



Bio-nano Synthesis Optimization of *Lysinibacillus* sp as Biostimulant and Biocontrol to Increased Rice Plants Production

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ABSTRACT

Background: The increasing demand for rice production in Indonesia necessitates innovative and sustainable approaches to enhance crop yield while minimizing environmental impact. Excessive use of chemical fertilizers and the prevalence of plant diseases remain major challenges in rice cultivation. This study aims to optimize the bio-nano synthesis of *Lysinibacillus* sp. for its potential application as a biostimulant and biocontrol agent to improve rice productivity.

Methods: Silver nanoparticles (AgNPs) were biosynthesized using *Lysinibacillus* sp. cultivated in different growth media. The synthesized AgNPs were characterized and evaluated for their biocontrol activity against major rice pathogens, including *Xanthomonas oryzae* and *Rhizoctonia solani*. In addition, the phytohormone-producing ability of *Lysinibacillus* sp. isolates was assessed through qualitative indole-3-acetic acid (IAA) detection using the Salkowski reagent.

Result: The optimized bio-nano synthesis process successfully produced AgNPs with significant biocontrol activity against *Xanthomonas oryzae* and *Rhizoctonia solani*. AgNPs synthesized in luria bertani (LB) medium at 50% concentration exhibited the highest inhibition zones, indicating strong biocontrol potential. Furthermore, *Lysinibacillus* sp. isolates demonstrated the ability to produce IAA, a key phytohormone involved in plant growth promotion. These results highlight the potential of *Lysinibacillus* sp. as an effective biostimulant and biocontrol agent for sustainable rice production.

Key words: Biocontrol, Bio-nano synthesis, Biostimulant, *Lysinibacillus* sp., Rice productivity, Silver nanoparticles.

INTRODUCTION

The increasing population with the demand for food in Indonesia, particularly rice, continues to grow each year. In 2022, rice production in Indonesia reached approximately 54.75 million tons of milled dry grain (MDG), marking an increase from the previous year's production of 54.42 million tons of MDG. Given the rising trend in food demand driven by population growth and increased per capita consumption, it is estimated that Indonesia will need to increase rice production by approximately 2-3% annually over the next five years to meet domestic needs (Anggraeni, 2020). Consequently, efforts to enhance rice productivity are imperative.

However, the endeavour to boost rice productivity often faces various challenges, one of the most significant being plant disease outbreaks, which can drastically reduce yields. Plant diseases are widely recognized as one of the major limiting factors in crop productivity worldwide because they reduce both yield and crop quality (War and Kayang, 2022). These diseases are typically caused by pathogens such as bacteria, fungi and viruses that attack different parts of the rice plant, including the roots, stems, leaves and grains. One of the most common and destructive diseases is bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae* (Marwan *et al.*, 2024). This disease is particularly damaging in areas with high rainfall, as it causes grayish-brown lesions on the leaves, which can spread rapidly, leading to leaf wilting and death (Singh *et al.*, 2024). If the

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infection occurs at the early stages of plant growth, yield losses can be as high as 50%. Additionally, sheath blight, caused by the fungus *Rhizoctonia solani*, poses a serious threat to rice production, especially in tropical and subtropical regions (Li *et al.*, 2021). This disease leads to greenish-gray spots on the stems, which eventually dry out and weaken the plant, making it prone to lodging, particularly under conditions of strong winds or rain. Severe infections by *Rhizoctonia solani* can reduce

yields by 30% or more and also degrade the quality of the harvested grain (Senapati *et al.*, 2022). Similar studies have also reported that bacterial leaf blight can cause yield losses of up to 30-40% in susceptible rice varieties (Marlina *et al.*, 2024).

These challenges often prompt farmers to intensify agricultural practices by using various chemical agents, including pesticides and synthetic fertilizers (Philpott, 2013). While these may be effective in the short term, they can have long-term negative impacts on the environment. Therefore, environmentally friendly approaches to enhancing rice production are necessary, one of which involves utilizing *Lysinibacillus* sp. as a biological agent. Rhizospheric bacteria have been widely reported to suppress plant pathogens while simultaneously promoting plant growth (Periyandavan *et al.*, 2025). In this context, *Lysinibacillus* sp. not only serves as a biocontrol agent capable of combating plant diseases but also functions as a bio-stimulant that supports overall plant growth (Kanbe *et al.*, 2024). This study focuses on enhancing the bio-nano synthesis capabilities of *Lysinibacillus* sp. to optimize its role as a bio-stimulant and biocontrol agent (Wei and Zheng, 2024).

