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OPTIMIZATION OF AMYLASE PRODUCTION BY BACILLUS AMYLOLIQUEFACIENS USING POTATO PEEL POWDER

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ABSTRACT

The current study was conducted to improve the production of amylase using Bacillus amyloliquefaciens using both traditional and statistical methods. A central composite design with 20 experiments was utilized to optimize three independent variables, which were selected using the one factor at a time (OFAT) method, to achieve maximum amylase production. The chosen factors, when utilized with this statistical optimization method, demonstrated 3 times increases in amylase production, in comparison to the OFAT technique. The analysis of variance (ANOVA) indicated a high coefficient of determination (R2) and was statistically significant ($p < 0.05$). The three-dimensional response graph revealed an interdependent interaction among the key variables.

Keywords: Amylase, Statistical Optimization, Bacillus, Food Industry

1. INTRODUCTION

Amylases are a category of enzymes that break down starch molecules by hydrolyzing α-1,4 glycosidic bonds, resulting in dextrin and various monomer products. These amylolytic enzymes play a crucial role in numerous biotechnological applications, including in the pharmaceutical, food, textile, fiber, detergent, brewing, oil drilling, and paper industries. The production of amylase (EC 3.2.1.1) from microbial sources has attracted significant interest from researchers due to the potential for large-scale production and the ease of manipulating microorganisms to generate enzymes with specific characteristics. Additionally, bacterial amylases are widely utilized in industries because of their robustness, high enzymatic activity under diverse conditions, and cost-effective production [1].

Members of the *Bacillus* genus are recognized for their ability to produce a diverse range of extracellular enzymes, with amylases being particularly significant in industrial applications. *Bacillus* species are commercially valuable microorganisms that have been utilized for their short fermentation cycles, dependable performance, safe handling, environmentally friendly attributes, ease of manipulation, and increased enzymatic activity under extreme conditions, economical enzyme production, and capacity to release secondary metabolites into the medium. Although various *Bacillus* species share similar growth patterns, the production of amylases varies based on the components of the medium, physical factors, and the specific strain used. There is a need to improve amylase production owing to its

extensive applications in multiple biotechnological fields. The enhancement of enzyme production can be achieved by fine-tuning the medium ingredients along with other key parameters. Recent research has concentrated on developing the process and scaling up the production of amylase by optimizing the necessary factors for bacterial growth in submerged fermentation (SmF)[2, 3]. SmF is often preferred over solid state fermentation (SSF) for cultivating bacteria aimed at enzyme production due to the ease of product purification. Generally, the production of amylase through SmF offers simplicity in sterilization and process management. Furthermore, SmF makes use of liquid substrates that flow freely, allowing bioactive components to be released into the fermentation medium. Importantly, SmF allows for a more extensive use of genetically modified organisms compared to SSF because of its controlled physical parameters. Recently, several studies have demonstrated the effective use of SmF for producing amylase from *Bacillus* sp. in a cost-efficient manner [4]. The "one factor at a time" (OFAT) approach for process optimization is not only laborious but can also lead to data misinterpretation due to the extensive number of experiments that must be conducted. The drawbacks associated with the OFAT method can be addressed by employing response surface methodology (RSM). RSM is a statistical model used to optimize the fermentation process to enhance enzyme yield by integrating all the factors involved in the experimental analysis [5].

RSM focuses on experimental approaches, model development, and statistical techniques to establish a relationship between a response variable and the design variables. The use of RSM in optimization research decreases the number of required experiments and creates a model that demonstrates how both dependent and independent variables, along with their interactions, affect the various responses. Given that the rate of enzyme production differs among bacterial strains, it is crucial to create a statistical model to optimize the fermentation process related to increased amylase production. Recently, various *Bacillus* species, including *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. polymyxa*, and *B. megaterium*, have been identified as significant producers of amylase [6]. Though there is still limited research focused on producing thermo-alkali stable amylase from Bacillus species, the increasing demand for amylase in biotechnological applications highlights the need to manufacture the enzyme on a large scale while minimizing costs and time. Considering the wide range of applications and significant requirement for thermo-alkali-tolerant amylase in industrial settings, particularly in the food sector, this study was conducted to optimize the production of amylase from Bacillus sp. under ideal conditions using Response Surface Methodology (RSM) [7]. Central composite design was selected to investigate the impact of three key variables—pH, agitation rate, and incubation period on the enzyme production.

