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Optimization and Molecular Docking insights of Alkaline Protease Production by *Bacillus safensis* Strain lab418 for Biocontrol of *Meloidogyne incognita*

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Abstract

Plant parasitic nematodes (PPN) cause significant losses in global agricultural production. The use of nematicides to prevent nematodes poses serious risks to both human health and the environment. Therefore, it is essential to find a safer alternative treatment. In this study, we explained the use of microbial bioagents such as alkaline protease to control *Meloidogyne incognita*.The isolate of lab418 was employed as a potential source of alkaline protease. By analyzing the sequence of the 16S rRNA gene, we identified the strain lab418 as *Bacillus safensis* lab418. This sequence has been assigned the accession number OR888822 in the NCBI database. To improve the production of alkaline protease by *B. safensis*, we employed the BOX-Behnken Design (BBD) and Plackett-Burman Design (PBD). Through optimization, we were able to significantly increase alkaline protease production to 245 U/mL, which represents a 1.92-fold improvement compared to the non-optimized production. This increase in alkaline protease production led to a mortality rate of 98% for *M. incognita*. Furthermore, positive effects on plant growth, specifically in terms of fresh weight and shoot and root length were recorded.These improvements can be attributed to the activation of biochemical characteristics related to plant defense mechanisms against nematodes, such as polyphenol oxidase, chitinase, glucanase, and phenolic compounds. Additionally, in silico analyses to investigate the anticipated protein interactions between alkaline protease and collagen present in the nematode cuticle was conducted, demonstrating the enzyme's binding and catalytic activity.

Keywords: Alkaline protease; Bacillus safensis; 16S rRNA; Optimization; Molecular docking; Nematode biocontrol.

1. Introduction

Plant parasitic nematodes (PPN) cause significant crop losses, estimated at 70% of annual crop production. Meloidogyne spp., which are soil-borne pathogens, pose a significant threat to horticultural and field crops worldwide, resulting in substantial economic damage [1-4]. The use of chemical nematicides has impacted ecosystems and public health. Therefore, there is a need for sustainable and eco-friendly alternatives for PPN management. In this regard, biological and biotechnological methods offer a promising solution, which represent in employment of rhizobacteria as biological factories for releasing potential bioagents

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for controlling plant nematodes[5].Several plant growth-promoting rhizobacteria (PGPR) possess nematicidal activities. These bacteria produce enzymes such as protease and chitinase, which are involved in the biocontrol of plant nematodes [6,7]. By inducing systemic plant resistance, they help limit nematode damage. Rhizobacteria, particularly certain strains of *Bacillus* spp., have been effective in reducing root-knot nematodes [8,9].The production of proteases by these bacteria has been widely utilized for nematode biocontrol [10-12]. Proteases play an essential role in the infection process of nematodes since the cuticle covering juveniles and the outermost coating of eggs consistof proteins [13].

Alternatively, various enzymes, including chitinase, polyphenol oxidase, and glucanase, have been associated with resistance development and the reduction of disease infection in plants [14]. This may occur through the release of phenolic compounds or metabolites related to the plant defense mechanisms [15-17]. Optimization of protease using response surface methodology (RSM) is a valuable tool for developing fermentation processes. RSM is a mathematical and statistical model that helps establish a functional interaction between multiple variables and a desired reaction [18,19].

Molecular docking is an in-silico analysis technique used to study the interaction between a protein and a substrate molecule, which can identify target protein binding sites and enhance understanding of fundamental metabolic processes [20]. It is frequently employed as a cost-effective and efficient method to predict the best binding affinity between receptor proteins and their substrates [21].

In this investigation, we isolated and identified a new strain that produces protease. We utilized the Plackett-Burman and central composite designs to optimize the cultural conditions and maximize the production of alkaline protease. Subsequently, we assessed the nematicidal activity of the alkaline protease from this new isolate against *M. incognita*, both in vitro and on infected eggplants under screen house conditions. Furthermore, we conducted molecular interaction studies between the alkaline protease and the collagen protein found in the cuticle layer of the nematode.

2. Material and Methods

2.1 Screening and isolation for protease-producing bacteria

Isolation was performed from milk samples freshly collected from an Egyptian farm.One millilitre of each sample was added to 9 mL of sterile distilled water and heated at 80°C for 10 minutes to eliminate most vegetative cells. The specimens were then placed on gelatin agar plates containing peptone (5 g), yeast extract (3 g), bacteriological agar (15 g), and gelatin (10 g) in 1000 mL of distilled water was adjusted to pH 7 [22]. The plates were incubated overnight at 37°C. Colonies that formed clear zones due to gelatin hydrolysis were identified as protease producers. The bacterial isolates were preserved in 15% glycerol at -80°C. Twenty-five strains were isolated, and the strain with the largest obvious zone of breakdown was selected for further investigation and designated as lab 418.

2.2. Molecular identification of the bacterial isolate

The bacterial DNA from the bacterial isolate lab 418 was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) and used as a template for amplifying the 16S rRNA gene through PCR.
The forward primer,
27F (5'AGAGTTTGATCCTGGCTCAG3'),
and the reverse primer,
1492R (5'GGTTACCTTGTTACGACTT3') [23], were used along with the Emerald AmpGT PCR master mix (2x premix) from Takara, Japan. The PCR program consisted of an initial denaturation at 94ºC for three minutes, followed by denaturation at 94ºC for thirty seconds, annealing at 50ºC for one minute, and extension at 72ºC for two minutes. A final extension at 72ºC was performed for ten minutes. The PCR products were purified using Qiagen, Germany's QIAquick Gel Extraction Kit. Electrophoresis on a 1.5% Agarose gel with a 100bp DNA Ladder (Enzynomics) was carried out to determine the molecular length of the target gene. The nucleotide sequence of the 16S rRNA gene was determined by the biological laboratory of colors (Cliniclab, Egypt) using the Sanger sequencing method with a big dye x terminator kit (Thermfisher, USA) and a 3500 genetic analyzer. The obtained sequences were aligned with available databases. The phylogenetic tree was created using the MEGA 11 program [24] by computing distances and grouping using the neighbor-joining method [25] with 1000 Bootstrap analysis [26].

2.3. Production and activity assay of serine alkaline protease

The production of proteases was conducted in a sterile 250-ml Erlenmeyer flask. The flask

contained the following components per 100 ml: 2.5 g defatted copra meal prepared according to AOAC 1990, 0.5 g peptone, and 0.3 g CaCO3. These were considered standardized conditions. Next, 3 ml of bacterial culture wasinoculated.For three days, the pH, temperature, and shaking intensity were set at 9, 30°C, and 150 rpm, respectively. After centrifuging the culture, the supernatant was collected as the source of protease enzyme [27]. Amount of 100 µl of the supernatant were added to 100 µl of casein solution prepared by combining 100 milliliters of 0.1 M Tris-HCl buffer with one gram of casein, then the pH was adjusted to 8.9. The reaction was allowed to proceed for 30 minutes at 37°C. To stop the reaction, 2.0 mL of trichloroacetic acid (TCA) was applied. TCA was prepared by mixing fifteen grams in 100 ml of distilled water. Simultaneously, a blank reaction was also running, which was stopped by TCA at the beginning. After completing the reaction, the mixture was centrifuged and kept in ice for 10 minutes [28,29]. An activity unit was defined as the amount of enzyme required to release 1µg of tyrosine per minute under the test conditions. The protein content released in the clear supernatant was quantified using reference levels of bovine serum albumin, following the method described by Lowry et al[30].

