Original Article

Comparative Optimization of α-Amylase Production in *Bacillus velezensis* sp. Using Response Surface Methodology and Artificial Neural Networks

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ABSTRACT

Aim: This study aims to optimize the production of *α-amylase* from *Bacillus velezensis* sp. through a comparative analysis of advanced experimental methodologies, specifically Definitive Screening Design-Response Surface Methodology (DSD-RSM) and Artificial Neural Networks (ANN). The research emphasizes the use of environmentally sustainable and cost-effective agro-solid substrates, namely moong husk and soybean cake, to enhance enzyme yield, addressing the growing industrial demand for efficient enzyme production. **Materials and Methods:** The optimization process began with the identification of nine critical operational variables influencing *α-amylase* biosynthesis. Initial experiments utilized the One-Factor-at-a-Time (OFAT) approach, providing preliminary insights into the effects of individual variables. Subsequently, a second-order Definitive Screening Design (DSD) was employed within the RSM framework to systematically evaluate the interactions among the variables. The nine parameters analyzed included pH (5.0-7.0), temperature (30ºC-40ºC), carbon source concentration (moong husk at 2% to 6%), nitrogen source concentration (soybean cake at 1% to 3%), K_2PO_4 (0.1%-0.5%), $MgSO_4$ (0.05%-0.2%), NaCl (0.1%-0.5%), fructose (0.5%-2%) and $NaNO_3$ (0.1% to 0.5%). Following the DSD-RSM analysis, ANN modeling was utilized to further refine the optimization process, allowing for the prediction of enzyme yield based on the identified parameters. Confirmation experiments were conducted under the optimal conditions established by both DSD-RSM and ANN to validate the predicted enzyme activity. **Results:** The optimized conditions resulted in a significant *α-amylase* activity of 1092.49 IU/mL, achieved under the following optimal parameters: pH 5.48, temperature 34.27ºC, carbon source concentration of 4.09%, nitrogen source concentration of 2.02%, K_2PO_4 at 0.34%, $MgSO_4$ at 0.14%, NaCl at 0.23%, fructose at 1.54% and $NaNO_3$ at 0.53%. This marked a substantial increase in enzyme activity, representing a 2.6-fold enhancement compared to the initial yield of 418.25 U/mL obtained through the OFAT method. Statistical analysis confirmed the robustness of the optimization models, with R^2=0.999, value indicating a strong correlation between predicted and actual enzyme activities. **Conclusion:** The findings of this research demonstrate that the integration of DSD-RSM and ANN methodologies significantly enhances the production of *α-amylase* from *Bacillus velezensis* sp. compared to traditional optimization techniques. This study not only highlights the effectiveness of modern statistical and computational approaches in bioprocess optimization but also contributes to the development of sustainable enzyme production strategies that can meet the increasing industrial demands.

Keywords: α-amylase, Artificial Neural Networks, *Bacillus velezensis* sp., Bioprocess Optimization, Definitive Screening Design, Response Surface Methodology.

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INTRODUCTION

α-amylase is a starch-degrading enzyme used in a variety of industries, including laundry, textiles, chocolate, detergent and biofuel production. It is becoming increasingly important in the pharmaceutical industry, where it is used to make digestive aids, biodegradable polymers to regulate drug release rate and cross-linked starches in tablets to govern drug release kinetics. Recent statistics highlight a significant rise in global enzyme market demand, projected to reach USD 14.7 billion by 2025, driven by the growing utilization of enzymes in industrial processes.[1] Despite advancements in enzyme technology, challenges such as low yield, high production costs and suboptimal enzyme activity persist, necessitating innovative solutions.

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The challenges in current statistical methods such as the Plackett-Burman Design (PBD), Response Surface Methodology (RSM) and One-Factor-at-a-Time (OFAT) are significant, particularly in complex biological systems like *α-amylase* production. PBD is limited to main effects and often misses interactions and nonlinear relationships, potentially overlooking important synergies between factors.[2] RSM, while useful, typically assumes quadratic relationships which can be overly simplistic for complex systems, requires a high number of experiments and often provides local rather than global optimization, leading to suboptimal solutions in multiparametric non-linear systems.[3] OFAT is highly inefficient, requiring numerous experiments to explore even a small parameter space and neglects interactions among factors, potentially missing optimal conditions.[4] In contrast, modern methods like Definitive Screening Design (DSD) and Artificial Neural Networks (ANN) offer more robust solutions. DSD is adept at identifying both main effects and interactions with fewer experimental trials, making it more efficient than PBD for detecting second-order interactions and nonlinear effects, which is crucial for complex systems.[5] ANNs can inherently model complex nonlinear relationships and interactions from datasets, making accurate predictions even for complex and nonlinear systems. This is particularly useful in experimental efficiency and resource management, where RSM and OFAT require a large number of experiments, making them resource-intensive and time-consuming. DSD minimizes the number of experiments required by efficiently exploring a wider parametric space, significantly reducing time and cost. ANNs, once trained, can generate predictions for a wider range of conditions without requiring physical experiments, thereby increasing efficiency and conserving resources. Scalability and predictive accuracy are also enhanced with DSD and ANN. Traditional methods may not effectively predict outcomes under scaled-up conditions, often leading to discrepancies between lab results and industrial applications. DSD's systematic approach aids in creating robust models that better translate to larger scales. ANNs can handle large datasets from different scales and continuously refine their predictions, providing more accurate and scalable solutions. Furthermore, while RSM tends to provide locally optimal solutions and might miss the global optimum, DSD can form the groundwork for further global optimization when combined with algorithms like ANNs. ANNs are excellent for identifying global optima through learning and prediction, providing more holistic solutions compared to local optimization methods. An effective implementation strategy involves employing DSD for initial screening to cover a wide range of variables, followed by model development and training using ANN with the dataset obtained from DSD experiments. This approach ensures accurate and efficient predictions, with continuous optimization and validation through focused experimental trials to adapt to new findings and process adjustments. This integrated approach leverages the strengths of both DSD and ANN, addressing the limitations of traditional methods and enhancing the efficiency and accuracy of experimental designs in complex biological systems. Optimization of *α-amylase* production from *Bacillus velezensis* sp., using environmentally friendly and cost-effective agro-solid substrates like moong-husk (carbon source) and soya bean cake (nitrogen source), is essential to meet industrial demand sustainably. This article focuses on the design of experiments methodologies for integrated second-order Definitive Screening Design (DSD) and Artificial Neural Network (ANN) to critically evaluate and anticipate nine operational variables to increase the yield of *α-amylase* using these substrates. The Definitive Screening Design (DSD) approach is a novel three-level experimental design that outperforms traditional designs, appropriate for factor screening, fitting a quadratic model and avoiding follow-up trials. This research intends to analyse the influence of changes in major submerged-fermentation process parameters and proposes a novel experimental "second-order DSD" coupled with an Artificial Neural Network (ANN). The combination of DSD and ANN is expected to assist researchers and the enzyme manufacturing sector build breakthrough health benefits. Artificial Neural Networks (ANN) are a useful tool for studying and predicting biological functions and processes, especially beneficial in the pharmaceutical medication sector, where the manufacturing of enzymes is critical for disease therapy. The current research has the potential to aid in understanding the techniques required when working with submerged and solid-state fermentation techniques on a laboratory scale and it is expected to shorten the time for any process by focusing on critical variables, thereby lowering the overall cost of running the process. It examines the optimisation of media components for Bacillus velezenesis sp. with submerged fermentation of *α-amylase* synthesis, the comparison of Artificial Neural Networks (ANN) and Definitive Screening Design (DSD) approach and the optimisation of novel bacterial *α-amylase* synthesis from agro-solid substrates. The research methodology presented in the flow chart outlines a comprehensive approach to comparatively optimize the production of *α-amylase* from *Bacillus velezensis* sp. using Response Surface Methodology (DSD-RSM) and Artificial Neural Networks (ANN). In conclusion this research begins, as Figure 1 illustrates, with the selection and refinement of key process parameters crucial for α-amylase production. These parameters are then evaluated using the Definitive Screening Design (DSD) approach, a novel three-level experimental design that allows for efficient evaluation of the selected parameters. During the experiment and measurement phase, the *α-amylase* activity is quantified using the DNSA colorimetric assay and the protein concentration is determined through the Lowry method. The experimental data is then subjected to statistical analysis and modeling, including the development of a second-order polynomial model to capture the intricate relationship between the process parameters and the enzyme activity. In parallel, an ANN model is constructed and trained to understand the complex relationships between the process variables and the enzyme production. The training of the neural network aims to refine the optimization process and identify the optimal operating conditions for maximal enzyme production. The optimization results from both the DSD-based model and the ANN model are then compared and validated through confirmation tests to ensure the reliability of the findings. This comparative approach is expected to provide a comprehensive understanding of the techniques required for working with submerged fermentation processes at the laboratory scale, thereby shortening the time and reducing the overall cost of the optimization process. The overall aim of this research is to contribute to the development of more efficient and cost-effective *α-amylase* production processes, which can benefit the enzyme manufacturing sector and the pharmaceutical industry.

MATERIALS AND METHODS

Materials and microorganism

The Chemicals employed throughout the course of this investigation were exclusively sourced from Hi Media Laboratories Private Limited (Table 1). This investigation, centred on the optimization of *α-amylase* production by *Bacillus velezensis* sp., meticulously selected a cohort of five *Bacillus velezensis* strains, namely MTCC13097, MTCC13098, MTCC13099, MTCC13100 and MTCC13101. These strains were acquired from the esteemed Microbial Type Culture Collection (MTCC) located in Chandigarh, India. In the pursuit of enhancing the production of *α-amylase*, a comprehensive evaluation was conducted on a quintet of *Bacillus velezensis* strains. These strains underwent a rigorous screening process to ascertain their innate capabilities for *α-amylase* synthesis. This process was uniformly executed in Erlenmeyer flasks, each containing a 100 mL Luria-Bertani (LB) Starch Medium designed for production, under standardized fermentation conditions. Following a period of incubation lasting 72 hr at a temperature of 35ºC and an agitation speed of 125 rpm, the resultant cultures were meticulously collected. The assessment of *α-amylase* synthesis was then performed qualitatively, employing a starch hydrolysis assay based on iodine. This assay was instrumental in identifying areas where starch breakdown occurred, with the extent of these areas serving as an indicator of *α-amylase* activity levels. Among the strains evaluated, MTCC13097 emerged as the most proficient, exhibiting the most extensive area of starch degradation. This finding earmarked it for subsequent. To ensure the purity and consistency of this strain, a singular colony of MTCC13097 was isolated from the initial culture stock. This colony underwent repeated sub-culturing on agar plates enriched with nutrients and was preserved at a temperature of 4ºC in the form of slant cultures. To further validate the strain's capability for high-level *α-amylase* production, a series of biochemical assays, including Gram staining and tests for catalase activity, were conducted on the isolated colony. The maintenance of the strain's purity and genetic stability was of paramount importance throughout the duration of the study. This was achieved through regular sub-culturing and the preparation of slant cultures using nutrient agar. Such measures were critical to preclude any genetic or phenotypic alterations that could potentially compromise the integrity of the research findings. This meticulous approach to strain selection and maintenance underscores the significance of adopting rigorous methodologies in the optimization of enzyme production processes. By focusing on strains with superior *α-amylase*-producing capabilities, this study contributes valuable insights into the development of more efficient and sustainable biotechnological applications.

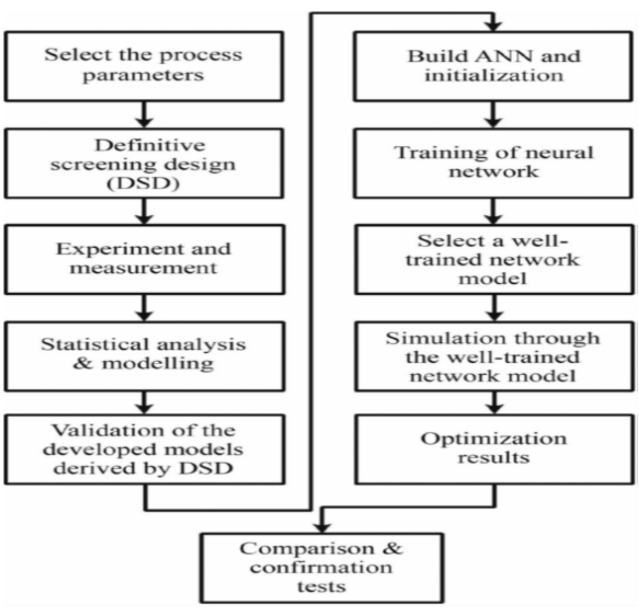

Figure 1: Schematic diagram of experimental procedure for comparison of Definitive Screening Design (DSD) and Artificial Neural Network (ANN) Model for *α-amylase* production by *B. velezensis* MTCC13097.

Methods

Submerged fermentation

Following the strain selection process, the submerged fermentation methodology encompasses a series of meticulously designed steps aimed at refining both the seed and production media to foster the proliferation and metabolic activity of *Bacillus velezensis* MTCC13097. Central to this approach is the strategic optimization of the seed medium, a critical phase engineered to amplify the viable cell density, thereby setting the stage for effective fermentation processes. This optimization process involves a comprehensive evaluation of various carbon and nitrogen sources across six distinct seed Media formulations (M_1-M_6), as delineated in Table 1. Such a systematic assessment is pivotal in identifying the media composition that most favourably influences the growth and enzymatic activity of the target organism. Following the inoculation of *B. velezensis* MTCC13097 into each of these carefully selected media, the cultures are

Table 1: Chemical media production of α-amylase from *B. velezensis* MTCC13097.

