

Antagonistic Potential of *Bacillus velezensis* and Sericin-Based Nanomaterials Against Mulberry Root Rot Pathogens

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Received: December 26, 2025 | Review began: February 03, 2026 | Review ended: May 16, 2026 | Published: May 19, 2026

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Abstract

Root rot disease in mulberry is primarily caused by the fungal pathogens *Lasiodiplodia theobromae* and *Rhizopus* sp., posing a significant threat to sericulture fields. The present study aimed to isolate bacteria from the rhizosphere of root-rot-affected mulberry plants and to evaluate their antagonistic potential against these fungal pathogens. Furthermore, the study assessed the biocontrol efficacy of bacterial crude metabolites in combination with sericin nanomaterials. Two pathogenic fungi and seven bacterial isolates were obtained from diseased roots and rhizospheric soil. The antagonistic activity of the isolates was initially screened against the target pathogens using the dual-culture technique. The most potent bacterial strain, isolated from the mulberry rhizosphere in Karnataka, was identified as *Bacillus velezensis* through 16S rRNA gene sequencing and phylogenetic analysis. *Bacillus velezensis* exhibited strong antagonistic activity against the root rot pathogens *L. theobromae* and *Rhizopus* sp., achieving mycelial growth inhibition of 64.79% and 35.38%, respectively, compared to the control ($p < 0.05$). Furthermore, sericin nanomaterials were synthesized and characterized using Scanning Electron Microscopy, Energy-Dispersive X-ray Spectroscopy, X-ray Diffraction, and Fourier Transform Infrared Spectroscopy to confirm their nanoscale size, elemental composition, and functional groups. The *in vitro* study demonstrated that sericin nanomaterials, in combination with bacterial crude extracts, exhibited high inhibitory potential against fungal mycelial growth. This approach represents a promising eco-friendly strategy for the effective management of mulberry root rot pathogens.

Categories: Plant-pathogen interaction, Soil microbiology, Nanotechnology for sustainable agriculture

Keywords: bacillus velezensis, lasiodiplodia theobromae, mulberry, rhizopus sp, root rot disease, sericin nanoparticles

Introduction

Mulberry (*Morus* spp.) is a deciduous perennial tree, belonging to the family Moraceae. It grows extensively in tropical and temperate regions of North America, Africa and Asia [1]. The major species of mulberry cultivated across the globe include *M. alba*, *M. nigra*, *M. rubra*, *M. indica* and *M. bombycis*. Mulberry leaves are the exclusive source of nutrition for silkworms (*Bombyx mori*), and hence the cultivation and management form a corner stone of sericulture. In India, Karnataka is the leading producer of mulberry and vanya silk fiber, producing nearly 11,823 metric tonnes of raw silk as of 2023, followed by Andhra Pradesh and Assam, which produced 8,834 and 5,700 metric tonnes, respectively [2]. Production of quality and quantity mulberry leaves and feeding them to silkworm is one of the crucial factors for quality silk production. Due to monocrop, perennial nature, poor crop management and repeated leaf harvest, mulberry is prone to soil-borne diseases such as root rot and root knot as they affect the root system and can cause considerable leaf yield and quality loss. Root rot is one of the most destructive diseases of mulberry, develops rapidly within a short period in

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almost all types of soils and can lead to disease epidemics. In India, black root rot and dry root rot are the two major types that have been reported. Dry root rot is primarily caused by *Fusarium solani* and *F. oxysporum* [3,4], whereas black root rot is primarily caused by *Lasiodiplodia theobromae* [5,6] and *Rhizopus* rot caused by *Rhizopus oryzae* [7]. Management strategies of these diseases employed traditionally includes chemical, biological as well as cultural practices. The harmful effects of chemical-based treatment by using a fungicide have led to increased attention towards ecofriendly alternatives. The use of potential microbes with antagonistic properties against phytopathogens responsible for crop diseases is a major management strategy [8]. Soils harbor beneficial microbial communities that naturally suppress plant pathogens through direct antagonism and also activate plant defense mechanisms. These beneficial microbes promote the development of disease-suppressive soils and ultimately reduce overdependence on harmful chemicals while maintaining healthy soil ecosystems, improving crop resilience and enhancing sustainable agricultural practices [9]. In addition, recent advancements in nanotechnology have positioned it as a promising approach in agriculture, offering innovative solutions to long-standing challenges while minimizing environmental impact. At the nanoscale, materials exhibit unique characteristics such as enhanced mechanical strength, increased surface-to-volume ratio and greater chemical reactivity compared to their larger-scale counterparts [10]. Several studies have reported nanoparticles as effective antimicrobial agents to combat soil-borne phytopathogens. Polymeric nanoparticles such as cellulose, lignin and chitosan possess antifungal and antibacterial properties, as well as plant growth-promoting abilities, and are considered environmentally friendly due to their biodegradability and natural abundance [11]. Therefore, it is important to evaluate nanoparticles as a novel approach for managing soil-borne diseases in mulberry. Based on this background, the present study aimed to (i) isolate and identify root rot pathogens and potential biocontrol agents from the mulberry rhizosphere; (ii) evaluate the *in vitro* antagonistic potential of the isolated bacteria; and (iii) assess the antifungal efficacy of a formulation combining bacterial metabolites with sericin-based nanomaterials.

Materials And Methods

Mulberry roots exhibiting root rot disease symptoms and soil samples from the affected rhizosphere were collected in three replicates from farmers' fields in Sathnoor Hobli, Kanakapura Taluk, Bengaluru South District. These samples were used separately to isolate pathogenic fungi and potential antagonistic bacteria.

Isolation of root rot pathogenic fungi

The root samples were carefully separated and cut into small fragments (2-3 cm in length) and then disinfected with 1.5% sodium hypochlorite (NaOCl) for 3 minutes, followed by three washes with sterile distilled water. The sterilized root material was then mixed in an isotonic sodium chloride solution (0.85%). After serial dilution, the solutions were plated onto potato dextrose agar (PDA) medium and incubated at 25°C for 5 days. For soil analysis, 3 g of soil samples were homogenized and sieved, after which 100 ml of isotonic solution (0.85% NaCl) was added and the mixture was thoroughly homogenized. Following serial dilution, 100 µl aliquots were spread onto PDA plates and incubated at 25°C for 5 days. Fungal isolates obtained from the incubated PDA plates were selected based on distinct morphological features and subcultured onto fresh PDA plates. Identification of *L. theobromae* and *Rhizopus* sp. was carried out based on macroscopic colony characteristics and microscopic observations using lactophenol cotton blue staining.

Isolation of antagonistic bacteria

The previously prepared solutions of both root and soil samples were used for bacterial isolation following serial dilution, particularly at 10^{-3} and 10^{-4} dilutions. One millilitre of each dilution was plated onto nutrient agar (NA) medium using the pour plate method and incubated at 28-30°C for 48 hours. A total of seven distinct bacterial colonies were selected and subcultured onto fresh NA plates, followed by Gram staining. Among the seven isolates, Gram-positive bacteria were selected for further studies.

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In vitro screening for antagonistic activity

In vitro screening for antagonistic activity was carried out using the dual culture method [12], with slight modifications. Four bacterial isolates were evaluated for their antagonistic activity against two mulberry root rot pathogens, *L. theobromae* and *Rhizopus* sp., on Sabouraud dextrose agar (SDA). Each bacterial isolate was streaked along one edge of the plate, while a 6 mm mycelial disc of the pathogen was placed on the opposite edge, approximately 4 cm apart. Control plates were inoculated with the pathogen alone. The plates were incubated at $27 \pm 2^\circ\text{C}$ until the fungal mycelia in the control plates reached the edge of the plate. Inhibition of mycelial growth towards the bacterial isolate indicated a positive antagonistic effect. The percentage of radial growth inhibition was calculated using the formula described by Kumar et al. [13]. All treatments were performed using three independent biological replicates.

$$GI\% = \frac{dc - dt}{dc} \times 100$$

where *dc* is the mean mycelial growth diameter of control sets, and *dt* is the mean mycelial growth of diameter of treatment sets. The data of all the parameters have been subjected to standard statistical methods.

Molecular characterization of positive antagonist bacteria

The potent bacterial isolate was identified and characterized through 16S rRNA gene sequence analysis. Total genomic DNA was extracted using the protocol described by Sambrook et al. [14]. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene fragment was performed using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3') [15]. The amplified products were checked for purity by agarose gel electrophoresis using a 0.8% agarose gel, and DNA concentration was measured using a NanoDrop spectrophotometer. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A consensus sequence of the 16S rRNA gene was generated from the forward and reverse reads. To confirm the taxonomic identity of the bacterial isolate and determine its evolutionary relationship with closely related species, a phylogenetic tree was constructed using the neighbour-joining method based on aligned 16S rRNA gene sequences. Based on maximum identity scores, 10 sequences were retrieved from the NCBI Basic Local Alignment Search Tool (BLAST) database and included in the analysis along with the test isolate sequence. Sequence alignment was performed using MUSCLE, and the phylogenetic tree was generated using MEGA XI software with 1,000 bootstrap replications to ensure statistical robustness.

Optimization of the growth parameters

Optimization studies were conducted to determine the optimal growth conditions for the bacterium with respect to temperature (25°C, 32°C and 50°C), pH (4.0, 7.0 and 8.0) and different carbon (starch, glucose and trehalose) and nitrogen sources (potassium nitrate, tryptone and ammonium sulphate). A one-factor-at-a-time approach was employed, wherein each parameter was varied independently while keeping the others constant to assess its effect on bacterial growth [16,17]. All experiments were performed using three independent biological replicates.

