ORIGINAL ARTICLE

Optimization of α-Amylase Synthesis by *Bacillus velezensis*: A Comparative Study of Taguchi Experimental Design and Box-Behnken Design for Enhanced Enzyme Production

Sasidhar Bhimana[1], Saravanan Ravindran[2]

[1]Department of Pharmacy, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu, India, [2]Department of Pharmaceutics, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu, India

Abstract

Objective: To improve the production yield of α-amylase from Bacillus velezensis sp. by utilizing the Box-Behnken Design (BBD) methodology. **Methods:** The research process involved a multi-stage approach with different media types selected for specific purposes based on their nutrient composition and the needs of *Bacillus velezensis* sp. at each stage. Initially, the seed media for *Bacillus velezensis* sp. was prepared using Luria Bertani (LB) broth, providing a broad range of nutrients for preliminary growth assessment and enzyme activity baseline. The LB broth, rich in proteins, carbohydrates, vitamins, and minerals, supported rapid bacterial growth and provided initial insights into α-amylase production. The growth medium was formulated using starch, NH_4NO_3, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3$, and $CaCl_2$, with chosen components aimed at optimizing enzyme production. Starch served as a primary carbon source, challenging the bacteria to produce α-amylase to break down complex carbohydrates. NH_4NO_3 supplied essential nitrogen for protein synthesis, while minerals and vitamins (K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3$, $CaCl_2$) supported optimal bacterial growth and enzyme activity. In the submerged fermentation stage, 2% of the seed media was inoculated into a basal medium containing starch to simulate controlled fermentation conditions. This approach minimized variability and focused on optimizing enzyme production. The initial α-amylase activity recorded was 2.78 U/mL, which indicated that process parameters were not yet optimized. Additional enhancement of enzyme activity was accomplished through the Box-Behnken Design (BBD). This method involved varying four significant parameters at two levels (2^4) to optimize the yield of α-amylase. **Results:** A peak of α-amylase concentration of 1092.92 U/mL.under optimized conditions, demonstrating the effectiveness of the staged media approach. The key process parameters influencing enzyme production include pH, temperature, moong husk ($P < 0.0001$). An optimal α-amylase activity of 1092.92 U/mL is achieved. **Conclusion:** The research demonstrates that a multi-stage approach, combined with Box-Behnken Design (BBD), can significantly enhance α-amylase production from *Bacillus velezensis* sp. By utilizing different media types and optimizing key process parameters, the study achieved a notable increase in enzyme yield. The systematic use of LB broth for initial growth, followed by tailored seed media and controlled submerged fermentation, set a solid foundation for optimization. The application of RSM, leveraging the Box-Behnken Design (BBD) model, effectively identified and optimized critical factors, leading to a substantial improvement in α-amylase activity. This optimized approach not only highlights the potential of RSM in enzyme production but also provides a framework for scaling up the process for industrial applications.

Key words: *α-amylase, Bacillus velezensis*, Optimization, Production medium, Submerged fermentation.

Address for correspondence:
Bhimana Sasidhar, Department of Pharmacy, Bharath Institute of Higher Education and Research, #173 Agharam Road, Selaiyur, Chennai-600073, Tamil Nadu, India. Phone: +91-8333017797, +91-8555926391. E-mail: bhimanasasidhar@gmail.com

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INTRODUCTION

Enzymes are indispensable in various industrial processes due to their remarkable attributes of specificity, efficiency, and environmental friendliness. Key sectors such as food, textiles, pharmaceuticals, and biofuels extensively harness the catalytic prowess of enzymes for diverse applications. Among these enzymes, α-amylase holds a prominent position as it catalyzes the hydrolysis of starch into maltose and other oligosaccharides, finding utility in food production, brewing, textiles, and pharmaceuticals.[1] Its unique capacity to modify starch polymers makes it indispensable across industries. Beyond its industrial applications, α-amylase is also gaining importance in the pharmaceutical field, where it is used in the development of digestive aids to improve nutrient absorption, in biodegradable polymers for controlled drug delivery systems, and in tablet formulations to regulate drug release kinetics.[2] In addition, α-amylase, in combination with lipase, is used to treat conditions related to pancreatic dysfunction, such as pancreatic exocrine insufficiency, pancreatitis, and cystic fibrosis, as well as to aid individuals with type I or type II diabetes in carbohydrate and fat digestion.[3]

Conventional optimization approaches typically entail altering one parameter at a time while keeping others constant. However, such methods inadequately account for the intricate interplay among multiple variables, limiting their efficacy in achieving true optimization.[4] In response to this challenge, statistical experimental designs [Figure 1], such as the Taguchi experimental design and Box-Behnken Design (BBD), have emerged as systematic methodologies that efficiently explore parameter spaces using orthogonal arrays and response surface methodology (RSM), respectively. These methods minimize the number of required experiments while capturing the effects of variable interactions, making them highly effective for bioprocess optimization.[5]

The Taguchi experimental design is particularly well-suited for initial screening of multiple parameters, as it allows for the efficient exploration of a wide range of factors with minimal experimental runs.[6] By employing Taguchi's orthogonal arrays, this study systematically investigated 13 significant parameters, including pH, temperature, agitation, inoculum size, aeration, carbon and nitrogen sources, and various nutrients, to identify the most influential factors for α-amylase production. This initial screening phase provided valuable insights into the optimal conditions for enzyme synthesis and laid the groundwork for further refinement.[7] Building on the findings from the Taguchi design, the BBD was employed to refine the optimization process by focusing on the most critical factors identified in the initial screening phase: pH, temperature, carbon source (moong husk), and nitrogen source (soybean cake).[8] The BBD approach, using

