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# Statistical Optimization of Medium Components for the Production of Chitinase by the Mimosa Pudica Root Nodule Isolate *Paenibacillus yonginensis*

**Akshada Bhadane, Shlok Mishra, Sayli Mandavkar, Sneha Bidvi, Rajendra Choure and Rupendra Jadhav***

*Department of Microbiology, Institute of Science, Mumbai, India*

**Corresponding author*

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**A B S T R A C T**

Chitinases – the primary chitinolytic enzymes have gained great attention due to its industrial, environmental, agricultural, medicinal and biotechnological applications. Nevertheless, the industrial-scale production of this enzyme remains in its early stages due to low yielding strains and high cost of production. This study focuses on enhancing chitinase production from low- cost chitin substrate through cumulative optimization, harnessing a high-yielding novel chitinase-producing strain isolated from Mimosa pudica root nodules. Among the various isolates, the best chitinase producer, identified as Paenibacillus yonginensis, exhibited an inherent high chitinase activity of 1.08 U/100 ml. Using Minitab statistical software, contemporary statistical optimization techniques were applied to optimize the media for Paenibacillus yonginensis chitinase production. Through Plackett-Burman design, it was determined that various factors including substrate (colloidal chitin), incubation time and pH were notably significant. Further optimization with a central composite design (CCD) approach using Response Surface Methodology (RSM) resulted in improved medium composition that yielded chitinase production up to 2.22 U/100 ml. It would be advantageous to use a high chitinase producing isolate in conjunction with the inexpensive and reliable nature of chitin as a carbon source to produce constant enzyme yields.

## Introduction

A number of bacteria have been reported to produce chitinases. Chitinases are the enzymes responsible for the biological degradation of chitin (Flach J. *et al.,* 1992). Chitin is made up of N-acetylglucos- amine through a β-(1 → 4) glycosidic bond to assemble poly (β-(1→4)-N-acetyl-d- glucosamine (GlcNaC) and it is by far the second most ample biopolymer present in nature which is insoluble and resistant to degradation, it requires chitinase (EC 3.2.1.14) or closely related enzymes for its breakdown. Glycosyl-hydrolase proteins, otherwise known as chitinases can cleave the 1, 4 bonds of the N-acetylglucosamine units, hydrolyze the chitin to

chitooligosaccharides [(GlcNAc)n] and act as a catalyst in chitin debasement. Chitinase is naturally produced by microbes, plants, insects, fungi and animals (Rathore A.S. *et al.,* 2015). Among them, microbially produced chitinase has received increasing consideration and may meet two purposes: i) reducing the environmental risks associated with waste management, and ii) raising the value of the degraded products.

In order to meet their nutritional needs, microbes produce chitinolytic enzymes. In insects, crustaceans, and fungi, these enzymes are involved in morphogenesis. In plants they play a role in defense mechanism against phytopathogens. Chitinases have found many industrial and pharmaceutical applications including biocontrol of plant pathogenic fungi and insects, production of chito-oligosaccharides, and management of chitinous wastes (Gohel V. *et al.,* 2006, Patil R. *et al.,* 2000). In microorganisms, chitinase production is controlled by a receptor-inducer system therefore, the composition of the culture medium can affect chitinase production.

Introduced by Plackett Burman in 1946 for culture condition optimization, these designs are two-level partial factorials most commonly employed for identifying important factors for further investigation. Plackett– Burman (PB) and central composite design (CCD) are multi-purpose examining tools comprising statistical methods for producing empirical models that assist in understanding enzyme kinetics and for optimizing the manufacturing conditions of expensive products such as enzymes. Response Surface Methodology (RSM) is the most broadly used technique (Chang C.-Y. *et al., 2006*).

It is a three-factor design that explains the relationship between one or more measured dependent responses with a number of input (independent) factors (Plackett R. *et al.,* 1946). These methods are widely used statistical experimental design in many biotechnological applications, enzyme manufacturing, biomass production and ethanol production because of its simplicity and most common saturated design. The unbiased estimation of main effects with the smallest variance can be done using saturated fractional factorial designs. In addition to this, the model is orthogonal, so the impact of individual variables will not interfere with the other variable interactions (Roberts W.K. *et al.,* 1988). Herein, the *Paenibacillus yonginensis* isolate from the root nodules of *Mimosa pudica* was used to study Chitinase production.

## Materials and Methods

**Colloidal chitin preparation -** Chitin flakes (20g) derived from shrimp shells were added slowly into 100 ml of concentrated HCl and left for vigorous shaking overnight at 4°C. The mixture was added to 250 ml of ice-cold 95% ethanol with rapid stirring and kept overnight at room temperature (25°C). The precipitate was collected by centrifugation at 10,000 rpm for 20 mins at 4°C and then washed with sterile distilled water until the pH of the colloidal chitin turned neutral (pH 7.0). Later, colloidal chitin prepared was stored at 4°C for further use as described earlier (Etesami, H. 2022).