2. MATERIALS AND METHODS

2.1. SUBSTRATE

Potato peel powder was used as the substrate for amylase production. Briefly, waste potato peels were sourced from the restaurant of Kerala, India. The potato peels were thoroughly washed to eliminate any dust, then sun-dried, followed by drying in an oven at 80 °C until they reached a constant weight. The dried peels were ground into fine particles (0.5 mm) using an electric grinder and stored in airtight containers for use in subsequent experiments.

2.2. INOCULUM PREPARATION

Bacillus amyloliquefaciens (*MTCC 1207) was obtained from microbial type culture collection, India.* A nutrient broth (50 mL, pH—7.0) was prepared in a 250-mL conical flask. After sterilization, the broth was allowed to cool. Under sterile conditions, a loopful of bacteria was aseptically added to the medium. The culture was then incubated overnight at 37 °C with an agitation speed of 150 rpm. Then, it was used as inoculums

2.3. AMYLASE PRODUCTION

To produce amylase, 250 ml flasks were utilized, and each Erlenmeyer flask was filled with 50 ml of potato peel medium (composed of 1% potato peel powder, 1% yeast extract, and a pH of 7.0). The media-filled flasks underwent autoclaving at 121 °C and 15 psi for 15 minutes. After autoclaving, the flasks were allowed to cool to room temperature. Under aseptic conditions, the flasks containing the media were inoculated with an inoculum size of 0.5%. Following inoculation, the flasks were kept at 28 °C with an agitation speed of 130 rpm for 24 hours. At the conclusion of the fermentation period (24 hours), the flasks were centrifuged for 10 minutes at 10,000g and 4 °C. The clear supernatant was utilized as the source of crude enzyme.

2.4. ENZYME ASSAY

The reaction mixture was composed of 1.0 ml of the supernatant, to which 1.0 ml of 1% soluble starch was added, and then incubated at 30°C for 30 minutes. Following the incubation, 0.5 ml of DNS reagent was introduced and placed in boiling water for5 minutes. The absorbance was recorded at 540 nm using a spectrophotometer. One unit of enzyme activity is defined as the quantity of enzyme that releases 1 µmol of glucose per ml per minute under standard assay condition.

2.5. OPTIMIZATION OF AMYLASE PRODUCTION BY THE TRADITIONAL ONE-VARIABLE-AT-A-TIME APPROACH

2.5.1. EFFECT OF CARBON SOURCES ON AMYLASE PRODUCTION

The production of amylase was assessed by cultivating the isolate in a potato peel medium (1%) that included different carbon sources such as glucose, starch, xylose, sucrose, and mannose. Each carbon source was added to the production media at a concentration of 0.5% (w/v) individually. All the inoculated flasks were kept at 37 °C for 24 hours. The quantitative assay for extracellular amylase utilized the cell-free supernatant from the culture.

2.5.2. EFFECT OF DIFFERENT NITROGEN SOURCES ON AMYLASE PRODUCTION

To evaluate the impact of various nitrogen sources on amylase production, the production medium was individually inoculated with different organic and inorganic nitrogen sources, including peptone, yeast extract, beef extract, potassium nitrate, and ammonium sulphate, at a concentration of 0.25% (w/v).

