Isolation and characterization of endophytic bacteria from Pennisetum purpureum Schumach

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Abstract. *Pennisetum purpureum* Schumach, commonly called elephant grass, found in tropical and subtropical climates is used as an energy crop as well as a forage grass. As part of an ongoing exploration of environmentally friendly agricultural approaches in Vietnam, which includes the utilization of endophytic species and endogenous bacteria in fertilizer synthesis, studies were conducted on elephant grass. Elephant grass specimens were gathered and examined to investigate the advantages of endogenous bacteria during elephant grass growth. Endogenous bacteria capable of generating IAA were identified from *Pennisetum purpureum* Schumach samples collected in the provinces of Hai Duong, Cao Bang, and Thai Nguyen. Five of these strains were chosen for their capacity to stimulate plant development and fight harmful bacteria. All selected strains were gram-negative and motile endophytic bacteria. After 72 hours in a medium with a pH of 7, 100 mg/l L-tryptophan, and 30°C on liquid NA media, the study identified five endogenous bacterial strains (HDR5, HDR9, TNT3, CBR1, and CBR2) that produced the maximum amounts of IAA. HDR5 and CBR2 strains demonstrated the ability to inhibit plant-pathogenic Xanthomonas strains. The investigation of HDR5's 16S rRNA nucleotide sequence revealed that it is closely linked to the genus Enterobacter. The study suggests the use of endogenous bacteria in fertilizer synthesis as an effective and eco-friendly approach.

1 Introduction

Elephant grass, *Pennisetum purpureum* Schumach, is a tropical grass species native to Africa. In Vietnam, elephant grass produces the most green matter and has great potential as fodder under intensive farming conditions. Several varieties of elephant grass, including Napier, Bela Vista, King grass, Merkeron, etc. are available in Vietnam with green leaves, smooth stems, and hairy stems and leaves [1]. Elephant grass is a tall perennial grass that can grow up to a height of 4-5 meters which is well-known for its high production and is frequently used for multiple purposes. One of the foremost benefits of elephant grass is its

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use as a feed crop due to its high production making it an excellent source of feed for animals, particularly cattle and sheep. The richness in nutrients, including protein, fiber, and energy in the elephant grass makes it a complementary grazing supplement.

Along with its use as cattle feed, elephant grass is also used to manufacture biofuels [2]. Its high cellulose concentration, which is a good source of lignocellulose, is used to generate ethanol and other biofuels. Additionally, the fibrous texture of the elephant grass, ideal for the production of pulp, is used as raw material in the paper industry [3, 4].

Elephant grass is often easy to grow on marginal land as it is a resilient grass that can thrive in a variety of soil types and climatic conditions. However, adequate water and fertilizer supplies are required for optimum growth and harvest. As a result, watering and fertilizing the ground may be essential to ensuring an excellent harvest. Therefore, researching and learning new farming methods are crucial to increase biomass yields, minimize costs, and be environmentally friendly.

Bacterial endophytes are the microorganisms that live inside plants without causing any harm to them. Rather they have been found to provide various benefits to plants, such as faster growth and disease resistance. One of the key advantages of bacterial endophytes in agriculture is their ability to support plants in surviving stressful situations by producing hormones and enzymes that help plants adapt to environmental challenges. As a result, crops can now be grown in previously unsuitable regions increasing food production. Another advantage of using bacterial endophytes in agriculture is their potential to protect plants against diseases and pests.

There are various advantages to using plant growth-promoting endophytes (PGPEs) in agriculture and environmentalism. They have the potential to increase crop yields, protect plants from diseases and pests, and help improve the soil. For instance, endophytic bacteria promoted plant growth when tested on clover and potato, resulting in a 63% increase in shoot height. Inoculating tomato plants with PGPEs resulted in a 22% increase in plant biomass and a 32% increase in fruit output compared to non-inoculated plants [5]. If beneficial endophytes are present, plants can enhance their ability to handle stress, improve nutrient absorption, activate plant defense mechanisms, and enhance rapid growth. Therefore, utilizing endogenous bacteria as probiotics and organic fertilizers to increase plant development and production is an effective technique.

The objective of this study was to isolate endogenous bacteria from elephant grass and evaluate their capacity to induce the production of the plant growth hormone Indole-3-acetic acid (IAA). Additionally, we assessed their capacity to combat the bacterial strain of Xanthomonas that infects plants. This study presents an option to use beneficial endophytic bacteria as probiotics to boost the biomass productivity of *Pennisetum purpureum*.

