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Screening of chitinase production medium by *Fusarium solani* isolated from endophytic karst using Two Level Factorial Design

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ABSTRACT. Chitinase is an important enzyme in various biotechnology applications, especially in biological control, so it requires increased induction efficiency. This study aims to determine the growth media factors that most influence the production of chitinase enzymes by the endophytic fungus *Fusarium solani* using the 2-Level Fractional method. Eight media variables are estimated to have an effect, namely the content of chitin colloid, ammonium sulfate, potassium sodium tartrate, KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, and pH. Chitinase activity was evaluated using the DNS technique after four days of liquid fermentation and analyzed using the design express DX6 4.1 application. The results of the analysis of variance (ANOVA) showed that the second-order polynomial regression model was very significant ($p < 0.0001$), with an R^2 value of 0.9515, which indicates that the model explains 95.15% of the data variation. Based on the screening results, it was found that ammonium sulfate, Na_2HPO_4, and $MgSO_4$ $7H_2$ were the most effective in increasing chitinase production. Optimization of media conditions resulted in an increase in chitinase activity of up to 18 U/mL. This research has the potential to be used as a basis for developing industrial-scale bioprocesses for the formulation of chitinase-based biopesticides and biopharmaceutical products.

Keywords: 2-level factorial design; chitinase; *Fusarium solani*; Response Surface Methodology; screening medium

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INTRODUCTION

Endophytic fungi are microorganisms that live within plant tissues without causing disease symptoms in their hosts. Their presence is known to provide various benefits, such as increasing plant resistance to biotic and abiotic stress (Kamran *et al*., 2022), secondary metabolites with potential antimicrobial activity, and producing important extracellular enzymes, one of which is chitinase. Chitinase enzymes play a role in the degradation of chitin, the main polymer that makes up the cell walls of pathogenic fungi and insect exoskeletons, thus offering potential as biological agents in plant disease control.

One endophytic fungus that has been extensively studied is *Fusarium solani* (Toghueo, 2020). Although some strains are pathogenic, endophytic strains originating from specific environments, such as karst areas, are thought to possess distinct physiological and metabolic characteristics (Ahmed *et al*., 2023). The extreme and nutrient-poor nature of karst ecosystems encourages plant-associated microorganisms to adapt, including through the production of specific enzymes. However, studies on the enzymatic potential, particularly chitinase production, of endophytic fungi from karst environments are still limited and tend to be descriptive. On the other hand, research related to enzyme production from endophytic fungi generally places more emphasis on optimizing production conditions (Nguyen *et al*., 2025). However, studies on optimization of chitinase production from karst-derived endophytic fungi using factorial design remain limited. This indicates a gap between descriptive studies of biological potential and more targeted approaches to understanding the factors that specifically influence enzyme production (Chandran *et al.*, 2025). Determining and establishing optimal conditions for these factors is crucial for developing an efficient and economical chitinase production process (Poria *et al*., 2021).

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In order to optimize chitinase production, an appropriate medium formulation strategy is required (Dukariya & Kumar, 2021). One approach that can be used is Two Level Factorial Design, a statistical method in experimental design that allows the determination of significant factors that affect enzyme production (Rachmawaty *et al*., 2018) Through this approach, combinations of medium variables can be systematically tested to obtain optimal conditions that support chitinase production by endophytic *Fusarium solani* (Rachmawaty *et al*., 2019).

This study aims to select chitinase production media from the endophytic fungus Fusarium solani isolated from karst environments using a two-level factorial design approach. The results are expected to fill the knowledge gap regarding the potential and variation in chitinase production from karst endophytic fungi and provide a scientific basis for the development of more efficient and environmentally friendly biotechnology applications.

MATERIALS AND METHODS

Isolate preparation. The endophytic fungus *F. solani* was grown on Potato Dextrose Agar (PDA) medium at 28 °C for 7 days (González-Pérez *et al.*, 2018). Spores were harvested with a 1% Tween 80 solution. The spore pellet was then centrifuged at 4,000 rpm for 20 minutes. The spores were mixed with distilled water to produce a number of spores as inoculum. The inoculum size used was $1x10^8$ spores/mL.