2.5.3. EFFECT OF PH ON AMYLASE PRODUCTION

To investigate the effect of pH on enzyme production, the media's initial pH was set between 5.0 and 10.0 prior to sterilization with 1N HCl and 1N NaOH. A 1% (w/v) concentration of potato peel medium was utilized as the carbon and nitrogen sources. Enzyme activities in each flask were measured as previously described after a 24-hour incubation period at 37 °C.

2.5.4. EFFECT OF TEMPERATURE ON AMYLASE PRODUCTION

To evaluate the effect of temperature on amylase production from the isolate, the inoculated potato peel medium containing carbon, and nitrogen sources at different temperatures (25–45 °C) for 24 h with constant agitation at 120 rpm. pH of each inoculated flask was maintained at 7.0. The enzyme activities were determined as described earlier.

2.5.5. EFFECT OF SHAKER SPEED ON AMYLASE PRODUCTION

To investigate how shaker influences speed enzyme production, the flask was kept on shaker for 80 to 130 rpm. A 0.5% (w/v) concentration of carbon and nitrogen source was utilized as the carbon and nitrogen sources, respectively. Enzyme activities in each flask were measured as previously described after a 24-hour incubation period at 37 °C.

2.5.6. EFFECT OF FERMENTATION PERIOD ON AMYLASE PRODUCTION

To investigate the effect of fermentation period on amylase production, the culture was incubated for 96 h at 30 ºC. Enzyme activities in each flask were measured as previously described for every 24 h interval.

2.6. OPTIMIZATION OF VARIOUS FACTORS FOR AMYLASE PRODUCTION USING RESPONSE SURFACE METHODOLOGY

Following the previous findings from the OFAT method, a statistical approach known as central composite design was utilized to optimize the key independent variables (pH, agitation speed, and incubation time) in order to enhance

amylase production, while keeping the carbon and nitrogen sources constant. Central composite design is a technique for optimizing a limited number of variables that estimates the best-fit parameters for quadratic models. It identifies not only the lack of fit within the model but also develops a sequential design for response surface methodology. The experimental framework consisted of 20 trials involving three variables (A, B, and C) at three levels (−1, 0, +1) to optimize the components of the medium. The coded values of −1 and +1 indicate the low and high levels of the variables based on prior experiments. All variables were maintained at a central coded value of zero.

Table 1 Presents The Experimental Strategy For The Selected Factors In Both Actual And Coded Formats.

Factor	Name	Units	Type	Low Actual	High Actual
A	pH		Numeric	6	8
B	Incubation time	h	Numeric	24	72
C	Agitation speed	rpm	Numeric	75	150

3. RESULTS

3.1. EFFECT OF CARBON SOURCE ON ENZYME PRODUCTION

The investigation into the influence of various carbon sources on the extracellular amylase production from *B. amyloliquefaciens* indicated that xylose served as the most effective inducer of amylase synthesis (72.5 ± 1.1 U/mL), followed by sucrose (60.3 ± 1.5 U/mL) (Fig. 1).

Figure 1

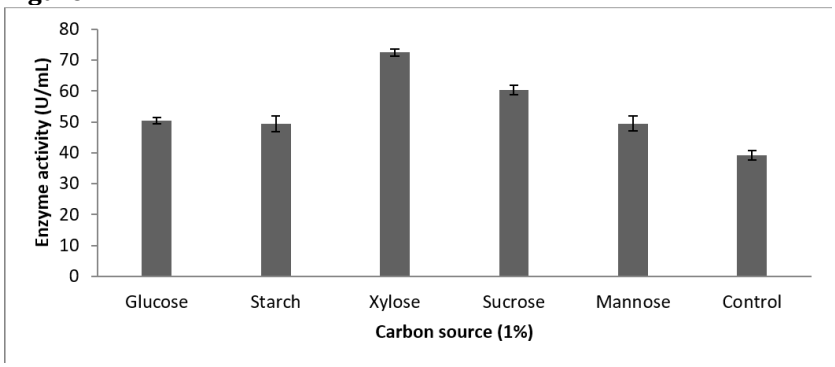

Fig. 1. Effect of Carbon Source on Amylase Production Using Potato Peel Medium.