2 Materials and Methods

2.1 Plant Material and Isolation of Bacterial Endophytes

Elephant grass samples were collected from Thanh Mien town, Hai Duong province; Trung Khanh district, Cao Bang province; and Pho Yen town, Thai Nguyen province. Bacterial endophytes were isolated from healthy and asymptomatic roots of elephant grass using the technique described by Hiep and Diep [6]. The stem, root, and leaf segments were washed with 96% alcohol for 3 minutes, followed by rinsing with autoclaved distilled water to remove the alcohol. Subsequently, they were washed with 1% sodium hypochlorite (NaClO) for 3 minutes and 3% hydrogen peroxide (H_2O_2) for 3 minutes. The segments were then rinsed four times with sterile distilled water to remove any residual chemicals. A 100 µl aliquot of the final sample wash was taken and inoculated onto a Petri plate

containing NA medium to test for surface sterilization. The Petri dishes were incubated at 30 °C for 2 days, and the absence of bacterial and fungal growth indicated effective sterilization. After plating on NA medium, the suspension was cultured for 48–72 hours at 28 °C.

2.2 Selection and Screening of Indigenous Bacteria Capable of Producing IAA

Endophytic bacterial strains were cultured in test tubes containing liquid NA medium supplemented with L-Tryptophan (100 mg/L) and shaken at 200 rpm in the dark at 30°C to prevent IAA degradation by light. After 72 hours, cultures of indigenous bacterial strains were collected and centrifuged at 4°C for 10 minutes at 10,000 rpm. Following centrifugation, 1 ml of the clear solution was carefully aspirated into test tubes, and 2 ml of Salkowski's reagent (300 ml of 98% H_2SO_4, 15 ml of 0.5 M $FeCl_3$) was added. The mixture was incubated in the dark for 20 minutes at room temperature to complete the reaction. The IAA concentration was determined using the Salkowski colorimetric technique at 530 nm.

2.3 Optimization of Endogenous Bacterial Strains for IAA Production

The selected strains were cultured in liquid NA medium enriched with L-Tryptophan at 200 rpm. pH values (4, 5, 6, 7, 8), Trp contents (50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L), culture periods (12, 24, 48, 72, 96 hours), and temperatures (30°C, 35°C, 40°C, 45°C, 50°C) were modified to identify an appropriate culture medium for the growth of endogenous bacteria. The culture liquid was centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant was collected, and the IAA concentration was determined using the Salkowski colorimetric technique at 530 nm.

2.4 Antagonistic Activity of Selected Endogenous Bacteria

The bacterial strains *Xanthomonas* and *Xanthomonas axonopodis* were obtained from the Faculty of Biotechnology, Vietnam National University of Agriculture. The agar-well diffusion technique, as described by Hadacek et al. [7], was used to examine the antibacterial ability. A petri dish with concentrated LB medium was prepared by spreading 100 µl of bacterial broth over the surface. Wells were created in the agar using a sterile pipette tip, spaced 2-3 cm apart. Five strains of bacteria were cultivated in liquid NA medium, and after 2 days, the solution was collected and cold-centrifuged at 4°C. Then, 50 µl of centrifuged bacteria was added to the wells, and the plates were kept at room temperature for 2 hours to allow the well extracts to diffuse into the bacterial growth medium. The plates were incubated at 37°C for 24 hours, and the zone of inhibition surrounding each well was measured to determine the antibacterial activity.

2.5 DNA Isolation and Sequencing

A sample (100 mg) was ground in 1 ml of extraction buffer and transferred to a 2 ml tube. The sample was incubated at 65°C for 10–60 minutes with stirring every 1-2 minutes. A mixture of Phenol:chloroform:isoamyl alcohol (25:24:1) in a 1:1 ratio was added to the suspension, mixed well, and centrifuged at 10,000 rpm for 8 minutes. Then, a mixture of Chloroform:isoamyl alcohol (24:1) in a 1:1 ratio was added, vortexed, and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new tube (1.5 ml), and ethanol was added in a 2:1 ratio (ethanol:supernatant) followed by gentle mixing. After

7 minutes of centrifugation at 10,000 rpm, the supernatant was removed, and the DNA precipitate was washed with 500µl of 70% ethanol solution. The precipitate was then centrifuged at 5400 rpm for 5 minutes at 4°C, the supernatant was removed, and the DNA was air-dried. The precipitate was stored at -20°C in 50-100 µl of TE buffer.

The 16S rRNA gene region was amplified using primers 27F and 1495R with the following sequences: 27F: 5' GAG AGT TTG ATC CTG GCT CAG 3'; 1495R: 5' CTA CGG CTA CCT TGT TAC GA 3' (Weisberg et al., 1991). The PCR reaction mixture contained 30ng of total DNA, 5µl of 1X buffer (Tris 10mM, KCl 50mM, pH 9.0, 0.1% Triton X-100), 2mM MgCl2, 20ng of each dNTP, 0.2 µM of 27F forward primer, 0.2µM of 1495R reverse primer, and 0.5 units of Taq DNA polymerase. The PCR reaction was performed with a heat cycle of 3 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 1 minute, primer binding at 55°C for 1 minute, and DNA synthesis at 72°C for 2 minutes. Finally, the reaction was held at 72°C for 10 minutes. The PCR products were visualized using a Biorad UV 2000 gel imager on a 1.5% agarose gel in TBE1X buffer at 100V for 90 minutes. The obtained sequences were compared to the NCBI gene bank (www.ncbi.nlm.nih.gov), and the evolutionary tree was inferred using the MEGA11 software [8] with the Saitou and Nei [9] method.

