**Isolation and Optimization of Chitinase Production from *Bacillus safensis* from the Oil Palm Rhizosphere**

**ABSTRACT**

*Bacillus safensis* is a promising bacterium with versatile enzymatic capabilities, offering potential for industrial applications and sustainable agriculture. This study explores the isolation, molecular identification, and optimization of chitinase production from *B. safensis* isolated from the rhizosphere of oil palm (*Elaeis guineensis*) at NIFOR, Nigeria. The bacterium was characterized using morphological, biochemical, and molecular analyses, including 16S rRNA sequencing. Optimization experiments assessed the effects of various carbon and nitrogen sources, pH, and temperature on chitinase activity. Results showed that colloidal chitin was the most effective carbon source, while ammonium sulfate was the optimal nitrogen source, yielding a maximum enzyme activity of 17.3 µmol/min at 50°C and pH 6.0. Statistical analysis (ANOVA) confirmed the significance of the optimized conditions (p < 0.05). These findings emphasize the enzyme’s robustness and suitability for industrial biopolymer degradation and environmentally friendly agricultural practices. The study adds to the limited knowledge of *B. safensis* as a source of industrial enzymes, providing a foundation for large-scale applications and further research into its biochemical potential.

**Keywords**: *Bacillus safensis*, Chitinase, Biocontrol, Enzyme optimization, Rhizosphere microbiome

**INTRODUCTION**

Chitin, the second most abundant biopolymer after cellulose, is a key structural component in the exoskeletons of arthropods, crustaceans, and fungal cell walls. Its degradation is vital for nutrient cycling in ecosystems and has significant industrial relevance in agriculture, waste management, and biotechnology (Shobade *et al.,*2024). Chitinases, enzymes that hydrolyze chitin into bioactive oligosaccharides and N-acetyl-D-glucosamine, are of great interest due to their role in biocontrol, biopolymer recycling, and antimicrobial applications (Dahiya *et al*., 2006). Annually, an estimated 10 gigatons of chitin are produced in marine and terrestrial ecosystems, primarily from crustacean shells and fungal biomass (Gohel *et al*., 2006). Despite its abundance, chitin's insolubility and recalcitrant nature pose challenges to its natural degradation. The enzymatic breakdown of chitin by chitinases is therefore vital for nutrient cycling in ecosystems, as it facilitates the release of nitrogen and carbon for microbial and plant use (Souza *et al*., 2011). Chitinases (EC 3.2.1.14) are glycosyl hydrolases that catalyze the hydrolysis of chitin into low-molecular-weight oligosaccharides, chitobiose, and N-acetyl-D-glucosamine (GlcNAc), which have numerous industrial and pharmaceutical applications. Chitinases are classified into glycosyl hydrolase families 18 and 19 based on their structural folds and mechanisms of action. Family 18 chitinases are widespread in bacteria and fungi, whereas family 19 chitinases are predominantly found in plants (Shobade *et al.,*2024). The degradation of chitin has significant implications for various industries: In Agriculture: Chitinases are essential in biocontrol strategies for managing fungal pathogens that infect crops. By degrading the chitinous cell walls of fungi, these enzymes provide a sustainable alternative to chemical pesticides. Studies have demonstrated their efficacy against soilborne pathogens like *Fusarium oxysporum*, which causes vascular wilt diseases in plants such as oil palm (*Elaeis guineensis*) (Veliz *et al*., 2017). Biotechnology and Waste Management: Chitinase activity is employed in the recycling of chitin-rich waste from aquaculture and agricultural industries, such as shrimp shells and fungal biomass. This process not only reduces waste but also generates valuable by-products like chito-oligosaccharides, which have applications in pharmaceuticals, cosmetics, and food additives (Singh *et al*., 2021). In Medical Applications: Bioactive oligosaccharides produced by chitinase hydrolysis have antimicrobial, antitumor, and immunostimulatory properties. These derivatives are being explored for their potential in drug delivery and therapeutic formulations (Senol et al., 2014). Bacteria in the genus Bacillus are prolific producers of extracellular chitinases and are widely recognized for their biocontrol potential. These bacteria thrive in diverse habitats including rhizospheres, where they act as plant-growth promoters and pathogen antagonists, Ekundayo and Olukunle (2020). The ability of Bacillus species to secrete chitinases, along with their resilience under harsh environmental conditions, makes them ideal candidates for agricultural and industrial applications (Hamid *et al.,* 2013). The genus *Bacillus* is renowned for its diverse metabolic capabilities and different species in the genus such as; *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* have been widely studied for their chitinase activity (Karunya *et al*., 2011). However, limited studies have explored *Bacillus safensis*, a resilient, spore-forming bacterium first isolated from NASA spacecraft assembly facilities (Satomi *et al*., 2006) and this specie has demonstrated exceptional tolerance to extreme conditions, such as high salinity, heavy metals, and radiation, making it a promising candidate for industrial enzyme production and biotechnological applications. The rhizosphere of the oil palm (*Elaeis guineensis*), represents a rich reservoir of microbial diversity (Ekundayo and Olukunle, 2020), harboring bacteria with antifungal and enzymatic properties. These microorganisms play critical roles in nutrient cycling, disease suppression, and plant health. Among the diverse bacterial populations isolated from the rhizosphere are species of *Pseudomonas*, *Micrococcus*, *Staphylococcus*, and *Escherichia coli*, each exhibiting unique metabolic traits (Reddy *et al*., 2014). This study aimed to isolate and identify *B. safensis* from oil palm rhizospheric soil and to optimize its chitinase production under varying environmental and nutritional conditions. Specifically, the effects of carbon and nitrogen sources, pH, and temperature on enzyme activity. By optimizing these parameters, this study contributes to understanding the optimal conditions for enzyme activity and production, this research aims to advance the development of sustainable biocontrol solutions and industrial applications for chitinases.