MATERIALS AND METHODS

Location and research period

This research was conducted at the Biotechnology Laboratory, Universitas Muhammadiyah Malang and Genetika Science Laboratory from February to December 2024.

DNA extraction, amplification and sequencing for bacterial identification

Bacterial DNA was extracted using the Quick-DNA Magbead Plus Kit, which includes cell lysis, selective DNA binding to magnetic beads, sequential washing to remove contaminants and elution in a low-salt buffer to obtain high-quality genomic DNA. The concentration and purity of the extracted DNA were assessed spectrophotometrically prior to PCR. Amplification of the 16S rRNA gene was performed using universal primers 27F and 1492R in a 25 µL PCR reaction. The thermal profile consisted of an initial denaturation at 95°C, followed by 35 cycles of denaturation (95°C), annealing (52°C) and extension (72°C). The resulting PCR products were then prepared for bidirectional sequencing to enable accurate bacterial species identification based on genetic signatures.

Biosynthesis of Ag nanoparticles (AgNP) and particle size analysis

AgNP were synthesized using *Lysinibacillus* sp. isolates following the method of (Rahman and Lalnunthari, 2024). Bacterial cultures grown in LB medium for 72 hours were centrifuged and the resulting supernatant was mixed with 5 mM AgNP. Negative and positive controls were prepared without AgNP and without supernatant, respectively. All mixtures were incubated at 37°C for 72 hours and nanoparticle formation was confirmed using UV-Vis spectroscopy at 350-650 nm (Wei and Zheng, 2024). Following

biosynthesis, the AgNP suspension was centrifuged again and the pellet was redispersed in deionized water before analysis. Particle size distribution was measured using a Particle Size Analyzer (PSA), which evaluates light diffraction patterns to determine average particle diameter and distribution profile. This analysis provides essential information on nanoparticle homogeneity and quality (Patel *et al.*, 2024).

The inhibition test of AgNP against *Xanthomonas oryzae* and *Rhizoctonia* sp.

The antibacterial activity of AgNP against *Xanthomonas oryzae* (Xoo) was tested using the disc diffusion method. Xoo cultures were spread on PSA plates and filter discs soaked in AgNP solutions (12.5-100% v/v) were incubated at 30°C for 48 h. Inhibition was determined by measuring clear zones, with efficacy calculated relative to the 100% AgNP stock solution (Nadhman, 2020; Sukorini *et al.*, 2021):

$$DH = \frac{\text{Diameter of clear zone in tretment}}{\text{Diameter of clear zone in standard control}} \times 100\%$$

The antifungal activity of AgNP against *Rhizoctonia* sp. was evaluated using the pour plate method by incorporating AgNP (12.5-100% v/v) into PDA. A fungal plug was placed at the plate center and growth reduction was quantified following Mahdizadeh *et al.* (2015):

$$IE = \frac{L_k - L_p}{L_k} \times 100\%$$

Where:

IE = Inhibition efficiency (%).

Lk = Colony growth area of fungi in the negative control (cm²).

CG = Colony growth area of fungi with AgNP addition (cm²).

Observed rhizoctonia solani fungi using FE-SEM

Rhizoctonia solani was inoculated with AgNPs to examine morphological changes, with untreated samples serving as controls. Fungal colonies grown on PDA were exposed to AgNPs and incubated for 24-48 hours. Samples were then mounted on SEM stubs, gold-coated and observed using FE-SEM (FEI Quanta FEG 650) to compare surface structural differences between AgNP-treated and untreated hyphae. This analysis provided detailed insight into the impact of AgNPs on fungal morphology.

Phytohormone and metabolite analysis of *Lysinibacillus* sp.

The phytohormone-producing potential of *Lysinibacillus* sp. isolates was assessed using a qualitative Salkowski assay. Isolates were cultured on NA medium supplemented with 100 ppm tryptophan and incubated for 48 hours. After adding Salkowski reagent and placing the cultures in darkness for 30 minutes, a pink color indicated positive indole-3-acetic acid (IAA) production (Karale *et al.*, 2024).

For further characterization, metabolites associated with plant growth regulation were analyzed using GC-MS. Liquid cultures were centrifuged and the supernatant was mixed with pre-cooled absolute ethanol, vortexed and recentrifuged. The resulting pellet was resuspended in ethanol and transferred to GC-MS vials for analysis of biostimulant and bioprotectant compounds (Pati and Rathore, 2024).