**Isolation of chitinase producing bacteria -** Root nodules from healthy *Mimosa pudica* leguminous plant were collected to isolate chitinase producing bacteria. 0.1% mercuric chloride (HgCl2) solution was used to surface sterilize the nodules. To ensure complete removal of mercuric chloride the nodules were rinsed thoroughly multiple times with sterile distilled water. The nodules were crushed in sterile saline. The endophytes were isolated by streaking on the nodular suspension on the Congo Red Yeast Extract Mannitol Agar (CRYEMA). The inoculated plates were incubated and monitored for bacterial growth over a period of 48 hours at 28°C. The colonies that developed on CRYEMA were further streaked on chitin agar plates (Colloidal chitin 1%, NaCl 0.5g, KH2PO4 3g, Na2HPO4 6g, yeast extract 0.05g, NH4Cl 0.5g, Agar, 1.5% per litre) to screen chitinase producers. The colloidal chitin agar plates were incubated at 30°C for 3 days. The bacterial colony that showed the maximum zone of chitin clearance was selected for further studies.

**Characterization of isolate -** The biochemical and morphological characteristics of chitin degrading isolates were studied, and the strains were identified using Bergey's manual of determinative bacteriology (Bhattacharya D. *et al.,* 2007). The potential bacterial strain was also characterized by 16S rDNA sequencing.

**Enzyme Assay -** The colloidal chitin culture broth was inoculated with the chitinase producing isolate. After incubation at 32°C for 10 days, the culture broth was centrifuged at 10,000 rpm for 10 mins and the supernatant was used as enzyme solution for determining enzyme activity. 0.6 ml of 1% colloidal chitin (substrate) mixed with 50 mM potassium phosphate buffer pH 7, and then 0.4 ml crude enzyme was added, and incubated at 50°C for 60 mins. The reaction mixture was added

with 1 ml Dinitrosalicylic (DNS) acid and was placed within a boiling water bath for 10 mins to stop the reaction. The supernatant was collected by centrifugation, and the reduced sugar was estimated at 540 nm (UV spectrophotometer) using the N-acetyl glucosamine (GlcNAc) standard curve. Unit of the chitinase enzyme was defined as, the quantity of enzyme that yields 1 µmol of reducing sugar / minute.

**Experimental design and statistical analysis for optimization -** Optimization of medium components for chitinase production by *Paenibacillus yonginensis* isolate was performed in two stages. At the first stage, the components that have significant effect on enzyme production were identified. At the second stage, the optimum values of these components for chitinase production were determined.

**Screening design -** The medium components for optimizing the chitinase production by *Paenibacillus yonginensis* isolate were screened by using Plackett–Burman design (PBD) (Singh A. K. *et al.,* 2009). Minitab software was used for these studies. The factors chosen for the present study included substrate concentration (colloidal chitin), yeast extract concentration, pH, agitation, incubation time, temperature and inoculum size, with each factor being represented at two levels, low (− 1) to high (+ 1). The values for Plackett-Burman experimental design are shown in Table 1. The basal culture medium was prepared accordingly with factors chosen with low and high values and incubated at 32°C for 10 days. After incubation, the culture filtrate was centrifuged at 10,000 g for 15 mins and the supernatant was tested for the enzyme activity.

**Optimization through RSM-** The components that showed a positive effect on the chitinase production (Table 1), were further optimized using RSM, by employing a central composite design (CCD) for maximum production of chitinase enzyme. The significant variables for chitinase production, i.e., substrate, inoculum size and pH and a total of 20 experimental runs were performed with these variables at three different levels (− 1, 0, 1). The full experimental plan, including the values of the significant variables, is given from Table 2 to Table 4. The enzyme activity in U/100ml (response value) was obtained by calculating the average of the triplicate in each trial. Statistical analysis was performed using Minitab software. ANOVA was used to evaluate the effect of independent variables on the response & significant results were identified by p-value of < 0.05 on the data obtained from RSM on chitinase production.

## Results and Discussion

### Isolation and identification of chitinase-producing bacteria

In this present study, the isolation and characterization of potent chitin degrading endophytes from the root nodules of *Mimosa pudica* was performed. Randomly picked four different bacterial isolates from the root nodules were tested for their ability to produce chitinase. Isolate showing highest zone of chitinase activity was selected for further studies. The morphological and biochemical test showed this strain belonged to *Paenibacillus* species. The 16S rDNA sequence and phylogenetic analysis showed that this bacterial isolate was closely similar to *Paenibacillus yonginensis*.

### Basal chitinase production

Colloidal chitin broth was inoculated with Paenibacillus yonginensis and culture supernatant was collected after 10 days incubation. The culture supernatant was used for testing the enzyme activity.

From the basal medium chitinolytic activity in the culture supernatant of *Paenibacillus yonginensis* reached a maximum of 1.08 U/100 ml.

### Screening using Plackett–Burman design

The PBD statistical tool is helpful in screening important factors which influence the production of maximum chitinolytic activity. When enzyme activities of all the 12 flasks were compared, it was observed that the composition / parameters used in flask no. 10, 1 and 5 were very effective for maximum chitinolytic activity (Table 5). The highest production 2.09 U/100 mL was observed with 1% colloidal chitin, 1% Yeast extract, pH 7, inoculum size 1.5%, temperature 28ºC, agitation of 100 rpm and incubation time of 8 days. Three factors out of the selected 7 factors are significantly important for maximum production of chitinase and they are Substrate, Inoculum size and pH (Fig. 1). These significant factors are decided on the basis of p value. The p-value of < 0.05 is considered to be significant and p value > 0.05 is consider as non- significant. Results in Table 6 shows the p value of all 7 factors.