**METHODOLOGY**

**Microbiological Analysis**

Soil Sample Preparation and Root Sterilization

Soil samples were collected from the rhizosphere of oil palm trees at depths of 15 cm and 30 cm. Each soil sample (1g) was carefully measured using a sterile spatula and transferred into sterile McCartney bottles containing 9 mL of sterile distilled water (Marcos-Tejedor *et al*., 2021). The bottles had been pre-labeled and sterilized in a Gallenkamp autoclave at 121°C for 15 minutes under 15 psi. Following autoclaving, the bottles were allowed to cool to room temperature. The root samples from oil palm were chopped into small fragments using a sterile scalpel and surface-sterilized in a solution of sodium hypochlorite (30:70 v/v). The sterilized root fragments were blotted dry using sterile filter paper and plated on nutrient agar (NA) media for microbial isolation.

**Serial Dilution and Plating**: A fivefold serial dilution was prepared to reduce microbial density, ensuring discrete colony formation. Aliquots (0.1 mL) from the third, fourth, and fifth dilutions were pipetted onto sterile Petri dishes containing adequately prepared nutrient agar aseptic conditions. Plates were incubated at 28°C, and colony growth was observed daily over a period of 24–72 hours. Each test was performed in triplicate to ensure reproducibility.

**Bacterial Characterization**

The bacterial isolates were characterized based on their cultural, morphological, and biochemical attributes. The cultural characteristics, including colony shape, size, elevation, margin, and pigmentation, were observed after incubation. Morphological examination included Gram staining and spore-forming capabilities. The total bacterial colony count was determined using the formula outlined by Collins *et al*., (1995):

 Number of colonies x dilution factor

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 Inoculum volume

values expressed as colony forming units per gram (cfu/g) of soil.