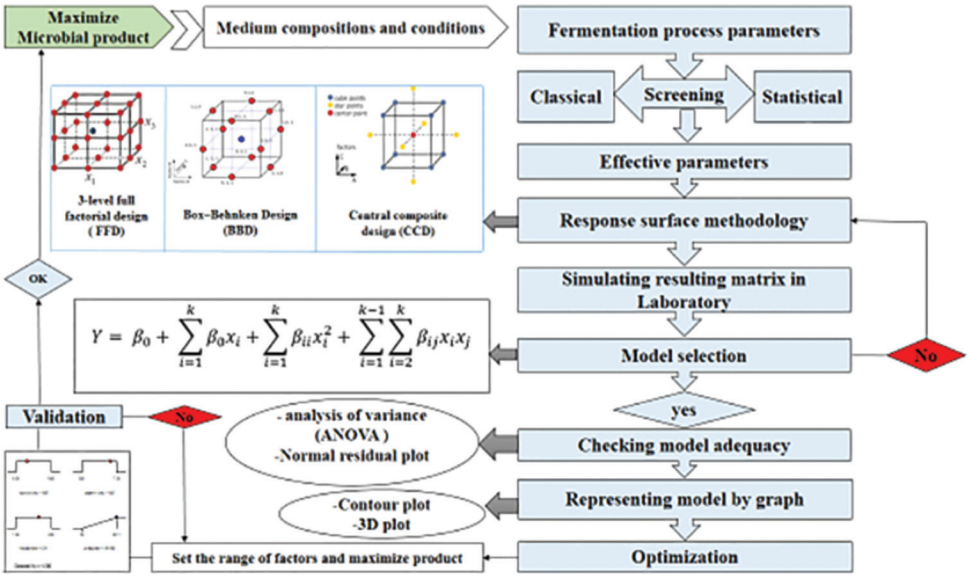

Figure 1: Description of optimization stages for maximizing microbial products

a 2^4-factorial design, allowed for a detailed exploration of the interactions between these factors and their optimal levels.[9] This refinement phase not only enhanced the precision of the optimization process but also provided a deeper understanding of the complex relationships between the key parameters influencing α-amylase production.[10]

The integration of Taguchi and BBDs into a single, unified optimization methodology offers a comprehensive framework for maximizing α-amylase production. This approach leverages the strengths of both methods: the Taguchi design efficiently screens a wide range of parameters, whereas the BBD refines the optimization by focusing on the most critical factors and their interactions. By combining these two phases, this study achieves a robust and systematic optimization process that significantly enhances enzyme yield.[6]

In addition to methodological innovation, this study also emphasizes the importance of utilizing agro-industrial waste as a sustainable and cost-effective substrate for enzyme production.[5] Agro-wastes, such as moong husk and soybean cake, are abundant in carbon and nitrogen, making them ideal substrates for microbial fermentation. These materials, often discarded as by-products of agricultural and food processing industries, can be repurposed to produce valuable enzymes, thereby minimizing waste and maximizing resource utilization.[7] The use of agro-waste not only reduces production costs but also aligns with the principles of circular economy and sustainable development.[4]

The principal aim of this study is to optimize α-amylase synthesis through *Bacillus velezensis* using a two-phase optimization approach that combines the Taguchi design for initial screening and the BBD for refined optimization. This integrated methodology seeks to ascertain the optimal conditions for maximizing α-amylase activity through a meticulous exploration of various parameters and their interactions. The findings are poised to provide invaluable insights into the influence of multiple parameters on enzyme production, thus furnishing a robust framework for optimizing other bioprocesses. Furthermore, this research highlights the potential of statistical optimization in enhancing enzyme yield and offers a scalable approach for industrial applications.[1]

By focusing on the optimization of α-amylase production using *B. velezensis*, this study contributes to the growing body of knowledge in enzyme bioprocess optimization. The results and conclusions of this research will not only advance our understanding of the factors influencing enzyme production but also provide practical guidelines for scaling up the process for industrial applications. The integration of Taguchi and BBDs into a single, unified methodology represents a significant step forward in the field of bioprocess optimization, offering a powerful tool for researchers and industry professionals alike.[9]

MATERIALS AND METHODS

Chemicals and microorganisms

All chemicals [Table 1] used in this study were purchased from HiMedia Laboratories Private Limited, a leading manufacturer and supplier of laboratory chemicals, media, and other scientific products. The *B. velezensis* strains (MTCC13097, MTCC13098, MTCC13099, MTCC13100, and MTCC13101) were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India, a recognized global repository of authenticated microbial cultures.[11] The five *B. velezensis* strains were screened for their natural amylase-producing ability under identical fermentation conditions in Erlenmeyer flasks containing 100 mL of production medium. After 72 h of incubation at 35°C and 125 rpm, the cultures were harvested, and amylase activity was qualitatively assessed using an iodine-based starch hydrolysis assay [Figure 2]. Larger zones of starch clearance indicated higher amylase activity, and strain MTCC13097 demonstrated the largest zone of hydrolysis, making it the selected strain for further experiments.[12] A single colony of the selected strain, MTCC13097, was purified by isolating it from the stock culture and repeatedly subculturing it on nutrient agar plates. The strain was maintained as slant cultures at 4°C to ensure genetic and phenotypic stability throughout the study.[13] Biochemical tests, including gram