## Optimization using response surface methodology

The three significant variables (substrate concentration, inoculum size and pH) selected based on the results of Plackett-Burman design (substrate, inoculum size and pH) were further optimized using the central composite design (CCD) of RSM, shown in Table 2 at 3 different levels (-1, 0, +1). After all the 20 experiments were completed, their enzyme activity U/100ml (response value) was obtained by calculating the average of the triplicate in each trial and the results in Table 7 suggest that composition of substrate, inoculum size and pH in flask no 8, 2, 16 and 9 are very effective for maximum chitinolytic activity.

From the Preto chart (Fig. 2), it is clear that the substrate is the dominant driver of the response. Most of the interaction effects (except pH and substrate, p value 0.049) are not significant, indicating factors largely act independently.

ANOVA was used to evaluate the effect of the independent variables on the response & significant results were identified by p-value of < 0.05 on the data obtained from RSM on chitinase production (Table 8).

Three-dimensional response plot showing interactive effect of inoculum size and pH at a fixed substrate concentration of 0.75% demonstrated a strong dependence on pH, with lower value (~5.0) resulting in reduced response levels, likely due to unfavorable acidic conditions affecting microbial growth and enzymatic activity (Figure 3). The response increased significantly as pH approached near- neutral conditions (~6.0–6.5), indicating enhanced metabolic efficiency. A decline in response at higher pH values suggests possible enzyme deactivation or physiological stress under alkaline conditions. Inoculum size exerted a pronounced effect on the response. Lower inoculum levels (~1.5%) resulted in diminished response, potentially due to insufficient biomass availability. Increasing the inoculum size to ~1.8–2.0% led to a marked enhancement in the response, reflecting improved substrate utilization and metabolic activity. However, further increases in inoculum size caused a reduction in the response, which may be attributed to nutrient depletion, oxygen limitation, or increased metabolic competition.

Three-dimensional response plot showing interactive effect of substrate concentration and pH at a fixed inoculum size of 1.75% demonstrated that at lower substrate concentrations (~0.50 g/L), the response remained relatively low, likely due to substrate limitation restricting microbial metabolism or product formation (Figure 4). As the substrate concentration increased to an optimal range (~0.80–0.90 g/L), the response improved significantly, reflecting enhanced substrate availability and metabolic activity. However, further increases in substrate concentration resulted in a decline in the response, possibly due to substrate inhibition, osmotic stress, or accumulation of inhibitory metabolites. The response exhibited a strong dependence on pH, with suboptimal values at lower pH (~5.0), suggesting unfavorable acidic conditions for microbial growth or enzymatic activity. The response improved markedly as pH approached near-neutral values (~6.0–6.5), indicating optimal physiological conditions. A decline in response at higher pH levels suggests reduced enzyme stability or metabolic inefficiency under alkaline conditions.

Three-dimensional response surface plot showing the interactive effect of inoculum size and substrate at a fixed pH of 6.25 showed that at lower substrate concentrations (~0.50 g/L), the response was limited, likely due to insufficient carbon availability for efficient microbial metabolism and product formation (Figure 5).

Increasing the substrate concentration up to an optimal range (~0.80–0.90 g/L) resulted in a substantial enhancement of the response. However, further increases led to a reduction in response, which may be attributed to substrate inhibition, osmotic stress, or accumulation of inhibitory metabolites. Lower inoculum size (~1.50%) resulted in reduced response levels, possibly due to inadequate biomass for rapid substrate utilization. Increasing the inoculum size to an optimal range (~1.80–1.90%) improved the response significantly, reflecting enhanced metabolic activity. A decline at higher inoculum sizes suggests increased competition for nutrients, oxygen limitation, or metabolic crowding.

Optimization of medium composition is essential for maximizing enzyme yield and ensuring process feasibility for industrial-scale production. In the present study, Plackett–Burman Design (PBD) was employed as a statistical screening tool to identify the key factors influencing chitinase production by *Paenibacillus yonginensis*. Among the evaluated variables, substrate concentration, inoculum size, and initial pH were identified as the most significant contributors to enzyme production.

**Table.1** Values for Plackett-Burman Design

| Variables | Components | -1 Value (g/L) | +1 Value (g/L) |
|---|---|---|---|
| **X1** | Substrate | 0.5% | **1.0%** |
| **X2** | Inoculum Size | 1.5% | **2.0%** |
| **X3** | Temperature | 28°C | **32°C** |
| **X4** | Incubation Time | 08 (D) | **12 (D)** |
| **X5** | Agitation | 100 RPM | **125 RPM** |
| **X6** | Yeast Extract | 0.5 | **1.0** |
| **X7** | **pH** | **5.5** | **7.0** |

**Table.2** Values for Response Surface Methodology (Central Composite Design)

| Variables | Components | -1 Level of Variable (g/L) | 0 Level of Variable (g/L) | +1 Level of Variable (g/L) |
|---|---|---|---|---|
| **X7** | pH | 5.5 | 7.0 | **8.5** |
| **X1** | Substrate | 0.5% | 1.0% | **1.5%** |
| **X2** | **Inoculum Size** | **1.0%** | **1.5%** | **2.0%** |