Preparation of colloidal chitin. Chitin were used in the experiment. Twenty grams were added to 200 mL of HCl (37%) and stirred with a glass rod at 40 °C for 5 minutes (Gîjiu *et al*., 2022). Distilled water was added to reach 1000 mL and the resulting chitin filtrate was washed with water until the pH reading of the filtrate was between pH 6.5 and 7.0 (Eddya et al., 2020). hen, it was dried until it reached 98% humidity. The colloidal chitin was sterilized at 121 ºC and 15 psi for 15 minutes and stored for further use.

Enzyme production medium. The production medium contains (g/L): chitin, 30; ammonium sulphate, 1.5; potassium sodium tartrate, 1.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4$-$7H_2O$, 1.5; $CaCl_2$, 0.1; and trace elements, 1.0 mL with a pH of 6.5. The medium composition and initial pH value are varied according to the design. One mole per litre of NaOH or 1.0 M HCl is used to adjust the initial pH value. The trace element composition included (g/L): $FeCl_3$-$6H_2O$, 8.0; $ZnSO_4$-$7H_2O$, 0.1; $CuSO_4$-$5H_2O$, 0.1; $Co(NO_3)_2 \cdot 6H_2O$, 0.1; and $MnSO_4$-$5H_2O$, 0.1. Trace elements were sterilized and added to the medium after autoclaving.

Chitinase production in liquid medium. Approximately 10% (v/v) (Reginatto *et al*., 2022) of 1×10^8 spores/mL spore suspension was transferred to 50 mL of sterile production medium. The broth culture was incubated at 28°C on a rotary shaker at 200 rpm (Akeed *et al*., 2020). Samples were taken aseptically on the 4th day of fermentation. The samples taken were centrifuged at 4,000 rpm for 10 minutes. The supernatant was used for chitinase testing and protein analysis.

Chitinase activity assay. Chitinase activity was determined using 1.0% (w/v) colloidal chitin as a substrate (Kidibule *et al.*, 2018). One milliliter of supernatant was mixed with 1.0 mL of 1.0% (w/v) colloidal chitin in 2.0 M phosphate buffer, pH 6.5. The mixture is incubated at 50°C for 1 hour. The final product of the reaction is determined using the DNS method. One unit (U) of chitinase activity is defined as the enzyme unit required to catalyze the release of 1 μmol of N-acetyl-D-glucosamine under the test conditions.

Experimental design using 2-level fractional factor. The experimental design in this study used a fractional factorial approach with eight independent variables (A–H) each set at two levels (low and high), but equipped with a center point (level 0) so that it does not fully represent a two-level factorial design. The fractional design was chosen to reduce the number of experiments compared to a full factorial, with a total of 128 runs resulting from treatment combinations, as well as the addition of replication at the center point to evaluate response stability and detect possible non-linear effects. The center point was determined as the middle value of each variable and serves to test for curvature in the system. The observed response was chitinase enzyme activity (U/mL), and

analysis was carried out to identify factors that had a significant effect at the 95% confidence level ($P < 0.05$).

Table 1. Coded values and variables used in the 2-level fractional factor design

Variable	Components	Level		
		Low level (-1)	0	High level (+1)
A	Colloidal chitin	1.0%	2.0%	3.0%
B	Ammonium sulphate	0.5 g	1.5 g	2.5 g
C	Potassium sodium tartrate	0.5 g	1.0 g	1.5 g
D	KH2PO4	6.0 g	7.0 g	8.0 g
E	Na2HPO4	1.5 g	2.0 g	2.5 g
F	MgSO4.7H2O	1.0 g	1.5 g	2.0 g
G	CaCl2	0.05 g	0.1 g	0.15 g
H	pH	3.5	5	6.5

Data analysis. Data obtained from chitinase activity measurements were analyzed using Design Expert DX6 4.1 software. A regression model was developed based on the responses to the tested variables, with ANOVA analysis used to assess the model's significance and the contribution of each variable. RSM and contour plots were used to determine the optimum conditions for chitinase production. Model validation was performed through repeated experiments at the predicted optimum conditions to confirm the accuracy of the resulting model.

RESULTS AND DISCUSSION

Two-level fractional factorial design. A two-level fractional factorial design (2-LFD) was used in this study as an initial step to screen for variables that significantly influence chitinase production, rather than for direct optimization of conditions. Through this approach, initial relationships between media factors and responses can be identified and mathematically modeled (Rachmawaty *et al.*, 2019). The variables studied included colloidal chitin, ammonium sulfate, potassium sodium tartrate, KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, and pH at two treatment levels. All experiments were conducted randomly to minimize bias due to uncontrolled variables.