Table 1: Materials

Media	Composition	%
Luria-Bertani starch medium:	Tryptone	1.0
	Yeast extract	0.5
	Sodium chloride	1.0
	Starch	0.25
Basal medium:	Starch	0.5
	Peptone	2.0
	$MgSO_4$	0.1
	K_2HPO_4	0.3
Seed medium:	Starch	2.0
	NH_4NO_3	0.7
	K_2HPO_4	0.1
	$MgSO_4.7H_2O$	0.01
	$FeCl_3$	0.005
	$CaCl_2$	0.002
Production medium:	Moong husk	3-5
	Fructose (Fru)	1.5
	Soybean cake (Soy)	1-3
	$NaNO_3$	0.5
	KH_2PO_4	0.3
	$MgSO_4.7H_2O$	0.1
	NaCl	0.2

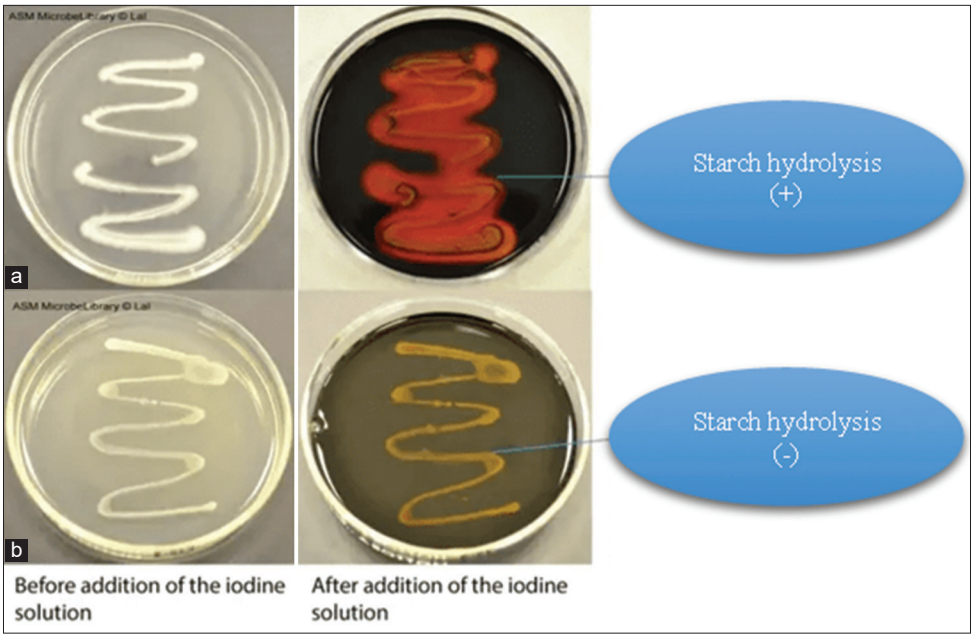

Figure 2: (a and b) α-amylase activity (+) of *Bacillus velezengis* spp. on starch agar plate: α-amylase activity (–) negative control

staining and catalase activity, were performed to confirm the strain's identity and its high amylase-producing capacity.[14]

Submerged fermentation

The cultivation process involved the optimization of both seed and production media to promote the growth of *B. velezensis* MTCC13097 and maximize α-amylase production.[15]

Seed media optimization

The seed medium was optimized to ensure a high density of viable cells for subsequent fermentation. Six seed media formulations (SM1-SM6) with varying carbon and nitrogen sources were evaluated [Table 1]. *B. velezensis* MTCC13097 was inoculated into each medium and incubated at 35°C with agitation at 150 rpm for 72 h.[16] Optical density at 600 nm (OD600) and α-amylase activity were measured periodically. Analysis of variance (ANOVA) was used to determine which formulations resulted in statistically significant ($P < 0.05$) differences in growth and enzyme production. The optimized seed medium, which supported the highest growth and enzyme activity, was selected for further experiments.[17]

Production media optimization

The basal production medium was designed to provide a foundation for submerged fermentation. Its composition was carefully selected to maintain bacterial growth and enable enzyme synthesis. The optimized seed medium was used to inoculate the basal production medium, which contained 0.5% starch as the carbon source and 2% peptone, 0.1% $MgSO_4$, and 0.3% K_2HPO_4 as nitrogen sources.[16] The production cultures were incubated under the same conditions as the seed medium (35°C, 150 rpm), and α-amylase activity was measured periodically up to 120 h using the 3,5-dinitro salicylic acid (DNSA) method. ANOVA was used to determine if the activity was significantly different ($P < 0.05$) between the optimized seed medium and a control lacking carbon/nitrogen sources.[18]

Optimization of α-amylase production using Taguchi and BBDs

To further enhance enzyme production, a two-phase optimization approach was employed, combining the Taguchi experimental design for initial screening and the BBD for refined optimization.[19] This integrated methodology allowed for a systematic exploration of multiple parameters and their interactions, ensuring efficient and effective optimization of α-amylase production.[15]