2.4. Statistical optimization of alkaline protease production

The optimization study comprised two stages. In the first stage, the Plackett-Burman design (PBD) was utilized to identify the influential factors that significantly impact protease production [31]. The subsequent phase involved the prediction of the optimal conditions for protease production utilizing response surface methodology (RSM) of BOX-Behnken Design (BBD) [32,33]. By determining the three variables that exhibit the greatest positive effect on protease production, the ideal point was established.

2.5. Plackett – Burman experimental design

Protease was produced by *B. safensis* lab418 in submerged fermentation. The PBD matrix was used to combine seven independent variables into nine combinations for screening; for each variable, a high (+) and low (-) level was assessed. The first-order linear model (Eq. 1) served as the foundation for PBD.

$$Y = B0 + \Sigma Bi\ Xi \quad (1)$$

where Y is the activity (production of proteases), Bi is the linear coefficient, B0 is the model intercept, and Xi represents the independent variable's level. Eq. (2) was used to identify each variable's primary effect:

$$E(Xi) = 2(\Sigma Mi+ - Mi-)/N \quad (2)$$

Where the tested variable's influence was denoted by E(Xi). The variables Mi+ and Mi–, which denote the total number of trials (N), and the high and low levels of the independent variable (Xi) evaluated in those trials were used to describe protease production. The student t-test was employed to determine each examined variable's significance level (p-value).

2.6. BOX–Behnken Design (BBD)

For the RSM of BOX–Behnken Design (BBD), three variables (Time, Temperature, and Peptone) were chosen, there were 17 runs in the experimental design, and three different levels of analysis were performed on the factors that are independently. Through fitting the second-order polynomial function in Eq. (3), It was determined how the independent variables and the anticipated response, Y, related to one another.

$$YActivity = ß0 + ß1X1 + ß2X2 + ß3X3 + ß11X12 + ß22X22 + ß33\ X32 + ß12X1X2 + ß13X1X3 + ß23X2X3 \quad (3)$$

The predicted protease production (U/mL) is called YActivity; the model constant was β0; the independent variables were Time, Temperature, and Peptone, respectively; the linear coefficients are β1, β2, and β3; the cross-product coefficients are β12, β13, and β23; and the quadratic coefficients are β11, β22, and β33. The gathered experimental data were subjected to regression analysis using “Expert Design 11”, or the coefficient of determination, was utilized to show how closely the information matched to the equation for the polynomial model. Threeduplicates of the tests were run, and the mean values were noted.

2.7. Enzyme excretion and partial purification

The bacterial strain lab418 was cultivated using optimized conditions to maximize serine protease production. Subsequently, the cultures were centrifugated at 14000 rpm for 10 minutes to gather the supernatant containing the enzymes. These enzymes were then conveniently precipitated using 60-70% ethanol and quantitatively assessed using

Bradford reagent (BioRad, USA). The crude enzyme precipitate was obtained by centrifuging at 14000 rpm for 10 minutes [27].The partially purified serine alkaline protease was utilized as a bioagent to evaluate its nematicidal effect against *Meloidogyne incognita.*

2.8. SDS and zymogram analysis

SDS-PAGE was performed according to Laemmli [34], and the zymogram was performed according to Yasumitsu[35], using a protein marker 250 kDa (ThermoFisher).

2.9. Evaluation the nematicidal activity of serine alkaline protease

2.9.1. Preparation of *M. incognita* juveniles

Nematode egg masses were obtained from tomato roots cultivated in the screen house of the National Research Centre. The nematodes were identified as *M. incognita* based on the perineal pattern. The egg masses were manually collected after washing the rootsand removing thesoil. Subsequently, a 0.1% sodium hypochlorite solution was added, and the mixture was stirred and washed on a sieve with 26 µm pores using water. The eggs were then incubated for 3-5 days to allow the development of second-stage juveniles (J2) [36].

2.10. Evaluation of Bacterial Culture and alkaline protease's in vitro nematicidal potential on *Meloidogyne incognita.*

2.10.1. Preparation of Protease and Bacterial Culture for biocontrol of *M. incognita*

Two formulas of bioagents were used in this study. The first formula consisted of the protease enzyme, while the second one consisted of a culture suspension of the bacterial strain lab418, known to be the best proteolytic isolate. The protease enzyme from strain lab418 was used in two forms: the first included the crude enzyme, while the second was partially purified. To prepare the enzyme solutions, an initial concentration of 245 U/mL was made using both the crude and partially purified enzyme. This concentration was achieved by dissolving 100 mg of precipitated protein from each form of the enzyme in 5 mL of distilled sterilized water. This concentration was referred to as the stock concentration (S). The bacterial culture used as the source of the protease enzyme was taken at the same growth phase as the culture assigned as the bioagent. The optical density of the bacterial growth was measured to be 1.9.

2.11. In vitro evaluation of Protease and BacterialCulture for biocontrol of *Meloidogyne incognita*

Both crude and partially purified samples were used at two concentrations: the stock concentration (S) and half of that concentration (S/2). These concentrations were applied to the bacterial cultures. In a glass tube containing 300 µl of nematode suspension with approximately 100 ± 5 *M. incognita* juveniles, 1700 µl of each concentration was added to achieve a total volume of 2 ml. A control sample, containing only distilled water, was also prepared. All tubes were incubated at 25°C ± 5°C for 24 hours. The reduction in the number of J2S juveniles was assessed using a Hawksley counting slide under a light microscope [37]. Nematode mortality rates were calculated after 24 hours of exposure using Abbott's formula [38].

2.12. Screen house experiment

Two kilograms of sterilized sandy clay soil (1:1 w/w) were placed into 20 cm diameter plastic pots. One-month-old eggplant seedlings (cv. Alabaster) were transplanted into each pot. Five milliliters of each treatment, along with 2000 *M. incognita* J2S juveniles, wereinoculated to each pot simultaneously. After three months, the eggplant roots were carefully removed and cleaned of soil. Using a sieve and decanting process, the second-stage juveniles (J2S) were extracted from the soil(250 g), and counted under a light microscope [39]. The reduction in nematode numbers was calculated relative to the untreated control. Plant growth parameters, including fresh weight (g) and length (cm) of shoots and roots, were measured and compared to the untreated control.

2.13. Biochemical parameters of eggplant

2.13.1. Determination of the total phenol content

To find the total phenolic contents, one gram of freshly picked leaves from every treatment was blended in 10 milliliters of 80% methanol and agitated for fifteen minutes at 70°C, then 250 µl of the addition of 1 N ofFolin-Ciocalteu reagent to 1 mL of each extract in 5 mL of distilled water. The absorbance of the sample was detected at 725 nm, and the phenolic content was expressed in milligrams

of catechol equal per hundred grams (mg CE/100 g) [40].

2.13.2Extracting determination of enzymes in eggplant leaves

Forty milliliters of phosphate buffer were used to chop and homogenize four-gram plant samples.The extract was centrifuged for 10 min at 4°C at 10,000g and the supernatant was used as the source of enzyme.

2.13.3. Polyphenol oxidase activity

To measure the activity of polyphenol oxidase, the reaction components including 2.5 milliliters of phosphate buffer (0.1 M, pH 7.0), 0.5 mL of the crude enzyme extract, and 1 mL of catechol (0.05 M) as the substrate were mixed [41]. The absorbance was regularly determined every 30 seconds at 540 nm.

