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# Chitinase from Bacillus sp. SRTI8: production, purification and biocontrol activities

**Sara Sahnoun[1*], Bilal Yahiaoui[1], Aïcha Benlounissi[3], Hassiba Laribi-Habchi[2], Abdenacer Mouffok[1]**

[1] *Laboratory of Applied Microbiology, Faculty of Microbiology, Ferhat Abbas University, Setif, Algeria*
[2] *Laboratory of Functional Analysis of Chemical Processes, Faculty of Technology, Saad Dahleb University, Blida, Algeria*
[3] *Higher National School of Biotechnology, Taoufik Khaznadar University, Constantine, Algeria*

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**Abstract**

From sand in the Algerian Sahara, an isolated strain of Bacillus called Bacillus sp. SRIT8 showed little chitinase activity when grown in a minimal medium supplemented with chitin (2.36 U). Using Plackett-Burman and Box-Behnken statistical plans, we could maximize chitinase synthesis, which led to a notable increase in this enzymatic activity (112 U). The purification of the resulting enzyme involved three steps: ammonium sulfate precipitation, molecular exclusion chromatography, and anion exchange chromatography. This process yielded a specific activity of 5437.14 U/mg with a purification yield of 22.44%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis examination revealed a protein band of about 31 kDa, and optimum enzyme activity was found at pH 5 and 40 °C. Enzyme activity was boosted by Ca+2, Na+, and Mn+2 ions but was suppressed by Hg+2 ions. The purified enzyme inhibited the growth of the plant pathogen Fusarium graminearum on wheat in both in vitro tests. So, it might prevent fungal infections in wheat throughout the germination process. The enzyme was also effective as a bioinsecticide, killing up to 52% of the larvae of Sitophilus granarius Linnaeus, an insect pest of stored grain. Our chitinase's capacity to hydrolyze fungus cell walls as well as insect cuticles can be utilised as biological control agent.

**Keywords:** *Bacillus sp* SRIT8, Chitinase, Statistical optimization, Purification, Biological control agent.

## 1. Introduction

Chitin is a biopolymer resulting from the polymerization of N-acetylglucosamine units linked together by a β-1,4 type bond. This molecule has been identified in the shells of crustaceans, the skeletons, and the peritrophic membranes of insects, and is part of the structure of fungal cell walls [1-3]. Some microorganisms produce several chitinases with different but complementary functions, where one activity complements the activity of the other until the complete degradation of this complex polymer [4,5]. Random internal cleavage by endochitinases produces low-molecular-weight multimers like chitotriose, chitotetraose, and diacetylchitobiose from this polymer. The enzyme chitobiase (EC 3.2.1.30) converts the latter into N-acetylglucosamine. N-acetylglucosamine (NAG) breakdown by β-N-acétylglucosaminidases yields NAG monomers [6]. Chitinases have been identified in several bacterial and fungal strains, such as *Aeromonas*, *Actinomyces*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Aspergillus*, and Trichoderma. They also exist in nematodes, plants, insects, and fishes. These enzymes have various functions in nutrition, morphogenesis, parasitism, and defense according to the needs of the producing organism [7,8].

Chitinases have been exploited industrially in agriculture and medicine. Microbial hydrolysis of the chitin chain generates products of medical importance: chitooligosaccharides and N-acetylglucosamine [9]. Chitinase participates in the manufacture of ophthalmic preparations and the control of malaria, the isolation of fungal and yeast protoplasts, and the production of single-cell proteins (SCP) [10,11]. Chitinase is part of the biocontrol of fungi and phytopathogenic insects by its ability to degrade chitin [12-14].

This research aims to identify and characterize a bacterium responsible for synthesizing extracellular chitinase and establish optimal fermentation conditions for the enhanced production of this enzyme. This chitinase has been the subject of research into its purification and characterization, as well as its potential application as a molecule with fungicidal and insecticidal action.

## 2. Materials and methods

### 2.1. Microorganism

The strain of *Bacillus sp.* was isolated from a rhizospheric soil of the plant species *Calotropis procera*, the

* Corresponding author.
E-mail address: Sarra.sahnounn@gmail.com (S. Sahnoun).
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wilaya of Illizi, Algeria. The chitinase activity of the SRTI8 strain was tested quantitatively on an agar medium amended with chitin (g/L): $Na_2HPO_4$, 6; $KH_2PO_4$, 3; $NH_4Cl$, 1; NaCl, 0.5; yeast extract, 0.05; chitin, 10, and quantitatively on a mineral salt broth supplemented with chitin (1% w/v), and incubated at 30 °C, 100 rpm. After each 48 h of incubation, the cultures were centrifuged at 12,000 rpm, 10 min at 4°C. The supernatant was analyzed for chitinase activity.

### 2.2. Chitinase assay and protein estimation

A mixture of chitin (0.2% w/v) and culture supernatant (0.5 mL) in a 50 mM sodium citrate buffer (pH 5) was made. The mixture was heated to 40 degrees for 15 minutes to speed up the process. Using the DNS approach, the amount of N-acetylglucosamine was calculated. The quantity of chitinase enzyme necessary to liberate one mole of N-acetylglucosamine per minute was designated as one unit (U) [15]. Bovine Serum Albumin (BSA) was used as a reference protein and 750 nm as the absorbance wavelength to calculate protein concentration, as described by Lowry et al. [16].

### 2.3. Effect of carbon sources on chitinase production

A variety of "chitins" have been used in this work, namely powdered chitin (Sigma), colloidal chitin and swollen chitin. Colloidal chitin was prepared according to the method used by Roberts and Selitrennikoff [17], The swollen chitin preparation followed the method of Monreal and Reese [18]. In order to determine the effect of these different forms of chitin on the production of chitinase, three different broths were used, namely M1 (0.3% chitin, 0.1% $MgSO_4$, $7H_2O$, 0.02% $K_2HPO_4$, 0 1% yeast extract and 1.5% agar), M2 g/L (Luria Bertaini broth): tryptone, 10; yeast extract, 5; NaCl, 5; chitin, 10), M3 g/L: $(NH_4)_2SO_4$, 1.0; $MgSO_4$,. 7H, 0, 0.3; $KH_2PO_4$, yeast extract 1.36, 0.5, chitin 10). These media were amended with different forms of chitin. Each medium was inoculated with the SRIT8 strain and incubated at 30° C with shaking (100 rpm) for 3 days. Chitinase activity was estimated as described below.