3.2. EFFECT OF NITROGEN SOURCE ON ENZYME PRODUCTION

Amylase production was assessed in the presence of different organic and inorganic nitrogen sources. Adding potassium nitrate to the medium resulted in the highest enzyme productivity (78.1 ± 1.9 U/mL) (Fig. 2).

Figure 2

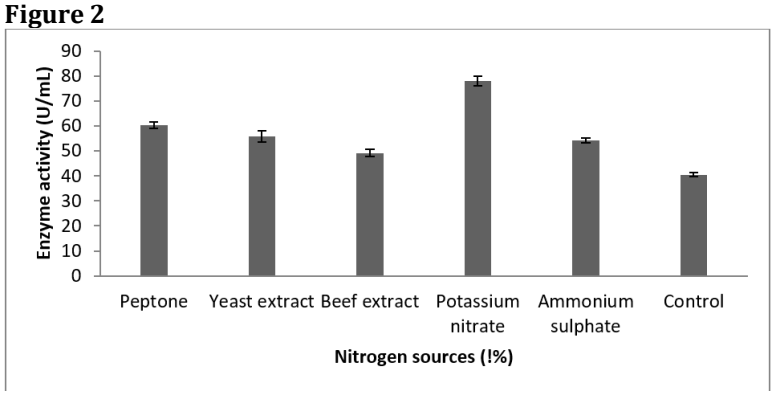

Fig. 2. Effect of Nitrogen Sources on Amylase Production Using Potato Peel Medium.

3.3. EFFECT OF PH ON AMYLASE PRODUCTION

The pH of the production medium, varying from 5 to 10, had a substantial impact on amylase activity. A neutral pH (7.0) was identified as the optimal condition for achieving the maximum enzyme productivity (80.4 ± 1.5 U/mL). Amylase activity showed a significant decrease at higher alkaline pH levels (Fig. 3).

Figure 3

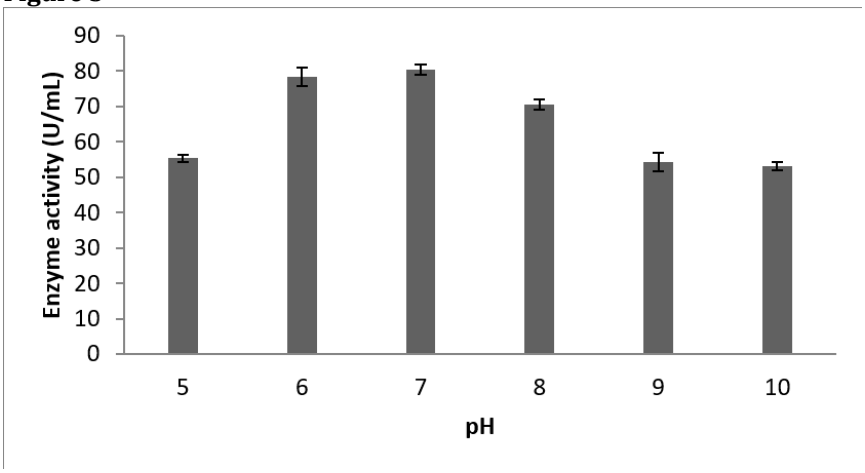

Fig. 3. Effect of Ph on Amylase Production Using Potato Peel Medium.

3.4. EFFECT OF TEMPERATURE ON ENZYME PRODUCTION

Temperature is a key physical factor that influences enzyme production. *Bacillus* demonstrated the highest enzyme titer (90.4 ± 1.9 U/mL) at 30 °C. An increase in temperature to 35 and 45 °C resulted in inhibited amylase production, indicating the organism's mesophilic nature (Fig. 4).