Extraction of crude extract

A loopful of the antagonistic bacterial isolate was inoculated into freshly prepared, sterilized nutrient broth under aseptic conditions and incubated at 28-30°C for 24 hours. The culture was then centrifuged at 10,000 rpm for 15 minutes in sterile tubes. The supernatant was filtered through a 0.5 µm Whatman filter to obtain a cell-free crude extract of bacterial metabolites [18].

In vitro evaluation of antifungal activity of crude extract by agar well diffusion

Different concentrations of the crude secondary metabolite extract (10%, 25%, 50% and 75%) were prepared using sterile distilled water. Four wells of 6 mm diameter were punched into each SDA plate using a sterile cork borer and labelled according to the respective concentrations. Subsequently, 70 µl of each concentration was dispensed into the corresponding wells. A mycelial disc of the pathogenic fungus (*L. theobromae* or *Rhizopus* sp.) was placed at the centre of each plate. Additionally, 70 µl of 5% hexaconazole (chemical fungicide) and 70 µl of sterile distilled water were used as

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positive and negative controls, respectively, along with an absolute control containing only the pathogen without any treatment. The assay was performed using three independent biological replicates, and the plates were incubated at room temperature for 7 days. Following incubation, the diameters of the inhibition zones were measured.

Extraction of sericin from silkworm cocoons

One hundred grams of cocoons were weighed and boiled in 1,000 ml of deionized water at 100°C for 30 minutes. The resulting extract was filtered through muslin cloth, and the filtrate was collected in sterile Petri dishes and gently dried in a hot air oven at 40°C. The dried crude sericin extract was then ground using a mortar and pestle to obtain a fine powder.

Synthesis of sericin nanomaterial

The synthesis of sericin nanomaterial was performed following the protocol of Meerasri et al. [19], with slight modifications. Five grams of sericin was dissolved in 50 ml of sterile deionized water by heating at 100°C for 40 minutes with constant stirring [19]. The solution was cooled to room temperature and then mixed with an equal volume of dimethyl sulfoxide (1:1 ratio) at a rate of 10 ml/min under constant stirring. The mixture was further stirred for 20 minutes, followed by centrifugation at 9,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 10 ml of deionized water. The sample was centrifuged again and washed with deionized water. The pellet was dried at 35°C.

Characterization of sericin nanomaterials

The sericin nanomaterials were characterized using Scanning Electron Microscopy (SEM), Energy-Dispersive X-ray Spectroscopy (EDS) and Fourier Transform Infrared Spectroscopy (FTIR). SEM images were captured at 6,000× magnification using an accelerating voltage of 10 kV with a scale bar of 5.00 µm. FTIR analysis was performed to identify the functional groups present in the sericin nanomaterial [19].

In vitro evaluation of antifungal activity of the sericin nanomaterials

Different concentrations of sericin nanomaterial (300 mg/ml, 400 mg/ml, 500 mg/ml and 1,000 mg/ml) were prepared using deionized water. An aliquot of 0.1 ml of each concentration was evenly spread onto the surface of respective SDA plates using an L-shaped glass rod. A 6 mm mycelial disc of *L. theobromae* was placed at the centre of each plate. Similarly, a 6 mm disc of *Rhizopus* sp. was placed on another set of plates treated with different concentrations of the nanomaterial suspension. Control plates were inoculated with only the pathogen. All plates were incubated at room temperature for 7 days [20]. All treatments were performed using three independent biological replicates.

Assessment of the antifungal activity of a bacterial crude extract-sericin nanoparticle formulation

The crude extract of the antagonistic bacterium and sericin nanoparticle was combined in 1:1 ratio, and the same was utilized to evaluate antifungal activity using the following methods:

- 1) Spread plate method: An aliquot of 0.1 ml of the formulation was evenly spread onto the surface of SDA plates, and a 6 mm mycelial disc of the *L. theobromae* was inoculated at the centre of the plate.
- 2) Agar well diffusion method: An aliquot of 70 µl of the formulation was added into a 6 mm well made at one edge of the SDA plate, and a 6 mm mycelial disc of the fungal pathogen was inoculated 4 cm away from it. A plate inoculated with only the crude extract and a plate which included only nanomaterial served as controls along with an absolute control which was inoculated with only the fungal pathogen. The plates were incubated at room temperature for 7 days.

All experiments were conducted using three independent biological replicates.

Statistical analysis

Statistical analysis was performed using Infostat Software. All experiments were conducted using three independent biological replicates, and the results are expressed as mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post hoc test to determine statistically significant differences among treatments at $p < 0.05$.

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Results And Discussion

In the present study, roots of root-rot-affected mulberry plants and rhizospheric soil samples were used to isolate fungal pathogens and antagonistic bacteria. Crude extracts from promising bacterial culture media and sericin nanoparticles were used *in vitro* for their inhibitory activity against fungal pathogens.

Isolation of fungal pathogens and bacteria and their antagonistic study

The root rot was observed in mulberry plants, including decaying or rotting roots, peeling of the outer bark of the cortex and easy uprooting of plants. Similar observations were reported by Sharma et al. [5]. Fungal pathogens responsible for root rot disease were isolated from the rhizosphere of infected mulberry plants and identified as *L. theobromae* and *Rhizopus* sp. based on macroscopic and microscopic observations. *Lasiodiplodia theobromae* and *R. oryzae* have been reported as causative agents of black root rot and *Rhizopus* root rot in mulberry, respectively [7,21,22]. Macroscopic and microscopic observations of *L. theobromae* showed dark grey-black pigmentation and typical striated mature conidia. The colonies were initially white and fluffy, and turned dark olive or black upon maturation (Figure 1a-d).

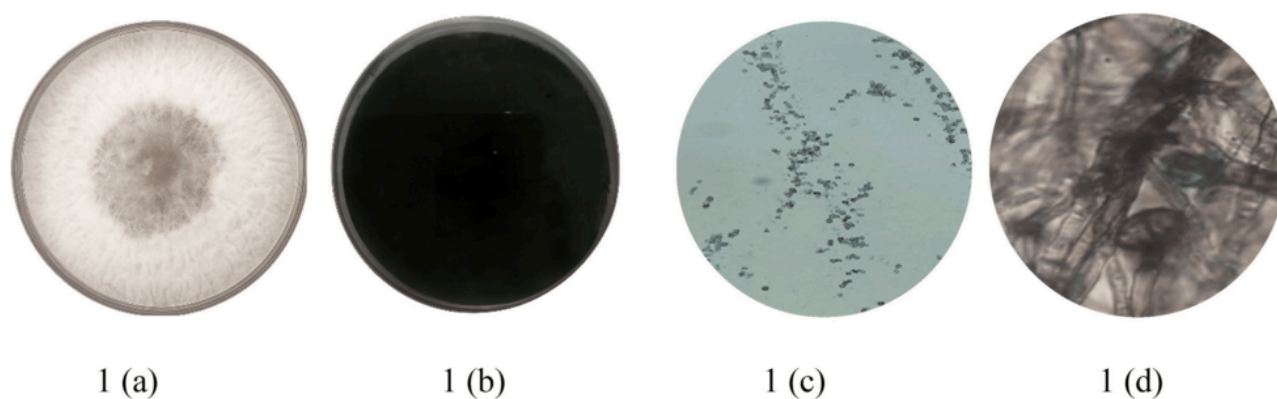


FIGURE 1: *Lasiodiplodia theobromae*. (a) Growth of *L. theobromae* on potato dextrose agar plates showing white, fluffy mycelia. (b) White, fluffy mycelia that turn blackish upon maturation. (c) Microscopic observation of *L. theobromae* spores at 10× magnification. (d) Striated sporangium observed at 40× magnification.

The morphological characteristics observed were consistent with those reported by Huda-Shakirah et al. [23], who observed white to greenish-grey colonies that eventually turn dark upon maturation, with hyaline conidia that become dark brown and striated as the fungus develops. This fungus has also been reported to cause root rot and collar rot in *Jatropha curcas* [24] and stem rot in citrus [25]. The other pathogen, *Rhizopus* sp., exhibited zygospore formation, rhizoid-like mycelial growth and cottony white colonies (Figure 2a-c).

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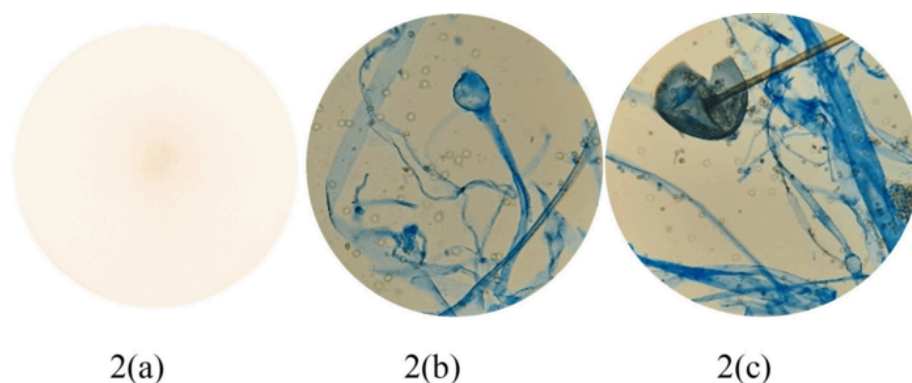


FIGURE 2: (a) The growth of *Rhizopus* sp. on potato dextrose agar plates showing cottony mycelia. (b) Hyphal structure of *Rhizopus* sp. with sporangium at the tip with domed structure at 10× magnification. (c) Sporangiospores and zygospore at 40× magnification.

This was consistent with the characteristics reported by Dolatabadi et al. [26]. The growth of the fungus was rapid, covering the entire plate within 4 days, indicating that it was an invasive fungus.