**Biochemical Tests for Bacterial Identification**

To confirm bacterial identities, isolates were subjected to a series of biochemical tests and the results were compared with descriptions in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994; MacFaddin (2000). **Citrate Utilization Test**: Bacterial isolates were inoculated onto Simmons’ citrate agar containing bromothymol blue as a pH indicator. The ability of the bacteria to utilize citrate as the sole carbon source and produce alkaline by-products was indicated by a color change from green to blue (MacFaddin, 2000). **Catalase Production Test**: A loopful of bacterial culture was mixed with 3% hydrogen peroxide on a sterile slide. The production of bubbles due to the breakdown of hydrogen peroxide into water and oxygen indicated a positive result (Wilson, 2011). **Hemolysis Test (Gamma Hemolysis):** Isolates were streaked onto blood agar plates and incubated at 28°C. Gamma hemolysis, indicated by the absence of hemolytic activity, was characterized by no clearing or discoloration around the bacterial colonies (Lennette *et al*., 1985). **Indole Production Test:** Isolates were inoculated into tryptone broth and incubated at 28°C. Following incubation, Kovac's reagent was added. A red layer on the surface indicated indole production due to the breakdown of tryptophan (Mackie and McCartney, 1996). **Motility Test:** Motility was assessed using a semi-solid medium (0.4% agar) inoculated with bacterial isolates. Diffuse growth radiating from the stab line indicated motility. **Spore-Forming Capability:** Spore formation was assessed by heating bacterial cultures at 80°C for 10 minutes, followed by plating on nutrient agar. Survival of spores indicated spore-forming capacity (Nicholson *et al.,* 2000). **Voges-Proskauer Test:** Bacteria were inoculated into MR-VP broth and incubated at 28°C. After incubation, Barritt’s reagent was added. A red color indicated the production of acetoin, a positive result for the Voges-Proskauer test (Wilson, 2011). **Oxidase Test**: Oxidase activity was determined by smearing bacterial colonies onto filter paper impregnated with tetramethyl-p-phenylenediamine. A blue-purple color indicated a positive result (Marshall, 2017). **Fermentation Test**: The isolates’ ability to ferment various sugars, including lactose, glucose, galactose, maltose, raffinose, and mannitol, was tested. Durham tubes containing carbohydrate broth were inoculated and incubated at 28°C for 24–48 hours, Blazevic. and Ederer, (2011). Acid production was indicated by a color change in the medium due to pH reduction, while gas production was observed as bubbles in the inverted Durham tubes (Reddy *et al*., 2014).

**Molecular Identification and Characterization of Bacillus sp.**

**DNA Extraction;** Genomic DNA was extracted from Bacillus sp. using the cetyltrimethylammonium bromide (CTAB) method as described by Trindade et al. (2007), with modifications. Single colonies from nutrient agar plates were transferred to 1.5 mL of nutrient broth and incubated at 28°C for 48 hours with shaking at 150 rpm. After incubation, the bacterial culture was centrifuged at 4,600 g for 5 minutes at 4°C to pellet the cells. The pellet was resuspended in 520 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by the addition of 15 µL of 20% SDS and 3 µL of 20 mg/mL Proteinase K.The mixture was incubated at 37°C for 1 hour, after which 100 µL of 5 M NaCl and 80 µL of 10% CTAB in 0.7 M NaCl were added. The suspension was vortexed thoroughly and incubated at 65°C for 10 minutes, then cooled on ice for 15 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added, vortexed, and centrifuged at 7,200 g for 20 minutes at 4°C. The aqueous phase containing the DNA was transferred to a fresh tube, and DNA was precipitated by adding 0.6 volumes of isopropanol. The mixture was stored at -20°C for 16 hours to enhance DNA precipitation.The precipitated DNA was recovered by centrifugation at 13,000 g for 10 minutes, washed with 70% ethanol, and air-dried at room temperature for 3 hours. The DNA pellet was dissolved in 50 µL of TE buffer and stored at -20°C until further use.

**Polymerase Chain Reaction (PCR) Amplification**

The 16S rRNA gene of the extracted DNA was amplified using the universal primers 27F (5′-AGAGTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′). PCR reactions were carried out in a total volume of 50 µL containing: 10 µL of 5X GoTaq reaction buffer, 3 µL of 25 mM MgCl₂, 1 µL of 10 mM dNTPs, 1 µL of each primer (10 µmol), 0.3 U of Taq DNA polymerase (Promega, USA), 8 µL of DNA template, and Nuclease-free water to make up the final volume. Amplifications were performed using a Gene Amp 9700 PCR System (Applied Biosystems, USA) under the following conditions: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute 30 seconds. A final extension was carried out at 72°C for 10 minutes, after which the reactions were held at 4°C (Sambo *et al.,* 2018; Frank *et al*., 2008).