**Table.3** Design Summary for Response Surface Methodology (Central Composite Design)

| | | | |
|---|---|---|---|
| **Factors:** | **3** | **Replicates:** | **1** |
| **Base runs:** | 20 | Total runs: | **20** |
| **Base blocks:** | **1** | **Total blocks:** | **1** |

α = 1.68179
Two-level factorial: Full factorial

**Table.4** Point Types for Response Surface Methodology (Central Composite Design)

| | |
|---|---|
| **Cube points:** | **8** |
| **Centre points in cube:** | **6** |
| **Axial points:** | **6** |
| **Centre points in axial:** | **0** |

**Table.5** Plackett-Burman Design Matrix

| Trials | X1 Substrate | X2 Inoculum Size | X3 Temperature | X4 Incubation Time | X5 Agitation | X6 Yeast Extract | X7 pH | Chitinase Activity (U/100 mL) |
|---|---|---|---|---|---|---|---|---|
| **1** | + | - | + | - | - | - | + | **1.24** |
| **2** | + | + | - | + | - | - | - | **0.76** |
| **3** | - | + | + | - | + | - | - | **0.21** |
| **4** | + | - | + | + | - | + | - | **0.88** |
| **5** | + | + | - | + | + | - | + | **1.12** |
| **6** | + | + | + | - | + | + | - | **0.62** |
| **7** | - | + | + | + | - | + | + | **0.59** |
| **8** | - | - | + | + | + | - | + | **0.84** |
| **9** | - | - | - | + | + | + | - | **0.83** |
| **10** | + | - | - | - | + | + | + | **2.09** |
| **11** | - | + | - | - | - | + | + | **0.48** |
| **12** | - | - | - | - | - | - | - | **0.56** |

X1 – X7 = Medium Components / Parameters, (-1) Low Value, (+1) High Value

**Table.6** ANOVA of Plackett-Burman Design

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| **Model** | 7 | 2.57617 | 0.368025 | 5.33 | **0.063** |
| **Linear** | 7 | 2.57617 | 0.368025 | 5.33 | **0.063** |
| **Substrate** | 1 | 0.92417 | 0.924169 | 13.38 | **0.022** |
| **Inoculum Size** | 1 | 0.64760 | 0.647605 | 9.38 | **0.038** |
| **Temperature** | 1 | 0.21261 | 0.212607 | 3.08 | **0.154** |
| **Incubation Time** | 1 | 0.00757 | 0.007569 | 0.11 | **0.757** |
| **Agitation** | 1 | 0.14322 | 0.143221 | 2.07 | **0.223** |
| **Yeast Extract** | 1 | 0.06812 | 0.068119 | 0.99 | **0.377** |
| **pH** | 1 | 0.57288 | 0.572882 | 8.30 | **0.045** |
| **Error** | 4 | 0.27619 | 0.069046 | | |
| **Total** | **11** | **2.85236** | | | |

F is Fisher's function; probability $p$ ($p \leq 0.05$) corresponds to significance

**Table.7** Central Composite Design Matrix

| Trial Run | X7 (pH) | X1 (Substrate) | X2 (Inoculum Size) | Chitinase Activity(U/100 mL) |
|---|---|---|---|---|
| **1** | 1.00000 | -1.00000 | 1.00000 | **0.98** |
| **2** | -1.00000 | 1.00000 | 1.00000 | **2.06** |
| **3** | 0.00000 | 0.00000 | 0.00000 | **0.39** |
| **4** | 0.00000 | -1.68179 | 0.00000 | **0.79** |
| **5** | 0.00000 | 0.00000 | 0.00000 | **0.49** |
| **6** | 0.00000 | 0.00000 | 0.00000 | **0.53** |
| **7** | 0.00000 | 1.68179 | 0.00000 | **0.74** |
| **8** | 1.00000 | 1.00000 | 1.00000 | **2.22** |
| **9** | -1.00000 | 1.00000 | -1.00000 | **1.08** |
| **10** | 0.00000 | 0.00000 | 1.68179 | **0.70** |
| **11** | 0.00000 | 0.00000 | -1.68179 | **0.71** |
| **12** | 1.68179 | 0.00000 | 0.00000 | **0.79** |
| **13** | 0.00000 | 0.00000 | 0.00000 | **0.09** |
| **14** | 1.00000 | 1.00000 | -1.00000 | **0.99** |
| **15** | -1.00000 | -1.00000 | -1.00000 | **0.52** |
| **16** | -1.00000 | -1.00000 | 1.00000 | **1.11** |
| **17** | 0.00000 | 0.00000 | 0.00000 | **0.54** |
| **18** | 0.00000 | 0.00000 | 0.00000 | **0.64** |
| **19** | 1.00000 | -1.00000 | -1.00000 | **0.98** |
| **20** | **-1.68179** | **0.00000** | **0.00000** | **0.70** |

X7, X1, X2 = Medium Components / Parameters, (-1) Low Value, (0) Middle Value, (+1) High Value