A total of seven bacterial isolates were obtained from root-rot-affected mulberry plants and its rhizosphere soil samples. Each of the isolate exhibited different colony morphologies, ranging from white to yellow pigmentation, with variations in margin, elevation and texture. Preliminary identification using Gram staining revealed that four isolates were Gram-positive and three were Gram-negative. Pure cultures of all isolates were maintained on nutrient agar slants at 4°C. Since most of the Gram-negative bacteria are known to be pathogens, they were excluded from further studies. Among the four isolates (SB-1 to SB-4), SB-1 exhibited significantly higher inhibitory activity against the test pathogens, i.e., *L. theobromae* and *R. oryzae*, compared to other isolates. The antagonistic effect of bacterial isolate of SB-1 achieved a maximum inhibition (64.79%), followed by SB-2 (37.69%) and SB-4 (21.30%), whereas SB-3 showed no inhibition against fungal pathogen *L. theobromae*. Similarly, against *Rhizopus* sp., the bacterial isolate of SB-1 recorded the highest inhibition (35.38%), followed by SB-2 (23.22%) and SB-3 (11.37%), while SB-4 exhibited no inhibitory effect (Table 1).

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Bacterial isolates	Inhibitory percentage	
	L. theobromae	Rhizopus sp.
SB-1	64.79±0.12 ^A	35.38±0.77 ^A
SB-2	37.69±1.82 ^B	23.33±2.69 ^B
SB-3	0.00 ^D	11.37±1.65 ^C
SB-4	21.30±5.12 ^C	0.00 ^D
f-value	302.22	263.79
CV	8.79	9.29

TABLE 1: Inhibitory percentage of bacterial isolates against *L. theobromae* and *Rhizopus* sp.

Values are presented as mean ± standard deviation of three independent biological replicates. Different superscript letters within the column indicate statistically significant differences according to Tukey’s Honestly Significant Difference post-hoc test (p < 0.05).

These results indicate that SB-1 has the potential to effectively suppress pathogen growth (Figures 3-4).

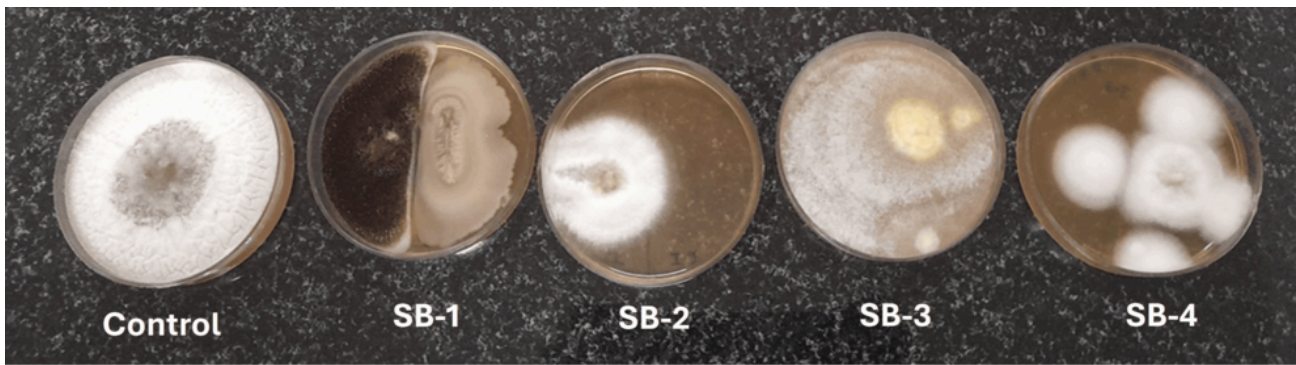


FIGURE 3: Antagonistic activity of bacterial isolates (SB-1 to SB-4) against *L. theobromae*.

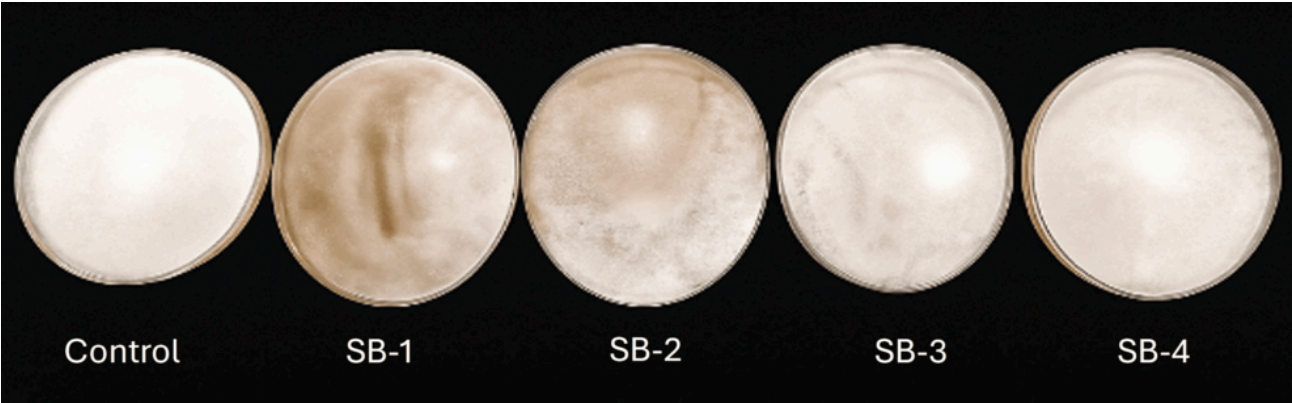


FIGURE 4: Antagonistic activity of bacterial isolates (SB-1 to SB-4) against *Rhizopus* sp.

Isolate SB-2 showed moderate inhibition against both pathogens, whereas isolate SB-3 exhibited no activity against *L. theobromae* and only minimal inhibition against *Rhizopus* sp. SB-4 displayed no inhibition against *Rhizopus* sp., but recorded a low level of inhibition against *L. theobromae* (Figures 5-6).

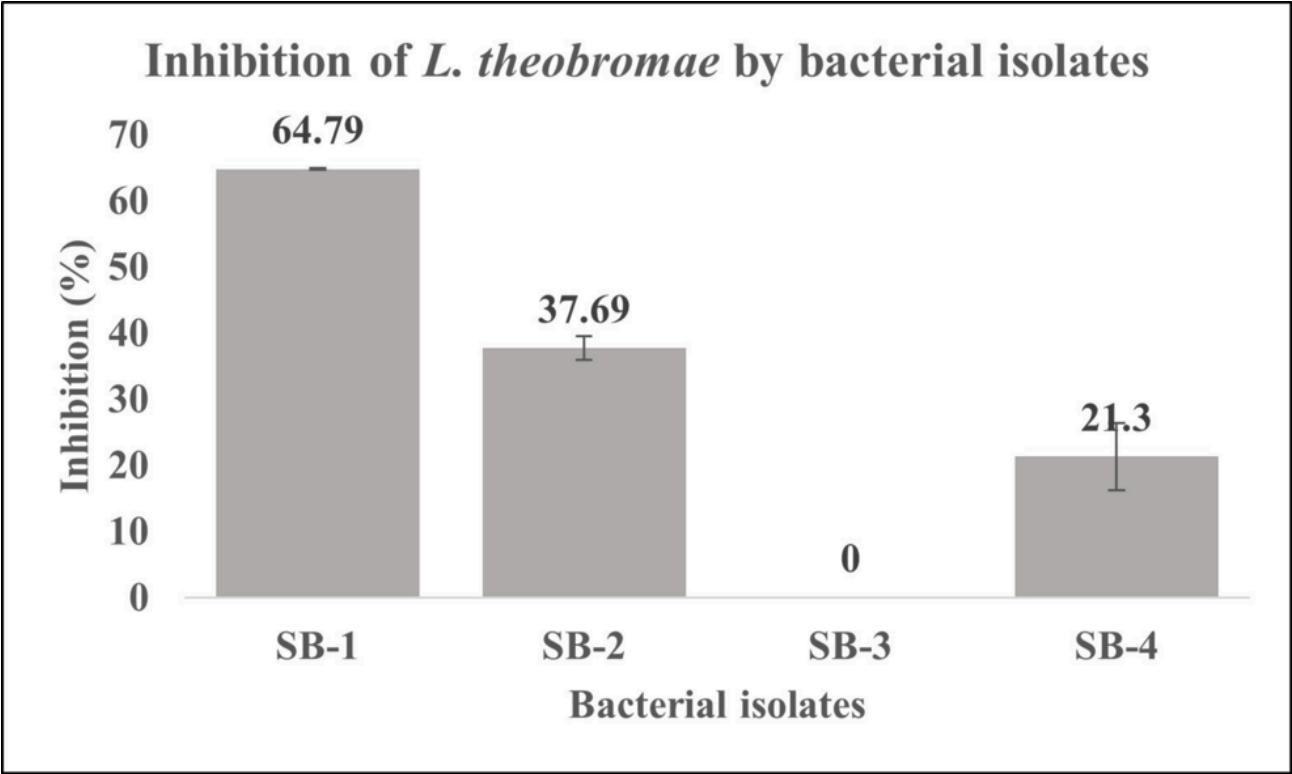


FIGURE 5: Mycelial growth inhibition of *L. theobromae* by bacterial isolates.