Figure 4

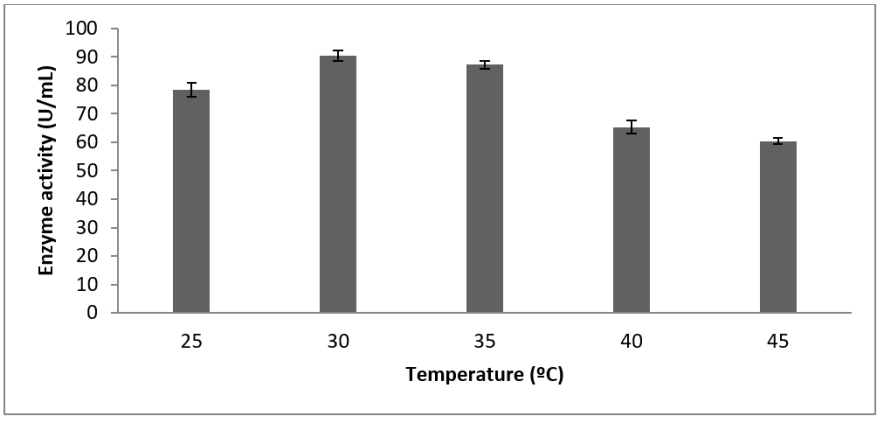

Fig. 4. Effect of Temperature on Amylase Production Using Potato Peel Medium.

3.5. EFFECT OF SHAKER SPEED ON AMYLASE PRODUCTION

In this study, amylase production was found to be high at 110 rpm and it declined at higher shaker speed (Fig. 5).

Figure 5

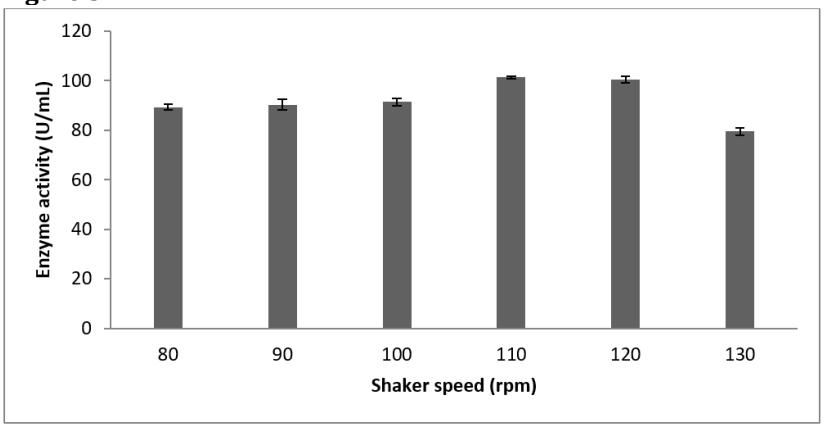

Fig. 5. Effect of Agitation Speed on Amylase Production Using Potato Peel Medium.

3.6. EFFECT OF FERMENTATION PERIOD ON AMYLASE PRODUCTION

Fermentation period is one of the important factors influencing on enzymes production. In this study, amylase production was maximum after 48 h incubation and declined at 96 h (Fig. 6).