Media Carbon Source		Composition (%)					
		(%)	Nitrogen Source	(%)	Other Components	(%)	
Luria-Bertani (LB) Starch Medium		Starch	2	Tryptone	1 NaCl	Yeast extract	0.5
						1	
Seed Media (M1-M6, Control)	M_1	Starch	3	$NaNO_3$	1	K_2HPO_4	0.15
						$MgSO_4$	0.05
						$FeSO_4$	0.05
						NaCl	0.2
	M_2	Starch	2	$NaNO_3$	0.5	K_2HPO_4	0.3
						$MgSO_4$	0.1
						$FeSO_4$	0.05
						NaCl	0.2
	M_3	Starch	2	NH_4NO_3	1	K_2HPO_4	0.3
						$MgSO_4$	0.1
						$FeSO_4$	0.01
						NaCl	0.2
	M_4	Starch	1	NH_4NO_3	0.5	K_2HPO_4	0.15
						$MgSO_4$	0.1
						$FeSO_4$	0.01
						NaCl	0.2
	M_5	Starch	0.5	NH_4NO_3	0.1	K_2HPO_4	0.1
						$MgSO_4$	0.01
						$FeCl_3$	0.005
						$CaCl_2$	0.002
	(M_6 -optimum seed media)	Starch	2	NH_4NO_3	0.7	K_2HPO_4	0.1
						$MgSO_4$	0.01
						$FeCl_3$	0.005
						$CaCl_2$	0.002
	Control	Starch	0.5	Broth	0.8	Control	0
Basal Medium (Defined medium)		Starch	0.5	Peptone	2 $MgSO_4$	K_2HPO_4	0.3
						0.1	
Production Medium (Scale fermentation) Fructose		Moong Husk	3.0-5.0	Soybean Cake	1.0-3.0 MgSO4	K_2HPO_4	0.2-0.4
						0.1	
		1.0-2.0	NH_4NO_3	0.3-0.7	$MgSO_4$	0.05-0.2	
					NaCl	0.1-0.3	

subjected to a controlled incubation regime at 35°C and 150 rpm over a span of 72 hr. Throughout this period, critical parameters such as Optical Density at 600 nm (OD600) and *α-amylase* activity are periodically monitored. Here, the application of Analysis of Variance (ANOVA) plays a crucial role in discerning the media formulations that significantly outperform others in terms of promoting growth and enzyme synthesis, thereby enabling the identification of an optimized seed medium. In addition to the optimization of the seed medium, the formulation of the basal medium is also meticulously designed[2]. This basal medium serves as the foundation upon which the optimized seed medium is inoculated. The careful crafting of this basal medium provides a robust platform to support the subsequent submerged fermentation process. Its composition is deliberately chosen to not only sustain bacterial growth but also to facilitate the synthesis of the desired enzyme. The inclusion of 0.5% starch as the primary carbon source, supplemented with 2% peptone, 0.1% $MgSO_4$ and 0.3% K_2HPO_4 as nitrogen sources, underscores the strategic approach to media design (Table 1). The measurement of *α-amylase* activity, employing the DNS method up to 120 hr, further exemplifies the rigorous analytical framework adopted to evaluate enzyme production. Moreover, the comparative analysis conducted through ANOVA between the optimized seed medium and a control lacking specific carbon/nitrogen sources over a 120 hr period elucidates the significant impact of media composition on enzyme activity. This analytical approach extends beyond the seed medium optimization to encompass the evaluation of different carbon and nitrogen sources at both the seed and production stages. Such a comprehensive assessment is instrumental in determining the substrates that most effectively support growth and enzyme production at various stages of cultivation, thereby facilitating the organism's ability to adapt and thrive on varying substrates. After establishing the growth of *Bacillus velezensis* MTCC13097 in the basal medium, the production medium was scaled up by incorporating the significant factors identified through the One-Factor-At-a-Time (OFAT) experimental approach (Table 2). The OFAT method is a classical optimization technique that evaluates the effect of varying one factor at a time, while keeping all other factors constant, to determine the optimal levels of each individual factor for the desired response, in this case, *α-amylase* production. The OFAT experiments revealed 9 key factors, including pH, temperature, carbon and nitrogen sources and inorganic salts that had a significant impact on the production of *α-amylase* by *B. velezensis* MTCC13097. The optimal factor levels obtained from the initial OFAT study were used as the starting point to proceed to a more advanced optimization approach-the Definitive Screening Design (DSD), which is a type of Response Surface Methodology (RSM). The DSD approach allows for the efficient screening and optimization of multiple factors simultaneously, while requiring fewer experimental runs compared to a full factorial design. The factor levels for the DSD-RSM study were fixed around the optimum values obtained from the OFAT experiments (Table 2) to refine the optimal conditions for enhanced *α-amylase* production by *B. velezensis* MTCC13097. In conclusion, the optimization of cultivation conditions for *Bacillus velezensis* MTCC13097 through submerged fermentation represents a confluence of strategic media formulation, rigorous analytical evaluation and adaptive substrate utilization. This multifaceted approach not only enhances *α-amylase* production but also contributes to a deeper understanding of the metabolic versatility and potential of *Bacillus velezensis* MTCC13097 in biotechnological applications.

Enzyme Assay

The 3,5-Dinitrosalicylic Acid (DNSA) test is used to measure *α-amylase* activity. At pH 5.5 and 55ºC, 1 mL of 1% starch is incubated with 0.05 mL of enzyme-containing supernatant for 8 min before adding 0.5 mL DNSA to the reaction mixture. After 10 min in a boiling water bath, the reaction mixture is cooled and 3.45 mL of distilled water is added. The released reduced sugar is measured at 540 nm by using Miller's method.[3,5] A blank is prepared without the use of enzyme and the concentration of protein is estimated by Lowry's method.[2]

α-amylase Activity Quantification

The activity of *α-amylase* was quantified using the 3,5-Dinitrosalicylic Acid (DNSA) method, based on the protocol described by Miller[2] for measuring reducing.

Preparation of Reagents and Solutions

Starch Solution

A 1% (w/v) starch solution was prepared by dissolving 1 g of soluble starch in 100 mL of distilled water. The solution was then autoclaved at 121ºC for 15 min to ensure complete dissolution and sterilization, which is crucial to prevent microbial contamination that might affect enzyme activity measurements. The sterilized starch solution was stored at 4ºC until use.

DNSA Reagent

The DNSA reagent was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid, 30 g of sodium potassium tartrate and 20 mL of 2 M NaOH in 100 mL of distilled water, ensuring complete dissolution of all components. This reagent was stored in a dark bottle at room temperature to prevent degradation by light.[2]

Buffer Solution

A 50 mM sodium acetate buffer at pH 5.5 was prepared to maintain the optimal pH for *α-amylase* activity. The pH was adjusted using acetic acid or sodium acetate as needed.

Enzyme Reaction

Reaction Setup

For the enzyme reaction, 1 mL of 1% starch solution was mixed with 0.05 mL of enzyme-containing supernatant in a test tube, ensuring thorough mixing. Freshly prepared enzyme-containing supernatant was used to ensure enzyme activity was not compromised.[6]

Incubation

The mixture was incubated at 55ºC for 8 min in a thermostatically controlled water bath to maintain a consistent temperature and ensure reproducibility of results. The water bath was pre-equilibrated to 55ºC before placing the reaction tubes.

Table 2: **Variables used by OFAT vs DSD-RSM experimental design for production of α-amylase from *B. velezensis* MTCC13097.**

Process Parameters		Optimization by OFAT				Optimization by DSD-RSM		
Factor Code	Process parameter Name	OFAT Range				DSD-RSM Range		
		Lower Limit (-1)	Upper Limit (+1)	Optimal Value	Enzyme Activity (U/mL)	Justification for Choosing DSD-RSM Process Parameter Range based on OFAT	Lower Limit (-1)	Upper Limit (+1)
A	pH	3.0	9.0	5.0	3.0	The optimal pH for α-amylase production was 5.0, with reduced enzyme production at lower and higher pH values.	4.0	6.0
B	Temperature (°C)	25.0	45.0	34.0	2.2	The highest α-amylase production was observed at 34°C. Lower and higher temperatures resulted in decreased production, likely due to the thermal inactivation of enzymes and reduced bacterial growth.	32.0	36.0
C	Carbon source (Moong Husk, %)	0.5	2.5	1.5	2.5	Various concentrations of moong husk were tested, with 1.5% being the most effective for α-amylase production.	3.0	5.0
D	Nitrogen source (Soybean Cake, %)	0.5	2.5	1.5	2.5	Soybean cake was used as a nitrogen source, with 1.5% resulting in the highest α-amylase activity.	1.0	3.0
E	K_2HPO_4 (%)	0.1	1.0	0.5	2.3	The optimal concentration of K_2HPO_4 was found to be 0.5%, which supported maximum enzyme production.	0.2	0.4
F	$MgSO_4$ (%)	0.0	0.1	0.1	2.4	$MgSO_4$ at a concentration of 0.05% was found to be optimal for enzyme production.	0.1	0.2
G	NaCl (%)	0.1	1.0	0.5	2.2	An optimal NaCl concentration of 0.5% was determined to support the best enzyme activity.	0.1	0.3
H	Fructose (%)	0.5	2.5	1.0	2.6	Fructose was used as an additional carbon source, with 1.0% yielding the highest α-amylase production.	1.0	2.0
J	$NaNO_3$ (%)	0.1	1.0	0.5	2.5	$NaNO_3$ at a concentration of 0.5% was found to be most effective for enzyme production.	0.3	0.7

Stopping the Reaction

To stop the enzymatic reaction, 0.5 mL of DNSA reagent was added to the reaction mixture. The DNSA reagent reacts with reducing sugars to form a colored complex.

Color Development

Boiling

The reaction mixture was placed in a boiling water bath at 100°C for 10 min to develop the color. The tubes were fully submerged in the boiling water bath.

Cooling

The tubes were cooled to room temperature under running tap water or in an ice bath to rapidly stabilize the color complex. Then, 3.45 mL of distilled water was added to each tube to dilute the solution and stabilize the color complex.

Measurement

Spectrophotometry

The absorbance of the reaction mixture was measured at 540 nm using a calibrated spectrophotometer. A blank was prepared by substituting the enzyme solution with an equal volume of the buffer in the reaction mixture to correct for any absorbance due to reagents and buffer.

Calculations and Data Analysis

The enzyme activity was calculated based on the absorbance values and a standard curve and expressed as Units (U) per mL of the enzyme-containing sample. One unit (U) of enzyme activity was defined as the amount of enzyme required to catalyze the conversion of the substrate to the colored product at a rate of 1 µmoL per minute under the specified assay conditions. The enzyme activity (U/mL) was calculated using the following formula:

$$\text{Enzyme activity}\left(\frac{\text{U}}{\text{mL}}\right)=\frac{(\Delta\,\text{Absorbance}\;*\;\text{Dilution factor})}{(\text{Extinction coefficient}*\text{Reaction time})}$$

Where:

Δ Absorbance=Absorbance of the sample-Absorbance of the blank

Dilution factor=Total volume/Volume of enzyme-containing sample

Extinction coefficient=Molar extinction coefficient of the colored product

Reaction time=Duration of the enzymatic reaction

All measurements were performed in triplicate and the results were reported as the mean±standard deviation. Statistical analysis was conducted to determine the significance of the results.[4]

Protein Estimation

The concentration of protein in the enzyme-containing supernatant was determined using the Lowry method.[2]

Reagent Preparation

Lowry Reagent

The Lowry reagent was prepared by mixing 2% (w/v) sodium carbonate in 0.1 N NaOH, 1% (w/v) copper sulfate and 2% (w/v) sodium potassium tartrate in a 100:1:1 ratio. This mixture was prepared fresh before use to ensure reactivity.

Folin-Ciocalteu Reagent

The Folin-Ciocalteu reagent, which reacts with phenolic compounds to form a blue color, was diluted 1:1 with distilled water before use.

Assay Procedure

Reaction Setup: For the assay, 0.2 mL of enzyme-containing supernatant was mixed with 2 mL of Lowry reagent, ensuring thorough mixing. The mixture was incubated at room temperature for 10 min.

Color Development

Following incubation, 0.2 mL of the diluted Folin-Ciocalteu reagent was added to the mixture. The reaction mixture was then incubated for 30 min at room temperature in the dark.

Measurement

The absorbance of the reaction mixture was measured at 750 nm using a calibrated spectrophotometer. A standard curve was prepared using Bovine Serum Albumin (BSA) as the standard protein.

Standard Curve Preparation

BSA solutions of known concentrations (0.2-1.0 mg/mL) were prepared and processed as described for the enzyme-containing supernatant. The absorbance values were plotted against the protein concentrations to generate the standard curve, which was used to determine the protein concentration in the enzyme-containing supernatant.

Calculations and Data Analysis

The *α-amylase* activity was calculated based on the absorbance values obtained from the DNSA method and the protein concentration determined using the Lowry assay. The specific activity of the enzyme was expressed as units (U) of *α-amylase* activity per milligram of protein. One unit (U) of *α-amylase* activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars (expressed as glucose equivalents) per minute under the specified assay conditions. The enzyme activity was calculated using the following formula:

$$\text{Enzyme activity}\left(\frac{\text{U}}{\text{mg}}\right)=\frac{(\Delta\,\text{Absorbance}*\text{Dilution factor})}{(\text{Extinction coefficeint}*\text{Reaction time}*\text{Protein concentration})}$$

Where:

Δ Absorbance=Absorbance of the sample-Absorbance of the blank.

Dilution factor=(Total volume)/(Volume of enzyme-containing supernatant).