RESULTS AND DISCUSSION

DNA extraction, amplification and sequencing for bacterial identification

Phylogenetic analysis using the Maximum Likelihood (ML) method was conducted to determine the evolutionary relationships of the isolates. The ML approach was selected for its high accuracy in estimating genetic relatedness.

The resulting phylogenetic tree (Fig 1) showed that Sample C1 clustered very closely with *Lysinibacillus fusiformis* (NR 112628.1; NR 042072.1), indicated by an almost zero genetic distance (0.000–0.001). This suggests that C1 may represent a new strain or a minimally diverged variant of *L. fusiformis*. Sample C2 grouped near

Lysinibacillus macroideus (NR 114920.1) and *L. boronitolerans*, indicating a slightly greater but still close evolutionary relationship, potentially representing a strain variation or a novel species within the genus. The tree structure showed a well-defined *Lysinibacillus* clade, while genera such as *Solibacillus* and *Ureibacillus* formed separate, distantly related clades, reflecting significant evolutionary divergence. These findings confirm the robustness of the ML method and highlight the genetic uniqueness of the isolates. Future work may include bootstrapping for tree reliability and further molecular or phenotypic characterization to explore the biotechnological potential of isolates C1 and C2 (Patel and Gupta, 2020).

Biosynthesis test of C1 and C2 bacteria using AgNP on UVV is Spectrophotometer

The biosynthesis of silver nanoparticles (AgNP) using bacterial strain C1 and C2 was evaluated through UV-Vis spectrophotometric analysis. The absorbance spectra provide insights into the formation and characteristics of the synthesized nanoparticles. The results, as illustrated in Table 1, indicate notable differences in absorbance

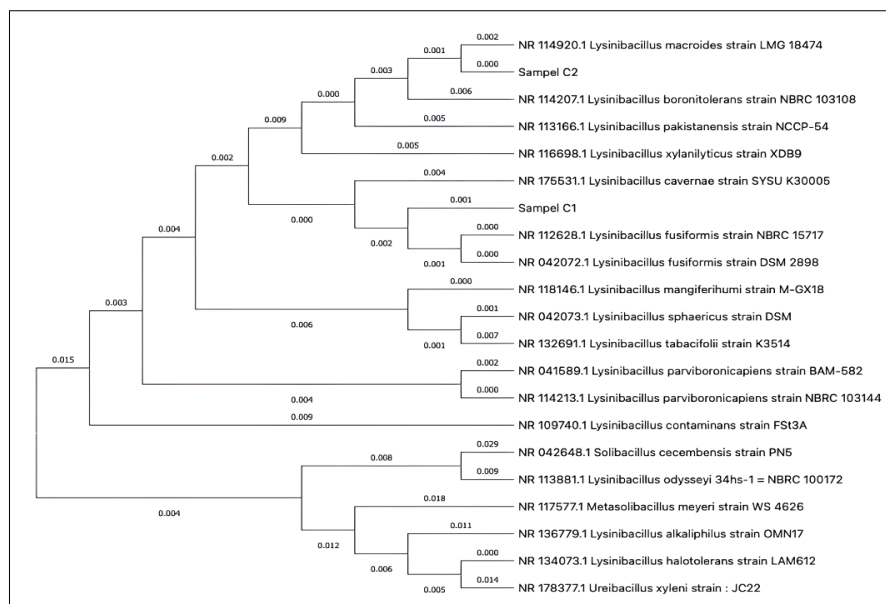


Fig 1: Phylogenetic tree analyzed using the maximum likelihood (ML) method.

Table 1: The biosynthesis of silver nanoparticles (AgNP) using bacterial strain C1 and C2 was evaluated through UV-Vis spectrophotometric.

Treatment	Wave length	Absorbance value
C1 Bacterial supernatant without AgNP	300 nm	0.6125
	650 nm	0.1964
C1 Bacterial supernatant + AgNP	300 nm	0.5914
	650 nm	0.2753
C2 Bacterial supernatant without AgNP	300 nm	0.5198
	650 nm	0.2007
C2 Bacterial supernatant + AgNP	300 nm	0.5098
	650 nm	0.2655

values between bacterial supernatants treated with and without silver nitrate (AgNO_3), which serves as the precursor for AgNP synthesis.