Model formation. The data obtained was highly consistent with the second-order polynomial model compared to other polynomial models. The responses obtained from the experiment were analysed using analysis of variance (ANOVA). The regression equation obtained is shown in Equation, without taking significance into account. The resulting equation is a second-order polynomial.

Y/(U/mL) = 0.029 A + 0.10 B -0.031 C – 0.060 D – 0.020 E + 0.10 F + 0.021 G – 0.062 H + 0.00081 A2 + 0.01 B + 0.000961 C + 0.0036 D + 0.0004 E + 0.1 F + 0.000441 G – 0.003844 H + 0.092 AB +0.014 AC -0.18 AD- 0.084 AE +0.028 AF +0.014 AG- 0.045 AH.

Y represents chitinase activity (U/mL), while A, B, C, D, E, F, G and H represent colloidal chitin, ammonium sulphate, potassium sodium tartrate, KH_2PO_4, Na_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2$, pH, respectively. This regression model was generated by Design Expert software after taking all variables into account. The predicted chitinase yield was obtained from the chitinase activity of *F. solani* at each experimental point using Equation.

The analysis of variance (ANOVA) for this model is presented in Table 2. The test results show that the regression model is highly significant, with a p-value of 0.0001, well below the significance threshold of 0.05. This indicates that the regression model is accurate in predicting the significant pattern of chitinase production by *F. solani* (Chen *et al*., 2024). The coefficient of determination (R^2) value of 0.9515 indicates the adequacy and validity of the model, and indicates that this model can be applied and is statistically accepted.

Table 2. Analysis of variance (ANOVA) of chitinase activity using a 2-LFD design

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	P > F	R^2
Model	151.0903	89	1.697643	8.384949	< 0.0001	0.9515
A	0.108803	1	0.108803	0.537399	0.4680	
B	1.330439	1	1.330439	6.571264	0.0144	
C	0.125652	1	0.125652	0.620616	0.4357	
D	0.467305	1	0.467305	2.3081	0.1370	
E	5.278849	1	5.278849	26.07313	< 0.0001	
F	1.363778	1	1.363778	6.735929	0.0134	
G	0.057022	1	0.057022	0.281641	0.5987	
H	0.490306	1	0.490306	2.421704	0.1280	

Based on Table 3, the resulting regression model is highly significant ($p < 0.0001$) with an R^2 value of 0.9515, indicating excellent model fit. The Na_2HPO_4 factor (E) has the most significant effect on chitinase activity ($p < 0.0001$), followed by ammonium sulfate (B) and $MgSO_4 \cdot 7H_2O$ (F) ($p < 0.05$). Other factors did not show a significant effect within the tested range.

Table 3. p-value of factors affecting chitinase activity

Factor	Variable	p-value
A	Coloidal chitin	0.4680
B	Ammonium sulfate	**0.0144**
C	Potassium sodium tartrate	0.4357
D	KH_2PO_4	0.1370
E	Na_2HPO_4	**< 0.0001**
F	$MgSO_4 \cdot 7H_2O$	**0.0134**
G	$CaCl_2$	0.5987
H	pH	0.1280
Model	–	**< 0.0001**
R^2	–	**0.9515**

Linear effect analysis showed that several single factors significantly influenced chitinase activity. Ammonium sulfate, as a nitrogen source, was shown to play an important role with a significant p-value, supporting biomass growth and enzyme synthesis. Furthermore, the availability of phosphate (Na_2HPO_4 or K_2HPO_4) was also a dominant factor, with a very low p-value, indicating the role of phosphate in metabolism and enzyme production (Kurniawan *et al*., 2022). Similar results were also found in other studies, where an optimal combination of carbon sources (such as colloidal chitin), nitrogen, and phosphate significantly increased chitinase activity (Aliabadi *et al*., 2016).

Plots generated from regression models, such as contour plots and response surfaces (e.g., in Fig. 1 through 8), specifically depict the interaction effects of two primary variables, while other variables are held constant. This provides a clear visual representation of optimal areas within the parameter space under study, facilitating data-driven decision-making.