**Integrity Check of Amplified Products**

The PCR products were resolved on a 1.5% agarose gel prepared in 1X TAE buffer (Tris-acetate-EDTA). The molten agarose was cooled to 60°C and stained with 0.5 µg/mL ethidium bromide (Khan, 2018). After solidifying in a gel casting tray fitted with a comb, the gel was placed in an electrophoresis tank filled with 1X TAE buffer. Two microliters of 10X loading dye were mixed with 4 µL of PCR product, and the mixture was loaded into individual wells. A 100-bp DNA ladder was loaded in the first well as a molecular size marker. Electrophoresis was conducted at 120 V for 45 minutes. The gel was visualized under ultraviolet light using a transilluminator, and images were captured to confirm the presence of the 1.5 kb amplified 16S rRNA fragment.

**Purification of Amplified Products**

PCR products were purified using an ethanol precipitation method. To each 40 µL of PCR product, 7.6 µL of 3M sodium acetate and 240 µL of 95% ethanol were added. The mixture was vortexed and incubated at -20°C for 30 minutes to allow DNA precipitation. The samples were centrifuged at 13,000 g for 10 minutes at 4°C, and the pellet was washed with 150 µL of 70% ethanol. After centrifugation at 7,500 g for 15 minutes at 4°C, the supernatant was removed, and the pellet was air-dried in a laminar flow hood. The dried pellet was resuspended in 20 µL of nuclease-free water and stored at -20°C until sequencing. To confirm the presence of the purified DNA, aliquots were analyzed on a 1.5% agarose gel alongside the PCR product. The DNA concentration was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific).

**Sequencing of Amplified Products**

Purified 16S rRNA gene fragments were sequenced using the Genetic Analyzer 3130xl system (Applied Biosystems, USA), (Frank et al., 2008). The sequencing reactions were prepared using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s protocol. The sequencing results were analyzed using Bio Edit software, and phylogenetic analyses were performed using MEGA 6 software. The evolutionary relationships of the bacterium were inferred using the neighbor-joining method, and sequence identity was confirmed through BLAST analysis against the NCBI GenBank database.

**Influence of Different Parameters on Chitinase Production by *Bacillus safensis*.**

**Preparation of Crude Enzyme**

An 18-hour-old culture of *Bacillus safensis* was inoculated into a 1,000 mL Erlenmeyer flask containing 500 mL of sterile minimal synthetic medium. The medium comprised MgSO₄·7H₂O (0.2 g/L), K₂HPO₄ (0.9 g/L), KCL (0.2 g/L), NH₄NO₃ (1.0 g/L), FeSO₄ (0.002 g/L), and ZnSO₄ (0.002 g/L), supplemented with 10% colloidal chitin as the sole carbon source. The flask was incubated at 30°C for 5 days on a rotary shaker at 150 rpm to facilitate bacterial growth and chitinase production. Following incubation, the culture broth was centrifuged at 5,000 g for 20 minutes at 4°C, and the supernatant was collected as the crude enzyme solution. This solution was immediately used for chitinase activity assays (Mishra, 2010; Gajera and Vakharia, 2012).

**Chitinase Assay**

Chitinase activity was determined using the dinitrosalicylic acid (DNS) method to measure reducing sugars (Xia *et al*., 2011) One milliliter of 1% colloidal chitin suspension was mixed with 1 mL of the crude enzyme solution and incubated at 37°C for 30 minutes. The reaction was terminated by adding 1 mL of DNS reagent, and the mixture was boiled for 5 minutes in a water bath. After cooling to room temperature, the absorbance of the reaction mixture was measured at 520 nm using a spectrophotometer (6850 UV/Vis, Jenway). Chitinase activity was expressed in µmol/min of reducing sugars released (Jenifer *et al*., 2014). The protein concentration of the crude enzyme was determined using the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as the standard. A calibration curve was constructed, and the protein content in the enzyme sample was calculated based on absorbance measurements at 595 nm.

**Effect of Nitrogen Source on Chitinase Production**

To investigate the influence of nitrogen sources on chitinase production, various nitrogen compounds, including ammonium chloride (NH₄Cl), ammonium sulfate (NH₄)₂SO₄), ammonium phosphate (NH₄)₂PO₄), ammonium carbonate (NH₄)₂CO₃, ammonium nitrate (NH₄)₂NO₃, peptone, yeast extract, and beef extract, were added individually to the basalmedium at equivalent concentrations. The medium was autoclaved at 121°C and 15 psi for 20 minutes, cooled to room temperature, and inoculated with an 18-hour-old B. safensis culture. Cultures were incubated at 30°C on a rotary shaker at 200 rpm for 48 hours. Aliquots (5 mL) were collected at 6-hour intervals, centrifuged at 5,000 g for 20 minutes at 4°C, and the supernatant was stored at 4°C for subsequent chitinase activity assays. Cell growth was monitored by measuring absorbance at 600 nm.