Phase 1: Taguchi experimental design for initial screening

The Taguchi experimental design was utilized to screen and evaluate 13 significant parameters influencing α-amylase production, including pH, temperature, agitation, inoculum size, aeration, carbon source, nitrogen source, and various nutrients.[17] This design employed an orthogonal array L27 (3^{13}), which enabled the efficient investigation of multiple factors with a minimal number of experiments. The Taguchi design is particularly advantageous for identifying the most influential factors while minimizing experimental effort.[18]

Signal-to-noise (S/N) ratios were calculated for each factor level combination to assess the impact of different parameters on α-amylase activity.[19] The larger-is-better principle was applied to maximize enzyme production, whereas the smaller-is-preferable principle was used for factors where lower values were desirable. The experimental data were analyzed using ANOVA to determine the significance of each factor and their interactions.[20] Design-Expert software facilitated the statistical analysis and model validation.[21]

The Taguchi design identified the optimal conditions for α-amylase production as pH 5, temperature 34°C, 4% moong husk as the carbon source, and 2% soybean cake as the nitrogen source.[22] These conditions were determined to be the most influential factors, forming a strong foundation for further refinement in the second phase.[2]

Phase 2: BBD for refined optimization

Building on the findings from the Taguchi design, the BBD was employed to refine the optimization process by focusing on the four most critical factors identified in the initial screening phase: pH, temperature, carbon source (moong husk), and nitrogen source (soybean cake).[23] The BBD approach, using a 2^4-factorial design, allowed for a detailed exploration of the interactions between these factors and their optimal levels.[24]

The experimental design [Table 2] and execution involved testing each factor at two levels: low (−1) and high (+1). The pH was varied between 4.0 and 6.0, whereas the temperature was set at either 34°C or 36°C. The carbon source, moong husk, was tested at concentrations of 4% and 6%, whereas the nitrogen source, soybean cake, was evaluated at levels of 1% and 3%.[25] To support bacterial growth and enzyme production, the production medium was supplemented with 0.125% $MgSO_4$, 0.2% NaCl, 0.4% K_2HPO_4, 1.5% fructose, and 0.5% $NaNO_3$.[26] The cultures were incubated for 70 h at 125 rpm with an aeration rate of 2.5 LPM, ensuring optimal conditions for α-amylase production.[27]

Data analysis and model development

The dependent variable (Y) measured in each experimental run was the average α-amylase activity, expressed in units per milliliter (U/mL). The experimental data were analyzed using multiple regression, applying a second-order polynomial equation to the results:

$$Y = \beta o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 + \beta_4\beta_4 D^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_1\beta_4 AD + \beta_2\beta_3 BC + \beta_2\beta_4 BD + \beta_3\beta_4 CD \quad (1)$$

Here, Y represents the estimated α-amylase yield in U/mL; A, B, C, and D denote the coded independent variables; β_1, β_2, β_3, and β_4 indicate the linear coefficients; β_0 is the intercept; β_1^2, β_2^2, β_3^2, and β_4^2 are the quadratic coefficients; and $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_2\beta_3$, $\beta_2\beta_4$, and $\beta_3\beta_4$ represent the interaction coefficients.[19]

Table 2: Variables selected for two-level factorial design

Factor	Name	Units	Coded low	Coded high
A	pH		−1↔4.00	+1↔6.00
B	Temp	°C	−1↔32.00	+1↔36.00
C	Carbon	%	−1↔3.00	+1↔5.00
D	Nitrogen	%	−1↔1.00	+1↔3.00

Design-Expert software (version 13) was used to create a trial of 29 experimental runs and to analyze the data. The model's predictive capability was validated by comparing the predicted and observed α-amylase activity values, demonstrating a strong correlation ($R^2 = 0.9999$).

Statistical validation and model accuracy

The ANOVA results indicated that the model was highly significant ($P < 0.0001$), with a high F-value, confirming that the observed variations in α-amylase activity were not due to random chance. The lack of fit was non-significant, indicating that the model effectively captured the relationship between the input variables and α-amylase production. The S/N ratio exceeded 4.0, demonstrating adequate model accuracy and reliability.

Enzyme assay

The activity of α-amylase was quantified using the DNSA method, a colorimetric assay that measures the enzymatic hydrolysis of starch and the subsequent release of reducing sugars. The assay was performed by combining 1 mL of a 1% starch solution with 0.05 mL of the enzyme-rich supernatant at pH 5.5 and 55°C for 8 min. After incubation, 0.5 mL of DNSA reagent was added to the mixture, which was then heated in a boiling water bath for 10 min to promote color development. The reaction mixture was cooled, and 3.45 mL of distilled water was added for dilution before measuring the absorbance at 540 nm using a spectrophotometer. The absorbance was related to the amount of reducing sugars generated from starch hydrolysis by α-amylase, following Miller's method. A blank sample, prepared without the enzyme-containing supernatant, was used as a control to measure any background absorbance or reducing sugars in the reaction mixture. One unit of α-amylase activity was defined as the amount of enzyme that generates 1 micromole of reducing sugar per minute under the specified assay conditions.

The protein concentration in the enzyme supernatant was determined using the Lowry method, a common technique for measuring protein in biological samples. This provided valuable information about the enzyme concentration in the crude extract and allowed for the calculation of specific enzyme activity.