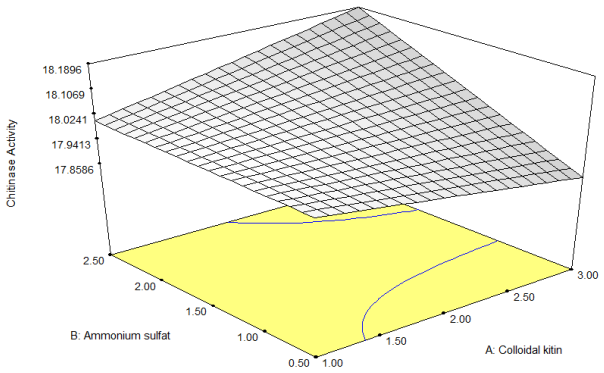


Fig. 1. Response surface plot of chitinase activity from the model equation: Effect of colloidal chitin and ammonium sulfate

The interaction between colloidal chitosan (substrate) and ammonium sulfate (nitrogen source) on chitinase activity was evaluated while other factors were kept constant (Adnan *et al*., 2024). The results showed that chitinase activity increased with increasing concentrations of both variables, reaching a peak at the corner of the plot (Pramesti & Puspita, 2020). Colloidal chitin acts as the main inducer that stimulates chitinase production, while ammonium sulfate provides essential nitrogen which is very necessary for fungal biomass growth and enzyme biosynthesis. Research Pramesti & Puspita (2020), colloidal chitosan functions as an inducer of enzyme production, while ammonium sulfate supplies nitrogen essential for fungal growth and biosynthesis. These results indicate that optimal chitinase activity requires a balanced increase in substrate and nitrogen availability.

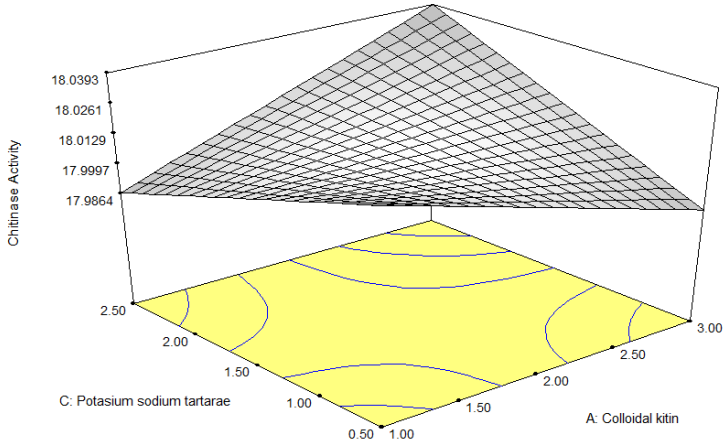


Fig. 2. Surface plot of chitinase activity response from the model equation: Effect of colloidal chitin and potassium sodium tartrate

The response surface in this plot appears nearly flat with a slight increase towards the leading edge. The increase in chitinase activity (from approximately 17.97 to 18.04 U/mL) is very small, indicating that the interaction between Colloidal chitin and Potassium sodium tartarae is not very significant or its influence is relatively weak compared to other factors in the model. Maximum activity is achieved when both variables are at the highest levels tested, indicating that high availability of the inducing substrate (chitin) together with adequate potassium/sodium source concentrations can create optimal conditions. However, because the difference between the minimum and maximum values is very small, the influence of these two factors, especially their interaction, can be considered minimal in influencing the results.

Colloidal chitin has consistently been identified as the primary and most effective industrial substrate for chitinase biosynthesis and activity (Xie *et al*., 2021). Increasing the concentration of colloidal chitin typically positively correlates with enzyme production and activity in both bacteria and fungi. Optimizing the concentration of colloidal chitin in the culture medium directly increases chitinase production. Potassium is an essential macronutrient and can act as an enzyme cofactor, but data show that varying the concentration of potassium sodium tartrate (as a source of K+ and Na+) does not significantly affect chitinase activity. Several studies have reported that K+ ions (Xie *et al.*, 2021). No significant interaction was found between the concentration of chitin colloid and potassium sodium tartrate in increasing chitinase activity (Zihad *et al*., 2022).