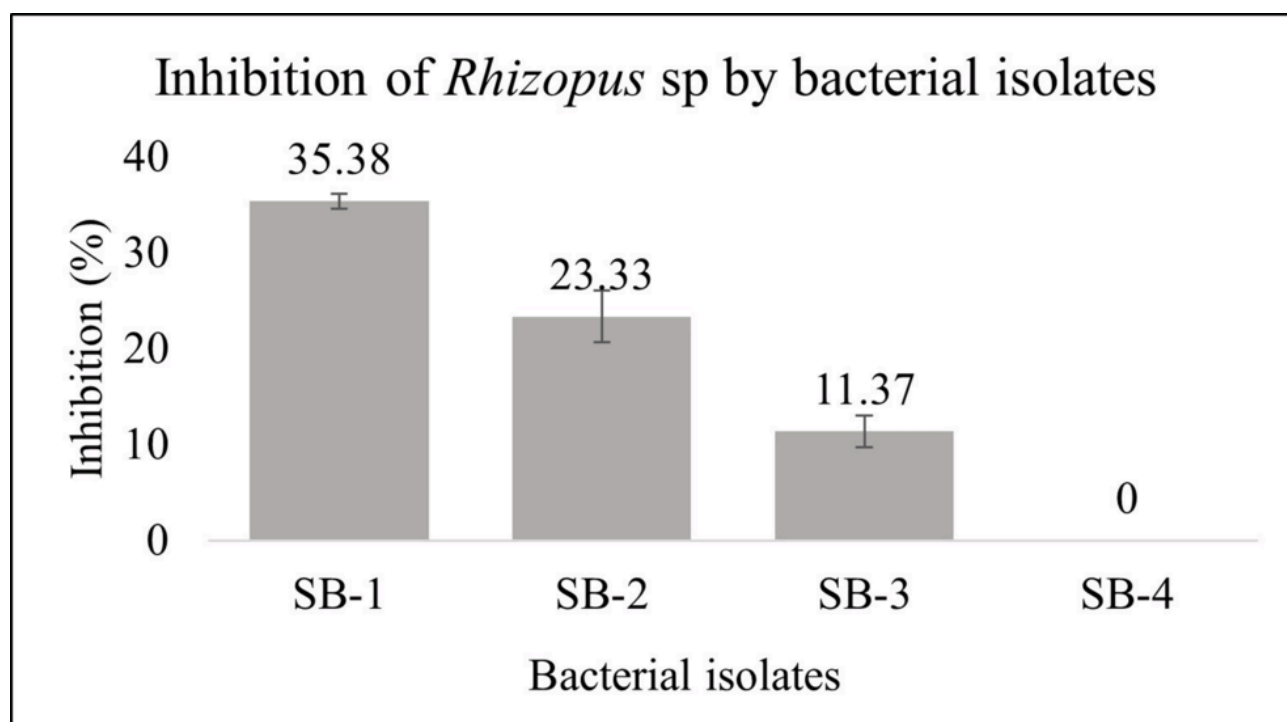


FIGURE 6: Mycelial growth inhibition of *Rhizopus* sp. by bacterial isolates.

Overall, statistical analyses confirmed distinct differences among the isolates. For *L. theobromae*, the analysis revealed a highly significant difference ($F = 302.22$, $p < 0.0001$), with an R^2 value of 0.99, indicating that 99% of the variation in inhibition was explained by the isolate tested; however, R^2 represents the proportion of variance explained by the model rather than a direct measure of accuracy. The coefficient of variation ($CV = 8.79$) indicates relatively low variability among observations. Similarly, for *Rhizopus* sp., significant variation was observed among the isolates ($F = 263.79$, $p < 0.0001$), with an R^2 value of 1.00 and a CV of 9.29. Inhibition against *Rhizopus* sp. was lesser than *L. theobromae* by SB-1, which could be attributed to the more rapid growth rate of *Rhizopus* sp., leading to competition for space and nutrients. The bacterial isolate SB-1 consistently demonstrated the strongest inhibitory activity against both fungal pathogens, suggesting it is the most effective bacterial isolate tested. The high F -values for both fungi confirm that the observed differences are statistically significant, while the relatively low CV values indicate consistent experimental observations based on experiments conducted in triplicate.

Molecular characterization of positive bacterial isolate

The promising bacterial strain SB-1, which exhibited strong antagonistic activity against the fungal pathogens *L. theobromae* and *Rhizopus* sp., was subjected to molecular identification using molecular tools. The isolation of DNA was done, and the agarose gel electrophoresis revealed a single intact high-molecular-weight DNA band. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers, and consensus sequence of 16S rRNA gene was generated, which was used to carry out NCBI-BLAST database. The 16S rRNA gene sequence of the isolate showed highest similarity to *Bacillus velezensis* strain HBUAS65246, partial 16S rRNA gene sequence, based on the BLAST analysis in the National Center for Biotechnology Information database (GenBank accession number ON954547.1). To elucidate the phylogenetic position of the isolate, sequence analysis of the 16S rRNA genes of the SB-1 and related species were used to construct a phylogenetic tree using the Neighbour-Joining method (Figure 7).

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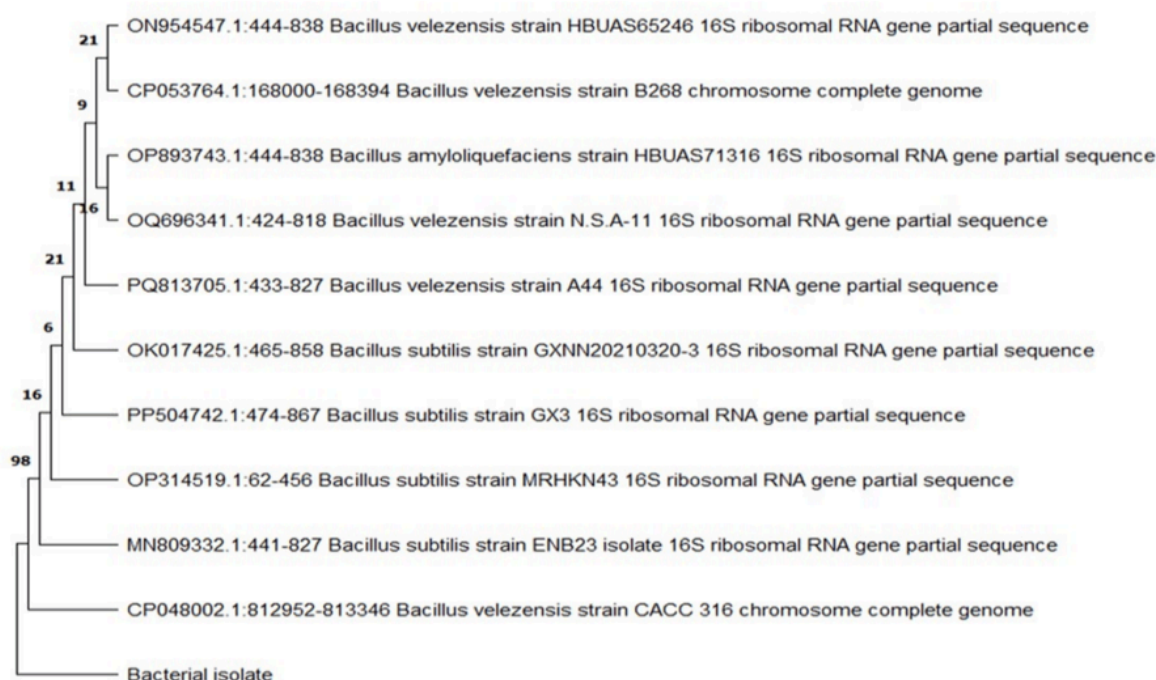


FIGURE 7: Phylogenetic tree based on 16S rRNA sequences constructed using the Neighbour-Joining method.

The resulting phylogenetic tree revealed that the isolate clustered closely with *B. velezensis* strain with a high bootstrap support value, confirming its identity. Thus, the molecular tools confirmed that SB-1 was *B. velezensis*. *Bacillus velezensis* has been reported to be a biocontrol agent against a wide variety of phytopathogens [27-29]. A similar antagonistic effect of *B. velezensis* and *B. amyloliquefaciens* activity against *L. theobromae*, a fruit-rot-causing fungal pathogen, was observed by Chuksatirote et al. [30]. Meena and Karthikeyan [31] found good antagonistic activity of plant growth-promoting rhizobacteria (PGPR) strains, including *B. velezensis* against a mulberry root-rot-causing fungus, *Macrophomina phaseolina*, *F. oxysporum* and *F. solani*. *Bacillus velezensis* has promising application in biological control against mulberry bacterial wilt disease, caused by *Ralstonia pseudosolanacearum* [32] and Sclerotinosis, and mulberry fruit rot caused by *Ciboria shiraiana*, *C. carunculoides*, *Scleromitrella shiraiana* and *Sclerotinia sclerotiorum* [33]. Cao et al. [34] demonstrated that *B. velezensis* possess strong antagonistic activity against bacterial phytopathogens of *R. solanacearum* and fungal pathogens *F. oxysporum*, which causes *Fusarium* wilt in banana.

Optimization of growth parameters of *B. velezensis*

Optimization studies were carried out to determine the optimum temperature, pH, carbon source and nitrogen source that supported the highest growth of *B. velezensis*. The isolate exhibited optimal growth at 50°C, indicating its thermophilic nature (Figure 8).