2.13.4. Chitinase and glucanase activity

According to Rustiguel et al [42], 0.05 M phosphate buffer pH 6 was used to assess the chitinase activity employing 0.1 percentage of the manufactured substrate 4-nitrophenyl-N-acetyl-β-d-glucosamine and the glucanase activity was evaluated [43].

2.14. Statistical analysis

Analysis of variance (ANOVA) was performed using Duncan's Multiple Range Test (DMRT), that was employed to distinguish significant means at the $P < 0.05$ significance level [44]. For the statistical study, SPSS (Design Expert-11) was utilized. The mean ± standard deviation (SD) was used to represent the data.

2.15. Homology modeling and structural validation of serine protease

The gene sequence encoding the serine protease in bacterial strain lab418 was retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore/OP610065). We used SWISSMODEL (https://swissmodel.expasy.org/interactive) to create the homology model. To authenticate the modeled protein structure, we employed stereochemical parameters from the SAVES server (https://saves.mbi.ucla.edu/), including PROCHECK, Verify 3D, and ERRAT. The cuticle collagen protein (ID: P34688) was retrieved from the UniProt database (https://www.uniprot.org/).

2.19. 2.16.Molecular docking

The PyMOL program prepared the alkaline protease model and the substrate protein. This was achieved by removing atoms and water molecules. Additionally, Autodock 1.5.7 was utilized to add polar hydrogen bonds and adjust the charge. The chosen model was then subjected to energy minimization, which was carried out using the YASARA Server (http://www.yasara.org/minimizationserver.htm). For molecular docking and interaction analysis between the protease and collagen, the Gramm web tool (https://gramm.compbio.ku.edu/) was employed [45]. The resulting protein-protein interaction was visualized using the PyMOL program.

3. Results

3.1. Isolation and molecular identification of protease-producing bacteria

Twenty-five bacterial isolates were successfully obtained from cow milk samples. Among these isolates, one showed a significant hydrolytic zone around its colony, making it the chosen isolate for further investigation (Figure 1).

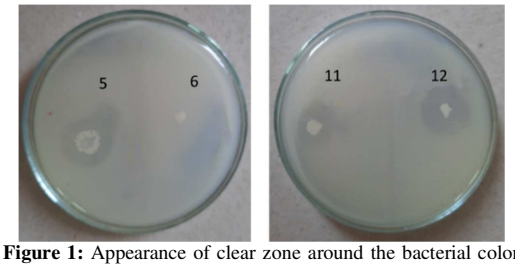

Figure 1: Appearance of clear zone around the bacterial colonies on gelatin agar plates indicating the proteolytic activity

The isolate was identified using 16S rDNA gene sequencing.The 27F and 1492R primers successfully amplified a 1400 bp DNA fragment representing the 16S rRNA region (Figure 2).

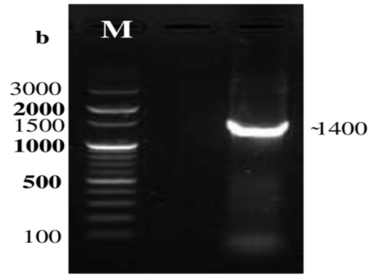

Figure 2: Agarose gel electrophoresis indicating the presence of 1400 bp DNA fragment corresponding to16S rDNA**.** M: 100 bp DNA ladder (enzynomics)

To determine its identity, the nucleotide sequence of the 16S rRNA fragment was compared to data in the NCBI GenBank database. The alignment results revealed that the bacterial isolate exhibited 100% identified with *B. safensis*, and the nucleotide sequence was deposited in the GenBank database under accession number OR888822. A phylogenetic tree was constructed using MEGA 11 (Figure 3), and the selected isolate was closely related to *B. safensis* FO-63b.

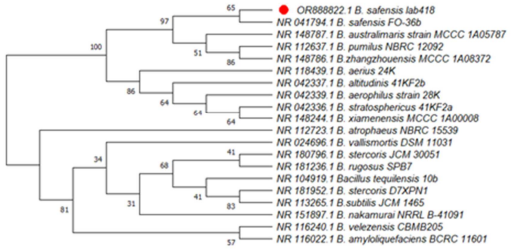

Figure 3: Constructedphylogenetic tree of *Bacillus safensis* Lab418 and its closely related based on the sequence of the 16S rRNA gene using MEGA 11. The values of bootstraps are placed on the branches

3.2. Optimization of alkaline protease production

3.2.1. Placket-Burman design

In order to improve the production of alkaline protease, nine combination matrices with seven independent factors were shown in (Table 1). The combination number 4 in Table 1 produced the highest yield. Increased from 48.69 ±0.98 to 209.99 ±2.42 U/mL. (Figure 4) displayed the primary effect of the variables and suggested that Defatted copra meal, peptone, $CaCO_3$, and time had a positive main effect while, inoculum, temperature, and pH had a negative main effect.

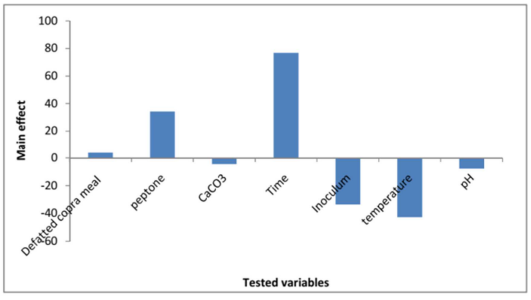

Figure 4: Main effects of independent variables on alkaline protease production by *Bacillus safensis*lab418 Based on the outcomes of the PBD

The regression coefficients, t-test, and p-value of seven independent variables in Table 2 indicated p-values of 0.006, 0.03, and 0.05 for Time, Temperature, and Peptone respectively. Such results enable Time, Temperature, and Peptone the most significant variables affecting protease production. The following first-order model (Eq. 1) may demonstrate the relationship between independent variables and the generation of proteases:

YActivity =200.552+ 1.902X1+68.475X2 -15.216X3+19.211X4-16.738X5-4.260X6 -3.804X7 (Eq. 1)

3.2.2. Box-Behnken Design (BBD)

Through 17 experimental cycles, a Box-Behnken scheme of response surface approach with three levels (- 1, 0, and + 1) was obtained in order to optimize the alkaline protease of *Bacillus safensis* lab418. Within the experimental design, the variables that were independent were evaluated at five distinct levels. Table 3 displays the expected and observed rise in alkaline protease based on a statistical examination of the test factors. Utilizing 0.75 (g/flask) peptone at 27˚C for 8 days, the greatest alkaline production of protease (245± 3.93U/ml) was reported (experimental run #1). Under these circumstances, the alkaline protease production that was predicted was almost exactly the same as the measured value, proving the accuracy of the model. An extremely significant F-value of 94.70 was found by the quadratic regressionmodel's analysis of variance (Table 4). The Predicted R^2 of 0.8697 is in reasonable agreement with the Adjusted R^2 of 0.9814; i.e. the difference is less than 0.2 (Tables 4 and 5).

The percentage of the observed response values' variability that can be accounted for by the tested factors and theirrelationship to one another is shown by the R-squared value.Through repeated regression analysis with the observed data, a second-order polynomial equation (Eq.3) was obtained, and the mathematical maximum point of that equation was found.