Maximum chitinase activity (approximately 18.1825 U/mL) was achieved when colloidal chitin (A) and KH2PO4 (D) were at the highest levels tested. Functionally, colloidal chitin acts as a substrate and inducer of enzyme production, while KH2PO4 provides phosphate and potassium which are essential for energy transfer processes and maintain cell osmotic balance. This finding confirms that to obtain optimal enzyme production, the availability of industrial substrates and essential mineral nutrients must be filled simultaneously at high concentrations. (Meriem & Mahmoud, 2017).

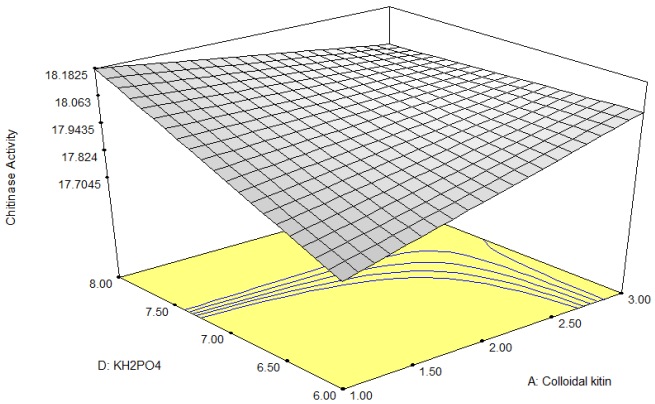


Fig. 3. Surface plot of chitinase activity response from the model equation: Effect of colloidal chitin and KH_2PO_4

Although the highest activity was achieved at high concentration combinations, the plot surface showed very minimal curvature (almost flat), indicating that the interaction between Colloidal chitin and KH_2PO_4 was not very strong. In other words, the effect of Colloidal chitin on chitinase activity was relatively stable across different KH_2PO_4 concentration levels, and vice versa. This means that changes in the concentration of one component did not drastically affect the impact of the other component. However, due to the consistently positive trend, the model recommends using the highest concentration combination of both components within the tested range to achieve optimal chitinase activity.

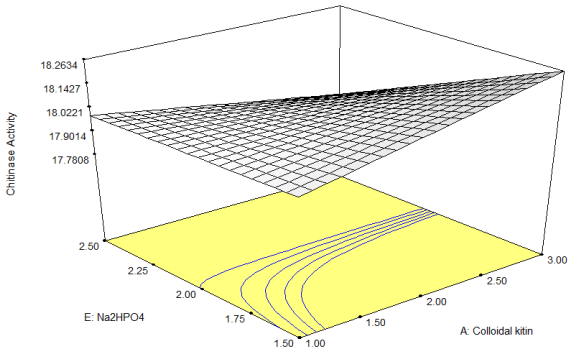


Fig. 4. Surface plot of chitinase activity response from the model equation: Effect of colloidal chitin and Na_2HPO_4

The response surface, which lacks extreme curvature or saddle points, confirms that both colloidal chitin as a substrate and Na_2HPO_4 as a supporting nutrient must be present simultaneously to achieve optimal chitinase activity. Maximum enzyme activity was only achieved when colloidal chitin was at the highest concentration allowed in this study (Kurniawan *et al*., 2022).

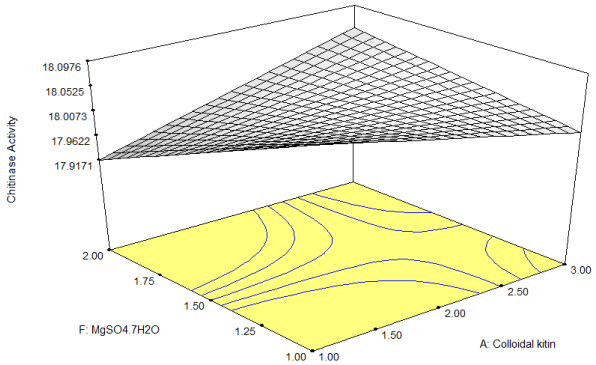


Fig. 5. Surface plot of chitinase activity response from the model equation: Effect of colloidal chitin and $MgSO_4 \cdot 7H_2O$

The nearly flat surface response with a slight slope towards the highest concentration of both variables indicates that both chitin and $MgSO_4 \cdot 7H_2O$ colloids contribute to the increase in chitinase activity, but the effect is relatively small and not statistically significant in the tested range (Widodo *et al*., 2022). Medium optimization studies showed that the combination of high concentrations of both components could increase chitinase activity.