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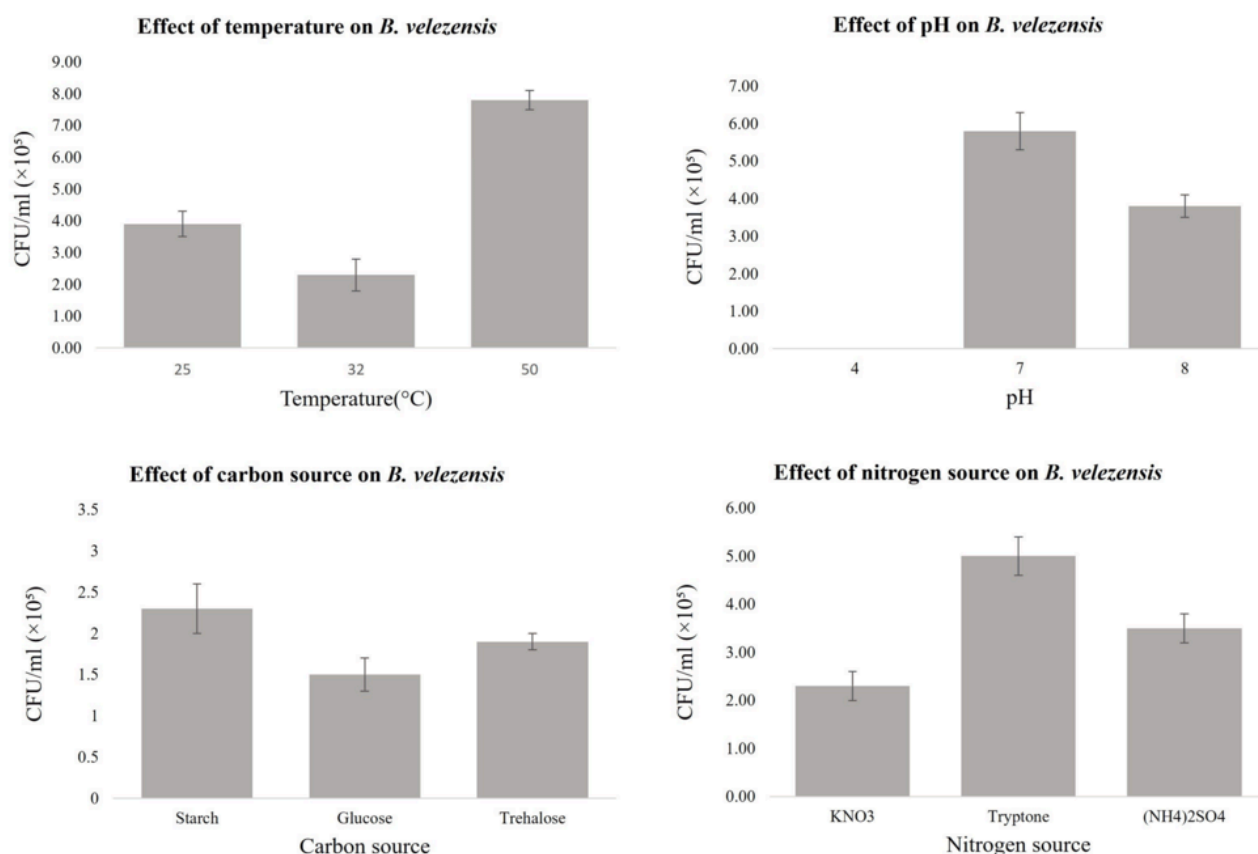


FIGURE 8: Optimum growth study of temperature, pH, carbon source and nitrogen source of *B. velezensis*.

The maximum growth was observed at pH 7.0, with starch as the preferred carbon source and tryptone as the nitrogen source. Temperature optimization results showed a steady increase in bacterial growth with increasing temperature, with the highest CFU/ml recorded at 50°C, indicating that it is a thermophilic strain. This finding is consistent with that of the study by Elegbeleye et al. [35], where this bacterium was used to extend the shelf life of milk processing lines and was able to grow at temperature above 50°C. The optimum pH was determined to be neutral (pH 7.0), at which the bacterial CFU/ml was higher compared to other pH conditions. This is in agreement with Cao et al. [36], who reported that pH 7.0 is optimal for the growth of *B. velezensis*. No growth was observed at acidic (pH 2.0) or alkaline (pH 10.0) conditions. Regarding carbon sources, the highest CFU/ml was observed with starch, although glucose and trehalose also supported bacterial growth. For nitrogen sources, highest CFU/ml of bacteria was seen in tryptone. These findings are consistent with Zhang et al. [37], who reported that the optimal growth medium for *B. velezensis* includes starch as the carbon source and tryptone as the nitrogen source.

In vitro* evaluation of antifungal growth by crude extract of *B. velezensis

Based on the growth optimization studies, *B. velezensis* was cultured under optimal conditions (50°C, 7.0 pH, starch and tryptone) to obtain the crude extract containing potential bioactive metabolites. Based on the average diameters of inhibition zones at different concentrations, the minimum inhibitory concentration (MIC) of the crude extract was estimated (Table 2).

The present study demonstrated that the crude extract of *B. velezensis* exhibits effective antifungal activity in a dose-dependent manner, with stronger inhibition at higher concentrations (75%). *Lasiodiplodia theobromae* showed slightly greater sensitivity to the crude extract compared to *Rhizopus* sp. across all concentrations tested. A measurable zone of

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inhibition was still observed at the lowest tested concentration (10%) (Figures 9-11).

Sl. No.	Concentration of crude extract of <i>B. velezensis</i>	Average diameter of replicates	
		<i>L. theobromae</i>	<i>Rhizopus</i> sp.
1	75%	4.12	3.17
2	50%	3.27	2.77
3	25%	3.05	2.15
4	10%	2.27	1.2

TABLE 2: Agar well diffusion method of the crude extract.

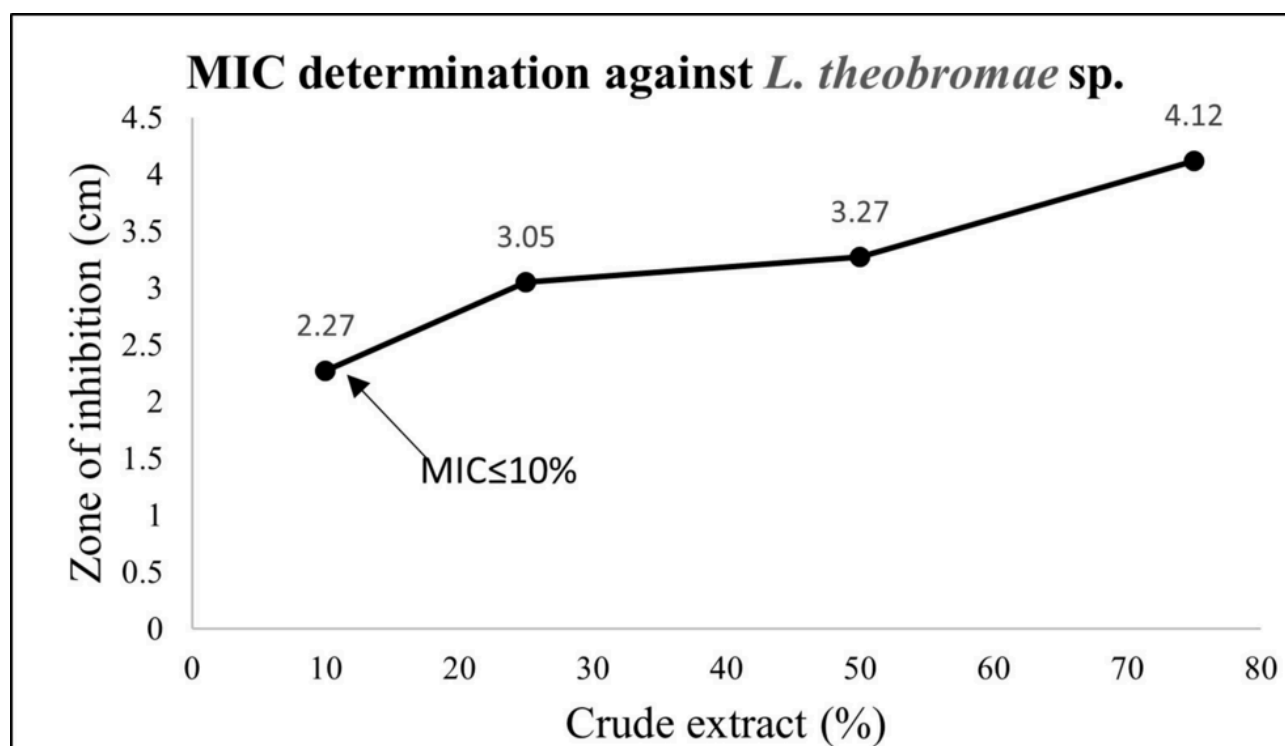


FIGURE 9: Evaluation of crude extract of *B. velezensis* against *L. theobromae*.

MIC, Minimum Inhibitory Concentration

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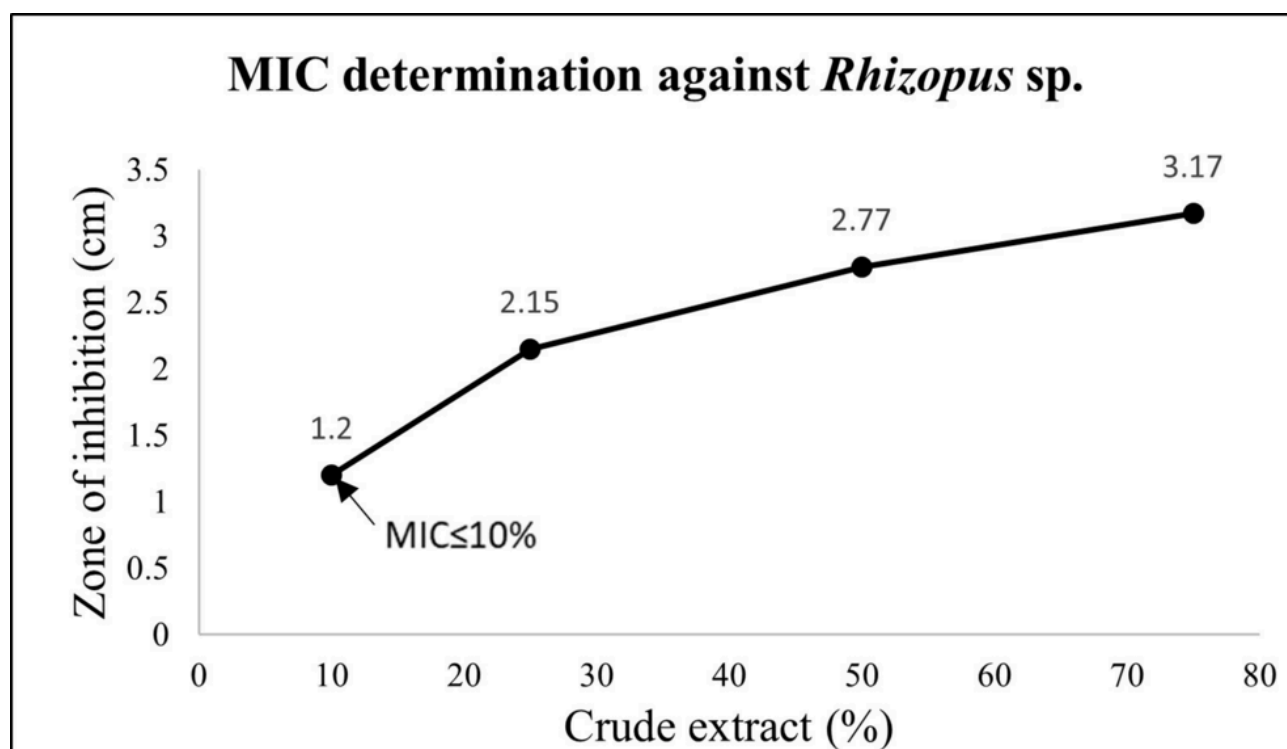