### 2.4. DNA extraction and identification of the isolate SRIT8

The Wizard Genomic DNA Purification Kit was used to isolate DNA from bacteria cultured in Luria Broth (LB) (Promega, USA). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with the following primers: 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (GGTTACCTTGTTACGACTT-3'). HOT FIREPol® (Solis Biodyne, Estonia) was used for amplification, and the protocol recommended by the manufacturer was followed. The temperatures used throughout the cycling process were as follows: activation at 95°C for 10 min, 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. An ABI 3730 XL gene analyzer was used for the sequencing (Applied Biosystem, ThermoFisher, USA). The sequencing information for the 16S rRNA gene was evaluated using the NCBI's advanced BLAST search tool (found here: http://www.ncbi.nlm.nih.gov/BLAST). Data about the nucleotide sequence has been submitted to a database to be assigned an accession number. A neighbor-joining approach was used for the phylogenetic analysis in MEGA X: Molecular Evolutionary Genetics Analysis on many Platforms [19].

### 2.5. Statistical optimization of strain SRTI8 chitinase production conditions

#### *2.5.1. Plackett-Burman design*

*Bacillus sp.* SRIT8's chitinase production was initially screened using a Plackett-Burman design to determine which factors were most influential (PBD). Ten different variables were used in this analysis: (A) chitin, (B) $MgSO_4$, (C) $CaCl_2$, (D) $MnCl_2$, (E) $K_2HPO_4$, (F) NaCl, (G) peptone, (H) yeast extract, (J) $(NH_4^+)_2SO_4$, and (K) temperature. Every variable has two possible values: high (+1) and low (-1). (-1). Three independent runs of each experiment were performed to ensure reliability, and Design-Expert was used to evaluate the data (version 11, State-Ease Inc., Minneapolis, USA). The following equation from the first-order model was used to assess the experimental results of this setup (Eq. 4):

$$Y = \beta_0 + \Sigma \beta_i x_i$$

(Eq. 1)

In this formula, Y represents the output (the amount of chitinase produced), $\beta_0$ represents the intercept term, $\beta_i$ represents linear coefficients, and $x_i$ represents the level of the independent variable. Variables having a confidence level of 95% or above were judged to have a substantial impact on chitinase production.

#### *2.5.2. Box-Behnken design*

After identifying the most influential factors in *Bacillus sp.* SRIT8 chitinase production, the Box-Behnken design was applied to optimize the screened components for maximum chitinase output [20]. The optimization study included both physicochemical, nutritional, and cultural parameters. The Box-Behnken design is plotted with three factors (A) yeast extract, (B) peptone, (C) chitin, and three levels for each factor (-1, 0, and +1). The general form of the polynomial equation (Eq. 2) of the second order is as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

**(Eq. 2)**

Where $Y$ is the response (chitinase activity), $\beta_0$ is the constant term; $\beta_1$, $\beta_2$, and $\beta_3$ are the coefficients of the linear terms; $\beta_{11}$, $\beta_{22}$, and $\beta_{33}$ are the coefficients of the quadratic terms; $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$ are the interaction coefficients.

### 2.6. Production of chitinase by *Bacillus sp.* SRTI8 in submerged fermentation

Chitinase production was initially performed in a growing colony of *Bacillus sp.* SRIT8 cultured in nutrient agar for 24 h was used to inoculate 50 mL of a nutrient broth with 0.2% chitin (medium used for the inoculum preparation) and incubated at 30 °C for 3 h. Then, 2.5% of inoculum consisting of $1.18\times10^7$ CFU/mL was transferred to a production medium containing 250 mL of optimized culture medium (chitin, 2; yeast extract, 10; peptone, 12; $(NH_4)_2SO_4$, 12; $K_2HPO_4$, 3.49; $CaCl_2$, 0.2; NaCl, 0.21; $MnCl_2$, 0.2). For optimal chitinase activity, the culture was incubated at 25 °C (20 h). The culture was incubated, centrifuged at 12000 rpm for 10 min, and the resulting supernatant was analyzed for chitinase activity and protein content. At 600 nm, the optical density was measured to assess the bacterial growth.

### 2.7. Purification of chitinase

#### *2.7.1. Precipitation of proteins with ammonium sulfate and acetone*

The culture filtrate was separated into fractions containing quantities of ammonium sulfate (from 30 to 100 % saturation). Then, the supernatant was chilled with acetone (-20°C) overnight and stirred at 4 °C. Different enzyme fractions were isolated at various acetone/culture filter ratios (0.5/1, 1/1, 1.25/1, 1.5/1, and 2/2). The protein precipitate was produced by centrifugation at 12,000 rpm for 20 min at 4 °C, dissolved in tris-HCl buffer (50 mM, pH 7), and dialyzed against the same buffer for 12 h at 4 °C. Protein and chitinase activity levels were calculated for each fraction.

#### *2.7.2. Gel filtration chromatography*

Dialysate was purified by passing through a 2.5 cm x 30 cm Sephadex G-50 gel filtration column pre-equilibrated in Tris-HCl buffer (50 mM, pH 7). The enzyme was separated into 4 mL fractions with the same elution buffer. Protein and enzyme activity were determined for each fraction.

#### *2.7.3. Anion exchange chromatography*

Tris-HCl buffer was used to equilibrate a DEAE-Sephadex ion exchange column (2.5 cm x 30 cm). Then the prior suspension was added to the column (50 mM, pH 7). 2 mL fractions were recovered and tested for chitinase activity and protein concentration after being washed with 2 volumes of starting buffer and eluted with a 0 to 0.5 M NaCl gradient, respectively. Chitinase-active fractions were combined and concentrated using ultrafiltration with an Amicon membrane (Millipore).

#### *2.7.4. Molecular weight determination*

The molecular weight of pure chitinase was estimated using a 12% polyacrylamide gel electrophoresis as established by Laemmli [86]. The molecular weight was calculated by comparing the sample's mobility to that of standard 15-120 kDa molecular weight markers (Thermo Scientific).

### 2.8. Biochemical analysis of purified chitinase

#### *2.8.1. Effects of temperature and pH on chitinase activity*

To determine the optimal temperature for enzyme activity, the enzyme was incubated at different temperatures ranging from 20 to 70 °C. The optimal pH for purified chitinase was tested over a pH range of 4-9 using sodium citrate (pH 4- 6) and Tris-HCl (pH 7- 9) buffers.