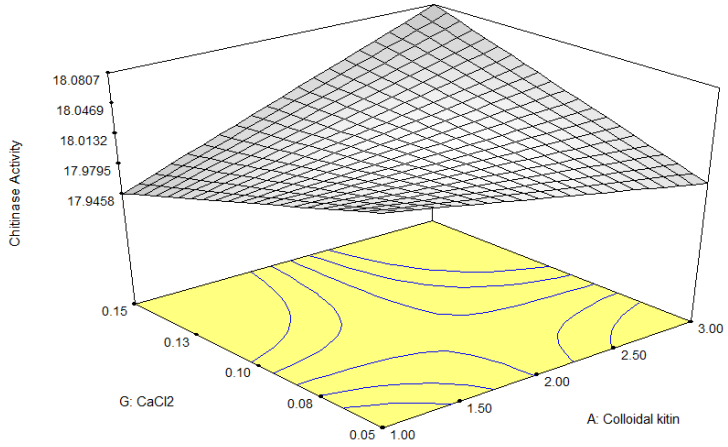


Fig. 6. Surface plot of chitinase activity response from the model equation: Effect of colloidal chitin and $CaCl_2$

Research shows that colloidal chitin does indeed act as a major inducer that stimulates the biosynthesis and activity of the chitinase enzyme, with the highest activity usually achieved at the optimal concentration of colloidal chitin. Meanwhile, $CaCl_2$ as a source of calcium ions (Ca^{2+}) can increase the stability and activity of chitinase, but the effect is highly dependent on the concentration; in some studies, the addition of Ca^{2+} has been shown to activate the enzyme, but if the concentration is sufficient, further addition does not provide a significant increase (Abu-Tahon & Isaac, 2020).

In other words, although calcium is required, increasing its level above a certain minimum point does not provide a significant increase in chitinase activity, even when the availability of the substrate (Colloidal chitin) is increased. Therefore, for this system, the optimization of chitinase activity is more dominantly determined by other factors in the model (such as the level of KH_2PO_4 or Na_2HPO_4 or the quadratic term of another variable) rather than the interaction between Colloidal chitin and $CaCl_2$.

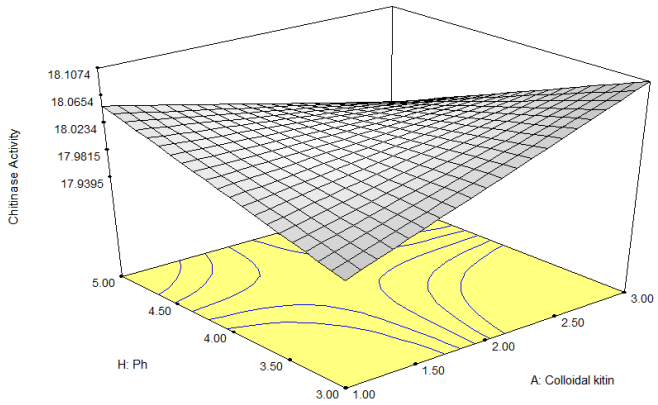


Fig. 7. Surface plot of chitinase activity response from the model equation: Effect of colloidal chitin and pH

Colloidal chitin has been consistently used as a substrate in chitinase activity assays, and the results indicate that this enzyme is highly efficient in hydrolyzing colloidal chitin into products such as N-acetylglucosamine (GlcNAc) and *chitooligosaccharides* (Deng *et al*., 2019). The combination of optimum pH and colloidal chitin availability is crucial to maximize enzyme activity, so adjustment of these two factors is necessary for biotechnological applications, such as chitin waste processing

and value-added oligosaccharide production (Guo *et al*., 2024). pH values and Colloidal chitin concentrations that are both in the middle (constant point) give a suboptimal response, explaining the need for extreme adjustments in one or both factors to achieve maximum chitinase activity.