In the C1 and C2 bacterial supernatant without AgNP, the absorbance values at 300 nm were recorded as 0.6125 and 0.5198, For 650 nm were recorded as 0.1964 and 0.2007 respectively. These values represent the baseline spectral properties of the bacterial metabolites and any naturally present chromophores. Upon introducing AgNO_3 to the bacterial supernatant (C1 + AgNP), the absorbance values at 300 nm and 650 nm changed to 0.5914 and 0.2753, respectively. The slight decrease at 300 nm and the notable increase at 650 nm suggest the formation of AgNPs, as the surface plasmon resonance (SPR) of AgNPs typically exhibits a characteristic peak in the range of 400-450 nm. The observed spectral shifts are consistent with the reduction of silver ions (Ag^+) to metallic silver (Ag^0) mediated by bacterial metabolites, which act as reducing and stabilizing agents. The higher absorbance at 650 nm in the treated sample is indicative of nanoparticle aggregation or variation in particle size, which can influence the SPR band broadening or shifting (Metryka *et al.*, 2023; Nakakimura *et al.*, 2012).

Particle size analysis result of biosynthesized nanoparticles by *Lysinibacillus* sp. *Lysinibacillus* sp (C1 isolate) in LB medium

The particle size analysis of nanoparticles biosynthesized by *Lysinibacillus* sp in Luria Bertani (LB) medium, as shown in Fig 2. revealed an average particle size of 1730 nm with a standard deviation of 99.94 nm. This indicates that the

biosynthesis process tends to produce microparticles rather than nanoparticles, with a relatively high consistency in particle size distribution.

The average particle size of 1730 nm suggests that biosynthesis in LB medium supports the formation of larger particles, which might be beneficial for specific applications requiring micron-scale particles. The standard deviation of approximately 100 nm reflects some variability in particle size, yet it remains controlled, showing that most particles fall within a similar size range. This level of variability, around 5.8% of the average size, indicates a well-controlled process, although further optimization could reduce this variability even more. The nutrient-rich LB medium likely contributes to the formation of these larger particles. Further research could explore how specific components of the medium, such as nutrients and cultural conditions like pH and temperature, influence particle size and consistency (Waseem *et al.*, 2024). The resulting 1730 nm particles could be ideal for applications that benefit from micron-sized particles, such as drug delivery systems and specific agricultural applications, where particle absorption and distribution are crucial.

Lysinibacillus sp (C2 isolate) in LB medium

In contrast, the particle size analysis for *Lysinibacillus* sp. cultured in LB medium as shown in Fig 3. For isolate C2 showed an average particle size of 189.7 nm with a standard deviation of 2.803 nm. This result places the particles firmly within the nanoparticle range, which is particularly relevant for various advanced applications, including drug delivery, electronics and nanostructured materials.

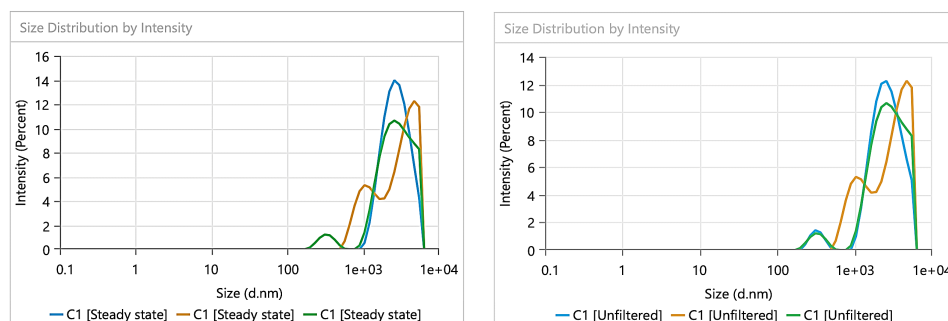


Fig 2: Size distribution by intensity *Lysinibacillus* sp (C1 isolate) in LB medium.

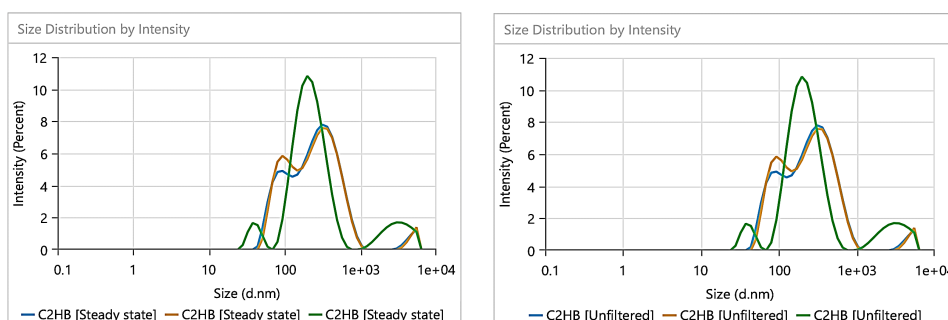


Fig 3: Size distribution by intensity *Lysinibacillus* sp (C2 isolate) in LB medium.