#### *2.8.2. Effect of selected activators and inhibitors on chitinase activity*

The effects of the metal ions $Mg^{+2}$, $Cu^{+2}$, $Fe^{+2}$, $Ca^{+2}$, $K^{+}$, $Zn^{+2}$, $Mn^{+2}$, $Hg^{+2}$, $Co^{+2}$, and $Na^{+2}$ were examined by incubating the enzyme in sodium citrate buffer (50 mM) with a metal ion (5 mM) for 30 minutes at 40 °C, and then determining the residual chitinase activity. In addition, the effects of surfactants (Tw 20, Tw 80, and Triton X-100 at 0.5%), methanol, and butanol were investigated (10 %).

### 2.9. Evaluation of the antifungal activity of purified chitinase

#### *2.9.1. Inhibition of fungal growth by purified chitinase*

An agar diffusion test of purified chitinase was performed to evaluate the antifungal activity of the enzyme on the growth of the pathogen *F. graminearum*. Wells were filled with 200 U of purified chitinase, and the boiled enzyme was used as a control.

#### *2.9.2. Protection of wheat grains with purified chitinase*

According to Gurav et al. [21], purified chitinase (20 U/mL) was used to treat wheat seeds. After washing and drying, the grains were kept on blotting paper in Petri dishes (10 grains per dish). The grains treated with distilled water are considered a negative control. The dishes were exposed to the external environment for 10 days (after each 24h, the same dose of chitinase was added), and the blotting paper was flooded regularly with 2 mL of distilled water to retain humidity. During the incubation, the development of the infection and the percentage of germination of the grains were monitored.

#### *2.9.3. Antifungal activity of chitinase in potato*

Holy potato tubers were rinsed with water, treated with 70 % ethanol, rinsed again with sterile distilled water, placed in dishes, and inoculated with $10^7$ spores of F. *graminearum* (1 lesion/tuber). After the appearance of disease on the tubers (8 days, the same dose of chitinase was added after each 24 h), and they were treated with purified chitinase (10 U/mL) and maintained at room temperature (25 °C). The size of lesions resulting from Fusarium head blight (mm) was measured.

### 2.10. Estimation of bioinsecticidal activity of purified chitinase

This protocol was performed according to the method of Laribi-Habchi et al. [22]. The bioinsecticidal activity of chitinase against the *Sitophilus granarius* insect was evaluated using different doses of purified chitinase (1, 3, 5, 7, and 10 U/mL) in petri dishes. Each enzymatic dose was homogeneously dispersed on 5 g of wheat, and the treated grains were put in contact with 10 insects aged 0-24 h. The number of dead insects in each dish was counted after 12, 24, and 48 h of contact, and the percentage of mortality was calculated by applying the following two formulas successively (Eqs. 3 and 4):

$$M\ (\%) = \text{dead insects/total insects treated} \times 100$$

$$CM\ (\%) = \text{test M} - \text{control M}\frac{\%}{100} - \text{control M}(\%) \times 100$$

**(Eq. 4)**

### 2.11. Statistical analyses

Design-Expert 11 software was used to analyze the results obtained by the Plackett-Burmann and Box-Behnken designs. The GraphPad program (version 9.2.0) was used for statistical analyses at the 5% level ($p = 0.05$). The data were subjected to analysis of variance (ANOVA), followed by a Dunnett test to determine the differences in means between treatments.

## 3. Results

### 3.1. Chitinase activity in a non-optimized culture medium

A rapid screening of the SRIT8 strain for the detection of chitinase activity was carried out by spreading the inoculum of the isolate on the agar medium supplemented with chitin; the strain remarkably hydrolyzed the chitin

and produced a clear zone around the bacterial colony, indicating enzyme activity.

Chitinase activity of the SRIT8 isolate was tested in liquid medium containing 1% chitin; the isolate exhibited low extracellular enzyme activity (4.4 U) when measured quantitatively after 36 h of incubation.

### 3.2. Molecular identification

The partial 16S rRNA gene sequence of isolate SRIT8 showed 98% identity with *Bacillus amylolequifaciens* (Figure 1) (GenBank accession no. OL742456).

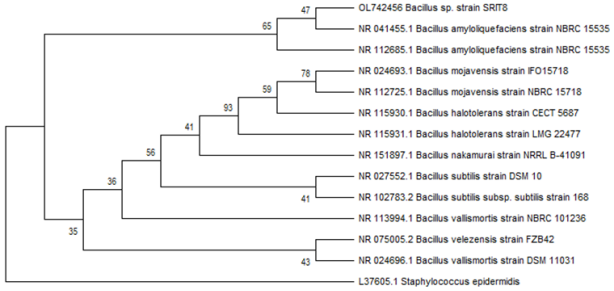


**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences obtained by the neighbor-joining (NJ) method showing the phylogenetic relationship of the *Bacillus sp.* SRIT8 with the related species. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA X. The tree is rooted with *Staphylococcus epidermidis* (L37605.1).

### 3.3. Selection of carbon source for chitinase production

To identify a suitable carbon source for chitinase production, chitinase production by isolate SRIT8 was tested in the presence of different chitin sources in three different fermentation media after 72 h of incubation. The results showed that enhanced levels of chitinase production were observed when powdered chitin was used as a carbon source. The yield of chitinase was 2.36, 3.53 and 4U in M1, M2 and M3, respectively, followed by medium containing swollen chitin and finally colloidal chitin. The powdered chitin, which showed the highest activity, was used in the following experiments as a carbon source. Similar results were obtained by Dai et al. [23] and Farag et al. [24] When chitin powder is used as a carbon source, maximum chitinase activity has been produced by *Paenibacillus sp* and *Aspergillus terrus*, besides, colloidal and swollen chitin are complicated to process and require huge volumes of acid, for this direct application of chitin is preferable in industrial production.

### 3.4. Statistical optimization of culture conditions for chitinase production by Bacillus sp. SRIT8

#### *3.4.1. Screening for significant variables*

*Bacillus sp*. SRIT8 produced 2.36 U of chitinase with the basal medium. A statistical technique for optimizing the medium was used to improve output levels here. Using a Plackett-Burman analysis, this study determined which aspects of culture impacted chitinase production most. Experiments were conducted using the Plackett-Burman method, and the results are shown in Table 1. The observed response (chitinase activity) is represented as a first-degree equation (Eq. 5) using the regression coefficients and the *P*-value.

$$Activity = 4.49 - 1.75\,A - 0.0683\,B + 0.7467\,C + 0.0317\,D + 1.58\,E - 1.14\,F + 1.79\,G + 0.43\,H + 0.3517\,J - 2.82\,K$$

**(Eq. 5)**

Where, A, chitin; B, $MgSO_4$;C, $MgSO_4$; D, $MnCl_2$; E, $K_2HPO_4$; F, NaCl; G, peptone; H, yeast extract; J, $(NH_4)_2SO_4$; K, temperature.