FIGURE 10: Evaluation of crude extract of *B. velezensis* against *Rhizopus* sp.

MIC, Minimum Inhibitory Concentration

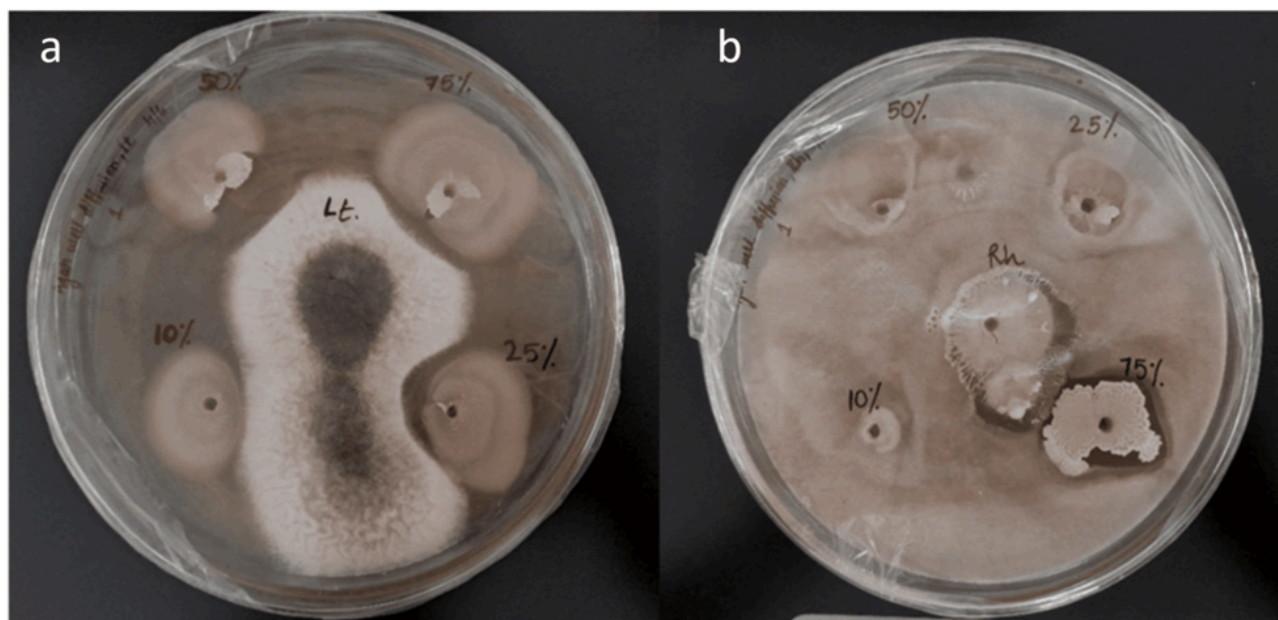


FIGURE 11: In vitro antifungal activity of the *B. velezensis* crude extract against *L. theobromae* (a) and *Rhizopus* sp. (b).

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The crude extract of *B. velezensis* exhibited promising antifungal activity against *L. theobromae*. Antifungal activity was also observed against *Rhizopus* sp. but to a comparatively lesser extent. This could indicate the presence of secondary metabolites produced by bacteria through zone of inhibition. However, bacterial cells were observed in the inhibition zone, which indicates that further advanced purification techniques are required to obtain the metabolites. Various species of the *Bacillus* genera such as *B. amyloliquefaciens*, *B. subtilis*, *B. velezensis* and *B. licheniformis* have been associated with the production of antimicrobial metabolites. Nifakos et al. [38] confirmed that *B. velezensis* can produce bioactive metabolites such as iturin A2, surfactin A-C13 and C-15 isoforms, bacillibactin and oxydificidin, among others.

Characterization of sericin nanomaterial using SEM, EDS, XRD and FTIR

Sericin, a silk protein derived from the cocoons of the silkworm (*B. mori*), represents a valuable biomaterial. It is a gelatinous, gummy and water-soluble protein that constitutes 15-35% of silk and is composed mainly of polar amino acids, particularly serine [39]. Dong et al. [40] emphasized the abundance of protease inhibitors and seroins in the sericin layers of *B. mori* silk fibers. They further reported that protease inhibitors mainly exhibit antifungal activity, whereas seroins possess broad-spectrum antimicrobial properties against bacteria, fungi and viruses. In the present study, sericin nanomaterials were synthesized to assess their antifungal activity against isolated mulberry fungal pathogens. Characterization of the nanomaterials was performed using SEM, EDS, XRD and FTIR analyses. SEM analysis of submicron particles revealed a rough and irregular surface texture, with evidence of partial agglomeration. The particle size ranged from 178 nm to 563 nm, indicating a polydisperse distribution, as observed by Teulon et al. [41] (Figure 12).

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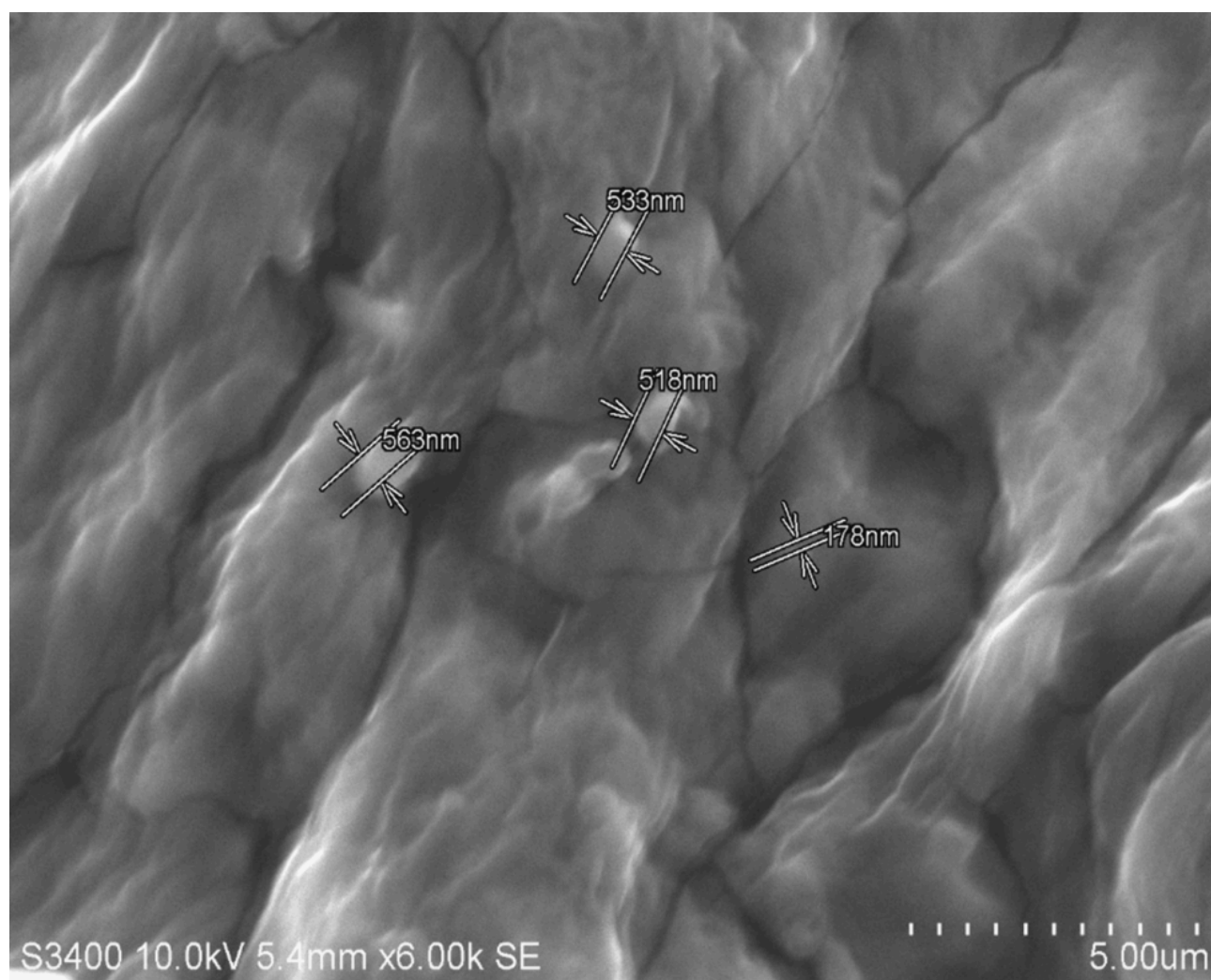


FIGURE 12: Scanning electron microscopy images of sericin nanomaterials.