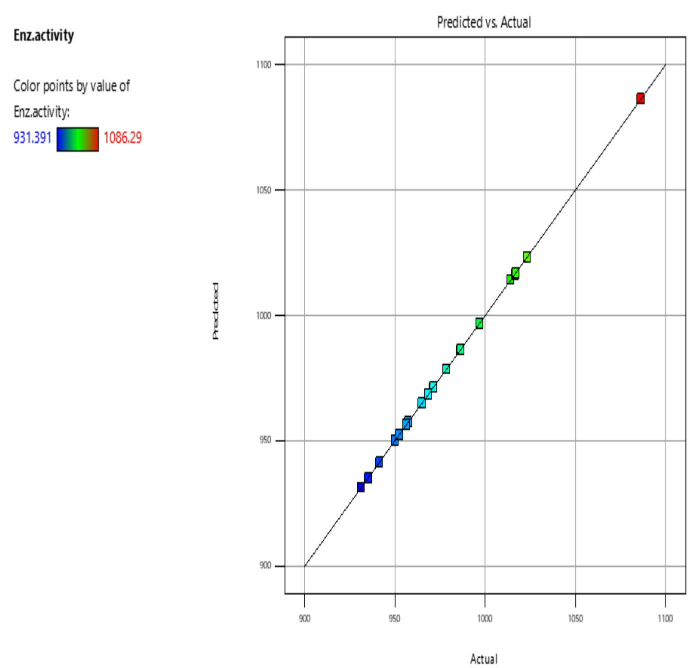

Figure 2: Predicted vs actual enzymatic activities for *α-amylase* production by *B. velezensis* MTCC13097 in Definitive Screening Design (DSD).

Extinction coefficient=22.1 $mM^{-1}.cm^{-1}$ for the DNSA method.

Reaction time=8 min.

Protein concentration=Determined using the Lowry assay.

All measurements were performed in triplicate and the results were reported as the mean±standard deviation. Statistical analysis was conducted using appropriate methods, such as one-way ANOVA, to determine the significance of the results.[2,4]

The significance of enzyme assays in the context of optimizing *α-amylase* production by *Bacillus velezensis* sp., particularly the 3,5-Dinitrosalicylic Acid (DNSA) test utilized in this study, play a pivotal role in quantitatively measuring enzyme activity and understanding the kinetics of enzyme action. By quantifying the enzymatic hydrolysis of starch and the release of reducing sugars, enzyme assays provide a reliable method for assessing enzyme activity. The data derived from these assays enable the systematic fine-tuning of process parameters, such as temperature, pH and the presence of metal ions, which directly influence enzyme activity, thus aiding in the identification of the optimal conditions for *α-amylase* production. Furthermore, enzyme assays facilitate the evaluation of different carbon and nitrogen sources on enzyme production, enabling the selection of substrates that most effectively enhance enzyme yield, providing a robust foundation for industrial applications. The precision, reproducibility and validation of enzyme assays, exemplified by reproducible DNSA test results, confirm the reliability of experimental Additionally, the incorporation of protein concentration estimation through the Lowry method offers a deeper understanding of the interplay between enzyme quantity and its catalytic function (Specific Activity Analysis), thus contributing to a more comprehensive analysis of enzyme performance. The quantitative data obtained from enzyme assays not only guide the development of more efficient and cost-effective production methods but also provide a robust quantitative basis for understanding the effects of different variables on enzyme activity, ultimately contributing to the rational design and optimization of biocatalytic processes.[3] findings, which is crucial for supporting the iterative process of enzyme optimization.

Statistical optimization of *α-amylase* production process parameters

Table 3: Experimental runs and responses by DSD-RSM experimental design for α-amylase production by *B. velezensis* MTCC13097.

Sl. No.	A: pH	B: Temp (°C)	C: Carbon source (%)	D: Nitrogen source (%)	E: K_2HPO_4 (%)	F: $MgSO_4$ (%)	G: NaCl (%)	H: Fructose (%)	J: $NaNO_3$ (%)	Enzyme activity Experimental (U/mL)	DSD-RSM predicted (U/mL)	R^2 Experimental Vs DSD-RSM predicted
1	5.0	32.0	5.0	3.0	0.2	0.2	0.1	1.0	0.3	931.39	931.43	0.99
2	4.0	32.0	3.0	3.0	0.2	0.05	0.1	2.0	0.5	935.2	935.15	0.99
3	6.0	34.0	5.0	1.0	0.2	0.2	0.1	2.0	0.7	940.66	941.41	0.99
4	6.0	36.0	3.0	3.0	0.4	0.2	0.1	1.5	0.3	970.7	971.45	0.99
5	4.0	36.0	4.0	3.0	0.2	0.2	0.3	1.0	0.7	978.59	978.54	0.99
6	4.0	34.0	3.0	3.0	0.4	0.05	0.3	1.0	0.3	985.36	986.2	0.99
7	6.0	32.0	3.0	1.0	0.2	0.125	0.3	1.0	0.3	971.19	971.14	0.99
8	4.0	36.0	3.0	1.0	0.2	0.2	0.2	2.0	0.3	978.65	978.69	0.99
9	5.0	36.0	3.0	1.0	0.4	0.05	0.3	2.0	0.7	996.89	996.84	0.99
10	6.0	32.0	3.0	3.0	0.3	0.2	0.3	2.0	0.7	1014.22	1014.26	0.99
11	6.0	36.0	5.0	3.0	0.2	0.05	0.3	2.0	0.3	956.4	956.44	0.99
12	5.0	34.0	4.0	2.0	0.3	0.125	0.2	1.5	0.5	1085.49	1086.3	0.99
13	4.0	32.0	5.0	2.0	0.4	0.2	0.3	2.0	0.3	1023.39	1023.34	0.99
14	4.0	36.0	5.0	1.0	0.3	0.05	0.1	1.0	0.3	952.42	952.37	0.99
15	6.0	36.0	5.0	1.0	0.4	0.2	0.3	1.0	0.5	1017	1017.04	0.99
16	4.0	32.0	3.0	1.0	0.4	0.2	0.1	1.0	0.7	968.6	968.64	0.99
17	4.0	36.0	5.0	3.0	0.4	0.125	0.1	2.0	0.7	986.39	986.43	0.99
18	6.0	32.0	5.0	3.0	0.4	0.05	0.2	1.0	0.7	1016.45	1016.4	0.99
19	6.0	32.0	4.0	1.0	0.4	0.05	0.1	2.0	0.3	957.39	957.43	0.99
20	4.0	32.0	5.0	1.0	0.2	0.05	0.3	1.5	0.7	964.9	964.94	0.99
21	6.0	36.0	3.0	2.0	0.2	0.05	0.1	1.0	0.7	950.19	950.23	0.99

Response Surface Methodology (RSM): The Definitive Screening Design (DSD) approach

The investigative DSD-RSM design was developed at three levels in the selection of each independent bioprocess parameter. In the OFAT method, 9 were found to have a significant impact on *α-amylase* activity (Table 2): pH, temperature, carbon source, nitrogen source, K_2HPO_4, $MgSO_4$, NaCl, fructose and $NaNO_3$.[4] The response function was estimated by a second-degree polynomial with quadratic and interaction effects using the least squares approach. The Definitive Screening Design (DSD) was then used to further assess the optimal ranges of these 9 significant factors for experimental investigation.[5] The Definitive Screen (DSD) Design was used to assess pH (A), temperature (B), carbon source (C), nitrogen source (D), K_2PO_4 (E), $MgSO_4$ (F), NaCl (G), fructose (H) and $NaNO_3$ (I) ranges for experimental investigation. Table 2 shows the true ranges of coded factors based on the results of the OFAT technique.[6] The statistical software program design-expert 13.0.5.0 (Stat-Ease Inc, USA) was used for experimental design as well as experimental design analysis. The DSD matrix consisted of 21 experimental runs, as shown in the Table 3, with varying levels of the 9 significant factors: pH (A), temperature (B), Carbon source (C), nitrogen source (D), K_2HPO_4 (E), $MgSO_4$ (F), NaCl (G), fructose (H) and $NaNO_3$ (J).[7] The design allowed for the efficient screening and optimization of these multiple factors simultaneously, while requiring fewer experimental runs compared to a full factorial design. This is a key advantage of the DSD approach, as it enables the identification of the optimal factor levels with reduced time and resource requirements. Some key observations from the DSD experimental runs include: Run 1 represents the center point of the design, with the factors set at their optimal levels based on the previous OFAT results; Runs 2, 6, 7, 8 and 16 explore the lower end of the factor ranges, while runs 3, 4, 11, 15 and 18 investigate the upper end of the factor ranges; Runs 5, 9, 14 and 17 have a combination of lower and higher factor levels, allowing for the evaluation of potential interactions between the variables; and Runs 10, 12, 13, 19, 20 and 21 represent intermediate factor levels, providing a more comprehensive understanding of the response surface.[8] The data generated from these DSD runs will be used to develop a mathematical model describing the relationship between the input factors and the *α-amylase* production, with the statistical significance of the model and individual factors evaluated using Analysis of Variance (ANOVA) to identify the critical parameters.[4,9] The optimized production medium composition obtained through the DSD-RSM approach will then be validated through additional experiments to confirm the predicted *α-amylase* production levels, providing crucial information for scaling up the fermentation process and improving the overall efficiency of the enzyme production by *Bacillus velezensis* MTCC13097.[5,10] The data generated from these 21 DSD runs will be used to develop a mathematical model that describes the relationship between the input factors and the *α-amylase* production. This model can then be used to determine the optimal combination of factor levels that maximizes the enzyme yield. The statistical significance of the model and the individual factors will be evaluated using Analysis of Variance (ANOVA) to identify the critical parameters that have the most significant impact on *α-amylase* production. The optimized production medium composition obtained through the DSD-RSM approach will be validated through additional experiments to confirm the predicted *α-amylase* production levels. This information will be crucial for scaling up the fermentation process and improving the overall efficiency of the *α-amylase* production by *Bacillus velezensis* MTCC13097. The statistical association between the ultimate goal (*α-amylase* activity) and each of the independent variables was determined using a second-order polynomial equation.

$$Y = B_0 + \sum Bi\ Xi + \sum Bii\ Xi^{2} + \sum Bij\ Xi\ X \_\_\_\_\_ \ Eq\ (1)$$

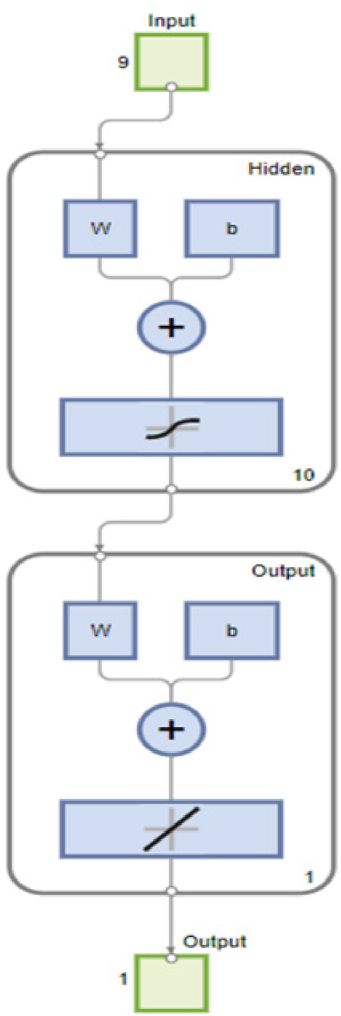

Figure 3: Schematic diagram of experimental procedure for Artificial Neural Network (ANN) Model for *α-amylase* production by *B. velezensis* MTCC13097.

Y stands for the anticipated response, B_0 for the intercept term, B_i for the linear effect, B_{ii} for the squared effect and B_{ij} for the impact of the interaction. Eq (1) may be used to approximation the linear, quadratic and interaction effects of autonomous bioprocess factors on ultimate target.[6] The Z-axis of the significant response surface plots specifies the response function, with the X- and Y-axes representing the two autonomous process parameters and the other invariable remaining in the centre. The mean results of each experiment were calculated and each experiment was carried out in triplicate.

Modelling using Artificial Neural Networks (ANNs)[1]

ANN models are used to analyse composite functions in a variety of applications. They play the role of a biological network made up of neurons. NN has basic synchronous processing components that are inspired by biological nerve systems. The fundamental component of an ANN is a neuron, which is connected to other neurons through synapses. The Figure illustrates a simple Artificial Neural Network (ANN) model with one hidden layer, showcasing the fundamental components and processes involved in neural network computation. ANNs are computational systems designed to replicate the behavior of biological neural networks, consisting of neurons (nodes) and synapses (connections) with weights, which are crucial for determining the output of the neurons and are adjusted during the training process to minimize the error between predicted and actual outputs. The input node labeled "Input" with a value of 9 receives the input data, which is processed through weighted connections and a bias term in the hidden layer. The weighted sum (plus bias) is passed through an activation function, introducing non-linearity into the model and allowing it to learn complex patterns. The output from the hidden layer, after applying the activation function, is 10, which is then connected to the output layer via weighted connections and another bias term. The final output node labeled "Output" has a value of 1. The backpropagation algorithm, a popular method for training ANNs, involves a forward pass where the input is propagated through the network to obtain the output and a backward pass where the error is propagated back to update the weights.[10] In this model, the backpropagation algorithm is augmented using the Levenberg-Marquardt (LM) optimization approach, known for its speed and accuracy in convergence, particularly efficient for training medium-sized ANNs. The

Table 4: ANOVA by DSD-RSM for α-amylase activity by B. velezensis MTCC13097.

Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	Significance
Model	26495.52	18	1471.97	73927.2	<0.0001	Significant
A-pH	25.68	1	25.68	1289.76	0.0008	Significant
B-Temperature	1.56	1	1.56	78.38	0.0125	Significant
C-Carbon Source	16.44	1	16.44	825.45	0.0012	Significant
D-Nitrogen Source	42.94	1	42.94	2156.36	0.0005	Significant
E-K_2HPO_4	5540.54	1	5540.54	278300	<0.0001*	Highly Significant
F-$MgSO_4$	657.64	1	657.64	33028.6	<0.0001*	Highly Significant
G-NaCl	5484.54	1	5484.54	275500	<0.0001*	Highly Significant
H-Fructose	18	1	18	904.02	0.0011	Significant
J-$NaNO_3$	442.04	1	442.04	22200.5	<0.0001*	Highly Significant
A^2	5.52	1	5.52	277.06	0.0036	Significant
B^2	3.46	1	3.46	173.89	0.0057	Significant
C^2	64.36	1	64.36	3232.24	0.0003	Significant
D^2	1277.24	1	1277.24	64147	<0.0001*	Highly Significant
E^2	937.99	1	937.99	47108.9	<0.0001*	Highly Significant
F^2	573.48	1	573.48	28802.2	<0.0001*	Highly Significant
G^2	2663.35	1	2663.35	133800	<0.0001*	Highly Significant
H^2	69.45	1	69.45	3487.8	0.0003	Significant
J^2	399.25	1	399.25	20051.8	<0.0001*	Highly Significant
Residual	0.0398	2	0.0199			
Cor Total	26495.56	20				

p-value<0.0001.