Table 2 displays the findings of the one-way ANOVA. Model significance was determined by the *F*-value (10668.25), the *P*-value (0.0075), and the $R^2$ value (coefficient of determination). Student's t-test was used to analyze variance to look for statistically significant effects. Chitinase production was substantially influenced by factors with confidence intervals larger than 95% ($P < 0.05$). There were a total of eight parameters that were determined to be significant: chitin, temperature, $K_2HPO_4$, NaCl, and peptone were the most important ($P$=0.0075), followed by $CaCl_2$ and yeast extract ($P$=0.01) and then $(NH_4)_2SO_4$ ($P$=0.02). Negative and insignificant impacts were seen for $MgSO_4$ and $MnCl_2$. They were eliminated from further testing.

**Table** 1. Plackett–Burman experimental design matrix with experimental and predicted response (Chitinase activity).

| Run | A (g/l) | B (g/l) | C (g/l) | D (g/l) | E (g/l) | F (g/l) | G (g/l) | H (g/l) | J (g/l) | K (°C) | Y (U) | Y' (U) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 15 | 0.1 | 0.2 | 0.2 | 3 | 0.5 | 0.5 | 0.5 | 7 | 30 | 7.28 | 7.27 |
| 2 | 3 | 0.5 | 0.05 | 0.2 | 3 | 3 | 0.5 | 0.5 | 1 | 37 | 0.51 | 0.4967 |
| 3 | 3 | 0.1 | 0.2 | 0.05 | 3 | 3 | 8 | 0.5 | 1 | 30 | 11.29 | 11.30 |
| 4 | 15 | 0.5 | 0.05 | 0.05 | 1 | 3 | 0.5 | 0.5 | 7 | 30 | 0.11 | 0.1233 |
| 5 | 3 | 0.1 | 0.2 | 0.05 | 1 | 3 | 0.5 | 5 | 7 | 37 | 0.48 | 0.4667 |
| 6 | 15 | 0.1 | 0.05 | 0.05 | 3 | 0.5 | 0.5 | 5 | 1 | 37 | 0.2 | 0.2133 |
| 7 | 3 | 0.1 | 0.05 | 0.2 | 1 | 0.5 | 8 | 0.5 | 7 | 37 | 4.03 | 4.04 |
| 8 | 3 | 0.5 | 0.2 | 0.2 | 1 | 0.5 | 0.5 | 5 | 1 | 30 | 7.62 | 7.63 |
| 9 | 15 | 0.1 | 0.05 | 0.2 | 1 | 3 | 8 | 5 | 1 | 30 | 4.08 | 4.07 |
| 10 | 15 | 0.5 | 0.2 | 0.05 | 1 | 0.5 | 8 | 0.5 | 1 | 37 | 1.14 | 1.13 |
| 11 | 15 | 0.5 | 0.2 | 0.2 | 3 | 3 | 8 | 5 | 7 | 37 | 3.62 | 3.63 |
| 2 | 3 | 0.5 | 0.05 | 0.05 | 3 | 0.5 | 8 | 5 | 7 | 30 | 13.54 | 13.53 |

A- Chitin, B- $MgSO_4$, C- $CaCl_2$, D- $MnCl_2$, E- $K_2HPO_4$, F- NaCl, G- Peptone, H- Yeast extract, J- $(NH_4)_2SO_4$, K- Temperature, Y- Activity, Y'- Predicted activity

**Table 2.** Analysis of variance of the response of chitinase production by *Bacillus. sp* SRIT8.

| Source | Sum of Squares | Degrees of freedom | Mean Square | *F*-value | *P*-value | Coefficient estimate |
|---|---|---|---|---|---|---|
| Model | 227.59 | 10 | 22.76 | 10668.25 | 0.0075 | |
| A-Chitin | 36.89 | 1 | 36.89 | 17292.25 | **0.0048** | - 1.75 |
| B-$MgSO_4$ | 0.0560 | 1 | 0.0560 | 26.27 | 0.1227 | - 0.0683 |
| C-$CaCl_2$ | 6.69 | 1 | 6.69 | 3136.00 | **0.0114** | 0.7467 |
| D-$MnCl_2$ | 0.0120 | 1 | 0.0120 | 5.64 | 0.2537 | 0.0317 |
| E-$K_2HPO_4$ | 30.02 | 1 | 30.02 | 14071.89 | **0.0054** | 1.58 |
| F-NaCl | 15.69 | 1 | 15.69 | 7353.06 | **0.0074** | - 1.14 |
| G-Peptone | 38.52 | 1 | 38.52 | 18056.64 | **0.0047** | 1.79 |
| H-Yeast extract | 2.24 | 1 | 2.24 | 1048.14 | **0.0197** | 0.4317 |
| J-$(NH_4)_2SO_4$ | 1.48 | 1 | 1.48 | 695.64 | **0.0241** | 0.0317 |
| K-Temperature | 95.99 | 1 | 95.99 | 44997.02 | **0.0030** | - 2.83 |
| Residual | 0.0021 | 1 | 0.0021 | | | |
| Cor Total | 227.59 | 11 | | | | |

**Table 3.** Box - Behnken experimental design matrix and results for the optimization of yeast extract, peptone and chitin for maximum chitinase production by *Bacillus sp.* SRIT8.

| Run | Factor 1 A:Yeast extract g/l | Factor 2 B:Peptone g/l | Factor 3 C:Chitin g/l | Response Activity (U) | Predicted Activity (U) |
|---|---|---|---|---|---|
| 1 | 10 | 10 | 3 | 47.38 | 51.22 |
| 2 | 10 | 8 | 2 | 70.8 | 63.39 |
| 3 | 5 | 8 | 2 | 68.49 | 69.29 |
| 4 | 10 | 12 | 2 | 107.16 | 106.36 |
| 5 | 7.5 | 10 | 2 | 78.75 | 74.89 |
| 6 | 7.5 | 12 | 1 | 63.54 | 59.97 |
| 7 | 10 | 10 | 1 | 50.22 | 54.59 |
| 8 | 7.5 | 12 | 3 | 58.54 | 55.51 |
| 9 | 7.5 | 8 | 1 | 40.34 | 43.37 |
| 10 | 7.5 | 10 | 2 | 69.68 | 74.89 |
| 11 | 5 | 10 | 3 | 34.04 | 29.67 |
| 12 | 7.5 | 10 | 2 | 76.25 | 74.89 |
| 13 | 5 | 12 | 2 | 45.13 | 52.54 |
| 14 | 5 | 10 | 1 | 32.06 | 28.22 |
| 15 | 7.5 | 8 | 3 | 42.34 | 45.91 |

### *3.4.2. Box-Behnken design*

Optimal chitinase activity was calculated by analyzing the Box-Behnken model, which considered the relationships between several factors. Chitin, yeast extract, and peptone concentrations were optimized with the help of this investigation. Fifteen separate tests were conducted. Table 3 displays the experimental setup and the resultant chitinase activity.