YActivity= 210.00+4.87X1+72.00X2-4.38 X3+13.50X12 +14.75X22 +22.50X32-33.37X1X2+ -30.12X1X3-34.88X2X3 (Eq. 3)

where response (YActivity) was predicted protease production and (X1, X2, and X3) were the codes of most effective variables Time, Temperature, and Peptone size respectively. The optimal activity was at trial number 1 which gave 245± 3.93 U/mL (1.92-fold from the basal medium) at 0.75 gm/L of peptone after 8 days at 27°C. A high significant influence of terms with smaller P-values ($P \leq 0.05$) on protease production is indicated by the pattern of interactions between the variables, which is indicated by the P-value, which was employed as a method for verifying the significance of each coefficient (Figure 5).

Additionally, the residual analysis (Figure 6) revealed that the residuals were uniformly distributed across the range and formed a symmetrical pattern, indicating that the average model explained all of the observed data. Plotting the observed-predicted values (residuals) against the response (optimization process) was the method used in this investigation.

The validity of *B. safensis* serine protease was predicted for each matrix trial, hence estimating the validity of the suggested model. The maximum observedprotease production (245 U/mL) was found to be relatively close to the expected value (236.88 U/mL) based on the experimental results presented in Table 3.

3.3. Statistical validation model

Table 1: Placket-Burman design for assessing the components influencing alkaline protease production from *B. safensis* lab418

Trial	Coconut waste (%)	Peptone (%)	$CaCO_3$ (%)	Time days	Inoculum (%)	Temperature ℃	pH	Enzyme activity (U/mL)
1	1.5(-)	0.25(-)	0.15(-)	6(+)	3.5(+)	35(+)	6.5(-)	95.87 ±1.36
2	3.5(+)	0.25(-)	0.15(-)	2(-)	1.5(-)	35(+)	8.5(+)	48.69 ±0.98
3	1.5(-)	0.75(+)	0.15(-)	2(-)	3.5(+)	25(-)	8.5(+)	88.26 ±1.94
4	3.5(+)	0.75(+)	0.15(-)	6(+)	1.5(-)	25(-)	6.5(-)	209.99 ±2.42
5	1.5(-)	0.25(-)	0.45(+)	6(+)	1.5(-)	25(-)	8.5(+)	159.78±0.72
6	3.5(+)	0.25(-)	0.45(+)	2(-)	3.5(+)	25(-)	6.5(-)	60.87 ±2.25
7	1.5(-)	0.75(+)	0.45(+)	2(-)	1.5(-)	35(+)	6.5(-)	82.17 ±1.21
8	3.5(+)	0.75(+)	0.45(+)	6(+)	3.5(+)	35(+)	8.5(+)	121.73 ±1.37
9	2.5(0)	0.5(0)	0.3(0)	4(0)	2.5(0)	30(0)	7.5(0)	127.52±1.53
10	2.5(0)	0.5(0)	0.3(0)	4(0)	2.5(0)	30(0)	7.5(0)	127.52±1.53
11	2.5(0)	0.5(0)	0.3(0)	4(0)	2.5(0)	30(0)	7.5(0)	127.52±1.53

F value = 10.88; *P*>F= 0.038; R^2: 0.962; R =0.980; Adjusted R^2=0.874

Table 2: Statistical analysis of PBD showed the coefficient values, *t* and *P* values for each variable affect alkaline protease production

Variables	Coefficients	Standard Error	t-Stat	P-value
Intercept	200.552	62.40851	3.213	0.04
Coconut waste (%)	1.902	5.757937	0.330	0.76
Peptone (%)	68.475	23.03175	2.973	0.05
$CaCO_3$ (%)	-15.216	38.38625	-0.396	0.71
Time (day)	19.211	2.878969	6.672	0.006
Inoculum (%)	-16.738	5.757937	-2.907	0.06
Temperature C	-4.260	1.151587	-3.699	0.03
pH	-3.804	5.757937	-0.660	0.55

Table 3: Examined the BOX–Behnken Design (BBD) experiment's results and the concentration of its most significant variables

Run	Factor 1 A:Time (Days)	Factor 2 B:Temperature (˚C)	Factor 3 C:Peptone (%)	Response 1 R1 (U/ml)	Predicted Value	Residual
1	8(+)	27(+)	0.75(0)	245± 3.93	236.88	8.12
2	4(-)	23(-)	0.75(0)	75± 1.37	83.13	-8.13
3	4(-)	25(0)	1(+)	116± 2.29	117.75	-1.75
4	6(0)	25(0)	0.75(0)	210± 1.04	210.00	0.0000
5	6(0)	25(0)	0.75(0)	210± 1.04	210.00	0.0000
6	8(+)	23(-)	0.75(0)	65± 2.12	65.88	-0.8750
7	8(+)	25(0)	1(+)	148± 1.30	157.00	-9.00
8	6(0)	25(0)	0.75(0)	210± 1.04	210.00	0.0000
9	6(0)	25(0)	0.75(0)	210± 1.04	210.00	0.0000
10	6(0)	23(-)	1(+)	56± 2.76	46.12	9.88
11	6(0)	25(0)	0.75(0)	210± 1.04	210.00	0.0000
12	4(-)	25(0)	0.5(-)	165± 2.64	156.00	9.00
13	8(+)	25(0)	0.5(-)	138± 2.39	136.25	1.75
14	6(0)	23(-)	0.5(-)	99± 1.19	99.88	-0.8750
15	4(-)	27(+)	0.75(-)	201± 2.75	200.13	0.8750
16	6(0)	27(+)	1(+)	236± 4.35	235.13	0.8750
17	6(0)	27(+)	0.5(-)	189± 3.28	198.88	-9.88

The model's P and F-values were determined to be 0.050 and 94.7, respectively, and it's R2 (coefficient of determination) was 99%, suggesting that the model's equation could account for the variability in the responses. Consequently, the relationship between the three variables—time, temperature, and peptone—and the generation of alkaline protease could be found using the second-order polynomial equation (Eq. 3). The maximal amount of protease produced was 245 U/mL, a 1.92-fold increase over the basal medium and after precipitation of crude enzyme at 60-70% ethanol saturation, 45% of the total regained activity with a particular activity increase of 9.3 times.

3.4. SDS and zymogram analysis of alkaline protease

Protein analysis was carried out for partially purified alkaline protease using SDS-PAGE and zymogram.The result illustrated in Figure 7 reveals that only one band, representing the serine protease with a molecular weight of 32 KDa, exhibited protease activity.

3.5. In vitro evaluation of Protease and Bacterial Culture for biocontrol of *Meloidogyne incognita*

After 24 hours of exposure, the results indicated that the treatment with partially purified protease had the greatest impact on *M. incognita* J2, resulting in the highest mortality percentage. Compared to the control, the partially purified protease demonstrated mortality percentages of 98% and 86% at concentrations of S and S/2, respectively. The crude protease, at the same concentrations, achieved mortality percentages of 82% and 93%, ranking second in comparison to the control. However, the culture suspension of *B. safensis* exhibited the lowest mortality rates compared to the control, with values of approximately 76% and 68% at concentrations of S and S/2, respectively (Table 6).