Figure 6

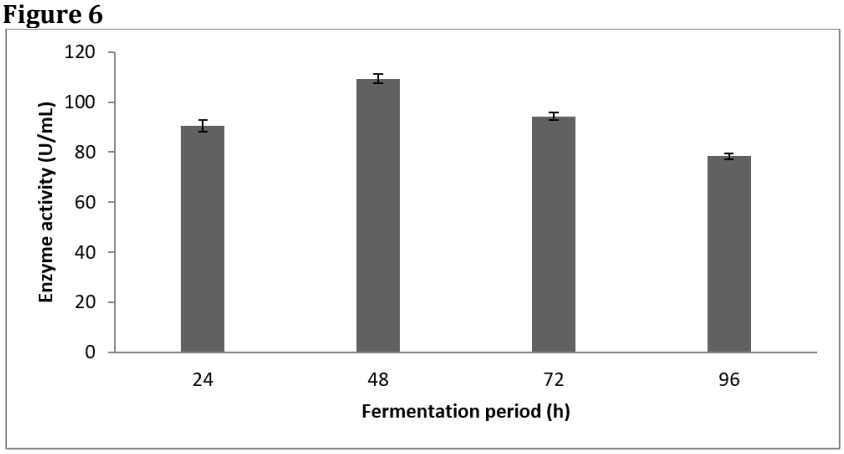

Fig. 6. Effect of Fermentation Period on Amylase Production Using Potato Peel Medium.

3.7. OPTIMIZATION OF INDEPENDENT VARIABLES USING RESPONSE SURFACE METHODOLOGY

The preliminary investigation identified independent variables such as pH, agitation rate, and incubation period as important parameters. These variables were further refined using Response Surface Methodology (RSM) with a central composite design. The CCD involved 20 experiments for three variables, detailed with actual variable level, and response Y (Table 2). Table 2 outlines the experimental range, levels, and original values for the independent variables utilized in the CCD model. The Model F-value of 12.06 implies the model is significant. There is only a 0.03% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant (Table 3). In this case C, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 0.64 implies the Lack of Fit is not significant relative to the pure error. There is a 68.00% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.The "Pred R-Squared" of 0.6348 is not as close to the "Adj R-Squared" of 0.8397 as one might normally expect. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response tranformation, outliers, etc. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 9.258 indicates an adequate signal.

Amylase production showed considerable variation when the levels of independent variables were adjusted. Maximum enzyme production occurred at the central values of these factors. Figure 7 displays the response surface plot representing the interaction between selected variables.

Table 2 Production of Amylase by Bacillus Amyloliquefaciens in Potato Peel Medium.

Run	pH	Incubation time	Agitation speed	Amylase (U/mL)
1	6	24	150	149.1
2	6	24	75	17.2
3	7	48	112.5	300.3
4	6	72	150	104.3
5	7	7.63	112.5	150.5
6	7	48	49.4	70.5
7	8	72	75	40.8
8	7	48	112.5	280
9	7	48	175.56	270.3
10	5.3	48	112.5	2.7
11	7	88.36	112.5	130.4
12	8	24	150	87.5
13	7	48	112.5	303.4
14	7	48	112.5	304.6
15	7	48	112.5	300
16	8	72	150	220.3
17	8	24	75	70.2
18	7	48	112.5	179.3
19	6	72	75	30.2
20	8.6	48	112.5	5.42

Table 3 Analysis of Variance for the Production of Amylase Using A Low Cost Medium.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	215413.5	9	23934.84	12.05853	0.0003
A-pH	1100.144	1	1100.144	0.55426	0.4737
B-Incubation time	104.602	1	104.602	0.052699	0.8231
C-Agitation speed	39969.55	1	39969.55	20.13692	0.0012
AB	2284.88	1	2284.88	1.151138	0.3085
AC	10.58	1	10.58	0.00533	0.9432
BC	1362.42	1	1362.42	0.686396	0.4267
A^2	137797.6	1	137797.6	69.42335	< 0.0001
B^2	35401.52	1	35401.52	17.83552	0.0018
C^2	21890.69	1	21890.69	11.02867	0.0077

Residual	19848.89	10	1984.889		
Lack of Fit	7769.214	5	1553.843	0.643164	0.6800
Pure Error	12079.67	5	2415.935		
Cor Total	235262.4	19			