RESULTS

Initial screening and optimization using Taguchi experimental design

The Taguchi experimental design was adeptly applied to optimize the cultivation conditions for α-amylase production by *B. velezensis* MTCC13097. In the initial phase, the organism was cultured in Luria-Bertani broth, where it entered the log phase after 8 h, reaching maximum growth (OD600 of 1.2) at 24 h. Amylase activity peaked at 72 h

with an enzyme activity of 2.8 U/mL, corresponding to the transition from the log to the stationary phase.[28] One-way ANOVA revealed a significant effect of incubation time on amylase activity, biomass, and total protein concentration ($P < 0.05$), with maximum values observed at 72 h.[29] To further optimize the culture conditions, six modified seed media formulations (SM1-SM6) were screened by varying the carbon source (1–3% starch) and nitrogen source (0.5–1.5% peptone). Among these, SM5 and SM6 supported the highest growth (OD600 of 5–7 at 24 h) and amylase activities (15–17.1 U/mL at 72 h).[30] One-way ANOVA and Tukey's HSD test showed that SM6 yielded significantly higher amylase activity than other media ($P < 0.05$), leading to its selection for subsequent experiments.[31]

The optimized SM6 seed medium was used to inoculate the basal production medium, resulting in amylase activity peaking at 11 U/mL at 72 h, representing a 1.5-fold increase over the control.[32] Two-way ANOVA revealed a significant effect of seed medium and incubation time on amylase production. *Post hoc* analysis showed that SM6 significantly increased amylase levels between 72 and 120 h compared to the control ($P = 0.043$), validating its effectiveness for transferring high enzyme-producing cells to production-scale fermentations.[33]

Taguchi experimental design's response analysis

A Taguchi L27 orthogonal array design was employed to evaluate the effects of 13 process parameters on α-amylase activity and total protein concentration. Twenty-seven fermentation runs were conducted according to the design, and the responses were measured for each run. The mean enzyme activity across all 27 runs was 1015.6 U/mL with a standard deviation of 46.1 U/mL, while the mean total protein concentration was 1135.2 mg with a standard deviation of 51.4 mg. These results indicate acceptable reproducibility in the fermentation responses. A strong correlation was observed between the predicted and observed values for each response, with a correlation coefficient (R) of 0.99 for enzyme activity and 0.98 for total protein. This confirms that the Taguchi design effectively captured the key factor interactions governing response behavior. Linear regression analysis yielded a very high R^2 value of 0.9856 for enzymatic activity and 0.9765 for total protein, demonstrating a near-perfect correlation between predicted and observed values. The regression lines closely followed the line of equivalence, with data points lying very close to this line, indicating that the Taguchi design accurately modeled and predicted the enzymatic activity and total protein outcomes.

ANOVA, factor effects, and interactions

One-way ANOVA identified all factors except Residual as highly statistically significant ($P < 0.0001$) for both responses. This indicates over 99% confidence that each factor influenced α-amylase activity and protein production. Factors A (pH), B (Temperature), F (Carbon source), and G (Nitrogen source) exhibited the lowest *P*-values (<0.0001), suggesting the strongest effects. These factors accounted for over 65% of the activity variation and more than 70% of the protein yield changes.

Main effects Pareto charts further validated the significance of these factors. For α-amylase production, pH, temperature, carbon source, and nitrogen source were the most influential, while inoculum size, aeration, and $MgSO_4$ had moderate effects. Similarly, for total protein concentration, pH, temperature, carbon source, and nitrogen source dominated, with inoculum size, aeration, $MgSO_4$, and $NaNO_3$ also showing moderate impacts.

The optimal settings identified through the Taguchi design were pH 5 (level 2), temperature 34°C (level 2), 4% moong husk as the carbon source (level 3), and 2% soybean cake as the nitrogen source (level 2). These conditions were predicted to yield the highest α-amylase activity of 1097 U/mL and a total protein concentration of 1230 mg. The desirability value of 0.997 indicated an almost ideal fit between the predicted and achievable optima, demonstrating the effectiveness of the optimization approach.

Refinement of parameters using BBD

Building on the findings from the Taguchi design, the BBD was employed to refine the optimization process by focusing on the four most critical factors: pH, temperature, carbon source (moong husk), and nitrogen source (soybean cake). The BBD approach, using a 2^4-factorial design, allowed for a detailed exploration of the interactions between these factors and their optimal levels.

The BBD experiments were conducted by varying four key factors at two levels: pH (4.0 and 6.0), temperature (34°C and 36°C), carbon source in the form of moong husk (4% and 6%), and nitrogen source in the form of soybean cake (1% and 3%). The results of these experiments, presented in Table 3, illustrate the observed and predicted α-amylase activities for each experimental run. A second-order polynomial equation was derived from the experimental data to model the relationship between these factors and α-amylase production.

$$Y = 151.46 + 1.15A + 0.2933B + 0.9783C + 1.48D - 1.67A2 - 0.04667B2 - 5.49C2 - 24.29D2 + 0.0000AB + 0.000AC + 0.000AD - 0.0650BC - 0.0650BD + 0.000CD$$

Where:

- Y represents the estimated α-amylase yield in U/mL
- A, B, C, and D denote the coded independent variables (pH, temperature, carbon source, and nitrogen source, respectively)

ANOVA

The ANOVA results [Table 4] indicated that the model was highly significant ($P < 0.0001$), with a high F-value,