3.6. Screen house experiment

Under screen house conditions, all enzyme treatments, whether crude or partially purified, significantly reduced *M. incognita* reproduction on eggplant. The outcomes demonstrated that each therapy decreased the nematode parameters significantly ($P \leq 0.05$), these parameters including a reduction in the number of J2s in soil, galls, and egg-masses per root as compared to the original *B. safensis* lab418 strain and the untreated control (Table 7). In general, the result indicates that the partially purified enzyme achieved the best reduction in J2s, galls, and egg-masses, as recorded at 71%, 83 %, and 84%, respectively, at concentrations of 245 U/mL. Followed by the partially purified enzyme at a concentration of 122 U/mL, which recorded 60 %, 79 %, and 71 % at the same parameters, respectively.While the crude enzyme recorded 52%, 65%, and 60% at S concentrations, the concentration of S/2 recorded 43%, 57%, and 52% in the same parameters, respectively. The original strain, *B. safensis* demonstrated the lowest reduction in all nematode parameters. All treatments had significant ($P \leq 0.05$) suppressive effects on different eggplant criteria and enhanced growth parameters (fresh weight (g) and length (cm) of shoots and roots)compared to control (Table 8).The partially purified enzyme recorded the highest increase in length (83%), fresh weight (284%) of shoots, length (108%), and fresh weight of roots (383%) at a concentration of S (Table 7 and Figure 8) as compared to untreated control. Followed by a concentration of S/2, which recorded 57 %, 237%, 76%, and 299% in shoot length, shoot fresh weight, root length, and fresh weight of roots, respectively. In the same trend, crude enzyme recorded the third rank in increasing eggplant parameters and finally original strain *B. safensis* lab418.

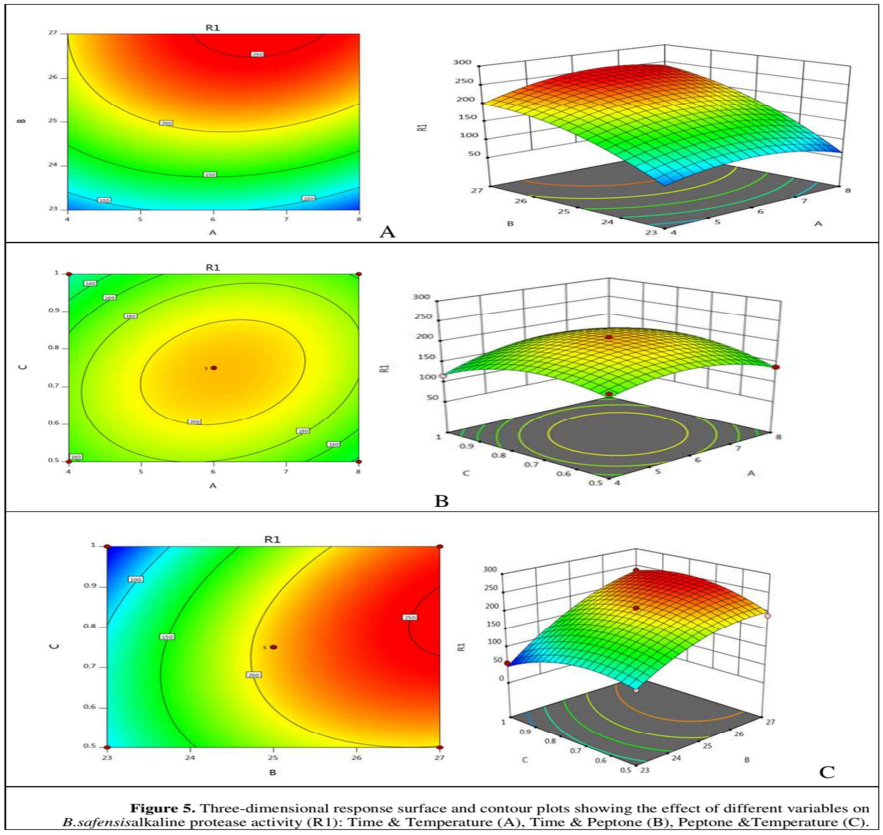

Figure 5. Three-dimensional response surface and contour plots showing the effect of different variables on *B.safensis*alkaline protease activity (R1): Time & Temperature (A), Time & Peptone (B), Peptone &Temperature (C).

3.7. Biochemical parameters of plants.

Protease enzymes support several plant biochemical parameters that are responsible for the plant's defense against diseases. These parameters include phenolic compounds and some enzymes such as chitinase, polyphenol oxidase, and glucanase.The effect of bacterial suspension, crude enzyme, and partially purified enzyme on some plant biochemical parameters was studied, and the obtained results are presented in (Figure 9).

The results revealed that the partially purified enzyme at S concentration demonstrates a significant ($P \leq 0.05$) increase in both chitinase and polyphenol oxidase activities compared to the control and other treatments. Also, the partially purified enzyme (S and S/2) has a significant difference in glucanase activity and phenolic compound production in contrast to the control group and the other groups receiving treatment, but there was no discernible distinction between the two concentrations of the partially purified enzyme. Generally, the partially purified enzyme (S concentration) produced higher enzymatic activity in leaves compared to that treated with bacteria and crude enzyme. The activities of polyphenol oxidase, glucanase, and chitinase were 225.6, 2.46, and 2.36 U/mL respectively, and also the total phenolic compounds reached (2.79 mg/gm).

Table 4: Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	210.00	1	3.77	201.08	218.92	
A-Time	4.87	1	2.98	-2.18	11.93	1.0000
B-Temperature	72.00	1	2.98	64.95	79.05	1.0000
C-Peptone	-4.38	1	2.98	-11.43	2.68	1.0000
AB	13.50	1	4.22	3.53	23.47	1.0000
AC	14.75	1	4.22	4.78	24.72	1.0000
BC	22.50	1	4.22	12.53	32.47	1.0000
A^2	-33.37	1	4.11	-43.10	-23.65	1.01
B^2	-30.12	1	4.11	-39.85	-20.40	1.01
C^2	-34.88	1	4.11	-44.60	-25.15	1.01

Table 5: ANOVA for Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	60667.28	9	6740.81	94.70	< 0.0001	significant
A-Time	190.13	1	190.13	2.67	0.1462	
B-Temperature	41472.00	1	41472.00	582.65	< 0.0001	
C-Peptone	153.13	1	153.13	2.15	0.1859	
AB	729.00	1	729.00	10.24	0.0151	
AC	870.25	1	870.25	12.23	0.0100	
BC	2025.00	1	2025.00	28.45	0.0011	
A^2	4690.07	1	4690.07	65.89	< 0.0001	
B^2	3821.12	1	3821.12	53.68	0.0002	
C^2	5121.12	1	5121.12	71.95	< 0.0001	
Residual	498.25	7	71.18			
Lack of Fit	498.25	3	166.08			
Pure Error	0.0000	4	0.0000			
Cor Total	61165.53	16				

Std. Dev.	8.44	R^2	0.9919
Mean	163.71	Adjusted R^2	0.9814
C.V. %	5.15	Predicted R^2	0.8697
		AdeqPrecision	29.4791

Table 6: Percentages of mortality of *M. incognita* J2 after 24h of the treatment exposure

Treatments	% Nematode mortality
Control	0 g
B.S. (S)	76 e
B.S. (S/2)	68 f
Crude (S)	93 b
Crude (S/2)	82 d
Partial purified (S)	98 a
Partial purified (S/2)	86 c

*Every value is a mean of five replicates, according to Duncan's multiple range test, the same letter (s) in different columns reveals no significant differences at ($P \leq 0.05$).