**Table.8** ANOVA of Response Surface Methodology (Central Composite Design)

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| **Model** | 9 | 3.79926 | 0.42214 | 5.85 | **0.005** |
| **Linear** | 3 | 1.75196 | 0.58399 | 8.09 | **0.005** |
| **pH** | 1 | 0.09285 | 0.09285 | 1.29 | **0.283** |
| **Inoculum Size** | 1 | 0.00271 | 0.00271 | 0.04 | **0.850** |
| **Substrate** | 1 | 1.65640 | 1.65640 | 22.94 | **0.001** |
| **Square** | 3 | 1.28434 | 0.42811 | 5.93 | **0.014** |
| **pH*pH** | 1 | 0.00047 | 0.00047 | 0.01 | **0.938** |
| **Inoculum Size*Inoculum Size** | 1 | 0.48498 | 0.48498 | 6.72 | **0.027** |
| **Substrate*Substrate** | 1 | 0.67312 | 0.67312 | 9.32 | **0.012** |
| **2-Way Interaction** | 3 | 0.76296 | 0.25432 | 3.52 | **0.057** |
| **pH*Inoculum Size** | 1 | 0.20027 | 0.20027 | 2.77 | **0.127** |
| **pH*Substrate** | 1 | 0.36242 | 0.36242 | 5.02 | **0.049** |
| **Inoculum Size*Substrate** | 1 | 0.20027 | 0.20027 | 2.77 | **0.127** |
| **Error** | 10 | 0.72209 | 0.07221 | | |
| **Lack-of-Fit** | 5 | 0.46599 | 0.09320 | 1.82 | **0.264** |
| **Pure Error** | 5 | 0.25610 | 0.05122 | | |
| **Total** | **19** | **4.52135** | | | |

F is Fisher's function; probability $p$ ($p \leq 0.05$) corresponds to significance

