene sequence for *Geobacillus kaustophilus* ML-1 (GenBank accession number: OP897632).

Amylases, cellulases, chitinases, pectinases, xylanases, proteases, lipases, and DNA polymerases are thermostable enzymes produced by thermophilic microorganisms with an optimal growth temperature of 50°C or higher. In this investigation, a thermophilic isolate was found in the hot springs of Rajwadi Hot Spring, Rajwadi, Maharashtra. With a steadily growing industrial interest in thermostable gene products, the genus *Geobacillus* and its members have emerged as viable candidates for applications in biotechnology and bioremediation.

B. RSM mediated optimization of α-amylase activity

(a) Response surface analysis. The BBD under RSM was used to maximize α-amylase activity after an initial pilot study revealed its effectiveness. The second-order polynomial equation below was utilized in a regression analysis to determine the optimum levels of α-amylase activity for the available experimental conditions:

$$Y\ (\alpha\text{-amylase activity}) = +26.45 + 16.83A + 0.28B - 0.39C - 0.96D + 10.0AB + 3.45AC + 0.43AD - 0.24BC + 0.35BD - 0.16CD + 2.11A^2 - 1.27B^2 + 2.0C^2 - 0.24D^2$$

For verification of the model's accuracy, analysis of variance (ANOVA) was implemented (Table 2). The model was highly significant for α-amylase activity, as indicated by the F-value of 484.53. Additionally, the p-value could be used to examine the importance of each coefficient, as it provides quantitative evidence for the degree of interaction present in each parameter. The

model's p-value in this analysis was < 0.0001, demonstrating its significance. There was a statistically significant relationship between all the factors and α-amylase activity, and the square effects of all the parameters as well as the interaction between pH and temperature (AB), temperature and salt concentration (BC), temperature and nitrogen source (BD), and salt concentration and nitrogen source (CD) (p-values < 0.05). With an F-value of 0.26 indicating that the lack of fit was not statistically significant, and an R^2 value of 0.9979 indicating a good relationship between the predicted (0.9934) and adjusted values (0.9959), it was determined that this response surface design could be used for modeling the design space. Further, a signal-to-noise ratio of 78.12 was found, indicating an appropriate signal and demonstrating that this model could be utilized to navigate the design (Sutar *et al.*, 2019; Patil *et al.,* 2021).

Table 2: ANOVA for the fitted quadratic polynomial model for α-amylase activity.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3534.84	14	252.49	484.53	< 0.0001	**significant**
A-pH	3396.97	1	3396.97	6518.89	< 0.0001	
B-Temperature	0.9804	1	0.9804	1.88	< 0.0001	
C-Salt Concentration	1.84	1	1.84	3.53	< 0.0001	
D-Nitrogen Source (Tryptophan)	11.12	1	11.12	21.33	< 0.0001	
AB	0.0420	1	0.0420	0.0806	< 0.0001	
AC	47.75	1	47.75	91.63	< 0.0001	
AD	0.7482	1	0.7482	1.44	0.2507	
BC	0.2450	1	0.2450	0.4702	0.5041	
BD	0.4970	1	0.4970	0.9538	0.3453	
CD	0.1056	1	0.1056	0.2027	0.6594	
A^2	28.82	1	28.82	55.31	< 0.0001	
B^2	10.48	1	10.48	20.10	0.0005	
C^2	26.02	1	26.02	49.94	< 0.0001	
D^2	0.3920	1	0.3920	0.7523	0.4004	
Residual	7.30	14	7.30			
Lack of Fit	2.85	10	2.85	0.2571	0.9629	not significant
Pure Error	4.44	4	4.44			
Cor Total	3542.13	28	3542.13			

(b) Interplay between variables. Response surfaces (3D surface plots and contour plots) were created to investigate the combined effects of independent variables on α-amylase activity (AB, AC, AD, BD, BC, and CD). The best concentrations for α amylase activity can be quickly determined from the 3D response surface plots, which also show the interactions between the independent variables (Ademakinwa *et al.*, 2019). The perfect interaction between the variables is represented by the occurrence of elliptical contours in the response surface plots shown in Fig. 1 (a-f).