3.8. Homology modeling and validation of alkaline protease model

Since the protease protein from *B.subtills* (3WHI) shows higher similarity with serine alkaline protease from *B. sefensis* (67%), therefore protease 3WHI was chosen as a template to perform homology modeling for building the 3D structure (Figure 10a,b). Structure alignment between the alkaline protease model and the template (3WHI) was conducted by PyMol software as illustrated in Figure 10c, the calculated root-mean-square deviation (RMSD) was 0.164 which exhibited a higher level of structural similarityThen the selected model was evaluated using the following stereochemical parameters ERRAT, VERIFY3D, and PROCHECK of the SAVES server., the Ramachandran plot of the alkaline protease model was generated (Figure 11).Analysis of the Ramachandran plot for both the 3D modeled of alkaline protease and the template model are presented in Table 9, the analysis of alkaline protease generated model showed that 86.8% of residues fell in the most favored regions, 12.2% fell in the additional allowed regions, 1% in the generously allowed regions and no residues found in the disallowed regions, indicating the good quality of alkaline protease generated model. Moreover, ERRAT and VERIFY3D were used to evaluate the model for alkaline protease, the results were 93.09 and 86.34% respectively. Thus, the results support the reliability of the alkaline protease model. Then, the constructed alkaline protease model was subjected to the YASARA program for energy minimization, the energy was minimized from -118406.3 KJ/mol to -167079.5 kJ/mol.

3.9. Molecular docking

The molecular docking interaction between the generated model of serine alkaline protease from *B.safensis* Lab418 and the cuticle collagen was performed, various poses of protein-protein interactions were generated, and the interaction with the best binding affinity was chosen. The interaction between the alkaline protease and cuticle collagen is illustrated in Figure12. To validate the result of molecular docking interaction, the collagenic activity of the protease was measured. The activity was 85 and 67 U/mL for crude enzyme and partially purified enzyme respectively. So, the result is consistent with the molecular docking study.

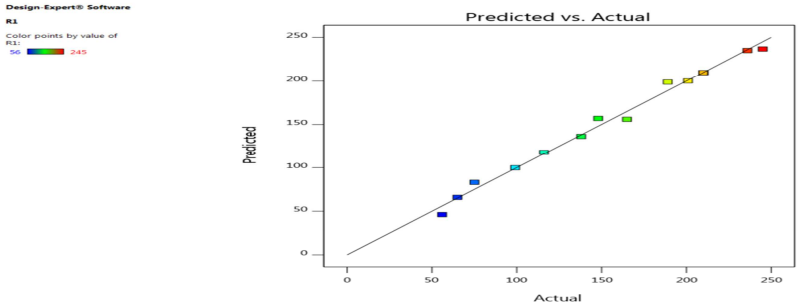

Figure 6: Residual plot of the observed-predicted values (residuals) versus the response (optimization process) of alkaline proteasefrom *Bacillus safensis*lab418

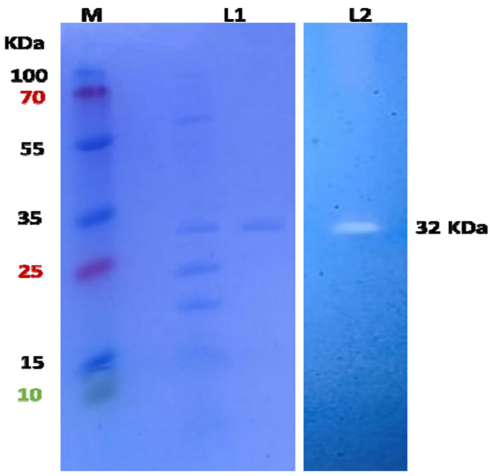

Figure 7: SDS and zymogram gel electrophoresis of partially purifiedfrom *B. safensis* lab418; **M**: protein marker; **L1**: partially purified alkaline protease; **L2**: zymogram of partially purified alkaline protease

Table 7: Effect of the alkaline protease enzyme on *M. incognita* parameters under screen house conditions

Treatments	Count of J2 in Soil	% Reduction	Count of galls/root system	% Reduction	Count of egg-masses / root system	% Reduction
Untreated control	80 a	---	136 a	---	54 a	---
B.S. (S)	51 c	36	69 c	49	29 b	46
B.S. (S/2)	64 b	20	108 b	23	32 b	41
Crude (S)	38 e	52	48 e	65	21 d	60
Crude (S/2)	42 d	43	57 d	58	25 c	53
Partial purified (S)	26 g	71	23 g	83	8 f	84
Partial purified (S/2)	32 f	60	28 f	79	15 e	71

*Every value is a mean of five replicates, according to Duncan's multiple range test, the same letter (s) in different columns reveals no significant differences at ($P \leq 0.05$)

Table 8: Effect of alkaline protease enzyme on plant growth parameters of eggplant infected with *M. incognita* under screen house conditions

Treatments*	Shoots				Roots			
	Length (cm)	% Increase	Fresh weight(g)	% Increase	length (cm)	% Increase	weight (g)	% Increase
Untreated control	15 e	---	11 e	--	15 c	--	4 c	--
B. S (S)	18 d	20	26 c	143	23 b	54	16 b	268
B. S (S/2)	18 d	17	19 d	73	16 c	11	15 b	235
Crude (S)	23 b	55	35 b	227	30 a	100	17 b	286
Crude (S/2)	21 c	40	27 c	151	24 b	61	16 b	273
Partial purified (S)	28 a	83	42a	284	31 a	108	19 a	333
Partial purified (S/2)	24 b	57	37 b	237	26 a	76	18 a	299

Figure 8: Effect of serinealkalineprotease enzyme on eggplant growth under screen house conditions after one month from seedlings inoculated with *M. Incognita.*