## Isolation and identification of chitinase-producing bacteria

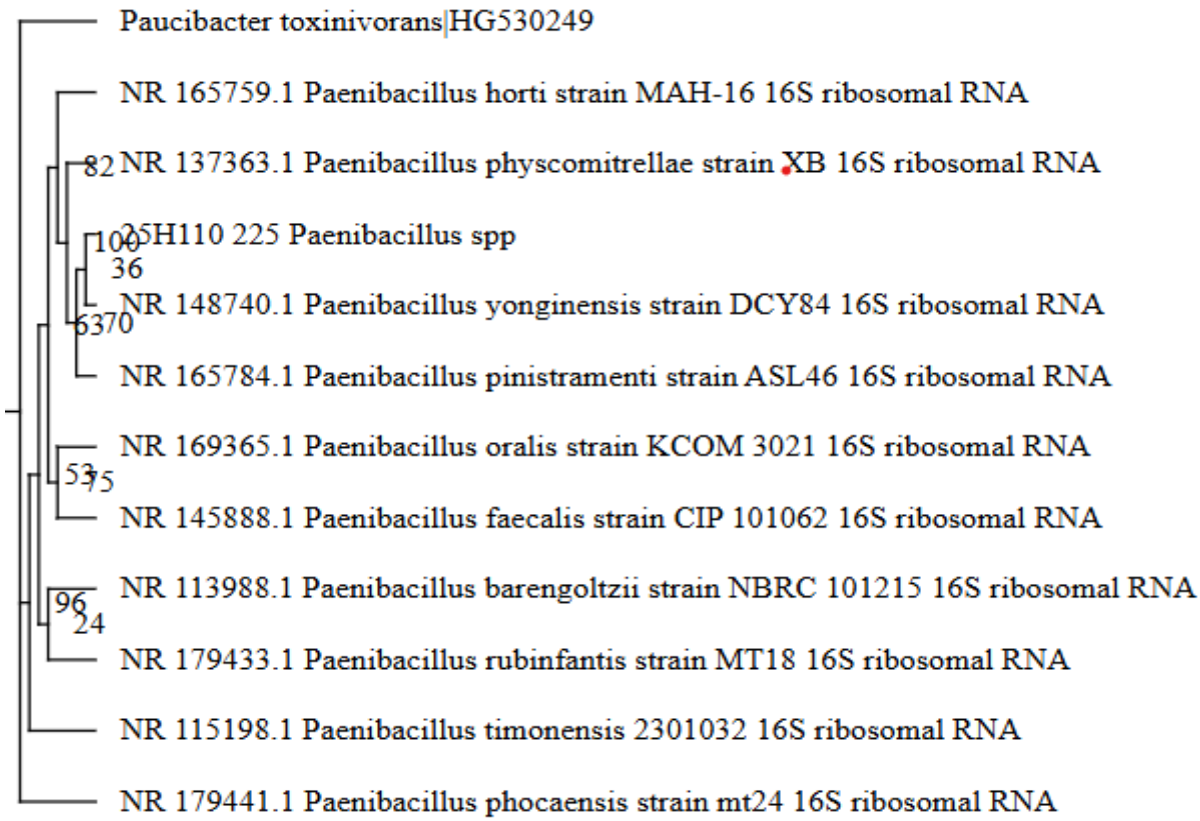



**Figure.1** Pareto Chart of Plackett-Burman design according to the enzyme activity obtained

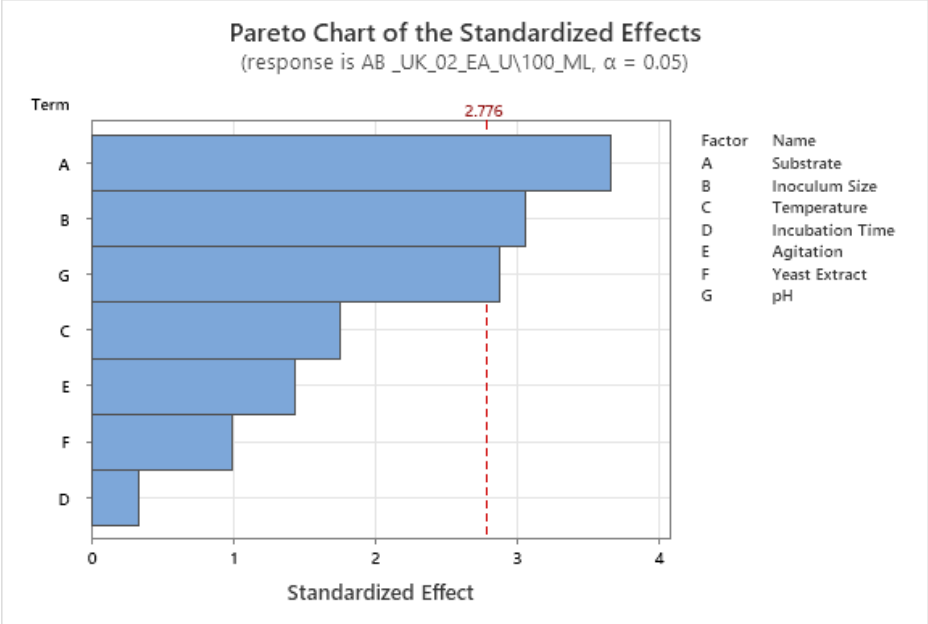


**Figure.2** Pareto Chart of Response Surface Methodology (Central Composite Design)

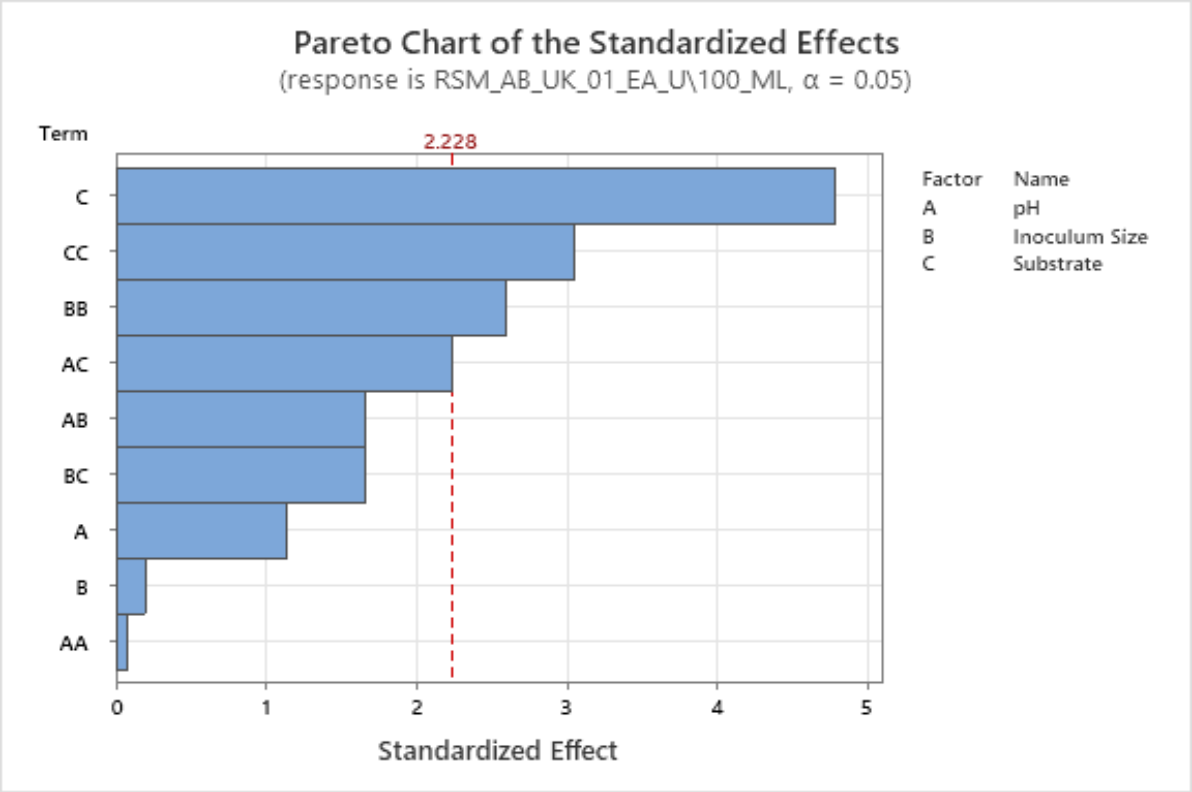



**Figure.3** Three-dimensional response surface plot showing the interactive effect of inoculum size and pH at a fixed substrate concentration of 0.75% on chitinase production by *Paenibacillus yonginensis.*