A thorough investigation into the impact of AB, AC, AD, BD, BC, and CD on enzyme activity was carried out by observing the interaction effects of the two variables. Each figure illustrates the result of adjusting one variable while maintaining all others constant. Fig. 2a shows the combined effect of low to high changes in pH and temperature, which leads to an increase in enzyme activity, as expected. As the contour plot is elliptical, the relationship between pH and temperature is highly significant. Temperature and pH levels, in particular, are crucial for bacterial growth. The microbial growth was dramatically influenced by both low pH and high temperature. At a pH of 7 (pH Optima) at 55°C temperature, the enzyme activity was at its highest (temperature optima). Fig. 2b below shows the combined influence of salt concentration (C) and pH (A) on enzyme activity. The response was mostly affected by NaCl, which displayed a quadratic response. Our research shows that our bacteria can only grow at a concentration of 5% before they begin to die. Good enzyme activity was achieved at moderate NaCl and pH values, as indicated by the contour plot's roughly circular shape. The elliptical shape of the contour plot of Fig. 2c reveals the highly significant interactions between pH (A) and tryptophan (D). Tryptophan is an essential amino acid that provides a nitrogen supply for the development of bacteria and other microorganisms. For this experiment, we found that the highest enzyme activity could be achieved by adjusting the nitrogen source. Tryptophan concentrations above 0.6 gm are not optimal for growth. At a tryptophan concentration of 0.4 gm, enzyme activity was at its highest. Whereas lower and higher values for both factors reduced enzyme activity, intermediate values improved it. Fig. 2d shows the interaction between temperature (B) and salt concentration (C), which had a significant effect on enzyme activity when increased or decreased (Fig. 2e). The contour plot is also elliptical in shape. The highly significant and elliptical contour plot of enzyme activity demonstrates the interaction effect of NaCl (C) and tryptophan (D) (Fig. 2f). The enzyme activity was reduced to a lesser extent both by the lowered quantity of NaCl and the increased concentration of tryptophan.