Table 3: BBD with 29 experimental and predicted values of independent variables

Run	Factor 1 A: pH	Factor 2 B: Temperature	Factor 3 C: (Carbon) Moong husk	Factor 4 D: (Nitrogen) Soy cake	Observed α-amylase activity	Predicted α-amylase activity
		°C	%	%	U/mL	U/mL
1	4.0	34.0	4.0	3.0	125.86	125.83
2	5.0	32.0	4.0	3.0	127.9	127.95
3	5.0	34.0	4.0	2.0	151.46	151.46
4	6.0	34.0	4.0	1.0	125.16	125.17
5	6.0	34.0	4.0	3.0	128.16	128.13
6	4.0	34.0	4.0	1.0	122.86	122.87
7	4.0	34.0	3.0	2.0	142.16	142.17
8	4.0	36.0	4.0	2.0	148.4	148.47
9	5.0	34.0	5.0	3.0	124.16	124.14
10	5.0	34.0	4.0	2.0	151.46	151.46
11	4.0	32.0	4.0	2.0	147.9	147.88
12	5.0	34.0	3.0	1.0	119.16	119.23
13	6.0	36.0	4.0	2.0	150.7	150.77
14	5.0	34.0	5.0	1.0	121.16	121.18
15	5.0	32.0	3.0	2.0	144.2	144.17
16	6.0	34.0	3.0	2.0	144.46	144.47
17	5.0	34.0	4.0	2.0	151.46	151.46
18	4.0	34.0	5.0	2.0	144.16	144.13
19	5.0	34.0	3.0	3.0	122.16	122.18
20	5.0	36.0	4.0	3.0	128.4	128.41
21	6.0	32.0	4.0	2.0	150.2	150.18
22	5.0	34.0	4.0	2.0	151.46	151.46
23	5.0	32.0	4.0	1.0	124.9	124.87
24	5.0	34.0	4.0	2.0	151.46	151.46
25	5.0	32.0	5.0	2.0	146.2	146.25
26	6.0	34.0	5.0	2.0	146.46	146.43
27	5.0	36.0	5.0	2.0	146.7	146.71
28	5.0	36.0	4.0	1.0	125.66	125.58
29	5.0	36.0	3.0	2.0	144.96	144.88

confirming that the observed variations in α-amylase activity were not due to random chance. The lack of fit was non-significant, indicating that the model effectively captured the relationship between the input variables and α-amylase production. The S/N ratio of 844.527 demonstrated adequate model accuracy and reliability.

Optimal conditions

Under the refined conditions of pH 5, temperature 34°C, 4% moong husk, and 2% soybean cake, a peak α-amylase concentration of 1092.92 U/mL was achieved. This represented a significant improvement over the initial unoptimized conditions and validated the effectiveness of the two-phase optimization approach.

Interaction effects and model validation

Interaction plots

The interaction effects between the most significant factors were analyzed using 3D surface graphs and 2D contour plots [Figure 3], providing valuable insights into the relationships between key parameters influencing α-amylase production. The analysis revealed that the optimal combination for maximum enzyme production was achieved at pH 5 with a temperature of 34°C, whereas increasing the temperature beyond this point led to a decline in enzyme activity. Similarly, at pH 5, a carbon source concentration of 4% moong husk yielded the highest enzyme production, with both lower and higher levels resulting in reduced responses.

Table 4: ANOVA for α-amylase production from *Bacillus velegensis* spp.

Source	Sum of squares	df	Mean square	F-value	*P*-value	
Model	4055.96	14	289.71	1.029E+05	<0.0001*	Significant
A-pH	15.87	1	15.87	5634.32	<0.0001*	
B-Temp	1.03	1	1.03	366.58	<0.0001*	
C-Carbon	11.49	1	11.49	4077.74	<0.0001*	
D-Nitrogen	26.23	1	26.23	9310.88	<0.0001*	
AB	0.0000	1	0.0000	0.0000	1.0000	
AC	0.0000	1	0.0000	0.0000	1.0000	
AD	0.0000	1	0.0000	0.0000	1.0000	
BC	0.0169	1	0.0169	6.00	0.0281	
BD	0.0169	1	0.0169	6.00	0.0281	
CD	0.0000	1	0.0000	0.0000	1.0000	
A^2	18.13	1	18.13	6435.37	<0.0001*	
B^2	1.41	1	1.41	501.52	<0.0001*	
C^2	195.44	1	195.44	69388.40	<0.0001*	
D^2	3826.79	1	3826.79	1.359E+06	<0.0001*	
Residual	0.0394	14	0.0028			
Lack of Fit	0.0394	10	0.0039			Not significant
Pure Error	0.0000	4	0.0000			
Cor Total	4055.99	28				

*P<0.0001, ANOVA: Analysis of variance

The interaction between temperature and carbon source showed that a temperature of 34°C, in combination with 4% moong husk, was most favorable for α-amylase synthesis. Furthermore, the best temperature and nitrogen source combination for maximal enzyme activity was 34°C with 2% soybean cake. The interaction between carbon and nitrogen sources indicated that the highest enzyme yield was obtained with 4% moong husk and 2% soybean cake. In addition, the optimal pH and nitrogen source pairing for maximum enzyme response was determined to be pH 5 with 2% soybean cake. These findings underscore the significance of optimizing multiple interacting factors to enhance α-amylase production efficiently.