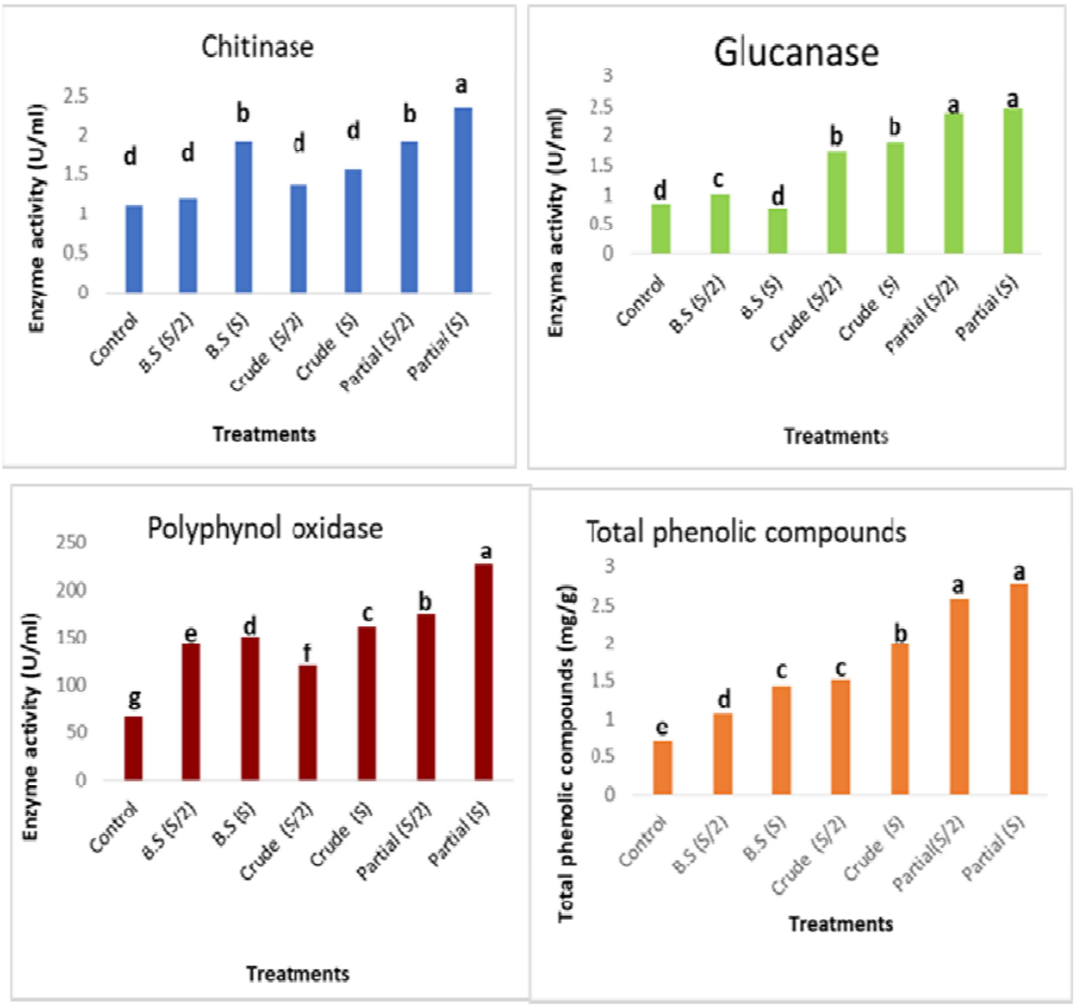

Figure 9: Effect of bacteria, crude enzyme, and partially purified enzyme on some biochemical parameters of eggplant leaves infected with *M. incognita.* such aschitinase, glucanase,polyphenol oxidase activity,andtotal phenolic compound. The concentration of 245 U/ml and123 U/ml corresponded to S and S/2 concentration.

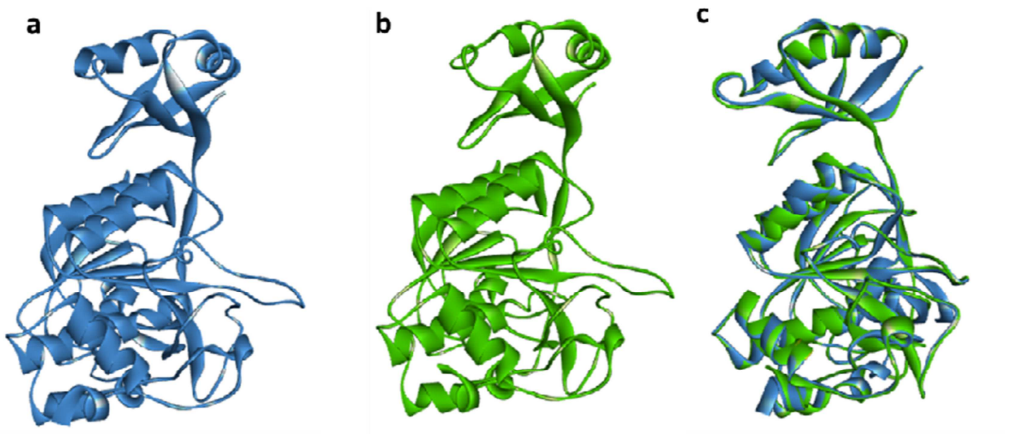

Figure10: 3D structure, **a**: template protease from *B.subtills,* 3WHI, (blue color), **b**: alkaline protease model from *B. safensis*lab418 (green color), **c**: structure alignment between template and model.

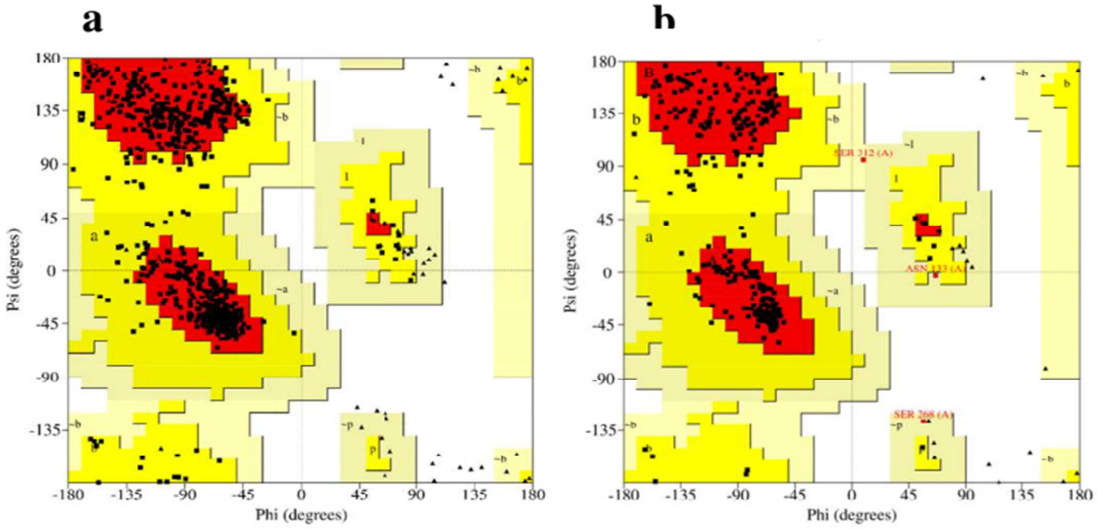

Figure 11: Ramachandran plot, **a**: template protease from *B.subtills* (3WHI): **b**: alkaline protease model from *B. safensis*lab418

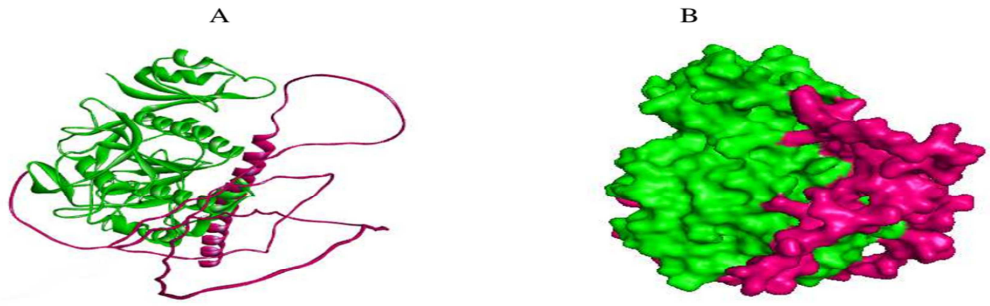

Figure 12: The molecular docking interaction between the alkaline protease from *B. safensis* lab418 (green color) and the cuticle collagen protein.**a**: Molecular interaction in 3D structure; **b**: Molecular interaction in surface complex structure.