Analysis of variance (ANOVA) and the Fisher *F* test validated the model's goodness of fit (Table 4). The numerical *F-value* came out to be 11.44. The low coefficient of variation (CV) and the *P*-values for the model (0.0077 and 0.2367, respectively) suggest that the acquired experimental data fit the model well, and the little lack of fit is appropriate for the model. Results from these trials may be trusted when the CV is less than 12.38 %. The $R^2$ score (0.9537) indicates a high agreement between the observed and expected results.

Each coefficient's significance is examined using its associated *P*-value. A significant coefficient is associated with a low *P*-value. Effects favor chitinase generation with positive coefficients, whereas the inverse is true for adverse effects. The coefficient A in the linear term (yeast extract) profoundly impacts the enzyme's efficiency. This suggests that this variable can alter chitinase synthesis and that even a slight variation in its concentration can have a noticeable effect. The model found the yeast extract, peptone interaction ($P<0.01$), and the chitin quadratic term ($P<0.05$) statistically significant. Important model terms in this scenario include A, AB, and $C^2$. Multiple regression analysis was used to characterize the connection between the experimental variables, their interactions, and the response (Eq. 6).

$$Activity = 33.29 + 5.34\,A + 2.89875\,B - 0.2138\,C + 6.61\,AB - 0.535\,AC - 0.7775\,BC - 2.71\,A^2 + 1.85\,B^2 - 12.38\,C^2$$

**(Eq. 6)**

Where A, yeast extract; B, peptone; C, chitin.

Using chitin as the carbon source and peptone and yeast extract as the nitrogen source, Figure 2 depicts the interaction reactions between process variables. The data show that enzyme synthesis is significantly boosted when yeast extract and peptone concentrations are kept high.

**Table 4.** Analysis of variance for the quadratic response surface.

| Source | Sum of Squares | Mean Square | *F*-value | *P*-value | Coefficient estimate | |
|---|---|---|---|---|---|---|
| Model | 5483.80 | 609.31 | 11.44 | **0.0077** | | Significant |
| A-Yeast extract | 1148.16 | 1148.16 | 21.55 | **0.0056** | 11.98 | |
| B-Peptone | 343.22 | 343.22 | 6.44 | 0.0520 | 6.55 | |
| C-Chitin | 1.86 | 1.86 | 0.0350 | 0.8590 | - 0.4825 | |
| AB | 891.62 | 891.62 | 16.73 | **0.0094** | 14.93 | |
| AC | 5.81 | 5.81 | 0.1090 | 0.7547 | - 1.21 | |
| BC | 12.25 | 12.25 | 0.2299 | 0.6518 | - 1.75 | |
| $A^2$ | 138.82 | 138.82 | 2.61 | 0.1674 | - 6.13 | |
| $B^2$ | 63.08 | 63.08 | 1.18 | 0.3262 | 4.13 | |
| $C^2$ | 2861.10 | 2861.10 | 53.70 | **0.0007** | - 27.84 | |
| Residual | 266.39 | 53.28 | | | | |
| Lack of Fit | 222.50 | 74.17 | 3.38 | 0.2367 | | Not significant |
| Pure Error | 43.89 | 21.95 | | | | |
| Cor Total | 5750.20 | | | | | |

As a result, the two factors substantially interact (Figure 2AB). Figure 2AC shows the significant reduction in chitinase activity at lower and higher concentrations of chitin. Chitinase activity increased when chitin increased from 1 to 2 g/L, but additional chitin content showed a decrease in chitinase production.

The maximum activity of chitinase has been obtained using the punctual prediction capacities of the design program of design to find the optimal values of the parameters. The maximum synthesis of chitinase was obtained at a chitin concentration of 2 g/L, 10g/L of yeast extract and 12 g/L of peptone. The equation of the model was validated and the production of the highest planned chitinase was 106.46 U. The results were resumed by testing the optimal nutritional concentrations. The average activity of chitinase was 112 U. The reliability of experimental design procedures is demonstrated by the high degree of concordance between the expected response and the acquired experimental data.

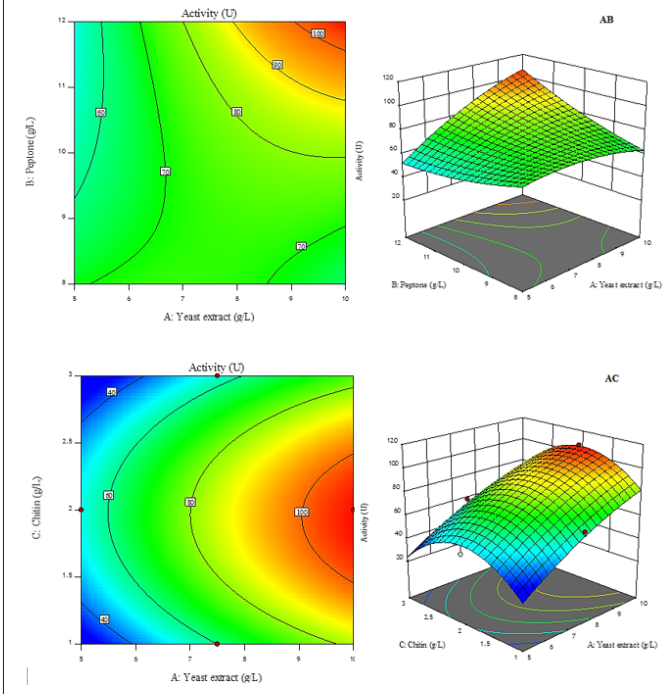


**Fig. 2.** Three-dimensional response surface curves and contours of chitinase production by *Bacillus sp.*SRIT8 showing interactions between peptone and yeast extract (AB), chitin and yeast extract (AC).