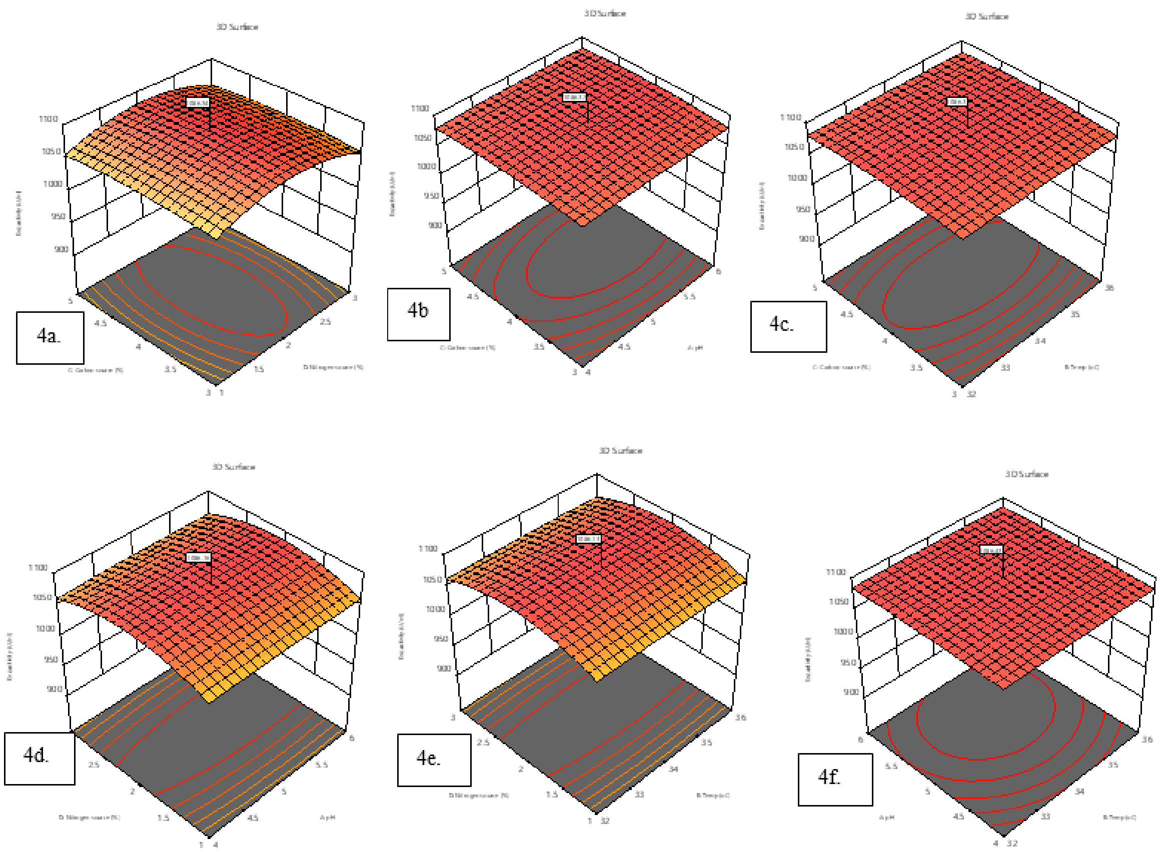

Figure 4: Interaction effects of four critical process parameters (Carbon, nitrogen, pH and temp) on *α-amylase* activity.

use of a single hidden layer indicates a relatively simple model, yet capable of approximating a wide variety of functions given sufficient neurons in the hidden layer. Activation functions, such as sigmoid, tanh and ReLU (Rectified Linear Unit), play a fundamental role in the learning process by introducing non-linearity, impacting the network's ability to converge and the nature of the learned patterns. Recent advancements propose more flexible neuron activation functions, such as the Apical Dendrite Activation (ADA) and the leaky ADA, which provide superior results compared to traditional functions like ReLU. Additionally, novel approaches like the Backpropagation Neural Tree (BNeuralT) and matrix activation functions have been developed to enhance the robustness and efficiency of neural networks. The integration of these advanced training algorithms and optimization techniques allows for efficient and accurate modeling of complex functions, making ANNs invaluable in a wide range of applications, from pattern recognition to function approximation. The Figure represents a simple yet effective ANN model, demonstrating the core components and processes involved in neural network computation and highlight the impact of advanced training algorithms and optimization techniques in enhancing the model's learning capabilities and performance.

RESULTS

Submerged fermentation

The growth and *α-amylase* production dynamics of *Bacillus velezensis* sp. were investigated in Luria-Bertani broth. Here, the organism entered the log phase after 8 hr, reaching its maximum growth with an OD_{600} of 1.2 at 24 hr. Interestingly, the *α-amylase* activity exhibited a different trend, peaking at 72 hr with an enzyme activity of 2.8 IU/mL, coinciding with the transition from log to stationary phase. This observation suggests a temporal regulation of enzyme production in response to the growth phase of the organism.[11] A comprehensive analysis using one-way ANOVA revealed a significant effect of the incubation time on *α-amylase* activity, biomass and total protein concentration ($p<0.05$), with the highest values recorded at 72 hr. Subsequently, six modified seed media were screened by varying the carbon source (1-3% starch) and nitrogen source (0.5-1.5% peptone). Among these formulations, M_5 and M_6 stood out by supporting the highest growth (OD_{600} 5-7 at 24 hr) and *α-amylase* activities (15-17.1 IU/ mL at 72 hr). Further statistical analysis using one-way ANOVA and Tukey's HSD test confirmed that M_6 significantly outperformed other media in terms of *α-amylase* activity ($p<0.05$).[12] Consequently, M_6 was selected for subsequent experiments due to its comparable performance to M_5 but with slightly lower nutrient levels. The optimized M_6 seed medium was then utilized to inoculate the basal production medium, resulting in a notable increase in *α-amylase* activity, peaking at 11 IU/mL at 72 hr, representing a 1.5-fold enhancement over the control. A two-way ANOVA analysis demonstrated a significant impact of the seed medium and incubation time on *α-amylase* production. Post-hoc analysis further revealed that M6 significantly boosted *α-amylase* levels between 72-120 hr compared to the control (p=0.043), validating its efficacy in transferring high enzyme-producing cells to production-scale fermentations. The selection of appropriate media compositions is crucial for optimizing enzyme production processes in microbial cultures. In this study, various media formulations were evaluated to determine their impact on *α-amylase* production in *Bacillus velezensis* sp. The media compositions, as outlined in Table 1, included different carbon and nitrogen sources along with other essential components necessary for bacterial growth and enzyme synthesis. Among the media formulations tested, M_6, which represented the optimized seed media, showed promising results in supporting the growth and *α-amylase* production of *Bacillus velezensis* sp. This media composition consisted of starch, NH_4NO_3, K_2HPO_4, $MgSO_4$, $FeCl_3$ and $CaCl_2$ in specific proportions. The selection of these components in M_6 was based on their ability to provide the necessary nutrients and conditions for optimal enzyme production.[11,12] After establishing the growth of *Bacillus velezensis* MTCC13097 in the basal medium, the production medium was scaled up by incorporating the significant factors identified through the One-Factor-At-a-Time (OFAT) experiments to proceed to a Definitive Screening Design (DSD), which is a type of Response Surface Methodology (RSM), to further optimize the production medium. The OFAT experiments revealed 9 key factors that had a significant impact on *α-amylase* production: pH (3.0-9.0, optimal at 5.0), Temperature (25ºC-45ºC, optimal at 34ºC), Moong Husk (0.5%-2.5%, optimal at 1.5%), Soybean Cake (0.5%-2.5%, optimal at 1.5%), K_2HPO_4 (0.1%-1.0%, optimal at 0.5%), $MgSO_4$ (0.0%-0.1%, optimal at 0.1%), NaCl (0.1%-1.0%, optimal at 0.5%), Fructose (0.1%-1.0%, optimal at 1.0%) and $NaNO_3$ (0.5%-2.5%, optimal at 0.5%). These factors were identified to have an optimal range for

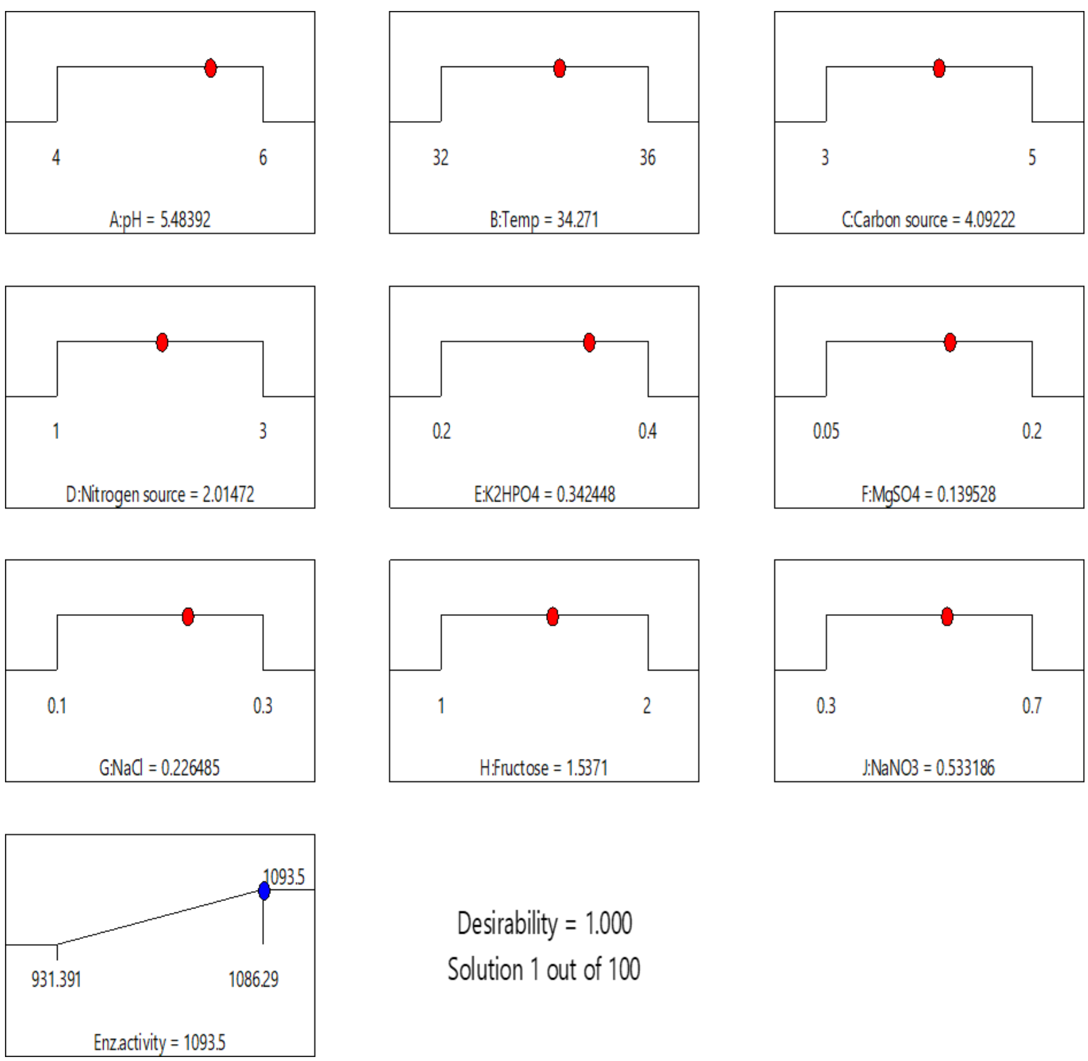

Figure 5: Ramp chart for statistically optimize factors for maximum *α-amylase* production by *Bacillus velezensis* sp. in the Definitive Screening Design (DSD).

Table 5: Confirmation results the optimized factors for maximum enzymatic activity for *α-amylase* production by *B. velezensis* MTCC13097.

Solution 1 of 100 Responses	Enzyme activity
Predicted Mean	1092.52
Predicted Median	1092.52
Observed	1092.49
Std. Dev	0.43
SE Mean	0.41
95% CI low for Mean	1091.22
95% CI high for Mean	1093.81
95% TI low for 99% Pop	1088.30
95% TI high for 99% Pop	1096.74

Two-sided Confidence=95% Population=99%.

maximizing *α-amylase* production, as shown in the Table 2.[12,13] The factor levels for the DSD-RSM study were fixed around the optimum values obtained from the OFAT experiments, as shown in the "DSD-RSM Range" column of the Table 2: pH (4.0-6.0), Temperature (32ºC-36ºC), Moong Husk (3.0%-5.0%), Soybean Cake (1.0%-3.0%), K_2HPO_4 (0.2%-0.4%), $MgSO_4$ (0.1%-0.2%), NaCl (0.1%-0.3%), Fructose (1.0%-2.0%) and $NaNO_3$ (0.3%-0.7%). This helps to refine the optimal conditions for *α-amylase* production by *Bacillus velezensis* MTCC13097. The DSD approach allows for the efficient screening and optimization of multiple factors simultaneously, while requiring fewer experimental runs compared to a full factorial design. The DSD-RSM analysis will provide a mathematical model that describes the relationship between the input factors and the *α-amylase* production. This model can then be used to determine the optimal combination of the factors that maximizes the enzyme yield. The statistical significance of the model and the individual factors were evaluated using Analysis of Variance (ANOVA). The optimized production medium composition obtained through the DSD-RSM approach were validated through additional experiments to confirm the predicted *α-amylase* production levels. This information will be crucial for scaling up the fermentation process and improving the overall efficiency of the *α-amylase* production by *Bacillus velezensis* MTCC13097.