The shape of the nanomaterials was slightly spherical, which is consistent with the study by Meerasri et al. [19], where the shape of sericin nanomaterials was found to be spherical based on SEM analysis. However, more enhanced and stable nanoparticles can be obtained through freeze-drying the nanoparticles and using stabilizer, which can preserve the structure of the nanoparticles. EDS revealed the presence of carbon (21.47%), nitrogen (22.67%), oxygen (53.66%) and sulfur (2.20%) as the major elements. The total (100%) refers to the weight percentage values obtained from the EDS analysis (Table 3).

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Element Line	Weight %	Weight % Error	Atom %
C K	21.47	± 4.21	26.18
N K	22.67	± 7.71	23.70
O K	53.66	± 3.46	49.11
S K	2.20	± 0.25	1.00
Total	100.00		100.00

TABLE 3: The amount of carbon, nitrogen, oxygen and sulfur present in the sericin nanomaterials.

The spectrum showed no traces of heavy metals or toxic elements, confirming the non-toxic and biocompatible nature of the synthesized sericin nanomaterial. The dominance of carbon, oxygen and nitrogen is characteristic of organic biomaterials such as sericin protein, while the presence of sulfur indicates amino acids like methionine and cysteine, which contribute to disulfide bond formation in β -pleated sheet structures. EDS analysis showed that oxygen, carbon and nitrogen were the major elements present, which is typical of sericin that is composed of amino acids, mainly serine. The XRD analysis of sericin nanomaterials showed a broad peak at $2\theta \approx 20-22^\circ$ (Figure 13), lacking the sharp spikes that would indicate a highly structured material. This shape reveals that sericin is mostly an amorphous, random coil matrix with only a few organized β -sheet domains hidden inside.

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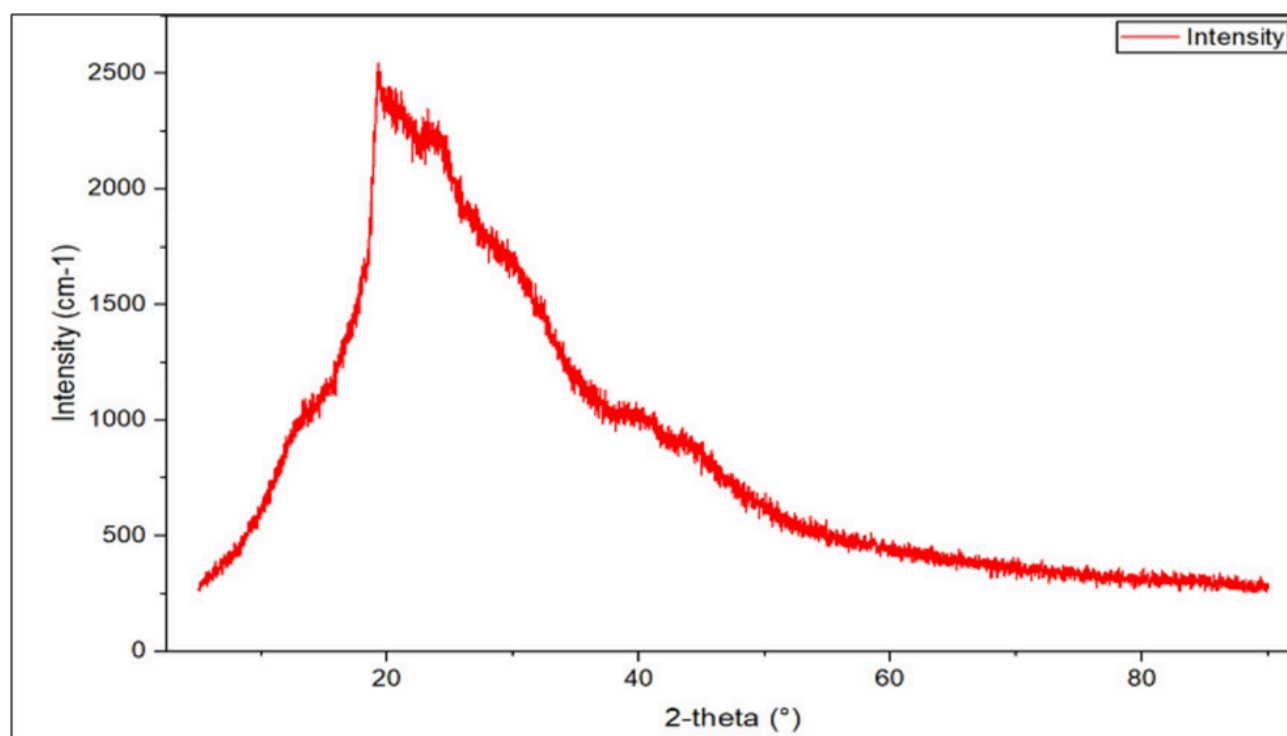


FIGURE 13: X-ray diffraction analysis of sericin nanomaterial.

In sericin, this peak typically corresponds to the β -sheet structure of the protein. A gradual decrease in intensity beyond 30° may be attributed to the presence of secondary structures. Overall, the XRD pattern indicates low crystallinity, which is typical of natural protein-based materials such as sericin. XRD pattern shows a peak obtained at around $2\theta = 20^\circ$. Other studies have reported 18.86° , 19.2° and 21.0° as the highest diffraction peak angles obtained for sericin [42-44].

The obtained FTIR spectra show a characteristic peak corresponding to amine ($3,284.69\text{ cm}^{-1}$), amide I ($1,620.42\text{ cm}^{-1}$), amide II ($1,519.30\text{ cm}^{-1}$), amide III ($1,316.93$ and $1,244.00\text{ cm}^{-1}$) and free carboxylate groups ($1,014.43\text{ cm}^{-1}$) (Figure 14). The peaks obtained confirmed the presence of protein-based functional groups. There was no disappearance of the peaks, indicating that the nanomaterial synthesis process preserved the original chemical structure of sericin. The FTIR results of the present study are consistent with those reported by Al Masud et al. [45], which showed the presence of amide and amine linkages in the obtained nanomaterials, indicating that there were no significant alterations in the chemical bonds present in the sericin nanomaterials and the bonds present in sericin. Sericin-based nanoparticles and nanoformulations have been widely explored for various promising applications, particularly in the biomedical sector, where they have been used as antimicrobial agents, tissue engineering and wound healing [41,46,47].

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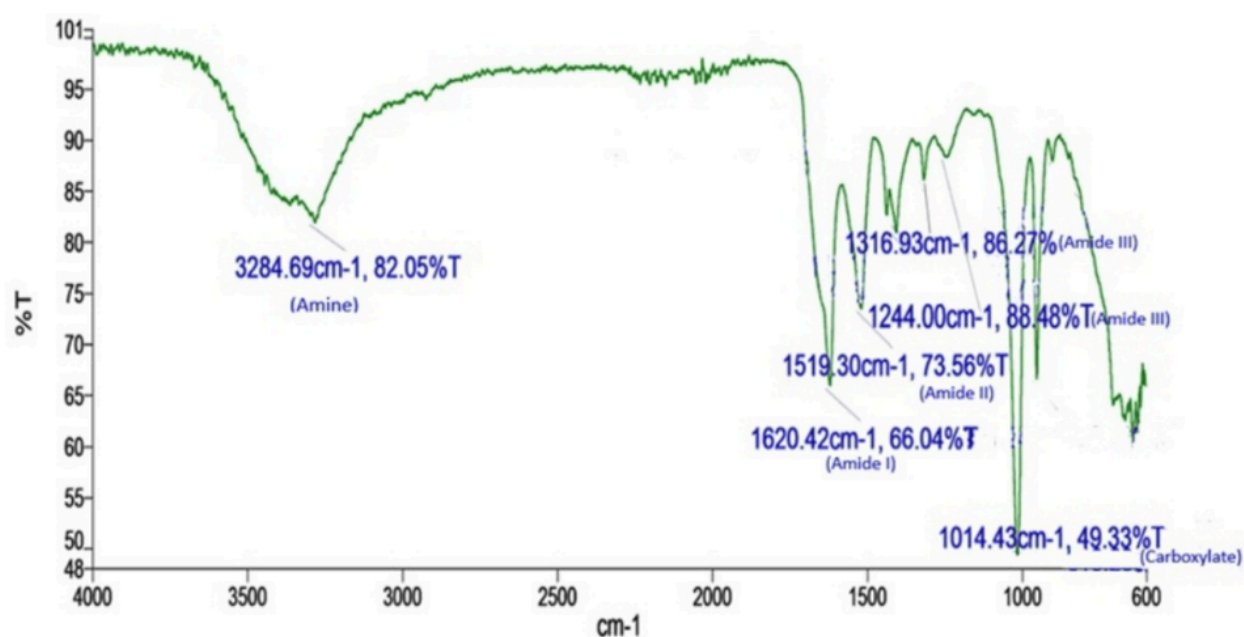


FIGURE 14: Fourier-transform infrared spectra showed characteristic peaks corresponding to amine, amide I, amide II, amide III and free carboxylate groups.

Evaluation of antifungal activity of sericin nanomaterials against *L. theobromae* and *Rhizopus* sp.

To evaluate the antifungal efficacy of sericin nanoparticles, different concentrations (300 mg/ml, 400 mg/ml, 500 mg/ml and 1,000 mg/ml) were tested. The observations revealed that the 1,000 mg concentration produced the highest inhibition against *L. theobromae* (27.73%) (Figure 15), whereas no inhibitory effect was observed against *Rhizopus* sp. (Figure 16) (Table 4).