### 3.5. Enzymatic purification

After the chitinase production step by strain SRIT8, protein precipitation was performed by ammonium sulfate and acetone, and adding ammonium sulfate to the supernatant results in better enzyme precipitation than acetone. The results obtained from the purification steps are presented in Table 5.

The dialyzed protein was subjected to gel filtration, and the chitinase yield was 59 %, with a purification factor of 3.70. The active fractions obtained were passed through the DEAE-Sepharose column. A purification at 16-fold of the enzyme, a recovery of 22.45% and a specific activity of 5437.14 U/mg were obtained in this stage. The obtained protein was subjected to SDS-PAGE: the appearance of a single band on the polyacrylamide gel confirms its purity, with a molecular weight of about 31 kDa (Figure 3).

**Table 5.** *Bacillus. sp* SRIT8 chitinase purification.

| Steps | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Culture supernatant | 339.13 | 1.015 | 334.11 | 1 | 100 |
| $(NH_4)_2SO_4$ precipitation | 287 | 0.401 | 715.71 | 2.14 | 84 |
| Sephadex-G50 | 201.83 | 0.163 | 1238.22 | 3.70 | 59 |
| DEAE-sepharose | 76.12 | 0.014 | 5437.14 | 16.27 | 22.44 |

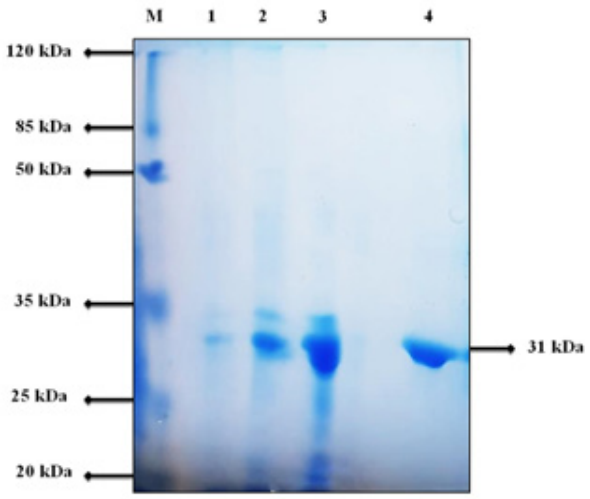


**Fig. 3.** SDS-PAGE analysis of chitinase purified from *Bacillus sp.* SRTI8. M, molecular weight markers; lane 1, proteins purified by Sephadex G-100; lane 2, ammonium sulfate precipitated proteins; lane 3, supernatant proteins; lane 4, ion exchange purified proteins.

## 3.6. Enzymatic characterization

### *3.6.1. Effects of pH and temperature on chitinase activity*

After the purification of the enzyme, its characterization allowed us to know the zone of activity of the enzyme at different pH and, therefore, the capacity of its use [3]. Our enzyme shows chitin hydrolysis efficiency at acidic pH values (4, 5, and 6), with optimal activity at pH equal to 5 (Figure 4A); a further increase in pH beyond 6 results in a progressive decrease in activity. Purified chitinase showed almost similar activity at 20, 30, 40, 50, and 60 °C, with a slight increase at 40°C (Figure 4B).

### *3.6.2. Effects of metal ions, organic solvents and detergents on chitinase activity*

The effect of various metal ions on chitinase activity was studied (Figure 4C). The enzyme activity was enhanced by $Ca^{+2}$, $k^{+}$, $Na^{+}$, $Mn^{+2}$, and slightly slowed by $Zn^{+2}$, $Mg^{+2}$, $Fe^{+2}$, and strongly inhibited by $Cu^{+2}$, $Co^{+2}$. $Hg^{2+}$ completely inactivated the enzyme. The influence of various non-ionic detergents (Tween 20, Tween 80, Triton X-100) and organic solvents was presented in Figure 4D. The addition of Tween 20, 80, and Triton X-100 caused some increase in enzyme activity. Adding methanol at a concentration of 0.5% decreased the initial chitinase activity to half, while the chitinase activity dropped sharply in the presence of butanol.

## 3.7. Potential antifungal role of purified chitinase in the biocontrol of *F. graminearum*

A good diffusion test was performed to show the effect of purified chitinase on the growth of *F. graminearum*, significant inhibition of mycelium growth was observed in purified chitinase with an inhibition zone diameter of 21 mm. At the same time, the control (boiled enzyme) showed no inhibitory activity against the same fungus.

### *3.7.1. Application of chitinase to prevent fungal infections in wheat grains*

For wheat grains coated with purified chitinase, a 100% germination rate was obtained, as well as extensive root development (≥6 cm) was recorded, with a slight fungal infection that appeared after the 6th day of exposure to the external environment (Figure 5a). In contrast, untreated grains were more susceptible to fungal infection; fungal mycelia appeared on the seeds after 2 days, with a reduction in germination rate of 60 % (Figure 5b).

### *3.7.2. Involvement of purified chitinase in treating Fusarium head blight in potato tubers*

We tested purified chitinase from strain SRIT8 for its ability to slow the growth of *F. graminearum* on potato tubers (Figure 6). After the appearance of *F. graminearum* filaments on the potato (8 days after spore infection), 10

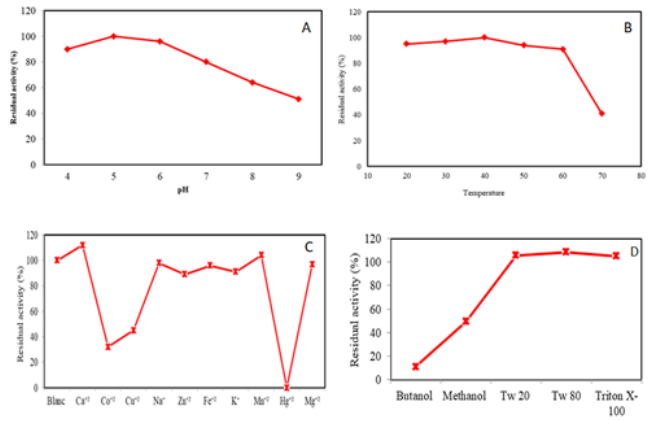


**Fig. 4.** Effects of pH (A), temperature (B), metal ions (C), organic solvents and detergents (D) on the activity of purified chitinase.