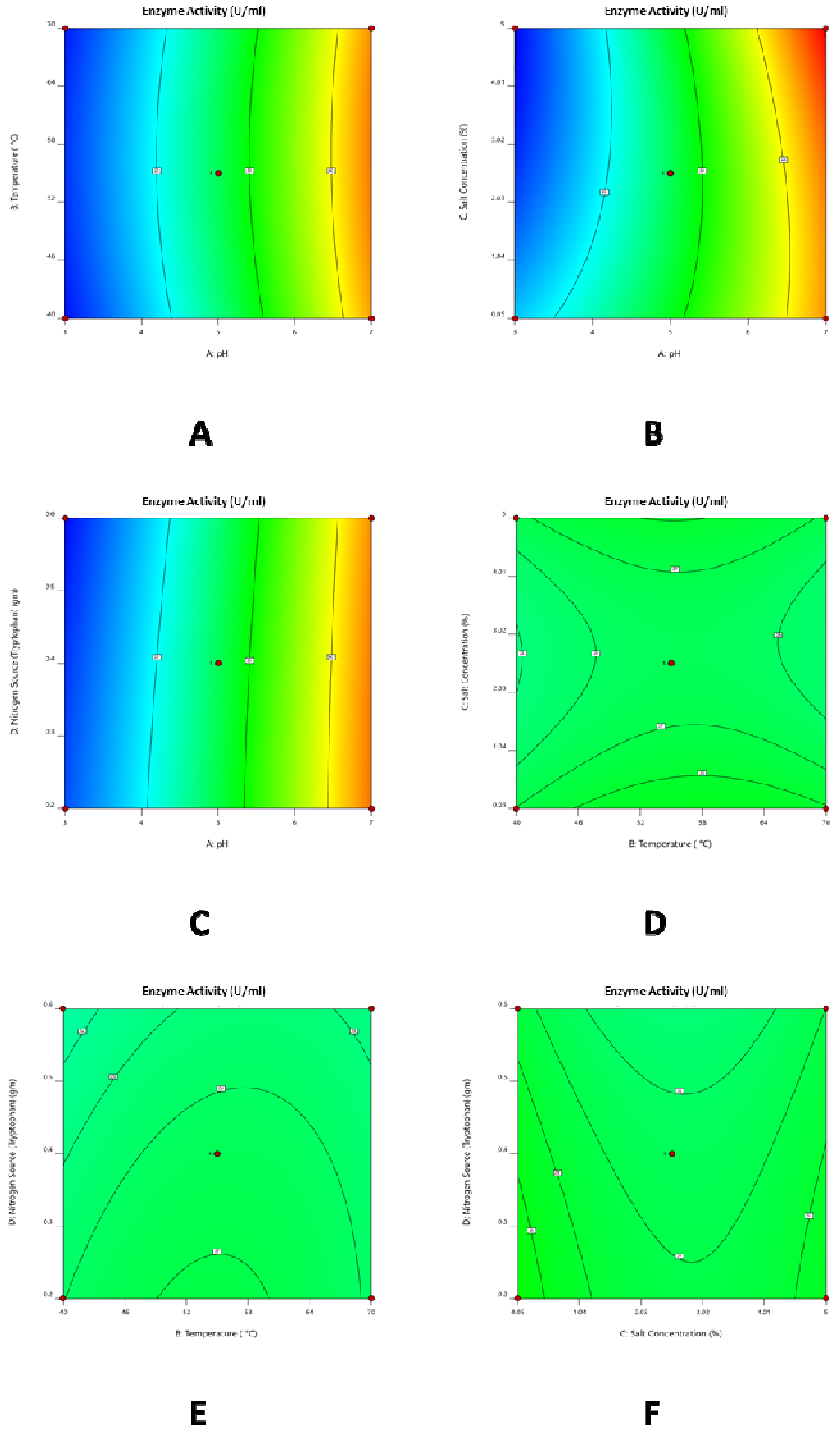

Fig. 2. Contour plots depicting the effect of interactions between: a, pH and temperature; b, pH and salt concentration; c, pH and nitrogen source; d, temperature, and salt concentration; e, temperature and nitrogen source; and f, salt concentration and nitrogen source on α-amylase activity.

B. α-amylase thermostability

The relationship between temperature and enzyme activity was found to be linear (Fincan & Enez 2014). As the temperature went up, the enzyme activity decreased (60-70^{o}C).

The application of thermostable amylolytic enzymes is crucial in current biotechnological applications (Özdemir *et al.*, 2012). They create a wide range of important goods for numerous industries, including maltose, maltodextrins, crystalline dextrose, glucose, and dextrose syrup.

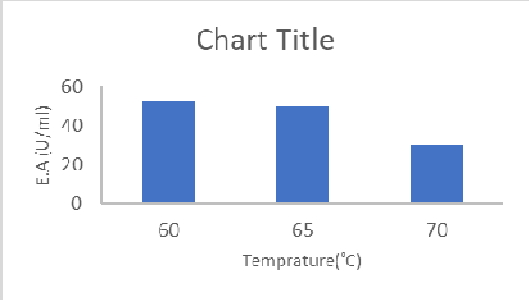

Fig. 3. Thermo-stability of the produced α-amylase enzyme at different temperatures.

CONCLUSIONS AND FUTURE SCOPE

Amylases account for thirty percent of the world's total requirement for enzymes and find applications in the paper, health care, and cleaning product industries, amongst many others. The newly identified bacterial strain *Geobacillus kaustophilus* strain AN11 can be employed to produce amylase because it is a potentially affordable source for the enzyme. Thermophilic amylase can be produced at a relatively low incubation temperature (60°C), and the enzyme was thermostable after being exposed to 70°C for 72 hours. RSM statistical software is quite helpful in scientific investigations comprising the identification of the key factors or the responses of these key factors on the multi-factor dependent variables. Based on the RSM findings, the enzyme activity was at its peak at a pH of 5 and temperature 40°C. In this study, we found that altering the nitrogen source resulted in the highest enzyme activity. Concentrations of tryptophan greater than 0.6 gm are not ideal for growth. Enzyme activity peaked at a concentration of 0.4 gm tryptophan. The optimal salt concentration for enzyme activity was 2.52 gm. As a follow-up investigation to validate the techno-feasibility of its mass commercial production, further method validation for the scaled-up production techniques is preferred.

Conflict of Interest. None.

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How to cite this article: Asama Nadaf and Nasir R. Shaikh (2023). Optimization of α-amylase Production from a Novel Bacterial Strain, *Geobacillus kaustophilus* Strain AN11, Employing Response Surface Methodology. *Biological Forum – An International Journal, 15*(5): 988-993.