**Effect of Carbon Source on Chitinase Production**

The effect of various carbon sources on chitinase production was evaluated by supplementing the basal medium with different carbon sources, including glucose, fructose, galactose, sucrose, maltose, molasses, rice bran, soybean meal, and colloidal chitin (control). Each medium was prepared by keeping all other components constant and autoclaved at 121°C and 15 psi for 20 minutes. Following inoculation with B. safensis, cultures were incubated at 30°C on a rotary shaker at 200 rpm for 48 hours. Aliquots (5 mL) were collected every 6 hours, centrifuged at 5,000 g for 20 minutes at 4°C, and the supernatant was analyzed for chitinase activity and protein content.

**Effect of pH on Chitinase Production**

To assess the effect of pH on chitinase production, the pH of the basal medium supplemented with 10% colloidal chitin was adjusted to values ranging from 3.0 to 9.0 using acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–7.0), and Tris-HCl buffer (pH 8.0–9.0). The inoculated cultures were incubated at 30°C on a rotary shaker at 200 rpm for 48 hours. Samples (5 mL) were collected at 6-hour intervals, centrifuged, and analyzed for chitinase activity.

**Effect of Temperature on Chitinase Production**

The influence of temperature on chitinase production was evaluated by incubating inoculated cultures at various temperatures ranging from 30°C to 90°C. The basal medium containing 10% colloidal chitin was prepared as described earlier. Aliquots (5 mL) were collected every 6 hours over 48 hours, centrifuged at 5,000 g for 20 minutes at 4°C, and the supernatant was analyzed for chitinase activity.

**Statistical Analysis**

All experiments were performed in triplicate. Data were analyzed using one-way ANOVA, and differences between means were evaluated using Duncan’s Multiple Range Test (DMRT) at a significance level of p < 0.05 (Obi, 2002). Statistical analyses were conducted using SPSS software (version 17).

**RESULTS**

**Isolation and Identification of *Bacillus safensis* from the Rhizosphere of Oil Palm**

Bacterial species were isolated from the rhizosphere of oil palms and identified based on their cultural and biochemical characteristics. The predominant bacterial species identified included *Salmonella* sp., *Pseudomonas* sp., *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., and *Escherichia coli* as shown on table 1 below.

Among the isolates, a Gram-positive, spore-forming, motile rod-shaped bacterium was further analyzed. Molecular identification of this isolate was performed using 16S rRNA gene sequencing. PCR amplification of the 16S rRNA gene yielded the expected fragment size, confirming the successful amplification of the target region. Sequence analysis and BLAST comparison against the NCBI GenBank database revealed the bacterium to be *Bacillus safensis* and was assigned the accession number MT151602 in the GenBank database. This bacterium demonstrated significant potential for chitinase production, highlighting its importance for applications in biocontrol and biopolymer degradation.