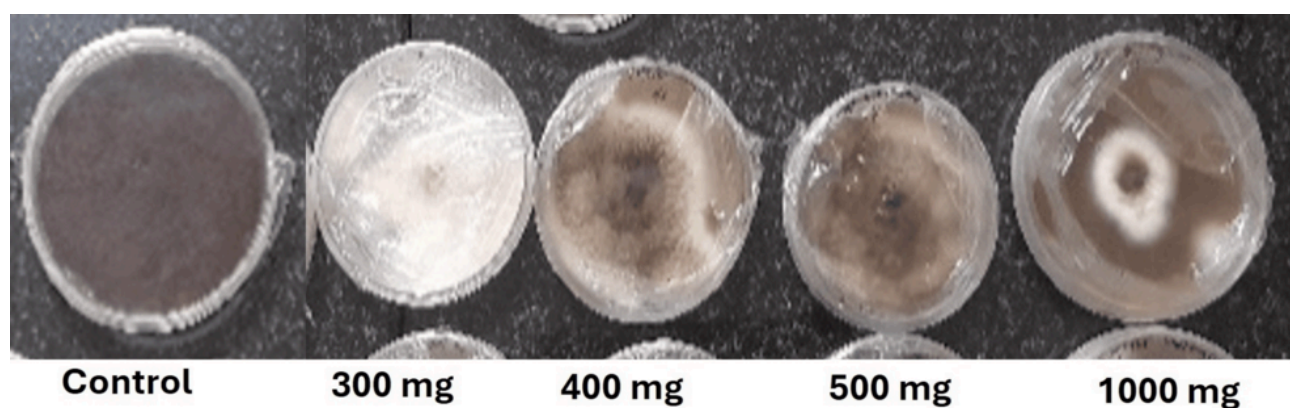


FIGURE 15: Evaluation of antifungal activity of sericin nanomaterials against *L. theobromae*.

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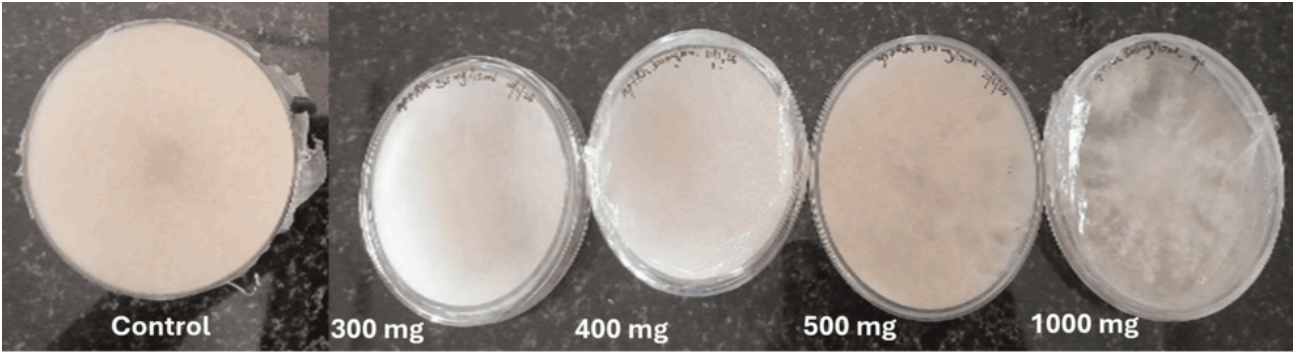


FIGURE 16: Evaluation of antifungal activity of sericin nanomaterials against *Rhizopus* sp.

Sl. No.	Treatment		Inhibitory percentage (%)	
			<i>L. theobormae</i>	<i>Rhizopus</i> sp.
1.	Sericin nanoparticles	300 mg/ml	--	--
		400 mg/ml	--	--
		500 mg/ml	--	--
		1,000 mg/ml	27.73 ^C	--
2.	Crude extract of <i>B. velezensis</i>		69.40 ^B	28.21 ^A
3.	Sericin nanoparticles formulation with crude extract of <i>B. velezensis</i> (1:1 ratio)		77.38 ^A	27.50 ^B

TABLE 4: Inhibitory percentage of sericin nano-formulations with crude extract of *B. velezensis* against *L. theobromae* and *Rhizopus* sp.

Values are presented as mean $\pm$ standard deviation of three independent biological replicates. Means followed by different superscript letters are significantly different according to one-way analysis of variance followed by Tukey's Honestly Significant Difference post-hoc test at $p < 0.05$. Means sharing at least one common superscript letter are not significantly different. "--" indicates no observable inhibitory activity.

Based on the results across the four tested concentrations, 1,000 mg of sericin nanoparticles was identified as the most effective in inhibiting the fungal mycelial growth of both the pathogens. Therefore, this concentration was selected for further formulation studies in combination with the antagonistic bacterium *B. velezensis*. A study conducted by Tahir et al. [48] showed that sericin-based silver nanoparticles were proven to be effective against bacteria such as *Escherichia coli* and *Staphylococcus aureus*. In the field of agriculture, nanoparticles have been utilized to combat root rot disease in plants. Kamel et al. [49] assessed the efficacy of copper oxide nanoparticles in combating root rot in cucumber. Silver

nanoparticles were used to combat root rot in common beans in a study carried out by Ibrahim et al. [50]. Studies where sericin nanomaterials were synthesized without metal salts are few. In most green synthesis of nanomaterials, sericin is used in conjunction with metal salts like silver nitrate, acting as a reducing and capping agent in the formation of metal-based nanoparticles. As per our knowledge, no research has been conducted with respect to sericin nanomaterials used as an antifungal agent to combat root rot disease in mulberry. The sericin nanomaterials synthesized in the present study were tested against *L. theobromae* and *Rhizopus* sp. It was observed that at a high concentration of 1,000 mg, the nanomaterials were able to inhibit black root rot fungus. However, it showed a negative effect against *Rhizopus* sp. Few studies have been conducted, where nanomaterials have been formulated with a beneficial bacterium and has produced promising effect, likely due to nanomaterials enhancing the controlled delivery of the active molecule to the target site [51]. As suggested by Li et al. [52], nanoparticles adhere to/penetrate the fungal cell wall (chitin, β -glucan matrix), causing wall poration and membrane depolarization. Membrane disruption causes cytoplasmic leakage and loss of turgor required for hyphal tip extension. This significantly inhibited mycelial growth and spore germination. Karunakaran et al. [53] have conducted studies on nano-bioformulations and have reported promising results in combating plant diseases and improving soil and plant health. The formulation was developed by combining sericin nanomaterial with the crude extract of *B. velezensis* and tested against *L. theobromae* and *Rhizopus* sp., exhibiting inhibition rates of 77.38% and 27.50%, respectively (Table 4). This formulation demonstrated the highest antifungal efficacy against *L. theobromae*, while showing comparatively low activity against *Rhizopus* sp., suggesting reduced sensitivity of this pathogen to the tested treatments. To the best of our knowledge, there are no direct reports to date in which this formulation has been developed; however, it can be related to the study by Mumtaz et al. [54], which demonstrated that sericin-based silver nanoparticles were effective in inhibiting *Aspergillus niger*, owing to the antimicrobial properties of the nanoparticles that inhibit pathogen growth.

Conclusions

In the present study, the root rot fungal pathogens (*L. theobromae* and *Rhizopus* sp.) and promising antagonistic bacteria (*B. velezensis*) were isolated from the diseased mulberry plant and the soil samples of its rhizosphere. The potential antagonistic bacteria against both the pathogens were identified and characterized through 16S rRNA sequencing. The isolate exhibited better antagonistic ability under *in vitro* study. A crude extract bacterium *B. velezensis* and sericin nanomaterials showed a promising antagonism against the growth of fungal pathogens. It can be concluded that the antagonistic bacterium, its crude metabolites and sericin nanoparticles are capable of inhibiting the mycelial growth of disease-causing fungi. However, further evaluation under *in situ* conditions in mulberry root rot systems, along with comparisons to other established biocontrol microbes, is necessary. This would be an eco-friendly and promising strategy to manage root rot disease in mulberry.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: A Mahadeva

Acquisition, analysis, or interpretation of data: A Mahadeva, Dholakiya Namratta, Deepti G, Archana S. Rao

Drafting of the manuscript: A Mahadeva, Dholakiya Namratta, Deepti G, Archana S. Rao

Critical review of the manuscript for important intellectual content: A Mahadeva, Dholakiya Namratta, Deepti G

Supervision: A Mahadeva, Archana S. Rao

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Disclosures

Plant subjects: All authors have confirmed that this study did not involve any herbarium specimen. **Human subjects:** All authors have confirmed that this study did not involve human participants or tissue. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

Data Availability Statements

The datasets (and/or code) supporting this study are available from the corresponding author upon reasonable request. All data generated or analyzed during this study are included in this published article and/or its appendices.

Acknowledgements

The authors wish to acknowledge the Director, Karnataka State Sericulture Research and Development Institute (KSSRDI), Bangalore. Gratitude is extended to Ms. Kavya Lakshmikanth, Department of Molecular Biology, MedAuxin, Bangalore, for her support in bacterial characterization. The authors also thank the Instrumentation Facility, IOE, University of Mysore, for SEM-EDS and FTIR analyses, and the Central Instrumentation Facility, CHRIST (Deemed to be University), Bangalore, for XRD analysis.

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How to cite this article:

Mahadeva A, Namratta D, G D, et al. (May 19, 2026) Antagonistic Potential of *Bacillus velezensis* and Sericin-Based Nanomaterials Against Mulberry Root Rot Pathogens. Curues J. Agri. Food Sci 2 : es44497-025-00068-3. DOI <https://doi.org/10.7759/s44497-025-00068-3>

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