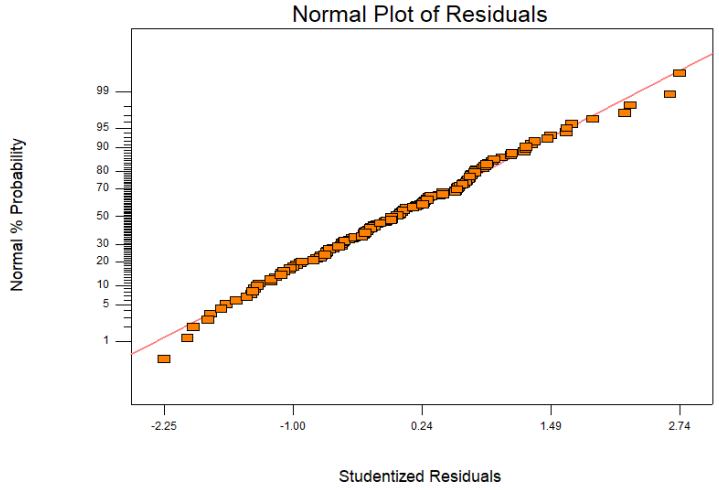


Fig. 8. Normal probability of studentized residuals for chitinase production

In this plot, all data points (residuals) are closely arranged along a straight diagonal line. A distribution that follows this line closely is a strong indication that the residuals are normally distributed. This is crucial because the normality of the residuals is one of the fundamental assumptions that must be met for the significance test (p-value in ANOVA) and confidence intervals generated by the regression model to be considered valid. If the residuals are spread far from the straight line, it would indicate problems, such as the presence of significant outliers, improper data transformation, or the presence of important variables not included in the model.

Several studies also emphasize the importance of checking the normality of residuals, not raw data, to ensure the reliability of regression models (Midway & White, 2025). If the assumption of normality is not met, measures such as data transformation or the use of nonparametric methods may be considered. Thus, a good normal plot of residuals provides statistical validation that the quadratic regression model used to predict and optimize chitinase activity is reliable and can be used optimally (Barker & Shaw, 2015).

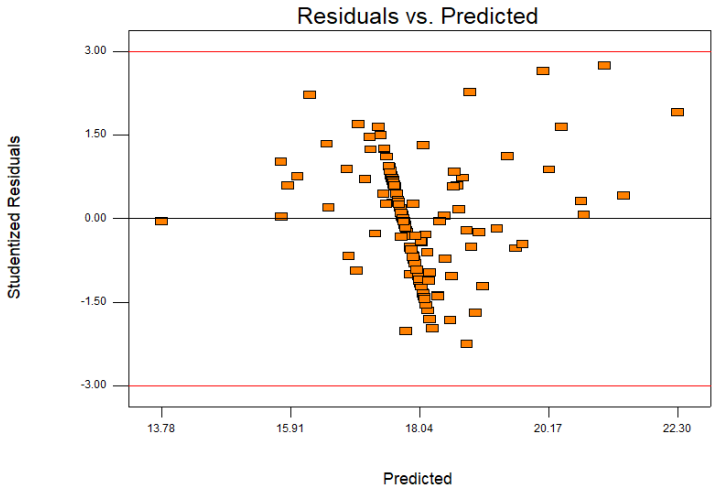


Fig. 9. Plot residuals versus predicted responses

Observation of the plot shows that the data points are randomly distributed around the horizontal zero line (0.00), and do not form a clear curved pattern (such as an inverted U-shape or parabola). This generally indicates that the quadratic regression model used (typical for Response Surface

Methodology) is quite appropriate and adequate in explaining the relationship between experimental factors and chitinase activity. This means that there is no need to add higher-order terms (such as cubic terms) to improve the model's fit. However, the residuals appear to be unevenly distributed along the α-axis. The residuals appear wider in the middle of the predicted values and narrower at the ends. This uneven distribution, or "inverted funnel" shape, indicates heteroscedasticity (non-constant residual variance). This implies that the model may have varying prediction accuracy depending on the predicted chitinase activity level

CONCLUSION

A two-level factorial design was employed in this study to screen for significant factors influencing chitinase production, including colloidal chitin, ammonium sulfate, potassium sodium tartrate, KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, and pH. Chitinase activity was used as the performance indicator. The analysis revealed that the derived mathematical model adequately explained the relationship between variables, with a coefficient of determination (R^2) of 0.9515. Three variables, ammonium sulfate, Na_2HPO_4, and $MgSO_4 \cdot 7H_2O$ were identified as significantly affecting chitinase production ($P < 0.05$). However, this study was limited to the factor-screening stage and did not include optimization of production conditions. Consequently, further research using approaches such as Response Surface Methodology (RSM) is required to more precisely determine the optimal conditions.

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