The small standard deviation of 2.803 nm indicates extremely high consistency in nanoparticle production, a critical factor for applications requiring uniform particle sizes. With a standard deviation of less than 2% of the average size, the process demonstrates remarkable stability and reliability, making it suitable for large-scale nanoparticle production (Patel *et al.*, 2024). The nutrient-rich environment of the LB medium appears to effectively support the synthesis of stable nanoparticles without leading to unwanted aggregation. This size and consistency make the nanoparticles ideal for applications that require precise control over particle characteristics, such as in sensor technology or energy storage systems.

Lysinibacillus sp (C1 isolate) in mineral medium

The particle size analysis of *Lysinibacillus* sp. cultured in a mineral medium as shown in Fig 4. for isolate C1 revealed

an average particle size of 2161 nm with a standard deviation of 106 nm. These findings suggest that the biosynthesis process under these conditions results in larger microparticles. The larger particle size indicates that the mineral medium may promote the aggregation or formation of larger particles, which could be advantageous or detrimental depending on the intended application. The relatively low standard deviation of 106 nm suggests consistent particle production, essential for commercial and industrial applications where uniformity is critical.

Further investigation into the specific ions and nutrients in the mineral medium could help optimize conditions for desired particle sizes. The average size of 2161 nm suggests potential applications in fields requiring significant surface area or specific mechanical properties, such as in filtration systems or drug delivery carriers (Jayaraj *et al.*, 2024).

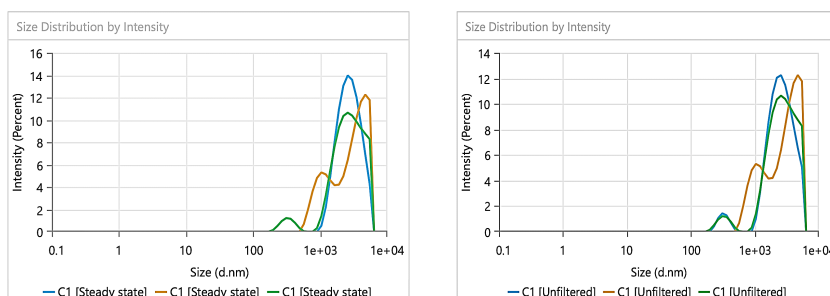


Fig 4: Size distribution by intensity *Lysinibacillus* sp (C1 isolate) in mineral medium.

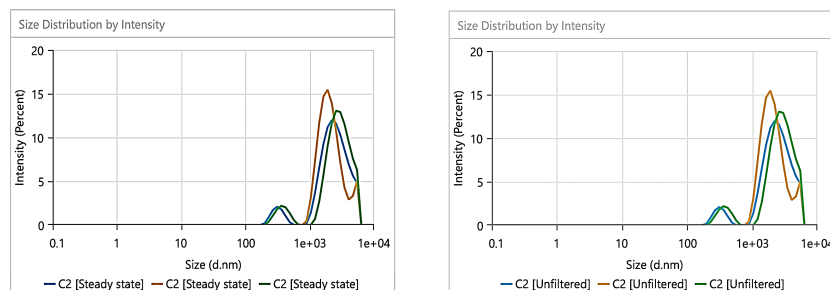


Fig 5: Size distribution by intensity *Lysinibacillus* sp (C2 isolate) in mineral medium.

Table 2: The inhibition zone AgNP against *Xanthomonas oryzae*.

Treatment	Side	Control	AgNP 10%	AgNP 20%	AgNP 30%	AgNP 40%	AgNP 50%
AgNP C1	Front						
	Behind						
AgNP C2	Front						
	Behind						

Lysinibacillus sp (C2 isolate) in mineral medium

For isolate C2, when cultured in a mineral medium as shown in Fig 5, the particle size analysis revealed an average size of 1981 nm with a standard deviation of 86.21 nm. This result also falls within the microparticle range, indicating that the mineral medium supports the formation of relatively large particles with limited size variation.