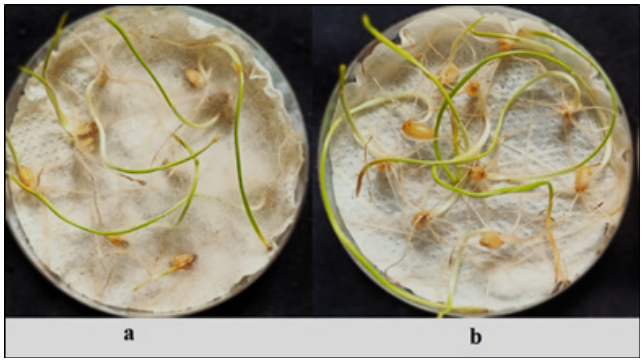


**Fig. 5.** Prevention of fungal infections and effect on the germination of wheat grains. (a) control, (b) grains treated with purified chitinase.

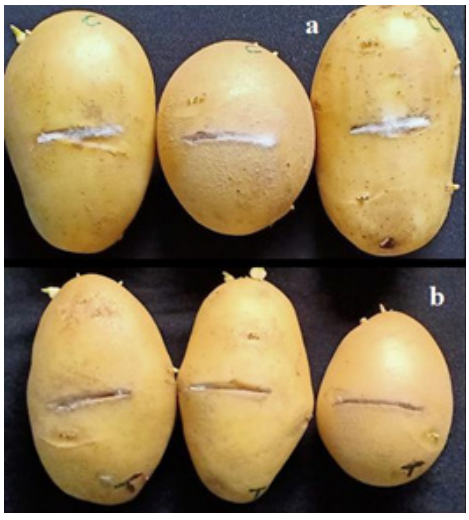


**Fig. 6.** Potato Fusarium wilt treatment, (a) control without chitinase, (b) tubers treated with 60 U/mL chitinase.

U of chitinase was sprayed on the lesions. After 7 days of treatment with the enzyme, the development of the fungus was partially inhibited lesion size was (1-3) mm compared to the control treated with distilled water (5-7 mm).

### 3.8. Insecticidal activity of purified chitinase

Figure 7 shows the bioinsecticidal effect of purified chitinase; exposure of *Sitophilus granarius* L. to wheat grains previously treated with chitinase causes a 52 % mortality after 24h of contact. The mortality rate of the insect was increased with the increase of the enzymatic dose until reaching its maximum at 10 U/mL.

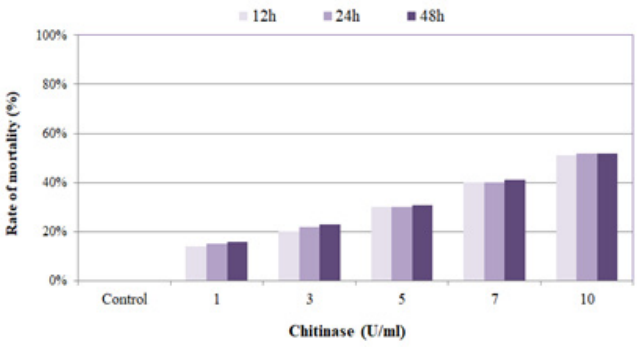


**Fig. 7.** The mortality rate of the insect pest *Sitophilus granarius* L under the effect of different concentrations of purified chitinase sprayed on wheat grains.

## 4. Discussion

*Bacillus sp.* is a bacterium well known for its ability to degrade chitin polymer, such as *B. subtilis* [25], *B. pumilus* [26], *B. licheniformis* [27], *and B. amyloliquefaciens* [28]. The composition of culture medium can significantly affect the enzyme production; for this, statistical methods of optimization of fermentation medium ingredients have been implicated in industrial biotechnology [29]. Little information is available regarding using the statistical conception for chitinase production by *Bacillus sp.* strains. Chitinases, an inducible enzyme, are synthesized exclusively when chitin is available as the carbon source [30, 31]. Sandhya et al. [32] indicated that induction of the chitinase enzyme requires a chitin concentration between 10-20 g/L, and our results (2 g/L) appeared lower than the previous range. On the other hand, a minimal substrate concentration with a higher enzyme production can be an advantage for industries [33]. Yeast extract has been shown to increase chitinase synthesis by a variety of microorganisms, including *Alcaligenes xylosoxydans* [34], *Stenotrophomonas maltophilia* [35], *Humicola grisea* [36], and *Bacillus pumilus* [26]. But, chitinase synthesis by *Streptomyces griseorubens C9* [37] and *Pseudomonas fluorescens* strain HN1205 [38] was considerably altered by the addition of yeast extract to the growth medium. Similarly, Wang et al. [39] articulated the positive effect of peptone and $K_2HPO_4$, increasing chitinase activity for better production by *Serratia marcescens*. Some other nitrogen sources, including ammonium sulfate, increase chitinase production in *Bacillus licheniformis* AT6 [9], *Lysinibacillus fusiformis* B-CM18 [40]. Minerals can also affect the amount of chitinolytic enzyme production. *Sanguibacter antarcticus* [41] and *Pantoea dispersa* [42] make more chitinase when the production medium contains $CaCl_2$ and NaCl.

The highest chitinase activity was obtained with the fractions precipitated with ammonium sulfate. Similar results to our result were obtained by Farag et al. [43], while other researchers show better precipitation with acetone than with ammonium sulfates, such as the precipitation of cellulase, pectinase, and xylanase from *Penicillium chrysogenum* [44], and polygalacturonase from *Aureobasidium pullulans* [45]. In this study, the chitinase was purified 16 times with a recovery of 22.45% of the initial activity, other results of purification of chitinases from *Bacillus* allowed obtaining a yield 58% with 15-fold purification [46], 3% with 15-fold purification [47], 35% with 11-fold purification [48], 15% with 16-fold purification [49]. Our enzyme presented a molecular mass of around 31 kDa, the same molecular weight was reported for *Bacillus subtilis* chitinase NPU 001 [50]. Different molecular weights have been recorded for other chitinases: *Bacillus sp* R2, 41 KDa [51]; *Bacillus licheniformis* B307, 42 KDa [52]; *Bacillus licheniformis* SSCL-10, 66KDa [53].