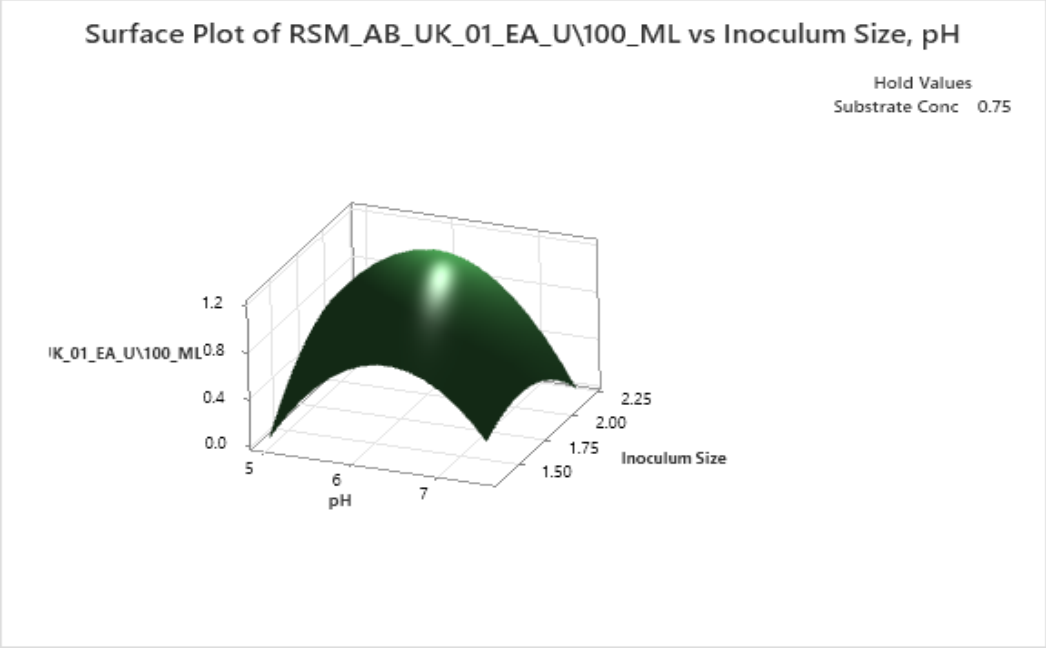


**Figure.4** Three-dimensional response surface plot showing the interactive effect of substrate concentration and pH at a fixed inoculum size of 1.75% on chitinase production by *Paenibacillus yonginensis*

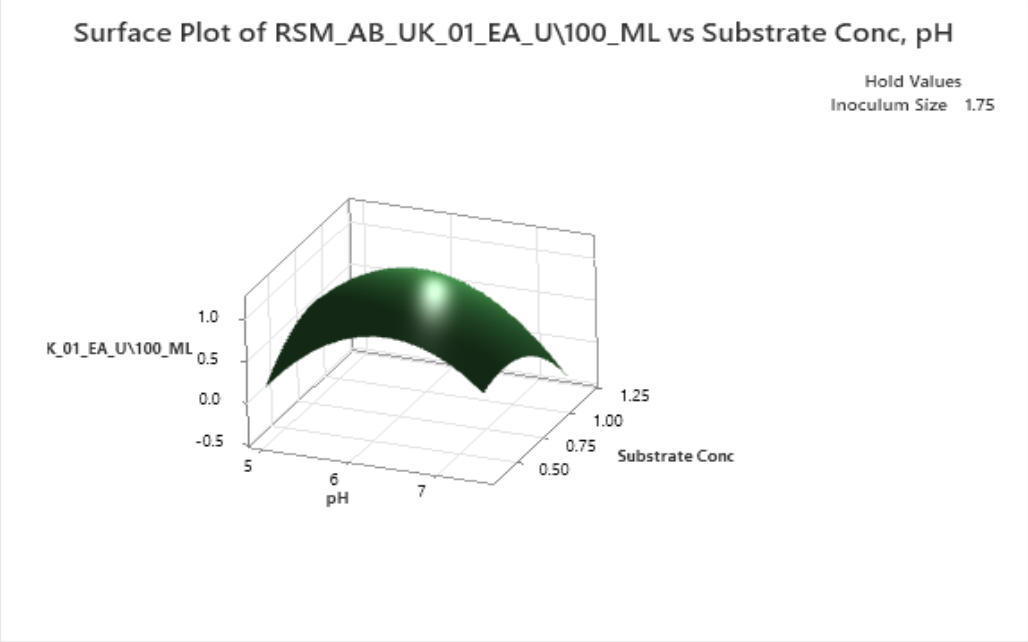
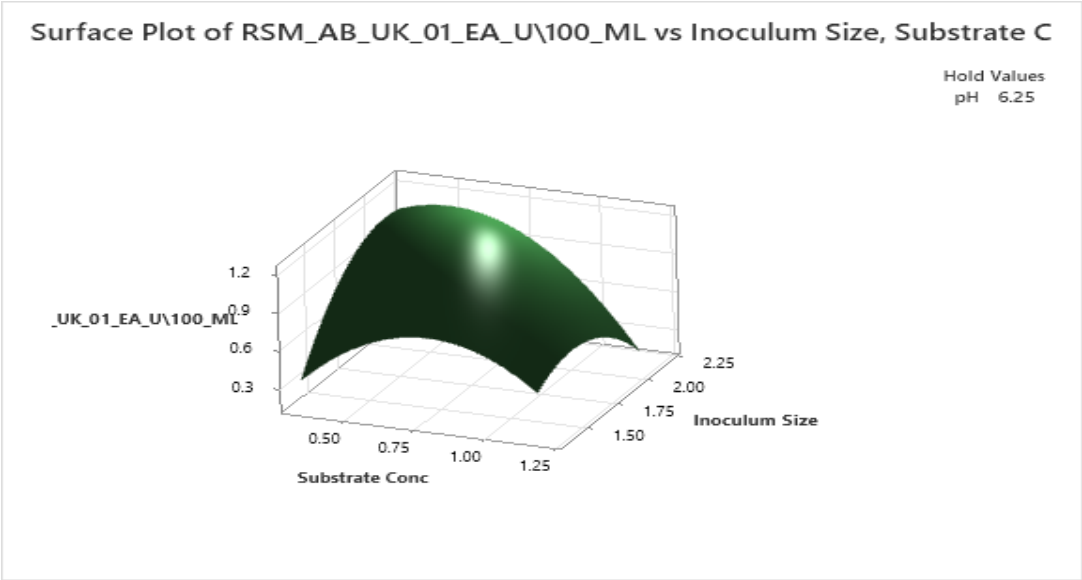