Figure 7

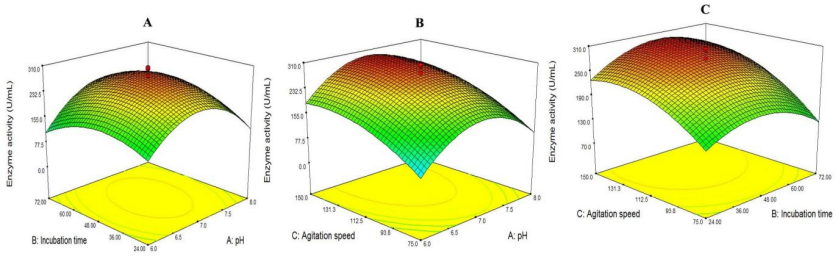

Fig. 7. 3D response surface graph for the roduction of amylase by *Bacillus amyloliquefaciens* (A) interactive effect of incubation time and pH, (b) agitation speed and pH, and (C) agitation speed and incubation speed.

4. DISCUSSION

The constituents of the medium and the conditions of the culture significantly affect the production of extracellular amylase. Carbon is not only a vital component of the medium but also a critical element for bacterial growth and metabolism, leading to increased enzyme production. Changes in the medium pH lead to decreased amylase activity due to the denaturation of proteins. The temperature significantly influences the production of amylase. Bacillus sp. exhibited peak amylase activity at 35 °C, while other studies have reported optimal temperatures ranging from 37 to 70 °C. The discrepancies between our findings and previous studies might stem from the different sources of bacterial isolation as well as strain variations [8, 9]. Reduced enzyme production at elevated temperatures indicates the isolate's sensitivity to temperature changes. Amylase must interact with and attach to the substrate at its active site to convert starch into products. Altering the temperature within a system can either enhance or reduce the frequency of enzyme-substrate collisions per time unit. Consequently, this impacts the reaction rate. Temperature shifts also influence bacterial growth, leading to variations in amylase production. For mesophilic bacteria, an increase in system temperature can trigger thermal denaturation of proteins and enzymes. In this study, maximum amylase production by *Bacillus* was achieved at an agitation rate of 110 rpm. Agitation rates between 100 and 250 rpm have also been noted for improved amylase production. The present research indicated that the highest yield of amylase from *Bacillus* sp. occurred after 48 hours of incubation. A marked decline in enzyme activity afterward was attributable to the progressive depletion of crucial nutrients in the culture medium, which created an unfavorable environment for the bacteria [10]. The outcomes of these experiments highlighted that enzyme production timing varies based on isolation sources, strain types, genetic characteristics, and cultivation conditions. Bacteria from the genus Bacillus are valuable organisms for biotechnological applications due to the alkali-tolerant properties of its amylase.The composition of the medium and its physical characteristics influence bacterial growth and the rate of enzyme production [11, 12]. Therefore, optimizing the physical conditions and medium components necessary for bacteria growth and utilization at an industrial scale is essential for enhancing enzyme productivity [13, 14]. In our research, optimizing pH, temperature, agitation, and incubation time using a CCD significantly affected amylase yield. The 3D response plot clearly illustrates that the model identifies the optimal region for amylase production at approximately the central values of the variables. Amylase production showed considerable variation when the levels of independent variables affecting *Bacillus* sp. growth were altered [15]. By employing response surface optimization of these independent variables, we were able to achieve a 3-fold increase in amylase production when compared to the one-factor-at-a-time (OFAT) method. Additionally, significant increases in enzyme production from microorganisms have also been documented through statistical optimization techniques [16] . The RSM approach using CCD resulted in a three-fold increase in enzyme yield from *Bacillus* sp. and indicated that the optimized variables derived from CCD could serve as ideal conditions for developing a cost-effective strategy to improve amylase production.

5. CONCLUSIONS

In this research, the selected *Bacillus amyloliquefaciens* strain utilized potato peel medium for the production of amylase in submerged fermentation process. By employing response surface methodology (RSM) with the Central Composite Design (CCD), the production of amylase was accelerated within a shorter time frame. The CCD demonstrated a 3-fold increase in amylase production compared to conditions that were not optimized, by engaging three different influencing factors: pH, agitation speed, and incubation duration.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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