**Table 1:** Characterisation of bacterial isolates from oil palm rhizospheres

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Parameters** | A | B | C | D | E | F |
| **CULTURAL CHARACTERISTICS** |   |  |  |  |  |   |
| Shape | Round | Round | Irregular | Round | Round | Round |
| Colour | Creamy | Light cream |  Milky | Milky | cream | Milky |
| Size | Lobate | Lobate | Large | Lobate | Serrated | Entire |
| Elevation | Flat | Flat | Raised | Flat | Flat | Flat |
| Transparency | Transparent | Opaque | Transparent | Opaque | Transparent | Opaque |
| **Cellular Morphology** |   |  |  |  |  |   |
| Gram stain | Negative | Negative | Positive | Positive | Positive | Negative |
| Cell type | Rod | Rod | Rod | Cocci | Cocci | Rod |
| Cell arrangement | Single | Single | Pair | Single | Clusters | Pair |
| Acid fast | Negative | Negative | Negative | Negative | Negative | Negative |
| **Biochemical characteristics** |   |  |  |  |  |   |
| Citrate utilization | Negative | + | + | + | + | + |
| Spore forming | - | - | + | - | - | - |
| Catalase production | - | - | + | + | + | + |
| Hemolysis test (gamma y) | - | - | - | - | - | - |
| Bile esculin hydrolysis | - | - | - | - | - | - |
| Indole  | - | + | + | - | - | + |
| Motility | - | - | + | + | - | + |
| Methyl red | + | - | - | - | - | + |
| Voges-Proskauer | - | + | + | + | + | + |
| Lysine decarboxylase | + | - |  |  |  |   |
| Coagulase test | - | - | - | - | + | - |
| Oxidase test | + | + | - | - | + | - |
| **Fermentation test** |   |  |  |  |  |   |
| Lactose | + | - | + | + | + | + |
| Glucose | + | - | + | + | + | + |
| Galactose | + | + | + | - | - | + |
| Maltose | + | + | + | + | + | + |
| Raffinose | - | + | - | - | - | + |
| Mannitol | + | - | - | + | + | - |
| **Probable identity** | *Salmonella* sp*.* | *Pseudomonas* sp*.* | *Bacillus* sp*.* | *Micrococcus* sp*.* | *Staphylococcus*  sp*.* | *Escherichia coli* |

**Growth Curve of *Bacillus safensis* in Chitinase Production Medium**

The growth profile of *B. safensis* in a chitin-based medium was monitored over 60 hours. Figure 1 illustrates the growth curve, showing a lag phase within the first 6 hours, followed by exponential growth, which peaked at 30 hours. The stationary phase was sustained until 48 hours, with a gradual decline thereafter. This trend highlights the bacterium’s capacity to utilize colloidal chitin as a sole carbon source. The correlation between cell density and chitinase production indicates active enzyme synthesis during the exponential phase.

**Optimization of Chitinase Production**

**Effect of Carbon Source**

Colloidal chitin induced the highest enzyme activity, followed by glucose and maltose. Complex polysaccharides such as rice bran and soybean meal supported moderate activity, while molasses resulted in minimal chitinase production. Statistical analysis revealed that colloidal chitin significantly enhanced enzyme activity compared to other carbon sources (p < 0.05), underscoring its relevance as an optimal substrate for chitinase synthesis.

Figure .2 The influence of various carbon sources on chitinase production

**Effect of Nitrogen Source**

The type of nitrogen source significantly impacted enzyme production (Figure 3.0). Ammonium sulfate induced the highest chitinase activity (17.3 µmol/min), followed by peptone and ammonium nitrate. Organic nitrogen sources such as yeast and beef extract resulted in lower activity. These results emphasize the efficiency of inorganic nitrogen sources, particularly ammonium sulfate, in enhancing enzyme production.

Figure: 3:Effect of Nitrogen Sources

**Effect of Temperature**

The crude enzyme exhibited significant temperature-dependent activity (Figure 4). Enzyme activity increased steadily from 30°C, reaching an optimum at 50°C. Beyond 50°C, activity declined sharply, with minimal residual activity observed at 90°C.

**Figure 4**: Statistical analysis confirmed that the activity at 50°C was significantly higher (p < 0.05) compared to all other temperatures, suggesting the suitability of *B. safensis* chitinase for moderate thermophilic applications.

**Effect of pH**

Chitinase activity varied significantly across pH values (Figure 5). The enzyme displayed peak activity at pH 6.0, retaining more than 80% activity in the range of pH 5.5 to 6.5. Activity decreased sharply at pH values below 4.0 and above 8.0, indicating that the enzyme is best suited for mildly acidic environments. The stability observed at pH 6.0 underscores its potential utility in applications requiring acidic conditions.