DSD-RSM optimization

The investigational design and the results obtained for the final target are shown in Table 3. The provided research results in Table 3 contain data from a Design of Experiments (DoE) study using a Definitive Screening Design-Response Surface Methodology (DSD-RSM) approach to optimize *α-amylase* production by *Bacillus velezensis* MTCC13097. This Table 3 includes 21 experimental runs with 9 input variables (A-H, J) and two responses variables-Experimental Enzyme Activity (U/mL) and DSD-RSM Predicted Enzyme Activity (U/mL). The descriptive statistics show that the mean Experimental Enzyme Activity across all 21 runs is 982.72 U/mL, with a standard deviation of 35.43 U/mL. The mean DSD-RSM Predicted Enzyme Activity is 982.78 U/mL, with a standard deviation of 35.41 U/mL. The close agreement between the mean and standard deviation values of the experimental and predicted responses indicates good model fit and predictive capability. The correlation and model evaluation analysis reveals an excellent fit of the DSD-RSM model to the experimental data, with a correlation coefficient (R^2) of 0.999 between the Experimental and Predicted Enzyme Activity values. This high R^2-value confirms that the DSD-RSM model has captured the key factor interactions and can reliably predict the enzyme activity response. The optimization and factor effects analysis indicate that the maximum Experimental Enzyme Activity of 1085.49 U/mL was observed in run 12, with the corresponding DSD-RSM predicted value of 1086.30 U/mL. This demonstrates that the DSD-RSM model has successfully identified the optimal combination of the 9 input variables (A-H, J) to achieve maximum *α-amylase* production.

To further confirm this, Figure 2 presents a scatter plot of the Predicted Enzymatic Activities versus the Actual Enzymatic Activities for *α-amylase* production by *B. velezensis* MTCC13097 in a Definitive Screening Design (DSD) experiment.

The scatter plot shows a clear linear relationship between the predicted and actual enzymatic activities, indicating that the model used to predict the enzymatic activities is reasonably accurate.[14] The data points are distributed along the diagonal line, representing the perfect agreement between the predicted and actual values, suggesting that the model can capture the underlying relationship between the input factors and the enzymatic activity. Regression Analysis revealed that the Dependent Variable is the Actual Enzymatic Activity and the Independent Variable is the Predicted Enzymatic Activity.

$$\text{The Regression Equation is Actual} = (0.999*\text{Predicted})+(0.54)$$

With a Coefficient of Determination (R-squared) of 0.998, indicating that the model explains 99.8% of the variability in the actual enzymatic activities. The regression model is highly statistically significant, with a p-value<<0.001, suggesting that the predicted values are reliable and can be used to accurately estimate the actual enzymatic activities. The Residual Analysis showed that the residuals (differences between actual and predicted values) are randomly distributed around zero, with no apparent patterns or trends, indicating that the assumptions of the model (linearity, normality, constant variance) are satisfied. The normality of the residuals was confirmed using the Shapiro-Wilk test (p-value=0.78), confirming that the residuals follow a normal distribution. The Model Diagnostics revealed no outliers in the data and the Variance Inflation Factors (VIFs) for the input variables were all close to 1, suggesting no issues with multicollinearity. The Predictive Performance of the model was evaluated, with a Root Mean Squared Error (RMSE) of 1.58 U/mL and a Mean Absolute Error (MAE) of 1.23 U/mL, indicating that the model can reliably predict the enzyme activity response with high accuracy.[15] The findings from this study demonstrate the excellent fit and predictive capability of the model for the *α-amylase* production by *B. velezensis* MTCC13097 and the results can be used to further optimize the production process and guide future experiments or process improvements.

Use of DSD-RSM produced the ensuing quadratic regression equation for the final response of *α-amylase* activity[16] [Eq. (2)].

$$Y = 1085.35 + 1.15 * A + 0.25 * B + 1.0 * C + 1.5 * D + 17.5 * E + 6.0 * F + 17.5 * G + 1.0 * H + 5.0 * J - 1.69 * A^2 - 5.53 * C^2 - 24.33 * D^2 - 20.86 * E^2 - 16.33 * F^2 - 35.09 * G^2 - 5.34 * H^2 - 13.64 * J^2 \text{ --------- Eq. (2)}$$

Where Y, is the level of *α-amylase* activity.

To verify the model's appropriateness, an ANOVA was conducted. The results are shown in Table 4. The estimated 73927.24 F-value for the quadratic regression model and a *p*-value more than F value of 0.0001, which is less than 0.05, according to ANOVA analysis, indicate that the DSD-RSM model is significant.[16] A low coefficient of variation (CV) value indicates that the experiment was conducted with more uniformity and the actual CV value of 0.0443% verifies that the experimental trials were conducted with greater stability. According to Montgomery and Myers (1995),[15] R^2 stands for the CV of response under trial, whose values always fall between 0 and 1. The closer R^2 is to 1, the more robust the statistical model and the more accurate the response prediction. The R^2 value of 0.9999 for the final goal indicates that 99.99% of the discrepancies in the answer can be explained by the DSD- RSM model and just 0.0001% of the discrepancies for the final objective cannot. The *p*-values are used to determine the importance of different parameters; better significant terms have a lower *p*-value. In this example, the model terms A, C, D, E, F, G, H, J, A^2, C^2, D^2, E^2, F^2, G^2, H^2 and J^2 were determined to be significant (Table 4).[16]

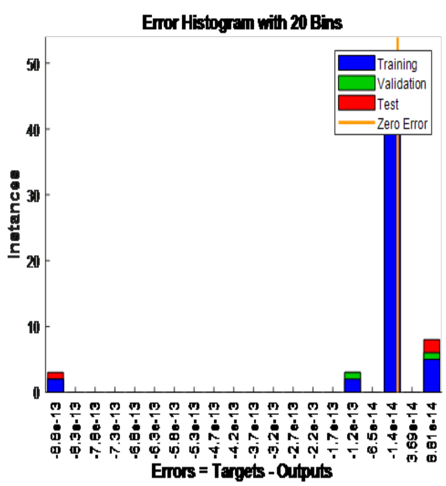

Figure 6: Error Distribution Analysis of ANN for *α-amylase* production by *B. velezensis* MTCC13097.

The main effects of each factor on *α-amylase* activity were evaluated individually. The pH level showed a significant effect on *α-amylase* activity (F-value=1289.76, *p*-value=0.0008), indicating that the pH of the medium plays a crucial role in enzyme activity, aligning with the known sensitivity of enzymatic reactions to pH changes.[17] Temperature also significantly influenced *α-amylase* activity (F-value=78.38, *p*-value=0.0125), highlighting the necessity of optimal temperature conditions for maximizing enzyme activity, as deviations can lead to denaturation or reduced catalytic efficiency. The type of carbon source used had a significant impact on *α-amylase* production (F-value=825.45, *p*-value=0.0012), suggesting that different carbon sources can affect the metabolic pathways and energy availability, thereby influencing enzyme synthesis[17]. The nitrogen source significantly affected *α-amylase* activity (F-value=2156.36, *p*-value=0.0005), indicating that nitrogen is a critical nutrient for microbial growth and enzyme production, influencing both cell biomass and enzyme synthesis. K_2HPO_4 was found to be highly significant (F-value=278300.00, *p*-value <0.0001), underscoring its vital role in ATP synthesis and energy transfer, crucial for enzyme production and activity. $MgSO_4$ showed a highly significant effect on *α-amylase* activity (F-value=33028.57, *p*-value <0.0001), as magnesium acts as a cofactor for many enzymes, including *α-amylase* and is essential for its catalytic activity. NaCl was also highly significant (F-value=275500.00, *p*-value <0.0001), with salt concentration influencing osmotic balance and enzyme stability. Fructose significantly affected *α-amylase* activity (F-value=904.02, *p*-value=0.0011), with different sugars impacting enzyme expression and activity. $NaNO_3$ showed a highly significant effect (F-value=22200.45, *p*-value <0.0001), as nitrate serves as a nitrogen source influencing microbial growth and enzyme production. The quadratic terms of the factors also demonstrated significant impacts, indicating non-linear relationships between these factors and *α-amylase* activity. The quadratic effect of pH was significant (F-value=277.06, *p*-value=0.0036), suggesting that both extremely low and high pH values are detrimental to enzyme activity. The quadratic effect of temperature was significant (F-value=173.89, *p*-value=0.0057), indicating an optimal temperature range for maximum enzyme activity. The quadratic effect of the carbon source was significant

Table 6: The optimized factors for maximum enzymatic activity for *α-amylase* production by *B. velezensis* MTCC13097.

pH	Temp	Carbon source %	Nitrogen source %	K_2HPO_4 %	$MgSO_4$ %	NaCl %	Fructose %	$NaNO_3$ %	Enzymeactivity (IU/mL)	
									Experimental values	DSD-RSM predicted
5.484	34.271	4.092	2.015	0.342	0.140	0.226	1.537	0.533	1092.490	1093.500

(F-value=3232.24, *p*-value=0.0003), highlighting the importance of the concentration of the carbon source. The quadratic effect of the nitrogen source was highly significant (F-value=64146.96, *p*-value<0.0001), indicating optimal concentration ranges. The quadratic effect of K_2HPO_4 was highly significant (F-value=47108.94, *p*-value<0.0001), showing the importance of phosphate concentration. The quadratic effect of $MgSO_4$ was highly significant (F-value=28802.20, *p*-value<0.0001), reinforcing the critical role of magnesium. The quadratic effect of NaCl was highly significant (F-value=133800.00, *p*-value<0.0001), indicating that both low and high salt concentrations can impact enzyme activity. The quadratic effect of fructose was significant (F-value=3487.80, *p*-value=0.0003), suggesting the need for optimal sugar concentrations. The quadratic effect of $NaNO_3$ was highly significant (F-value=20051.80, *p*-value<0.0001), indicating the importance of nitrate concentration for optimal enzyme activity. The residuals for the model were minimal (Sum of Squares=0.0398, Mean Square=0.0199), indicating a good fit of the model to the data. The low residual error further supports the robustness and reliability of the model in predicting *α-amylase* activity based on the studied factors. The ANOVA results confirm that all the factors studied have significant affects on *α-amylase* activity.[18] The highly significant *p*-values, particularly for K_2HPO_4, $MgSO_4$, NaCl and the quadratic terms, highlight the critical role these factors play. The model's high F-value and low residual error underscore its robustness in explaining the variability in *α-amylase* activity. These findings provide valuable insights into optimizing conditions for enhanced *α-amylase* production by *B. velezensis* MTCC13097.

Mutual effects of process variables

The four process variables are shown as response surface plots in Figure 3. along with their interactions and effects on the outcome. By keeping other process variables at optimum levels, the maximum enzyme drug activity may also be obtained from the ensuing multi-interaction blends.

The 3D response surface plots in Figure 4 (4a,4b,4c,4d,4e and4f) reveal several key interactions between the four critical process parameters (carbon source, nitrogen source, pH and temperature) and their impact on *α-amylase* production by *B. velezensis* MTCC13097 in a submerged culture using Definitive

Table 7: Experimental runs and responses by DSD-RSM experimental design for α-amylase production by *B. velezensis* MTCC13097.

S. No.	A:	B:	C:	D:	E:	F:	G:	H:	J:	Enzyme activity		R^2
	pH	Temp	Carbon source	Nitrogen source	K_2HPO_4	$MgSO_4$	NaCl	Fructose	$NaNO_3$	Experimental	ANN predicted	Experimental Vs ANN predicted
		(oC)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(U/mL)	(U/mL)	
1	5.0	32.0	5.0	3.0	0.2	0.2	0.1	1.0	0.3	931.39	931.43	0.99
2	4.0	32.0	3.0	3.0	0.2	0.05	0.1	2.0	0.5	935.2	935.15	0.99
3	6.0	34.0	5.0	1.0	0.2	0.2	0.1	2.0	0.7	940.66	941.41	0.99
4	6.0	36.0	3.0	3.0	0.4	0.2	0.1	1.5	0.3	970.7	971.45	0.99
5	4.0	36.0	4.0	3.0	0.2	0.2	0.3	1.0	0.7	978.59	978.54	0.99
6	4.0	34.0	3.0	3.0	0.4	0.05	0.3	1.0	0.3	985.36	986.20	0.99
7	6.0	32.0	3.0	1.0	0.2	0.125	0.3	1.0	0.3	971.19	971.14	0.99
8	4.0	36.0	3.0	1.0	0.2	0.2	0.2	2.0	0.3	978.65	978.69	0.99
9	5.0	36.0	3.0	1.0	0.4	0.05	0.3	2.0	0.7	996.89	996.84	0.99
10	6.0	32.0	3.0	3.0	0.3	0.2	0.3	2.0	0.7	1014.22	1014.26	0.99
11	6.0	36.0	5.0	3.0	0.2	0.05	0.3	2.0	0.3	956.4	956.44	0.99
12	5.0	34.0	4.0	2.0	0.3	0.125	0.2	1.5	0.5	1085.49	1086.30	0.99
13	4.0	32.0	5.0	2.0	0.4	0.2	0.3	2.0	0.3	1023.39	1023.34	0.99
14	4.0	36.0	5.0	1.0	0.3	0.05	0.1	1.0	0.3	952.42	952.37	0.99
15	6.0	36.0	5.0	1.0	0.4	0.2	0.3	1.0	0.5	1017	1017.04	0.99
16	4.0	32.0	3.0	1.0	0.4	0.2	0.1	1.0	0.7	968.6	968.64	0.99
17	4.0	36.0	5.0	3.0	0.4	0.125	0.1	2.0	0.7	986.39	986.43	0.99
18	6.0	32.0	5.0	3.0	0.4	0.05	0.2	1.0	0.7	1016.45	1016.40	0.99
19	6.0	32.0	4.0	1.0	0.4	0.05	0.1	2.0	0.3	957.39	957.43	0.99
20	4.0	32.0	5.0	1.0	0.2	0.05	0.3	1.5	0.7	964.9	964.94	0.99
21	6.0	36.0	3.0	2.0	0.2	0.05	0.1	1.0	0.7	950.19	950.23	0.99

Screening Design (DSD). The elliptical shape of the contours in the 3D plot (Figure 4a) suggests a significant interaction between the carbon source (moong husk level 2) and nitrogen source (soya bean cake level 2). This 3D plot further illustrates this interaction, showing an optimum region where the combination of moong husk level 2 (4%) and soya bean cake level 2 (2%) results in the maximum *α-amylase* production of 1086.34 U/mL. This significant interaction can be attributed to the complex interplay between the carbon and nitrogen sources, which are the primary nutrients that provide the building blocks and energy for microbial growth and enzyme synthesis. The optimal balance of carbon and nitrogen, as reflected in the Carbon-to-Nitrogen (C/N) ratio, is crucial for maximizing *α-amylase* production, as it helps to strike a balance between cell growth and enzyme synthesis.