The small standard deviation of 86.21 nm, relative to the average size, suggests a controlled and consistent biosynthesis process, which is crucial for applications where precise particle size is essential. The ability to produce uniform particles in the 2-micron range could be beneficial for various industrial applications, including those requiring significant surface area or specific chemical reactivity (Chadive *et al.*, 2024). This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature. Conclusions may be included in a final paragraph. The concluding comments should not be a summary of the method and the study as the Abstract provides this. The final paragraph of the paper should identify important outcomes and their implications for the area of study or recommendations for further research.

Biocontrol activity of AgNP Against *Xanthomonas oryzae*

The antibacterial activity of AgNP derived from isolates C1 and C2 was evaluated on Potato Sucrose Agar (PSA) at concentrations of 10-50%. As shown in Fig 6, both AgNP types inhibited the growth of *Xanthomonas oryzae* across all treatments. Quantitative data in Table 2 indicate that AgNP C1 at 50% produced the largest inhibition zone (4.5 mm), while AgNP C2 at 30-40% yielded inhibition zones of 4 mm. According to Athanasiadis *et al.* (2023), inhibition zones ≤ 5 mm are classified as weak, which corresponds to the inhibition observed in this study. The positive control (AgNP 100% without pathogen) showed no inhibition, confirming that AgNP activity is dependent on pathogen interaction rather than the nanoparticle solution alone. Although categorized as weak, the ability of AgNP to consistently inhibit *X. oryzae* suggests potential antibacterial activity worth further optimization (Nadhmam, 2020).

Biocontrol test results of AgNP against *Rhizoctonia solani*

The antifungal activity of AgNP synthesized from isolates C1 and C2 was evaluated on PDA medium at concentrations of

10-50%. As shown in Fig 7, both AgNP types inhibited *Rhizoctonia solani*. Table 3 indicates that AgNP C1 at 50% produced the largest inhibition zone (22 mm), classified as *very strong*, while AgNP C2 at 20% yielded its highest inhibition (15 mm), categorized as *strong* (Wang *et al.*, 2023).

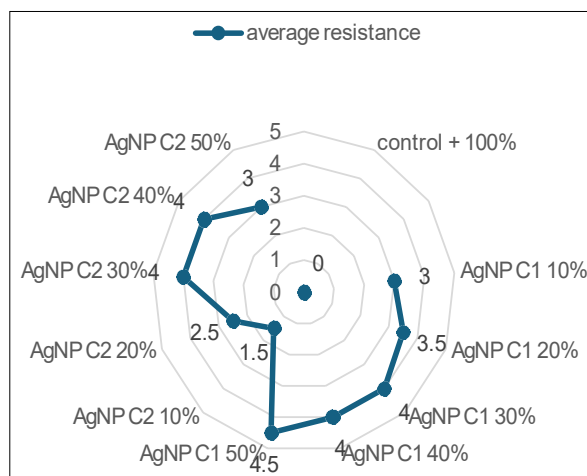


Fig 6: Biocontrol activity of AgNP against *Xanthomonas oryzae* on PSA Medium.

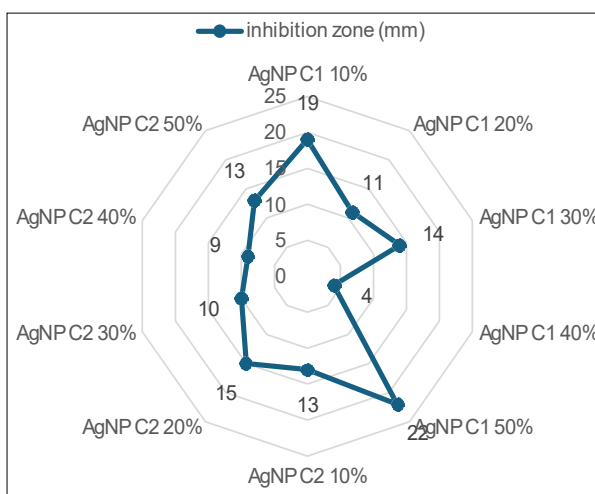
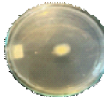


Fig 7: Biocontrol activity of AgNP against *Rhizoctonia solani* on PDA Medium.

Table 3: The inhibition zone AgNP against *Rhizoctonia solani*.