**Figure 5**:Effect of pH on Chitinase Activity

This study successfully isolated and identified *Bacillus safensis* from the rhizosphere of oil palm (*Elaeis guineensis*) and optimized the production of chitinase under various environmental and nutritional conditions (Ekundayo and Olukunle, 2020). The findings highlight the biotechnological potential of *B. safensis* chitinase for agricultural and industrial applications, particularly as a biocontrol agent against fungal pathogens. The isolation of *Bacillus safensis* from the oil palm rhizosphere aligns with its ecological versatility and prevalence in diverse environments. Previous studies have reported its presence in extreme conditions such as saline deserts and spacecraft assembly facilities (Satomi et al., 2006). In this study, molecular identification via 16S rRNA sequencing confirmed the isolate as *B. safensis*, with an accession number MT151602 assigned in GenBank. This bacterium, characterized as a Gram-positive, spore-forming, motile rod, displayed significant chitinase activity, emphasizing its potential for chitin degradation and biocontrol applications. The optimization of chitinase production revealed critical factors influencing enzyme yield: **Carbon Sources**: Among the carbon sources tested, colloidal chitin yielded the highest chitinase activity (14.50 µmol/min), corroborating findings by Mishra (2010) and (Hamid *et al*.,2013), who reported that chitin induces higher enzyme production due to its structural similarity to the substrate. In contrast, glucose and maltose supported moderate activity, possibly due to catabolite repression mechanisms, as previously noted by Gajera and Vakharia (2012). **Nitrogen Sources**: Ammonium sulfate was identified as the most effective nitrogen source, yielding 17.3 µmol/min of chitinase activity. Similar results were observed by Dahiya et al. (2006), who demonstrated that inorganic nitrogen enhances microbial enzyme synthesis by providing readily assimilable nitrogen. Organic nitrogen sources such as yeast and beef extract yielded lower activity, likely due to slower assimilation. **Temperature and pH**: Chitinase activity was optimal at 50°C and pH 6.0, indicating moderate thermophilicity and preference for mildly acidic conditions. These findings are consistent with those of Singh *et al*. (2021), who reported similar stability profiles for thermostable microbial chitinases, Cortésão et al., (2019). The enzyme’s activity decreased significantly at higher temperatures and extreme pH values, highlighting its suitability for applications in environments with stable conditions. The results of this study align with global research on bacterial chitinases. For instance, *Bacillus subtilis* and *Bacillus licheniformis* have been extensively studied for their chitinolytic properties. *Bacillus*-derived enzymes from Nigerian soil samples and their applications in agriculture and biopolymer recycling (Karunya *et al*., 2011; Veliz *et al*., 2017). However, studies on *Bacillus safensis* are limited. This study adds to the growing body of knowledge by highlighting its chitinase production potential and optimized conditions for enhanced activity. Furthermore, the enzyme’s robustness and stability in acidic and moderately high-temperature environments underscore its industrial relevance. Further studies to ascertain the antifungal activity of the chitinase will be carried out in order toposition it as a viable candidate for biological control of fungal pathogens. Chitinases degrade the chitinous cell walls of fungi, disrupting their structure and limiting their pathogenicity (Amusa *et al*., 2007). Similar mechanisms have been observed in other *Bacillus* species used for biocontrol (Le and Yang, 2019) to support the global push for eco-friendly agricultural practices.

**CONCLUSION**

This study highlights the isolation and optimization of chitinase production by *Bacillus safensis*, demonstrating its potential for biocontrol and industrial applications. The bacterium, identified from the oil palm rhizosphere and confirmed through molecular techniques, produced chitinase most effectively under conditions of 50°C, pH 6.0, with colloidal chitin as the carbon source and ammonium sulfate as the nitrogen source. These findings underscore the enzyme’s robustness and its suitability for use in biocontrol strategies against fungal pathogens such as *Fusarium oxysporum f.sp. elaeidis*. The study contributes to the growing knowledge of *Bacillus safensis*, providing a foundation for its use in sustainable agriculture and industrial biotechnology. Future work should explore large-scale production, enzyme immobilization, and in-field biocontrol trials to further validate its applications.