Model validation

Confirmation runs under the optimized conditions produced responses within 5% of the predicted values, validating the optimization methodology. The low standard deviations of 0.03–0.04 U/mL for enzyme activity and 0.05–0.06 mg for total protein demonstrated the robustness and reproducibility of the optimized process.

Comparative analysis and final optimization

The Taguchi design provided a broad exploration of multiple parameters, identifying the most influential factors for α-amylase production. The BBD refined the optimization process by focusing on the interactions between these critical factors, leading to a peak α-amylase concentration of 1092.92 U/mL. This represented a 393-fold increase compared to the unoptimized medium, demonstrating the effectiveness of the two-phase optimization approach.

The desirability value of 0.997 indicated an almost ideal fit between predicted and achievable optima, and the high R^2 value of 0.9999 confirmed the model's predictive capability. The optimized conditions of pH 5, temperature 34°C, 4% moong husk, and 2% soybean cake were found to be the most effective for maximizing α-amylase production.

DISCUSSION

This study systematically optimized the culture conditions for α-amylase production by *B. velezensis* MTCC13097 using a two-phase statistical experimental design approach, combining the Taguchi experimental design for initial screening and the BBD for refined optimization. The integration of these methodologies provided a comprehensive framework for maximizing enzyme yield, offering valuable insights into the key growth parameters and their optimal levels for industrial-scale applications.

Optimization of culture conditions using Taguchi design

The Taguchi experimental design was employed to evaluate the effects of 13 process parameters on α-amylase production and

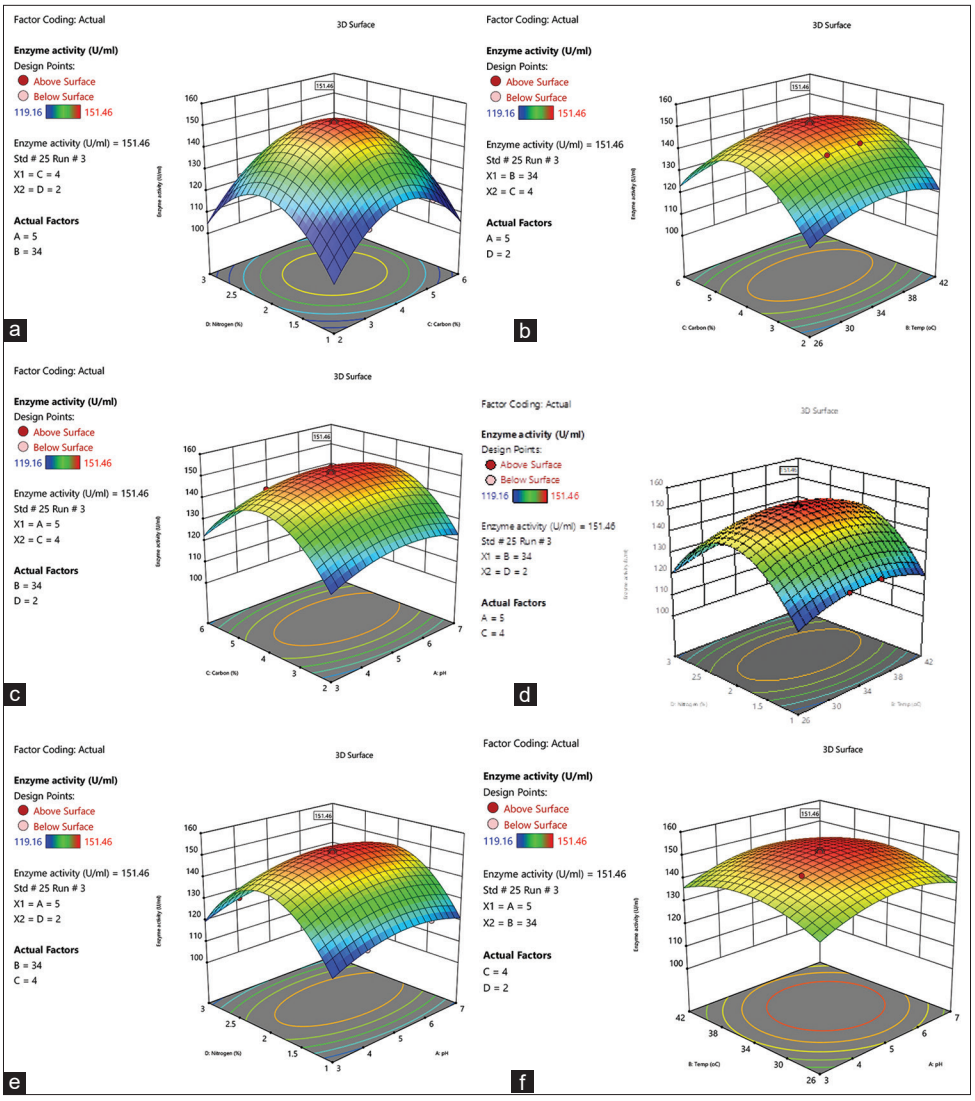

Figure 3: 3D and 2D surface with contour plots; (a) revealed non-significant interaction between carbon (moong husk) and nitrogen (soya bean cake), (b) illustrated significant interaction of carbon (moong husk) and temperature, (c) indicated significant interaction of carbon (moong husk) and pH, (d) indicated a significant interaction of nitrogen (soya bean cake) and temperature, (e) indicated a significant of nitrogen (soya bean cake) and pH. (f) indicated a non-significant temperature and pH