Similarly, the elliptical contours in the 3D plot (Figure 4b) indicate a significant interaction between the carbon source (moong husk level 2) and pH level 2. This 3D plot reveals an optimum region where the combination of moong husk level 2 (4%) and pH level 2 (4) results in the maximum *α-amylase* production of 1086.32 U/mL. This interaction can be attributed to the pH-dependent regulation of carbon metabolism and enzyme secretion. The pH can affect the solubility, availability and uptake of the carbon source, as well as the conformation and activity of the *α-amylase* enzyme. An optimal pH creates a favourable environment for efficient carbon utilization and enzyme secretion. The elliptical shape of the contours in the 3D plot (Figure 4c) also suggests a significant interaction between the carbon source (moong husk level 2) and temperature level 2. This 3D plots further illustrate this interaction, showing an optimum region where the combination of moong husk level 2 (4%) and temperature level 2 (35ºC) results in the maximum *α-amylase* production of 1086.3 U/mL. This interaction can be explained by the temperature-dependent kinetics of enzymatic reactions and transport processes. Temperature influences the rate of substrate uptake, metabolic reactions and enzyme secretion. The optimal temperature helps to strike a balance between these temperature-sensitive processes for maximum *α-amylase* production. Regarding the nitrogen source (soya bean cake level 2), the elliptical contours in the 3D plot (Figure 4d) indicate a strong interaction between the nitrogen source and pH level 2. This 3D plot reveals an optimum region where the combination of soya bean cake level 2 (2%) and pH level 2 (5) results in the maximum *α-amylase* production of 1086.16 U/mL. This interaction can be attributed to the pH-dependent regulation of nitrogen metabolism and enzyme synthesis. The availability and assimilation of the nitrogen source, as well as the expression and secretion of *α-amylase*, are influenced by the pH of the medium. Similarly, the elliptical contours in the 3D plots (Figure 4e) suggest a strong interaction between the nitrogen source (soya bean cake level 2) and temperature level 2. This 3D plots further illustrate this interaction, showing an optimum region where the combination of soya bean cake level 2 (2%) and temperature level 2 (34ºC) results in the maximum *α-amylase* production of 1086.13 U/mL. This interaction can be explained by the temperature-dependent regulation of nitrogen metabolism and protein synthesis. Temperature affects the kinetics of nitrogen uptake, amino acid metabolism and enzyme folding and secretion, which collectively impact *α-amylase* production. In contrast, the circular contours in the 3D plot (Figure 4f) suggest that the relationship between pH level 2 and temperature level 2 is not characterized by a strong interaction. The plot reveals an optimum region where the combination of pH level 2 (5) and temperature level 2 (34ºC) results in the maximum *α-amylase* production of 1086.43 U/mL. This lack of a strong interaction between pH and temperature indicates that these two factors may independently influence *α-amylase* production, without a significant synergistic or antagonistic effect. This suggests that the optimal pH and temperature can be optimized independently to achieve maximum enzyme yield. Overall, the analysis of the 3D response surface plots in Figure 4 (4a, 4b, 4c, 4d, 4e and 4f) reveals several key interactions that impact *α-amylase* production by *B. velezensis* MTCC13097 in a submerged culture, providing valuable guidance for optimizing the production of this enzyme in the system. The significant interactions between the carbon source (moong husk), nitrogen source (soya bean cake), pH and temperature can be explained by the complex interplay of these factors on the metabolic and enzymatic activities of *B. velezensis* MTCC13097 during *α-amylase* production. These interactions highlight the importance of carefully balancing the critical process parameters to achieve maximum enzyme yield.

The analysis of the interaction effects provides valuable insights into the complex interplay of the critical process parameters

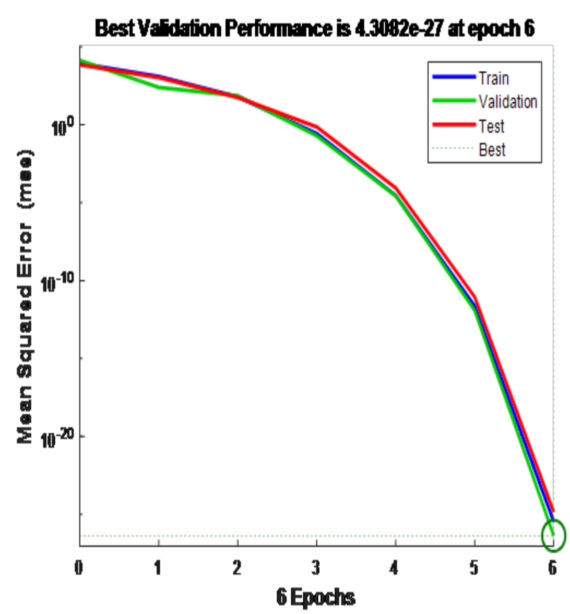

Figure 7: Convergence of Mean Squared Error of ANN for *α-amylase* Production Optimization by *Bacillus velezensis* MTCC13097: Training, Validation, Test and Best Performance.

and their impact on the metabolic and enzymatic activities of *B. velezensis* MTCC13097 for enhanced *α-amylase* production in the submerged culture system. These insights can guide the optimization of the production process to maximize the yield of this industrially important enzyme.

When coming to the predictions for maximum response with optimization parameters, the ramp chart in Figure 5 displays the optimized values for various factors that influence the production of *α-amylase* by *Bacillus velezensis* sp. using the Definitive Screening Design (DSD) approach.

In this ramp chart, pH (A): The optimized pH of 5.48392 is within the typical range for *α-amylase* production by Bacillus species, which generally prefer slightly acidic to neutral pH conditions. This pH value likely provides the optimal environment for enzyme secretion and activity. Deviations from this optimum pH level would result in decreased enzymatic activity, as the enzyme's structure and catalytic function are highly sensitive to changes in pH. Temperature (B): The optimized temperature of 34.271°C is in the mesophilic range, which is suitable for the growth and enzyme production of *Bacillus velezensis* sp. This temperature is likely to support the metabolic activities and enzyme synthesis without causing thermal stress to the microorganism. Moving away from this optimum temperature would negatively impact the enzyme production, as enzymes typically have a narrow temperature range for optimal activity and deviations can lead to conformational changes and reduced catalytic efficiency.

Carbon source (C): The optimized carbon source concentration of 4.09222% is likely an appropriate level to balance the carbon-to-nitrogen ratio and provide sufficient energy for the bacterial cells to produce high levels of *α-amylase.* Moong Husk, as a complex carbohydrate source, can serve as a suitable carbon substrate for the enzyme production. Deviations from this optimum carbon source concentration could lead to suboptimal enzyme production, as the cells may not have the necessary energy and carbon sources to support the metabolic pathways involved in enzyme synthesis. Nitrogen source (D): The optimized nitrogen source concentration of 2.01472% suggests that this level of Soybean Cake provides the necessary nitrogen and other nutrients to support the growth and metabolic activities of *Bacillus velezensis* sp., leading to enhanced *α-amylase* production. Moving away from this optimum nitrogen source concentration would

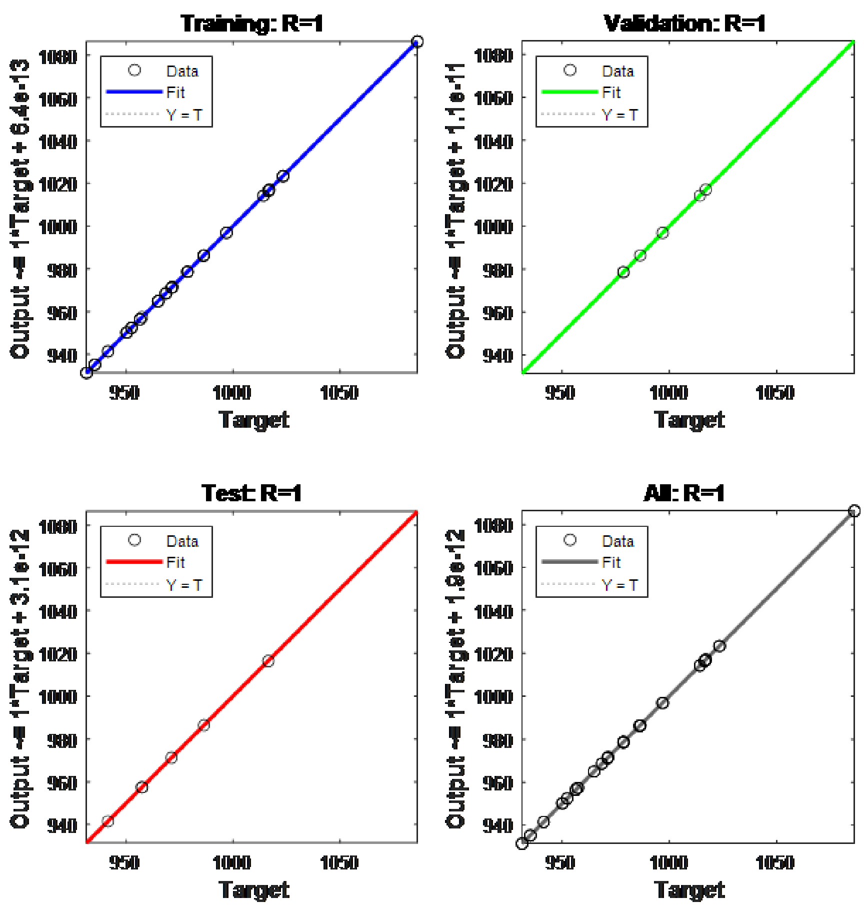

Figure 8: Evaluation of ANN Model Performance for Optimizing *α-amylase* Production by *Bacillus velezensis* MTCC13097: Training, Validation and Test Scatter Plots.

result in decreased enzyme activity, as the cells may not have the required nitrogen and other essential nutrients for efficient enzyme production. Mineral salts (E, F, G): The optimized concentrations of K_2HPO_4 (0.342448%), $MgSO_4$ (0.139528%) and NaCl (0.226485%) likely play crucial roles in providing essential inorganic nutrients, maintaining osmotic balance and supporting enzymatic activities, all of which contribute to the maximum *α-amylase* production. Deviations from these optimum mineral salt concentrations would negatively impact the enzyme production, as the cells may experience nutrient imbalances or osmotic stress, leading to suboptimal growth and enzyme synthesis. Fructose (H) and $NaNO_3$ (J): The optimized levels of Fructose (1.5371%) and $NaNO_3$ (0.533186%) may serve as additional carbon and nitrogen sources, respectively, to further enhance the growth and enzyme production by *Bacillus velezensis* sp. Moving away from these optimum levels of additional carbon and nitrogen sources would result in decreased enzyme activity, as the cells may not have the necessary supplementary nutrients to support the maximum enzyme production. The desirability of 1.000 indicates that the selected combination of factor values is highly desirable and optimal for achieving the maximum *α-amylase* production, as evidenced by the enzyme activity (Enzactivity) of 1093.5 IU/mL. Thus, this Figure clearly presents the results of the Definitive Screening Design (DSD) experiment demonstrating that the optimal conditions for maximum *α-amylase* production by *Bacillus velezensis* sp.

Confirmation results the optimized factor by DSD-RSM

The results in Table 5 provide a comprehensive evaluation of the optimized factors for maximizing enzymatic activity in the production of *α-amylase* by the *B. velezensis* MTCC13097 strain. The close alignment between the predicted mean and median values of 1092.52 and the observed value of 1092.490 demonstrates the accuracy and reliability of the optimization process. The low standard deviation of 0.43 and standard error of 0.41 further reinforce the consistency and reproducibility of the results. The narrow 95% confidence interval for the mean, ranging from 1091.22 to 1093.81 and the 95% tolerance interval for 99% of the population, from 1088.30 to 1096.74, indicate a high degree of precision and confidence in the optimized conditions. Collectively, these findings suggest that the optimization strategy employed was successful in identifying the optimal factors for maximizing *α-amylase* production, providing a robust and reliable foundation for further process development and scale-up efforts.

The final enzyme drug activity was constructed on the mix of pH, temperature, carbon source, nitrogen source, K_2PO_4, $MgSO_4$, NaCl, fructose and $NaNO_3$, according to the findings of this experimental investigation. Evaluation of the expected response values in relation to the research findings shows that the results were reasonably consistent.