Treatment	AgNP 10%	AgNP 20%	AgNP 30%	AgNP 40%	AgNP 50%
AgNP C1					
AgNP C2					

FE-SEM analysis (Fig 8) revealed morphological damage to fungal hyphae following AgNP exposure. Control hyphae appeared smooth and intact, whereas C1 + AgNP caused swelling and deformation. More severe structural collapse occurred in the C2 + AgNP treatment, suggesting a stronger synergistic antifungal effect. This enhanced disruption is likely due to combined actions of AgNP and metabolites produced by isolate C2, which compromise cell wall integrity and trigger oxidative stress (Islam *et al.*, 2024). Overall, the results demonstrate that AgNP-particularly those derived from isolates C1 and C2-show strong potential as effective biocontrol agents against *R. solani*.

Qualitative observation results of phytohormone testing on *Lysinibacillus* sp.

The qualitative Salkowski test showed a clear color change in isolates C1, C2 and the combination of C1 + C2 after reagent addition (Fig 9), indicating their ability to produce indole-3-acetic acid (IAA). As IAA is a key phytohormone involved in cell division, elongation and tissue differentiation (Pati and Rathore, 2024), its detection confirms the phytohormone-producing potential of these isolates. Individually, both C1 and C2 produced IAA and the combined treatment also resulted in a strong positive reaction, suggesting possible synergistic enhancement. The ability of these isolates to synthesize IAA aligns with the role of Plant Growth-Promoting Rhizobacteria (PGPR), which improve root development, nutrient uptake and stress tolerance (Ikhwan *et al.*, 2023, Marwan *et al.*, 2024). Overall,

the Salkowski assay demonstrates that *Lysinibacillus* isolates C1 and C2 individually and together possess significant potential as biostimulant and biofertilizer agents capable of enhancing plant growth.

Bioprotectant and biostimulant test using gas chromatograph mass spectrometer (GC-MS) on *Lysinibacillus* sp.

Biostimulants test result

GC-MS analysis (Fig 10) detected several metabolites with biostimulant potential. Key compounds included terpenes and terpenoids-such as trans-Pinocarveol, (+)-Nopinone, Isopinocampnone and Longiborneol-which are known to enhance plant growth by regulating physiological signaling, stimulating root development, improving nutrient uptake and increasing stress tolerance. The fatty acid derivative Methyl Ester of 3-Hydroxy-Undecanoic Acid was also identified, supporting cell membrane stability and stress-responsive signaling. These metabolites collectively indicate a strong biostimulant profile, contributing to plant vigor and sustainable crop productivity (Pati and Rathore, 2024).

Bioprotectants test result

The GC-MS analysis also revealed several compounds with potential bioprotectant properties as shown in Fig 11, which are essential for safeguarding plants against pathogens and pests. Benzaldehyde derivatives, such as Benzaldehyde, 2-methyl-, m-tolualdehyde and Benzaldehyde, 4-methyl-, are known for their strong antimicrobial

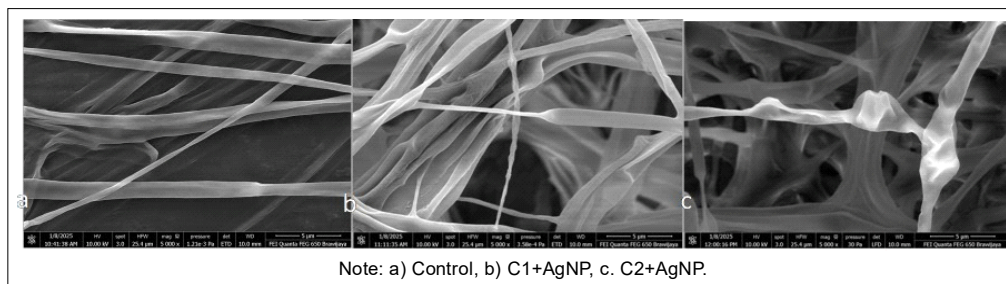


Fig 8: Observed *Rhizoctonia solani* fungi using FE-SEM.

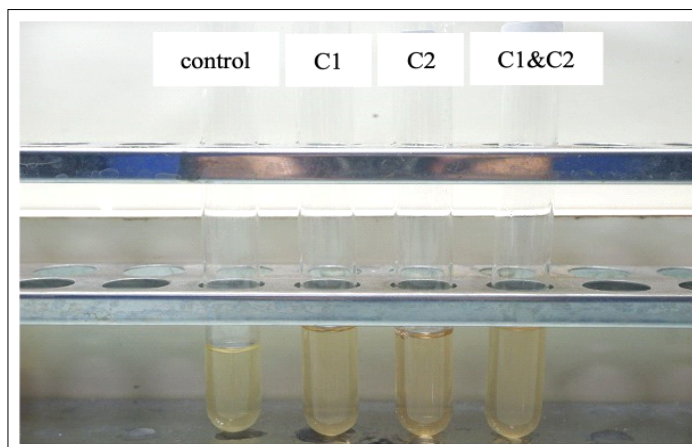


Fig 9: Bacterial Isolates C1, C2 and the combination of C1 and C2.