The purified chitinase had maximum activity at pH 5, a similar value was obtained for the optimal pH of chitinases from *Brevibacillus formosus* BISR-1 [54] and *Aeromonas veronii* B565 [55]. The optimum temperature for enzymatic activity was found to be 40 °C, which is similar to that found in the chitinase of *Bacillus pumilus* MCB-7 [56], *Bacillus circulans* No.4.1 [57], *Lecanicillium lecanii* [2] and *Alcaligenes faecalis* [4]. The presence of metal ions as components of the active site is necessary for the catalytic activity of several enzymes to preserve their stability [58]. The enzymatic activity of our chitinase is enhanced by $Ca^{+2}$, $k^{+}$, $Mn^{+2}$ and inactivated by $Hg^{+2}$, and this is in agreement with the results obtained in other research [1, 59-61]. $Cu^{+2}$, $Co^{+2}$ inhibit the activity of our chitinase while they increase the chitinase activity of *Aeromonas* sp. PTCC 1691 [62]. In enzymatic reactions, adding organic solvents to the reaction medium makes hydrophobic substrates more soluble, facilitating the formation of the substrate-enzyme complex [63]. The activity of purified chitinase is slightly enhanced by Tw 20, Tw 80, and significantly reduced by butanol and methanol. This result is similar to those obtained for chitinase from *Lecanicillium lecanii* [2], *Bacillus cereus* TKU027 [64], and *Aspergillus terreus* [43].

The main structural carbohydrate polymers in the cell wall of *Fusarium sp.* are chitin and glucan, and these polymers effectively contribute to the rigidity of the cell wall [65]. Studies by Okay et al. [66] show that the zone of growth inhibition of *Trichoderma harzianum* and *Alternaria citri* by *Serratia marcescens* MO-1 chitinase can reach 16 and 17 mm, respectively. Purified chitinase from *Aspergillus niger* LOCK 62 causes 60% inhibition of the hyphal extension of *Fusarium culmorum* [67]. Senol et al. [3] purified an antifungal chitinase from *Bacillus subtilis* TV-125A against *Fusarium culmorum*. An *Alternaria alternata* inhibitory chitinase was also isolated from *Serratia marcescens* [68], and *Cellulosimicrobium cellulans* 191 produces a chitinase that inhibits *Rhizopus oligosporus* [69]. Wang et al. [70] purified two enzymes (FI and FII) from *Bacillus amyloliquefaciens* V656 broth culture, and both enzymes showed antifungal activity on *Fusarium oxysporum*. Hungund et al. [8] noted the inhibition of germination of *Aspergillus niger* NCIM 1207 spores by *Bacillus cereus* BSH-4 chitinase. Inhibition at this stage is important because it prevents the asexual life cycle of

the fungus and therefore its propagation. Purified chitinase from *Stenotrophomanas maltophilia* showed a biofungicidal and bioinsecticidal effect against *Fusarium oxysporum* and the Colorado potato beetle *Leptinotarsa decemlineata* [71].

*F. graminearum* is the causative agent of the Fusarium head blight of cereals. It mainly infects wheat and barley [72]. At the time of germination, seeds are susceptible to infection by pathogens. To prevent these infections, different chemical and biological treatments are applied to the seeds [73]. It can be concluded that initial seed treatment with chitinase could be an excellent way to protect seeds against different fungal infections, as well as to minimize the number of chemical fungicides used. Similar results were obtained for chitinase from *Bacillus pumilus* RST25 on soft wheat grains [21] and chitinase from *Bacillus thuringiensis* on soybeans [74].

*F. graminearum*, the responsible pathogen of ear blight on wheat, barley, and maize, in recent years, *has been* considered among the 13 Fusarium species responsible for post-harvest dry rot of potatoes. It was isolated from lesions of tubers affected by the disease [75,76]. Loc et al. [77], used purified chitinase from *Trichoderma asperellum* PQ34 to treat anthracnose caused by *Colletotrichum sp.* on mango and chili. Greenhouse experiments by Li et al. [78], showed the efficacy of *Bacillus cereus* strain CH2 chitinase in controlling *Verticillium dahlia*-induced verticillium wilt on eggplant. It reduced the severity of verticillium wilt by 53.13% in 14 days. Shternshis et al. [79] tested three biological products, *Bacillus thuringiensis* (BACTICIDE), the metabolites of *Streptomyces avermitilis* (PHYTOVERM), and the chitinase of *Streptomyces sp.* It was shown that utilizing BACTICIDE® and PHYTOVERM® to manage raspberry midge illness caused by the pathogen *Thomasiniana theobaldi* reduced midge severity by a factor of 2. However, the latter was reduced by four after being sprayed with chitinase. Raspberry sting blight caused by the fungus Didymella applanata was decreased significantly in field experiments using *Streptomyces sp.* chitinase under natural circumstances [80].

Insect pests are considered the leading cause of post-harvest wheat yield losses; improper storage conditions facilitate the access of these pests. The grain weevil *Sitophilus granarius* L. is the most encountered storage pest [81]. This mortality can be explained by the ability of chitinase to bind to the chitin, constituting the insect's cuticles, inducing its hydrolysis, and thus the insect's death [82]. Laribi-Habchi et al. [83] show the insecticidal effect of ChiA-Si40 produced by *Shewanella inventionis* against *Sitophilus granarius* (the grain weevil). A 100% mortality rate of the insect was achieved. As well as chitinase extracted from red scorpion fish, *Scorpaena scrofa* offal resulted in 100% mortality of *Callosobruchus maculatus* [84]. The study by Rishad et al. [56] indicated that incubation of purified chitinase from *Bacillus pumilus* MCB-7 with *Scirpophaga incertulas* larvae causes 60% larval mortality after 12 days of incubation. Also, 100% mortality was observed after contact between *Helopeltis theivora*, the tea bug, with chitinase purified from *Pseudomonas fluorescens* MP-13 [85].

Our present study shows the effectiveness of the Plackett-Burman and Box-Behnken experimental designs in optimizing chitinase production by *Bacillus sp.* SRIT8, resulting in a 47-fold increase in chitinase production compared to a non-optimized medium. Purification of chitinase was also performed, and a 22% purification yield was obtained. The purified enzyme was characterized, the optimum temperature and pH were found to be 40 °C and 5. Our study also reveals that the purified chitinase from *Bacillus sp*. SRTI8 can serve as a biofungicide and bioinsecticide, by its ability to control the growth of the fungal species *Fusarium graminearum* and by increasing the larval mortality of *Sitophilus granarius* Linnaeus.

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**Conflict of interest**

The authors declare no competing interests.

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