total protein concentration. The results demonstrated that pH, temperature, carbon source (moong husk), and nitrogen source (soybean cake) were the most influential factors, accounting for over 65% of the activity variation and more than 70% of the protein yield changes. The optimal conditions identified through the Taguchi design were pH 5, temperature 34°C, 4% moong husk, and 2% soybean cake, which were predicted to yield a maximum α-amylase activity of 1097 U/mL and a total protein concentration of 1230 mg/mL. The S/N ratios and ANOVA confirmed the statistical significance of these factors, with $P < 0.0001$, indicating over 99% confidence in their impact on enzyme production. The desirability value of 0.997 further validated the model's accuracy, demonstrating an almost ideal fit between predicted and achievable optima. The low standard deviations observed in confirmation runs (0.03–0.04 U/ mL for enzyme activity and 0.05–0.06 mg for total protein) highlighted the robustness and reproducibility of the optimized process.

Refinement of parameters using BBD

Building on the findings from the Taguchi design, the BBD was employed to refine the optimization process by focusing on the interactions between the four most critical factors: pH, temperature, carbon source (moong husk), and nitrogen source (soybean cake). The BBD approach, using a 2^4-factorial design, allowed for a detailed exploration of the interactions between these factors and their optimal levels. The second-order polynomial equation derived from the experimental data effectively captured the relationship between the input variables and α-amylase activity, with a high R^2 value of 0.9999 and a S/N ratio of 844.527, indicating a robust and reliable model. The optimal conditions identified through the BBD were consistent with those from the Taguchi design: pH 5, temperature 34°C, 4% moong husk, and 2% soybean cake, resulting in a peak α-amylase concentration of 1092.92 U/mL. This represented a 393-fold increase

compared to the unoptimized medium, demonstrating the effectiveness of the two-phase optimization approach.

Interaction effects and model validation

The interaction effects between the most significant factors were analyzed using 3D surface graphs and 2D contour plots, providing a comprehensive understanding of the relationships between key variables influencing α-amylase production. The analysis revealed that the optimal combination for maximum enzyme production was pH 5 with a temperature of 34°C, as increasing the temperature beyond this point led to a decline in enzyme activity. At pH 5, a carbon source concentration of 4% moong husk yielded the highest enzyme production, with both lower and higher levels resulting in reduced responses. Similarly, the interaction between temperature and carbon source demonstrated that 34°C in combination with 4% moong husk was the most favorable condition for α-amylase synthesis. Furthermore, the best combination of temperature and nitrogen source for maximal enzyme production was determined to be 34°C with 2% soybean cake. The interaction between carbon and nitrogen sources indicated that the highest enzyme yield was achieved with 4% moong husk and 2% soybean cake, whereas the optimal pH and nitrogen source pairing for maximum enzyme response was pH 5 with 2% soybean cake. Confirmation runs conducted under these optimized conditions produced responses within 5% of the predicted values, validating the reliability of the optimization methodology. In addition, the low standard deviations observed in these runs further reinforced the robustness and reproducibility of the optimized process, confirming its effectiveness for large-scale applications.

Comparative analysis and broader implications

The Taguchi design provided a broad exploration of multiple parameters, identifying the most influential factors for α-amylase production. The BBD refined the optimization process by focusing on the interactions between these critical factors, leading to a peak α-amylase concentration of 1092.92 U/mL. This represented a 393-fold increase compared to the unoptimized medium, demonstrating the effectiveness of the two-phase optimization approach. The use of agricultural by-products, such as moong husk and soybean cake, as carbon and nitrogen sources not only lowered the cost of enzyme production but also promoted sustainability by repurposing waste. This approach aligns with the principles of circular economy and sustainable development, offering a cost-effective and eco-friendly alternative to synthetic medium ingredients. The findings of this study are consistent with previous research on the optimization of fermentation processes using RSM and the design of experiments (DoE). For example, similar approaches have been successfully applied to optimize the production of bioethanol from *Kappaphycus alvarezii* waste and biodegradable potted seedling trays from rice straw and cow manure. The integration of RSM with DoE methodologies, such as the BBD, has been shown to enhance the accuracy and efficiency of experimental processes, as evidenced by the optimization of biodiesel production conditions using hydrodynamic cavitation.

CONCLUSION

This comprehensive study makes a substantial contribution to the field of enzyme synthesis optimization. The two-phase optimization approach, combining the Taguchi and BBDs, successfully identified the optimal conditions for maximizing α-amylase production by *B. velezensis* MTCC13097. The identified optimal conditions and significant factors offer a pragmatic route to augment α-amylase production, with potential applications in various industries, including food, textiles, pharmaceuticals, and biofuels. The high R^2 value (0.9999) and desirability value (0.997) of the model confirm its predictive capability and reliability, providing a robust framework for scaling up the process for industrial applications. The use of agro-industrial waste as substrates not only reduces production costs but also aligns with the principles of sustainable development, making this approach economically and environmentally viable. As the landscape of enzyme synthesis continues to evolve, the findings presented in this research enrich the collective knowledge base and pave the way for further discoveries and advancements in the realm of biotechnology. Future studies could explore the application of this optimization approach to other microbial strains and enzymes, as well as the integration of advanced bioprocessing techniques to further enhance enzyme yield and production efficiency.

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