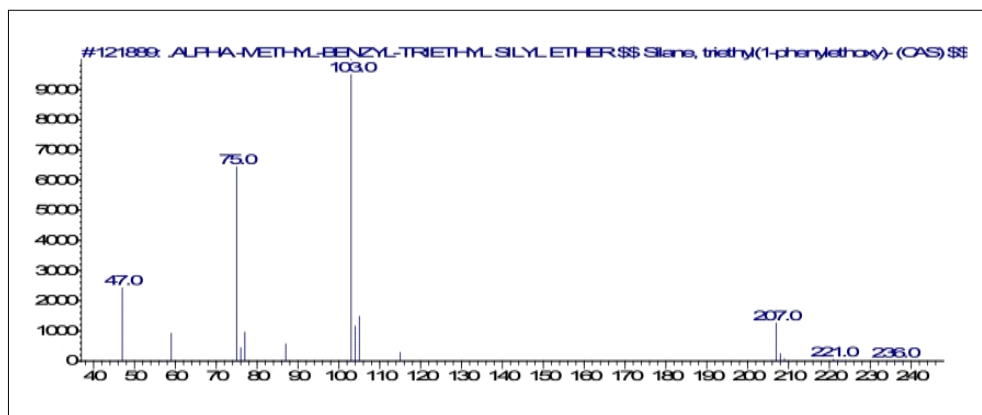


Fig 10: Biostimulants test result.

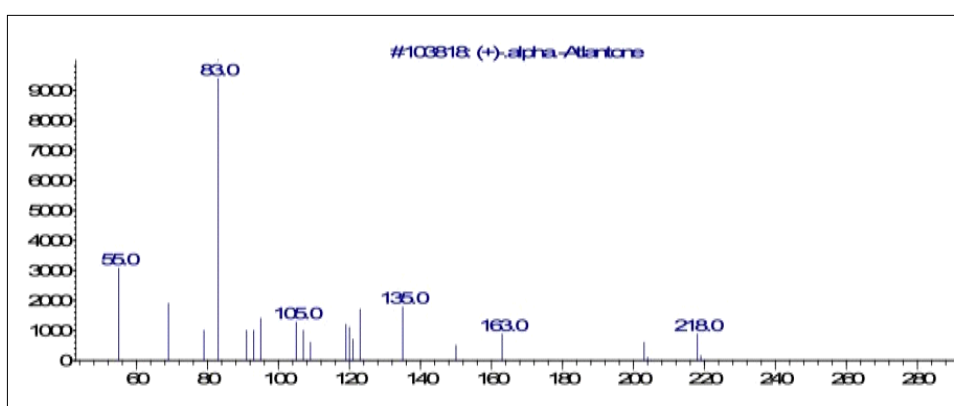


Fig 11: Bioprotectants test result.

properties. These aldehydes can inhibit the growth of bacteria and fungi, providing crucial protection against microbial infections. This bioprotective action is particularly valuable in preventing diseases that can devastate crops, thereby reducing the need for synthetic pesticides. Additionally, Jacobine, a pyrrolizidine alkaloid identified in the analysis, serves as a natural pesticide. Alkaloids like Jacobine deter herbivores and inhibit the growth of pathogens, acting as a chemical defense mechanism in plants. By incorporating these bioprotectants into agricultural practices, farmers can enhance crop protection in a more environmentally friendly manner, reducing reliance on chemical pesticides and contributing to sustainable farming practices (Senapati *et al.*, 2022).

CONCLUSION

This study demonstrates the strong potential of AgNP synthesized by *Lysinibacillus* sp. isolates C1 and C2 as biocontrol and biostimulant agents. AgNP-particularly those derived from isolate C1-showed notable inhibitory activity against *Xanthomonas oryzae* and *Rhizoctonia solani*, indicating their promise as eco-friendly alternatives to chemical pesticides. Both isolates also produced indole-

3-acetic acid (IAA), with C1 generating the highest levels and the C1-C2 combination showing consistent production. This phytohormone activity highlights their capacity to enhance plant growth, nutrient absorption and stress tolerance.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

This study did not involve any animals or animal experimentation. Therefore, ethical approval for animal use was not required.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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