Table 9: Ramachandran plot statistics of the generated model of alkaline protease from *B. safensis*lab418 and template model from *B.subtills* (3WHI)

Ramachandran Plot statics	Template model (3WHI)	Generated model of alkaline protease
Amino acids in most favored regions	499	257
Amino acids in additional allowed regions	94	36
Amino acids in generously allowed regions	0	3
Amino acids in disallowed regions	0	0
Number of non-glycine and non-proline residues	593	296
Number of end-residues (Gly and Pro)	9	2
Number of glycine residues (shown as triangles)	76	31
Number of proline residues	32	15

4. Discussion

Various effective natural enemies have been employed in the biocontrol of nematodes, there are still obstacles, such as limited hosts and cultural methods. For example, most bacteria are only effective against the larvae and eggs of plant-parasitic nematodes, fungi are difficult to cultivate and restricted by soil. These obstacles have inspired researchers to investigate novel or alternative strains for biological control. In this study, we describe the isolation of a novel strain of *Bacillus sp*. that exhibits nematocidal activity. The nematicidal factor identified in this strain is alkaline serine protease, which opens up new opportunities for research on bacterial biocontrol agents that regulate plant-parasitic nematodes. *Bacillus sp*. B16 utilizes a serine protease identified as a pathogenic factor to kill worms. [46].Discovering novel protease-producing strains with incredibly active and stable enzymes through screening for new protease-producfiging bacteria may prove beneficial for nematode biocontrol and plant growth promotion [47]. In this study, twenty-five alkaline protease-producing strains were isolated; *B. safensis* was the most potent strain, identified by the 16S rRNA gene, and given the name lab418. Generally, the majority of *Bacillus species* bacterial isolates are widely recognized for their effectiveness in producing extracellular proteases [48].

The alkaline protease gene from *B. safensis* lab418 was previously isolated, cloned, sequenced, and deposited in the GenBank database with accession number OP610065 [27], the analyses of the amino acid sequence of our protease revealed that it consists of 383 amino acids, this result agrees with the results obtained by ([49]. The alkaline protease's amino acid sequences from *B. safensis* lab418 was compared to other proteases from different *Bacillus* species in the NCBI protein database, the alignment results show 99% similarity with protease from *B. safensis* WP095408136.1 and 98% similarity with alkaline serine protease from *B. pumilus*ACM07731.1 this result agrees with the result obtained by [49].Who reported 97% similarity between protease from *B. safensis* RH12 and *B. pumilus* CBS strain.

Placket-Burman design was used for statistical optimization for increasing production of an alkaline protease from *B. safensis* lab418**,** the optimum yield was 122 U/mL, and the statistical analysis showed that time, temperature, and peptone are the most important factors influencing the formation of alkaline protease. The synthesis of alkaline protease was then further optimized using the BOX–Behnken Design (BBD)., and the results increased the alkaline protease enzyme productivity to reach 245 U/mL (1.92-fold from the basal medium) after the validation experiment using the following combination of 0.75 gm/L peptone for 8 days at 27°C. Prior research by [50] revealed that environmental factors such as temperature have a significant impact on the rate of enzyme production. For instance, at 35°C, the *B. subtilis* bacteria showed good output. Time was another physical component in order to maximize the produced protease enzymes [51]. [52] used PB design and Box Behnken design for the optimization of *P. aeruginosa* KU1's protease output which increased by approximately 1.32-fold. After optimization, *B. licheniformis* NK-based protease output rose 1.5 times [53]. Thus, the production of proteases can be increased with the aid of statistical tools.

In the present investigation, the nematicidal effect of alkaline protease from *B. safensis* lab418 on *M. incognita* J2 under laboratory conditions was studied, the result data revealed that the partially

purified protease (245 U/mL) had the greatest impact on the mortality percentage of *M. incognita* J2 (98%) after 24 h of exposure, in comparison to the control, thus the results indicated that the alkaline protease is crucial to the management of plant nematodes as enhancing the nematode death and these findings are consistent with earlier research on the biological control of nematodes through the employment of enzymes that show nematicidal activity [54,55,7]. [56] found that when *Xiphinema index* juveniles and adults were exposed to bacterial filtrates containing protease, chitinase, collagenase, and lipase enzymes for up to 72 hours, all of the filtrates showed 54–100% mortality. Under the screen house condition, the bacterial enzymes (crude or partially purified) had suppressive effects on plant nematodes infested eggplant and enhanced growth parameters (shoot length, shoot fresh weight, root length, and root fresh weight) when compared to untreated control (without bacterial addition), This outcome is with according to research by [57].Also, the effect of bacterial crude enzymes, and the partially purified alkaline protease enzyme on some biochemical parameters of eggplant leaves infected with *M. incognita* have been studied, these parameters include phenolic compounds and enzymes such as polyphenol oxidase, chitinase, and glucanase. The purified enzyme (245 U/mL) produced higher enzymatic activity in leaves compared to that treated with bacteria or crude enzyme, The activities of polyphenol oxidase, glucanase, and chitinase were 225.6, 2.46, and 2.36 U/mL respectively, and also the total phenolic compounds reached (2.79 mg/gm), this could be due to the specificity of the partially purified enzyme rather than crude enzyme and bacterial suspension. Proteases, in general, support the plant's enzymatic and non-enzymatic antioxidant systems, which are essential for the plant's defense against diseases, these enzymes include polyphenol oxidase, chitinase, and glucanase [58]. Also,plants with high polyphenol content exhibit resistance to an assortment of plant diseases [59-62].

The introduction of these chemical compounds acquired the plants a systemic resistance due to the lethal effect of these released chemical compounds on *M. incognita* J2 and nematode multiplication [63],[17]. In addition to disease control, the presence of defense-related components increased plant health and productivity [64,65].The plant growth increase was generally attributed to their content of secondary metabolites such as phenols, terpenes, alginate, sulfatedgalactan, and carrageen [66-68].

Finally, the interaction between alkaline protease from *B.safensis* lab418 and the cuticle collagen protein dpy-7 of nematode, which is a target nematode protein for degradation by protease [69] was studied by molecular docking. The interaction showed a binding affinity of -4.8. Also, the collagenic activity of the protease from *B.safensis* lab418 was studied and the results exhibited a collagenic activity with 85 and 67 U/mL for crude enzyme and partially purified enzyme respectively. Limeet al. indicated a collagenolytic activity of protease produced by *Bacillus*sp[70]. The collagen is found in the cuticle of the nematode, the cuticle is an outer membrane layer that coats nematode cells, and this layer serves as the primary interface with the environment and is crucial for maintaining the shape and movement of the body. Mutations in either the cuticle's structural components or the enzymes necessary for cuticle development cause changed body shape or nematode death, which is composed primarily of collagen [71].Hu et al. [72] reported that the crude extract of Bacillus cereus, which contains alkaline and neutral proteases, exhibits a strong nematicidal effect against Meloidogyne incognita juveniles. Additionally, several studies have highlighted the use of fungi as biocontrol agents against M. incognita, including species of Aspergillus and Penicillium [73-74]. Both of bacterial and fungal species, which are also involved in the bioremediation of industrial pollutants such as textile dye industrial wastes, exert their nematicidal action through cuticle damage caused by the crude enzymes they produce.

5. Conclusions

The present study revealed that *B. safensis* strain Lab418 is a potent organism for alkaline protease production, which recorded a high mortality percentage against *M. incognita*, improving the plant defense system and growth of plants. Thus, the alkaline protease from the *B. safensis* Lab418 strain could be a potential candidate and eco-friendly for biocontrol applications against root-knot nematodes.

6. Abbreviations

CCD	Central Composite Design
J2	Second-stage juveniles
PBD	Plackett-Burman design
PDB	Protein Data Bank
PGPR	Plant growth-promoting rhizobacteria
PPN	Plant parasitic nematodes
RSM	Response surface methodology
SD	Standard deviation
TCA	Trichloroacetic acid

7. Conflicts of interest

There are no conflicts to declare.

8. Acknowledgments

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