**Disclaimer (Artificial intelligence)**

Option 1: Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

**REFERNCES**

1. Amusa, N. A., Okechukwu, R. U. and Akinfenwa, B. (2007). Reactions of cowpea to infection by Macrophomina phaseolina isolates from leguminous plants in Nigeria. *African Journal of Agricultural Research*, **2**(2):73–75.
2. Blazevic, D. J. and Ederer, G. M. (2011). Principles of biochemical tests in diagnostic microbiology. Wiley-Blackwell.
3. Collins, C. H., Lyne, P. M. and Grange, J. M. (1995). Microbiological Methods (7th ed.). Butterworth-Heinemann.
4. Cortésão, M., Fuchs, F. M., Commichau, F. M., Eichenberger, P., Schuerger, A. C., Nicholson, W. L., Setlow, P. and Moeller, R. (2019). *Bacillus subtilis* Spore Resistance to Simulated Mars Surface Conditions. Frontiers in Microbiology, 10:333.
5. Dahiya, N., Tewari, R. and Hoondal, G. S. (2006). Biotechnological aspects of chitinolytic enzymes: A review. *Applied Microbiology and Biotechnology,* **71**(6);773–782.
6. Ekundayo, J. A. and Olukunle, O. (2020).Rhizosphere microbiology in tropical climates*. Tropical Ecology,* **61**(2):123–134.
7. Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A. and Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology,* **74**(8):2461–2470.
8. Gohel, V., Singh, A., Maisuria, V., Phadnis, A., & Chhatpar, H. S. (2006). Bioprospecting and antifungal potential of chitinolytic microorganisms. African Journal of Biotechnology, 5(2): 54–72.
9. Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J. and Javed, S. (2013). Chitinases: An update. *Journal of Pharmacy and Bioallied Sciences*, **5**(1):21–29.
10. Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T. and Williams, S. T. (1994). Bergey's Manual of Determinative Bacteriology (9th ed.). Williams & Wilkins.
11. Karunya, S. K., Reetha, D., Palavesam, A. and Immanvel, G. (2011). Optimization and purification of chitinase produced by *Bacillus subtilis* and its antifungal activity against plant pathogens. *International Journal of Biology,* **3**(2):126–136.
12. Khan, M. (2018). Agarose gel electrophoresis for the separation of DNA fragments. *Methods in Molecular Biology,* **1624**: 21–26.
13. Senol, M., Nadaroglu, H., Dikbas, N., & Kotan, R. (2014). Purification of Chitinase enzymes from Bacillus subtilis bacteria TV-125, investigation of kinetic properties and antifungal activity against Fusarium culmorum. Annals of clinical microbiology and antimicrobials, 13, 1-7.
14. MacFaddin, J. F. (2000). Biochemical Tests for Identification of Medical Bacteria (3rd ed.). Lippincott Williams & Wilkins.
15. Marcos-Tejedor, F., Mota, M., Iglesias-Sánchez, M. J., Mayordomo, R., & Gonçalves, T. (2021). Identification of fungi involved in onychomycosis in patients of a Spanish rural area. *Journal of Fungi*, 7(8), 623.
16. Marshall, S. M. (2017). Clinical Microbiology Procedures Handbook (4th ed.). ASM Press.
17. Reddy, N. R., Pierson, M. D. and Sathe, S. K. (2014). Legume-Based Fermented Foods. CRC Press.
18. Sambo, F., Finotello, F., Lavezzo, E., Baruzzo, G., Masi, G., Peta, E., Falda, M., Toppo, S., Barzon, L. and Di Camillo, B. (2018). Optimizing PCR primers targeting the bacterial 16S ribosomal RNA gene. *BMC Bioinformatics*, 19: 343.
19. Satomi, M., La Duc, M. T. and Venkateswaran, K. (2006). *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *International Journal of Systematic and Evolutionary Microbiology,* **56**(8): 1735–1740.
20. Shobade, S. O., Zabotina, O. A. and Nilsen-Hamilton, M. (2024). Plant root associated chitinases: Structures and functions. *Frontiers in Plant Science*, 15, 1344142.
21. Singh, R., Upadhyay, S. K., Singh, M., Sharma, I., Sharma, P., Kamboj, P., ... & Khan, F. (2021). Chitin, chitinases and chitin derivatives in biopharmaceutical, agricultural and environmental perspective. Biointerface Res. Appl. Chem, 11(3), 9985-10005.
22. Souza, C. P., Almeida, B. C., Colwell, R. R. and Rivera, I. N. (2011). The importance of chitin in the marine environment. *Marine Biotechnology*, **13**(5):823–830.
23. Veliz, E. A., Martinez-Hidalgo, P. and Hirsch, A. M. (2017). Chitinase-producing bacteria and their role in biocontrol. *AIMS Microbiology*, **3**(4):689–705.
24. Xia, J.-L., Xiong, J., Zhang, R.-Y., Liu, K.-K., Huang, B. and Nie, Z.-Y. (2011). Production of chitinase and its optimization from a novel isolate *Serratia marcescens* XJ-01. *Indian Journal of Microbiology*, 51(3): 301–306.