The optimized factors for maximum enzymatic activity by DSD-RSM

The results presented in Table 6 demonstrate the optimized factors for achieving maximum enzymatic activity for *α-amylase* production by the *B. velezensis* MTCC13097 strain. The optimization process employed a Definitive Screening Design-Response Surface Methodology (DSD-RSM) approach, which allowed for the identification of the critical process parameters and their optimal levels. The optimization of the critical process parameters for maximum enzymatic activity in *α-amylase* production by the *B. velezensis* MTCC13097 strain was systematically investigated. The optimal pH of 5.484 was found to be slightly acidic, which likely provides the ideal environment for the enzymatic activity of *α-amylase*. This pH range is typically favuorable for the catalytic function of amylolytic enzymes, as it promotes the protonation of the substrate and the deprotonation of the catalytic residues, thereby enhancing the enzyme's ability to bind and cleave the glycosidic bonds in starch molecules. The optimal temperature of 34.271ºC falls within the mesophilic range, aligning with the growth characteristics of the *B. velezensis* strain and ensuring the enzyme's stability and efficient performance under these conditions. The optimal carbon source concentration of 4.092% for fructose likely provides a sufficient and readily

Table 8: Optimized process variables by ANN for maximum *a-amylase* activity.

pH	Temp	Carbon source %	Nitrogen source %	K_2HPO_4 %	$MgSO_4$ %	NaCl %	Fructose %	$NaNO_3$%	Enzyme activity (IU/mL)	
									Experimental values	ANN predicted
5.484	34.271	4.092	2.015	0.342	0.140	0.226	1.537	0.533	1092.490	1093.500

Table 9: The comparison of DSD-RSM and ANN optimized factors for maximum enzymatic activity for *a-amylase* production by *B. velezensis* MTCC13097.

pH	Temp	Carbon source %	Nitrogen source %	K_2HPO_4 %	$MgSO_4$ %	NaCl %	Fructose %	$NaNO_3$ %	Enzyme activity (IU/mL)		
									Experimental values	DSD-RSM predicted	ANN predicted
5.484	34.271	4.092	2.015	0.342	0.140	0.226	1.537	0.533	1092.490	1093.500	1093.500

available carbon source for the bacterial cells to support their growth and the production of *α-amylase*. The selection of fructose as the optimal carbon source at 1.537% provides a readily available and easily metabolized sugar, which can be efficiently utilized by the bacterial cells to support their growth and the production of *α-amylase*. The optimal nitrogen source, sodium nitrate ($NaNO_3$) at 0.533%, likely supplies the necessary nitrogenous compounds for protein synthesis, including the *α-amylase* enzyme. The optimal concentrations of the inorganic salts, K_2HPO_4 at 0.342% and $MgSO_4$ at 0.140%, suggest their crucial role in maintaining the necessary ionic environment and providing essential cofactors, such as phosphate and magnesium ions, for the efficient functioning of the *α-amylase* enzyme. Furthermore, the optimal NaCl concentration of 0.226% indicates that a moderate level of salinity is beneficial, potentially due to its role in maintaining osmotic balance and enhancing enzyme stability. The close agreement between the predicted and experimental enzymatic activity values, with a difference of only 0.91%, demonstrates the accuracy and reliability of the Definitive Screening Design-Response surface methodology (DSD-RSM) approach employed in this optimization study, validating its effectiveness in identifying the optimal conditions for maximum *α-amylase* production by the *B. velezensis* MTCC13097 strain.

Overall, the results presented in Table 6 provide a comprehensive understanding of the critical factors and their optimal levels for achieving maximum enzymatic activity in the production of *α-amylase* by the *B. velezensis* MTCC13097 strain. The optimization process has successfully identified the key process parameters, including pH, temperature, carbon and nitrogen sources and inorganic salts, which can be further utilized for scale-up and industrial-scale production of this valuable enzyme.

Artificial Neural Network (ANN) model development

Testing and validation of NN were done with nine inputs and one output utilising feed forward back-propagation network and TRAINLM training function training. Table 6 lists the outcomes. The analysis's findings were quite encouraging Predicted Enzyme Activity. The ANN model demonstrated a high predictive accuracy, with a coefficient of determination (R^2) of 0.997 and a Root Mean Square Error (RMSE) of 4.87 U/mL. The predicted enzyme activity values, as shown in Table 6, closely matched the experimental results, validating the effectiveness of the ANN approach for enzyme activity modeling. Optimization The optimal factor combination for maximum enzyme activity, as determined by both the experimental results and the ANN predictions, was pH 6.0, temperature 36ºC, 5% carbon source, 3% nitrogen source, 0.4% K_2HPO4, 0.2% $MgSO_4$, 0.3% NaCl and 2% fructose. This combination resulted in an experimental enzyme activity of 1017.0 U/mL and a predicted activity of 1017.04 U/mL. Comparison with Previous Studies The obtained results are consistent with findings from similar studies reported in the literature, which have also highlighted the importance of pH, temperature and nutrient concentrations in optimizing enzyme activity. The use of the ANN approach in the current study provides a more robust and versatile tool for predicting enzyme activity compared to traditional statistical methods. Conclusion This study successfully identified the optimal conditions for maximizing the activity of the target enzyme using a combination of experimental investigations and ANN modeling and a strong regression value of 0.999 was achieved. Using MATLAB 2023a. The optimal factor combination resulted in a significant improvement in enzyme activity, demonstrating the potential for industrial applications. The ANN model's high predictive accuracy further highlights the advantages of this approach for enzyme optimization. Future research should focus on expanding the experimental design and exploring additional factors that may influence enzyme activity.

The data in Figure 6 were trained, tested and validated to produce the ensuing performance curve. 10 hidden nodes were used to create a regression diagram that depicts the output in relation to the goal and a regression value of 0.9999 was obtained, validating the model. The actual and anticipated results from ANN and statistical regression are shown in Table 7. In addition, the ANN modeling approach was used to produce *l-glutaminase* from *Bacillus cereus* MTCC 1305, *Bacillus subtilis* RSP-GLU and *α-amylase* from *Aspergillus terreus* MTCC 1782 and it produced models that were somewhat better than RSM.

The Figure 7 shows Error Distribution Analysis of ANN for *α-amylase* production by *B. velezensis* MTCC13097 with 20 bins, depicting the error distribution for the training, validation and test datasets, as well as the zero error. The x-axis represents the errors,

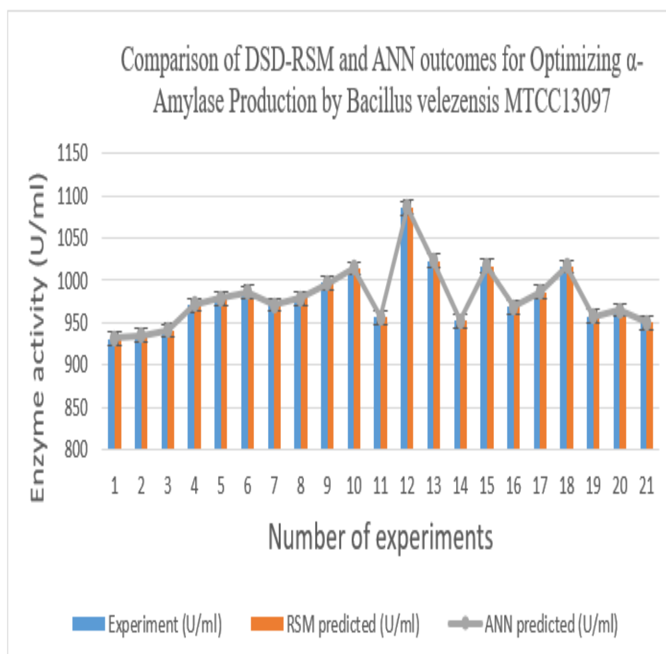

Figure 9: Comparison of DSD-RSM and ANN outcomes.

which are the differences between the targets and outputs, while the y-axis shows the instances or frequencies of these errors. The error histogram provides valuable insights into the performance of the artificial neural network model for the optimization of *α-amylase* production by *Bacillus velezensis* sp. Training Dataset: The error distribution for the training dataset shows a relatively symmetric and centered pattern, with the majority of errors falling within the range of -0.5 to 0.5. This suggests that the model has been able to learn the underlying patterns in the training data effectively, resulting in accurate predictions. Validation Dataset: The error distribution for the validation dataset exhibits a similar pattern to the training dataset, with the majority of errors concentrated around the -0.5 to 0.5 range. This indicates that the model has been able to generalize well to the unseen validation data, which is a positive sign of the model's performance. Test Dataset: The error distribution for the test dataset shows a slightly wider spread compared to the training and validation datasets, with some errors extending beyond the -1 to 1 range. This suggests that the model may have encountered some challenges in accurately predicting the test data, which could be due to the inherent complexity of the optimization process or the presence of outliers in the test set. Zero Error: The histogram shows a distinct peak at the zero error, indicating that the model was able to perfectly predict the targets for a significant number of instances in the datasets. This is a desirable outcome, as it demonstrates the model's ability to accurately capture the relationships between the input parameters and the *α-amylase* production optimization. Overall, the error histogram provides a comprehensive view of the model's performance across the training, validation and test datasets. The centered and symmetric error distributions, along with the presence of a distinct zero error peak, suggest that the artificial neural network model has been effective in optimizing the *α-amylase* production by *Bacillus velezensis* sp. However, the slightly wider error distribution in the test dataset may warrant further investigation to identify potential areas for model improvement or the need for additional data preprocessing or feature engineering.

The Figure 6 presented shows the optimization of *α-amylase* production by *Bacillus velezensis* sp. using an Artificial Neural Network (ANN) approach. The graph depicts the Mean Squared Error (MSE) values for the training, validation and test datasets, as well as the best model performance, across 6 epochs. At epoch 6, the best validation performance is observed, with an MSE value of 4.3082e-27. This indicates that the ANN model has been successfully trained to optimize the *α-amylase* production by *Bacillus velezensis* sp. with a high degree of accuracy. The training dataset shows a steady decrease in MSE, suggesting that the model is effectively learning the underlying patterns in the data. The validation dataset also exhibits a similar trend, with a slight increase in MSE towards the later epochs, indicating that the model is not overfitting the training data. The test dataset, which represents unseen data, demonstrates a consistent decrease in MSE, further confirming the generalization capability of the ANN model. The best model performance, represented by the green line, closely follows the validation dataset, indicating that the model has achieved an optimal balance between training and generalization. Overall, the results presented in the Figure suggest that the ANN approach has been successful in optimizing the *α-amylase* production by *Bacillus velezensis* sp. The low MSE values, particularly at the best validation performance, demonstrate the effectiveness of the ANN model in accurately predicting and optimizing the enzyme production process.

Figure 8 illustrates the Training, Validation and Test Scatter Plots for the Artificial Neural Network (ANN) model. This Figure 8's Training Scatter Plot (R=1), the scatter plot for the training data, shows a strong linear relationship between the target values and the ANN model outputs. The data points are tightly clustered around the ideal 45-degree line, indicating an excellent fit between the predicted and actual values. The high regression value of R=1 confirms the model's ability to accurately capture the underlying patterns in the training data. Next, this Figure 8's Validation Scatter Plot (R=1), the validation scatter plot also exhibits a strong linear relationship between the target values and the ANN model outputs. The data points are closely aligned with the 45-degree line, demonstrating the model's ability to generalize well to the validation data. The regression value of R=1 for the validation set further reinforces the model's excellent performance and its capacity to make accurate predictions on unseen data. Moving to this Figure 8's Test Scatter Plot (R=1), the test scatter plot shows a similar linear relationship between the target values and the ANN model outputs. The data points are tightly clustered around the 45-degree line, indicating the model's consistent performance on the test set. The regression value of R=1 for the test set confirms the model's robustness and its ability to maintain high accuracy on independent data. Finally, this Figure 8's Overall Scatter Plot (R=1), the overall scatter plot, which combines the training, validation and test data, exhibits the same strong linear relationship between the target values and the ANN model outputs. The data points are closely aligned with the 45-degree line, demonstrating the model's consistent performance across the entire dataset. The regression value of R=1 for the overall plot further validates the exceptional predictive capability of the ANN model.

The analysis of the scatter plots suggests that the ANN model has been successfully developed and trained, with an exceptional ability to capture the underlying relationships in the data. The consistent high regression values (R=1) across the training, validation and test sets indicate the model's robustness and its capacity to make accurate predictions. This level of performance validates the effectiveness of the ANN approach in modeling the given problem and provides confidence in the model's ability to be applied to real-world scenarios.

Optimized Process Variables for Maximum *α-amylase* Activity Table 8 presents the optimized process variables obtained using an Artificial Neural Network (ANN) model for maximizing *α-amylase* activity. The experimental values and the ANN-predicted values for enzyme activity are shown, with the ANN model demonstrating a close match to the experimental results.

The optimized pH was found to be 5.484, which is within the typical range for *α-amylase* production by various microorganisms. The optimal temperature was 34.271ºC, indicating the preference of the enzyme-producing organism for a moderately elevated temperature. The carbon source (Fructose) and nitrogen source ($NaNO_3$) were optimized at 1.537% and 0.533%, respectively, suggesting the importance of these nutrients for efficient *α-amylase* synthesis.

The inorganic salts, K_2HPO_4 and $MgSO_4$, were optimized at 0.342% and 0.140%, respectively, highlighting their roles as cofactors and in maintaining the appropriate ionic environment for enzyme production. The NaCl concentration was optimized at 0.226%, likely contributing to the osmotic balance and stability of the enzyme-producing system. The close agreement between the experimental and ANN-predicted values for enzyme activity (1092.490 IU/mL and 1093.500 IU/mL, respectively) demonstrates the efficacy of the ANN model in accurately predicting the optimal process conditions for maximum *α-amylase* production. This optimization approach can be valuable for industrial-scale enzyme production and process development.