**Figure.5** Three-dimensional response surface plot showing the interactive effect of inoculum size and substrate at a fixed pH of 6.25 on chitinase production by *Paenibacillus yonginensis.*




These parameters play critical roles in regulating microbial metabolism, enzyme induction, and secretion efficiency. Previous studies have similarly reported that chitinase biosynthesis is strongly influenced by physicochemical conditions, particularly pH, nutrient availability, and microbial density. The remaining medium components showed no statistically significant contribution within the tested range and were therefore excluded from further optimization, improving model accuracy and experimental efficiency.

Since PBD does not evaluate interaction and quadratic effects, Central Composite Design (CCD) under Response Surface Methodology (RSM) was applied to determine the optimal levels and interaction effects of the selected variables. The observed variation in enzyme activity across the experimental runs confirmed the presence of significant interaction effects. The three-dimensional response surface plots exhibited characteristic dome-shaped curvature, indicating the existence of optimal conditions within a defined experimental range.

These findings demonstrate that chitinase production is governed by complex interactions between variables rather than independent effects. Similar interaction-dependent optimization patterns have been reported in chitinase-producing *Paenibacillus* and *Bacillus* species, highlighting the effectiveness of RSM in improving enzyme yield. The statistical significance of the quadratic model confirms its suitability for describing the experimental system and predicting optimal conditions.

Among the optimized parameters, pH was identified as a major determinant of chitinase production, with maximum enzyme activity observed within a slightly acidic range of pH 6.2–6.5. This optimal range likely supports enzyme stability, proper protein folding, and efficient secretion. Deviations from the optimal pH resulted in reduced enzyme production, possibly due to enzyme instability and disruption of cellular metabolic processes. Similar pH-dependent production profiles have been reported in other Gram-positive chitinolytic bacteria, indicating that slightly acidic conditions are favorable for chitinase synthesis.

Inoculum size also significantly affected enzyme production, with maximum activity observed at 1.8–1.9%. Lower inoculum levels may result in insufficient biomass for effective enzyme synthesis, whereas higher inoculum densities can cause rapid nutrient depletion and oxygen limitation, leading to reduced enzyme production. This observation highlights the importance of maintaining optimal microbial density to balance cellular growth and enzyme synthesis. Similar moderate inoculum-dependent production trends have been reported previously (Adnan M. *et al.,* 2024).

Substrate concentration exhibited a nonlinear effect on enzyme production, with maximum activity observed at 0.8–0.9 g/L. Chitin functions as both a carbon source and an inducer for chitinase synthesis; however, excessive substrate concentrations may lead to substrate inhibition, feedback repression, and increased medium viscosity. These effects can reduce oxygen and nutrient transfer, thereby limiting microbial metabolism and enzyme secretion efficiency (Sashiwa H. *et al.,* 2004). Such substrate-dependent limitations are commonly observed in submerged fermentation systems.

The second-order polynomial model developed using CCD effectively described the relationship between the selected variables and enzyme production. The strong agreement between predicted and experimental values confirms the reliability of the model. The observed reduction in enzyme activity at extreme variable levels further highlights the sensitivity of the production process and the importance of precise optimization.

Overall, the sequential application of PBD and RSM enabled systematic identification and optimization of the critical factors influencing chitinase production by *P. yonginensis*. This approach significantly improved enzyme yield and established a reliable operational window for production. The optimized conditions identified in this study provide a strong foundation for future scale-up and industrial application of chitinase production.

## Author Contributions

Akshada Bhadane: Investigation, formal analysis, writing—original draft. Shlok Mishra: Validation, methodology, writing—reviewing. Sayli Mandavkar:—Formal analysis, writing—review and editing. Sneha Bidvi: Investigation, writing—reviewing. Rajendra Choure: Resources, investigation writing—reviewing. Rupendra Jadhav: Validation, formal analysis, writing—reviewing.

## Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethical Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent to Publish** Not applicable.

**Conflict of Interest** The authors declare no competing interests.

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