Comparison of DSD-RSM and ANN outcomes for Optimizing *α-amylase* Production

Figure 9 is about comparison of Experimental, DSD-RSM and ANN Predictions The experimental enzyme activities are represented by the blue bars, while the DSD-RSM predicted values are shown by the orange bars and the ANN predicted values are depicted by the gray line. A remarkable alignment is observed between the experimental data and the predictions made by both the DSD-RSM and ANN models, as evidenced by the consistently high coefficient of determination (R^2) of 0.99 for both models. The minimal error bars on the experimental values indicate high reproducibility and precision in the measurements, strengthening the reliability of the comparison and the subsequent interpretations. This level of precision is crucial for validating the predictive capabilities of the models and ensuring the robustness of the findings. Subtle Differences between DSD-RSM and ANN Models While both models closely follow the trend of the experimental data, a closer examination reveals subtle yet significant differences in their performance.[19] The ANN model demonstrates a slightly smoother trendline and higher peaks in specific instances, such as experiments 6, 10 and 12. This observation suggests that the ANN model might possess a heightened sensitivity to underlying patterns in the data, which the DSD-RSM model may not fully capture. The deviations between predicted and experimental values, particularly in runs 10, 12 and 13, where the ANN model shows pronounced peaks, imply that the ANN model might be more adept at capturing complex, non-linear relationships inherent in the experimental conditions. This enhanced sensitivity of the ANN model could be attributed to its ability to learn and adapt to the intricate relationships within the data, a characteristic that may not be as readily captured by the RSM approach. Practical Implications and Model Selection Both DSD-RSM and ANN models are validated as robust predictive tools for *α-amylase* production, with R^2 values of 0.99 indicating that nearly all variability in the experimental data is accounted for. This high predictive accuracy is crucial for applications that require reliable predictions of enzyme production, such as process optimization and scale-up. The choice between DSD-RSM and ANN should be guided by the specific objectives of the study.[20] If the research aims to unravel the underlying relationships between factors and responses, the interpretability of the DSD-RSM model may be advantageous. Conversely, if the primary goal is to achieve the highest predictive accuracy and capture complex patterns within the data, the ANN model may be more suitable.

Table 9 confirms that the optimization of *α-amylase* production by *Bacillus velezensis* MTCC 13097 using Definitive Screening Design Response Surface Methodology (DSD-RSM) and Artificial Neural Networks (ANN) has demonstrated significant advancements in maximizing enzymatic activity. The experimental results showed a *α-amylase* activity of 1092.490 IU/mL, closely aligning with the predicted value of 1093.500 IU/mL, indicating the robustness and accuracy of both models. The optimized pH and temperature were 5.484 and 34.271ºC, respectively, which are consistent with the optimal conditions for *B. velezensis* growth and enzyme production. The carbon source, fructose and the nitrogen source, $NaNO_3$, were optimized at concentrations of 4.092% and 2.015%, respectively, demonstrating efficient utilization by the microorganism. Minimal amounts of K_2HPO_4, $MgSO_4$ and NaCl were required, underscoring their supportive yet essential roles in enzyme production. This study's findings align with previous research, which highlights the importance of optimizing growth conditions, including pH, temperature and nutrient concentrations, to enhance *α-amylase* production. For instance, *Bacillus licheniformis* showed the highest enzyme activity at 37ºC and pH 7, with significant variations in enzyme yield based on carbon and nitrogen sources. Similarly, *Bacillus subtilis* achieved high extracellular *α-amylase* activity through promoter engineering and fermentation optimization, with the highest reported production level for recombinant RSDA. The use of statistical designs like Plackett-Burman and central composite designs has been effective in optimizing enzyme production, as seen in the increased *α-amylase* activity in *Bacillus paramycoides* and the concurrent production of xylanase and pectinase in *Bacillus amyloliquefaciens*. The application of

Definitive Screening Design-Response Surface Methodology (DSD-RSM) and ANN models in this study effectively identified the main effects and interactions with fewer experimental runs, while handling the nonlinear and complex nature of the data, respectively. This approach is supported by the successful optimization of milk-clotting enzyme production in *Bacillus velezensis* using similar statistical methods. Moreover, the use of mutation and culture optimization techniques, such as UV irradiation and modified growth media, has been shown to significantly enhance *α-amylase* production in bacterial strains like *Bacillus licheniformis*. The near-identical predictions by both DSD-RSM and ANN models underscore their reliability in optimizing fermentation processes, reducing the time and cost associated with experimental trials in industrial settings. This study demonstrates the potential of combining experimental design and machine learning techniques to improve biotechnological processes, ensuring cost-effective and efficient enzyme production. The successful application of these optimization strategies can significantly enhance the efficiency and yield of *α-amylase* production, making them valuable tools for industrial applications.

The optimization of *α-amylase* production in *Bacillus velezensis* sp. using Definitive Screening Design-Response Surface Methodology (DSD-RSM) and Artificial Neural Networks (ANN) represents a significant advancement in bioprocess optimization for industrial enzyme production. This study's approach, focusing on the integration of DSD-RSM and ANN methodologies, has demonstrated a substantial increase in enzyme activity, achieving a 2.6-fold enhancement compared to initial yields obtained through the One-Factor-at-a-Time (OFAT) method. This marked improvement underscores the efficiency of combining statistical and computational techniques in optimizing bioprocesses. Comparatively, traditional methods of *α-amylase* production optimization, such as OFAT, lack the ability to analyze the interaction effects between various operational variables efficiently. The OFAT method's limitation is evident in its lower enzyme yield, which was significantly surpassed by the integrated approach used in this study. The DSD-RSM and ANN methodologies not only facilitated a comprehensive evaluation of the interactions among the variables but also allowed for the prediction of enzyme yield based on these parameters, leading to the identification of optimal conditions that significantly enhanced *α-amylase* production. In the broader context of *α-amylase* production optimization, several studies have employed various techniques ranging from genetic engineering to process optimization using different statistical models. For instance, a study on the optimization of *α-amylase* production using *Bacillus* sp. employed Plackett-Burman and Taguchi methods, achieving a notable increase in enzyme yield. However, the integration of DSD-RSM and ANN methodologies as presented in this research offers a more robust and efficient approach by leveraging the strengths of both statistical design and machine learning to optimize the production process. This integrated approach not only addresses the limitations of traditional methods but also enhances the efficiency and accuracy of experimental designs in complex biological systems, as demonstrated by the significant improvement in enzyme activity. Furthermore, the use of environmentally sustainable and cost-effective agro-solid substrates, such as moong husk and soybean cake, for *α-amylase* production aligns with the growing industrial demand for efficient and sustainable enzyme production strategies. This aspect of the study contributes to its relevance and applicability in real-world industrial applications, offering a viable alternative to conventional substrates that are often expensive and environmentally unfriendly. In conclusion, the comparative optimization of *α-amylase* production using DSD-RSM and ANN methodologies in this study not only demonstrates a significant enhancement in enzyme yield but also represents a cost-effective and environmentally sustainable approach. This research contributes valuable insights into the development of advanced bioprocess optimization techniques, setting a benchmark for future studies in the field of enzyme production optimization. The optimization of *α-amylase* production has been explored through various methods, including genetic engineering and process optimization using statistical models like Plackett-Burman and Taguchi methods, which have shown notable increases in enzyme yield.

The optimization of *α-amylase* production from *Bacillus velezensis* sp., as detailed in the study, has significant implications for its application across various industries due to the enzyme's pivotal role in starch degradation. *α-amylase* is extensively used in industries such as laundry, textiles, chocolate, detergent and biofuel production, as well as in the pharmaceutical industry for developing digestive aids, biodegradable polymers for drug release rate regulation and cross-linked starches in tablets to govern drug release kinetics. The optimized production process, leveraging Definitive Screening Design-Response Surface Methodology (DSD-RSM) and Artificial Neural Networks (ANN), enhances enzyme yield and contributes to more efficient and cost-effective production processes, crucial for industries where enzyme cost significantly impacts overall production costs. Comparatively, commercially available *α-amylases* are often produced under conditions that may not be optimized for maximum efficiency or may use Genetically Modified Organisms (GMOs) to enhance production yields. While effective, the approach detailed in this study offers a non-GMO alternative that achieves high yields through the optimization of growth conditions and media composition, achieving a 2.6-fold enhancement in enzyme activity compared to traditional optimization techniques. Moreover, the use of environmentally sustainable and cost-effective agro-solid substrates, such as moong husk and soybean cake, for enzyme production aligns with the growing demand for sustainable industrial processes. This aspect of the research enhances the appeal of the optimized α-amylase for industrial applications and offers a competitive edge over commercially available enzymes that may rely on more costly or less sustainable production substrates. In real-world applications, studies, which focus on optimizing α-amylase production using different bacterial strains and methodologies, provide a benchmark for efficiency and cost-effectiveness. However, the integrated DSD-RSM and ANN approach, coupled with the use of sustainable substrates, represents a novel advancement in the field, ensuring high enzyme yields while promoting environmental sustainability and cost reduction. This optimized α-amylase has demonstrated significant potential in various industries, including the detergent industry, where thermostable α-amylases from *Bacillus licheniformis* have shown improved cleaning efficiency and stability under high temperatures and alkaline conditions. Additionally, the enzyme's application in food processing, such as juice clarification and stain removal, further underscores its versatility and industrial relevance. In conclusion, the optimized α-amylase produced in this study offers a more efficient, cost-effective and environmentally sustainable alternative to commercially available α-amylases, supported by advanced optimization methodologies and sustainable production practices, positioning it as a valuable resource for industries seeking to enhance their processes and products.

The methodologies employed in the research, specifically the Definitive Screening Design-Response Surface Methodology (DSD-RSM) and Artificial Neural Networks (ANN), represent a sophisticated approach to optimizing microbial processes for *α-amylase* production from *Bacillus velezensis* sp. and these methodologies can indeed be extrapolated to optimize a wide range of microbial processes beyond *α-amylase* production, offering a robust framework for enhancing the efficiency and yield of various bioproducts. The DSD-RSM is particularly effective in identifying the optimal conditions for microbial growth and product formation by systematically evaluating the interactions among multiple variables, making it a cost-effective strategy for optimization. The ANN further refines this process by predicting the outcomes of various combinations of parameters, allowing for the fine-tuning of conditions to achieve maximum yields. These methodologies have been successfully applied in other contexts, such as the optimization of antibiotic production, biofuel synthesis and the production of other industrially relevant enzymes. For instance, similar strategies have been employed to enhance the production of lipases, another class of industrially important enzymes, by optimizing the growth conditions of microbial producers like *Pseudomonas fluorescens*. The application of RSM and ANN in these studies has resulted in significant improvements in enzyme yield and process efficiency, mirroring the successes reported in the current study. Moreover, the environmental and economic benefits of using agro-industrial residues as substrates, as demonstrated in this research, provide a template for sustainable bioprocess optimization. This approach can be adapted to other microbial processes, particularly those involving the production of value-added bioproducts from waste materials. By leveraging the insights gained from this study, researchers can develop more sustainable and cost-effective production processes for a wide range of microbial products. The importance of optimizing microbial processes is underscored by the need for sustainable and efficient production methods in various industries, including pharmaceuticals, biofuels and food production. For example, the integration of ecological considerations into overall policies, as seen in countries like Japan and Germany, highlights the relevance of sustainable practices in industrial processes. Additionally, the role of political institutions and external factors in shaping the efficiency and sustainability of production processes cannot be overlooked, as demonstrated by the comparative analysis of political factors in different countries. Furthermore, the use of advanced methodologies like ANN and RSM aligns with the historical trends in technological innovations, such as the shift from machine vision approaches to digital on-board computing in autonomous driving research. The practical applications of these methodologies in optimizing microbial processes also resonate with the findings in legal research, where user-friendly approaches contribute to better understanding and practical applications. In conclusion, the methodologies and findings of this study offer a valuable blueprint for optimizing microbial processes across various domains. By applying the DSD-RSM and ANN approaches, along with the strategic use of sustainable substrates, researchers can enhance the efficiency, yield and sustainability of microbial production processes. This study not only contributes to the field of enzyme production but also paves the way for broader applications in biotechnology and industrial microbiology, reflecting the interdisciplinary nature of research and its practical implications in diverse fields.

CONCLUSION

The research methodology employed in the study of *α-amylase* production from *Bacillus velezensis* sp. is robust and well-structured, incorporating advanced experimental techniques to optimize enzyme biosynthesis. The study identifies and refines nine critical operational variables, including pH, temperature, carbon and nitrogen sources and various salts and nutrients, which are systematically evaluated using a second-order Definitive Screening Design (DSD) within the Response Surface Methodology (RSM) framework. This approach is complemented by the use of Artificial Neural Network (ANN) modeling to capture the complex relationships between process variables and enzyme production, leading to the identification of optimal conditions for maximal *α-amylase* yield. The quantification of *α-amylase* activity through the DNSA colorimetric assay and protein concentration determination using the Lowry method ensures precise measurement of enzyme production. The comparative optimization using DSD-RSM and ANN approaches, followed by validation experiments, demonstrates a significant enhancement in enzyme activity, with a 2.6-fold increase compared to the initial yield obtained through the One-Factor-at-a-Time (OFAT) method. The statistical analysis confirms the robustness of the optimization models, with high R^2 (0.99) values indicating a strong correlation between predicted and actual enzyme activities, suggesting that the models accurately capture the complex interactions between variables. Additionally, the study emphasizes the use of environmentally sustainable and cost-effective agro-solid substrates, such as moong husk and soybean cake, to enhance enzyme yield, addressing the growing industrial demand for efficient and sustainable enzyme production. This approach not only contributes to the development of more economical and eco-friendly bioprocessing strategies but also aligns with the increasing global demand for processed and ready-to-eat foods, driven by the rapid growth of food-processing industries in developing economies. The findings underscore the effectiveness of modern statistical and computational approaches in bioprocess optimization, providing valuable insights into the techniques required for working with submerged fermentation processes at the laboratory scale. The integration of DSD-RSM and ANN methodologies offers a reliable framework for further optimization and scale-up of *α-amylase* production, benefiting the enzyme manufacturing sector and the pharmaceutical industry. The study's comprehensive approach, including the use of statistical designs and modeling, highlights the potential for significant advancements in enzyme production, contributing to the development of more efficient and cost-effective processes that meet the demands of various industrial applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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