3 Results and Discussions

3.1 Isolation of bacterial endophytes

Due to the potential utilization of endophytic bacteria in various applications, including biofertilizers and bioremediation, their discovery is crucial. In this study, 32 strains of endogenous bacteria were isolated from elephant grass samples. Among them, 16 strains were obtained from the roots (Hai Duong: 10 strains; Cao Bang: 4 strains; Thai Nguyen: 2 strains), 10 strains were extracted from the stem (Hai Duong: 5 strains; Cao Bang: 2 strains; Thai Nguyen: 3 strains), and 6 strains were recovered from the leaves (Hai Duong: 3 strains; Cao Bang: 2 strains; Thai Nguyen: 1 strain). The majority of the colonies were bacilli, gram-negative, milky white, or light yellow in color, with a smooth and spherical surface. Hiep and Diep [6] observed similarities between the morphology of endophytic bacteria on chrysanthemum plants and the morphology of endogenous bacteria recovered in this study.

3.2 Selection and screening of endogenous bacteria capable of producing IAA

Endogenous bacterial strains were cultured on liquid NA media with an additional 100 mg/l of L-tryptophan. They were shaken at 200 rpm at 30 degrees for 72 hours in the dark. The bacterial culture was then collected at 4°C and centrifuged for 10 minutes at 10,000 rpm. After centrifugation, the clear solution was collected and 1 ml was added to a test tube. To this, 2 ml of Salkowski's reagent was added. The test tubes were kept in the dark at room temperature for 20 minutes. The quantity of IAA generated was measured using the colorimetric approach at 530 nm. Each bacterial strain produced a varying amount of synthesized IAA under the same growing conditions. Among the 32 strains examined, 5 strains (TNT3, HDR5, HDR9, CBR1, CBR2) produced IAA (>50 µg/ml) at concentrations of 67.13 µg/ml, 63.70 µg/ml, 53.25 µg/ml, 50.85 µg/ml, and 56.27 µg/ml, respectively. 17 bacterial strains produced IAA concentrations less than 20 µg/ml, while 10 bacterial strains produced IAA concentrations above 20µg/ml.

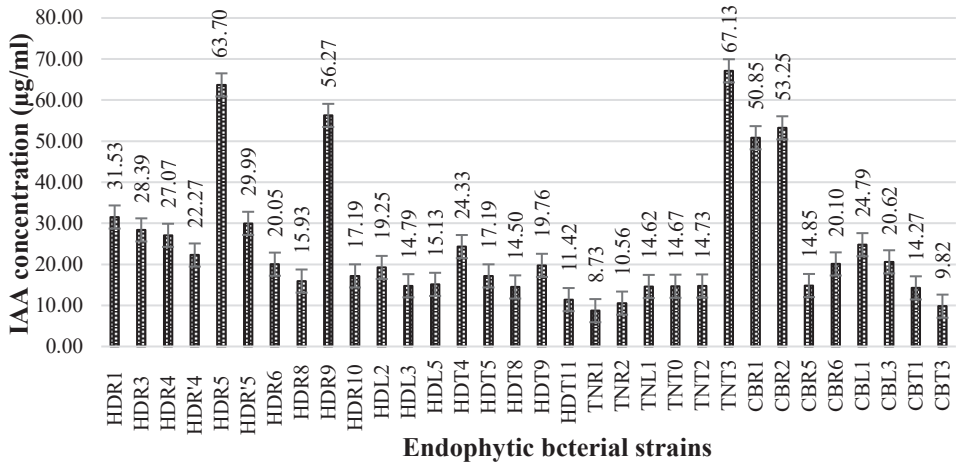


Fig. 1. Endogenous bacteria capable of producing IAA

Hieu and Hiep [10] assessed the quantity of IAA generated by endogenous bacteria isolated from virgin trees, and the two endogenous bacterial strains with the highest ability to produce IAA were RH5 (51.8 µg/ml) and LH6 (51.2 µg/ml). When compared to the five selected indigenous bacterial strains, strain CBR1 produced less IAA than strains TNT3, HDR5, HDR9, and CBR2. The experimental results revealed that geographical location has a significant impact on the ability of endophytic bacteria to thrive and produce growth stimulants [11].

3.3 Effect of pH on the ability to produce IAA of selected strains

The pH of plant tissue varies from species to species and part to part. Therefore, determining the optimal pH range for endogenous bacterial strains that can survive and produce maximal IAA is critical.

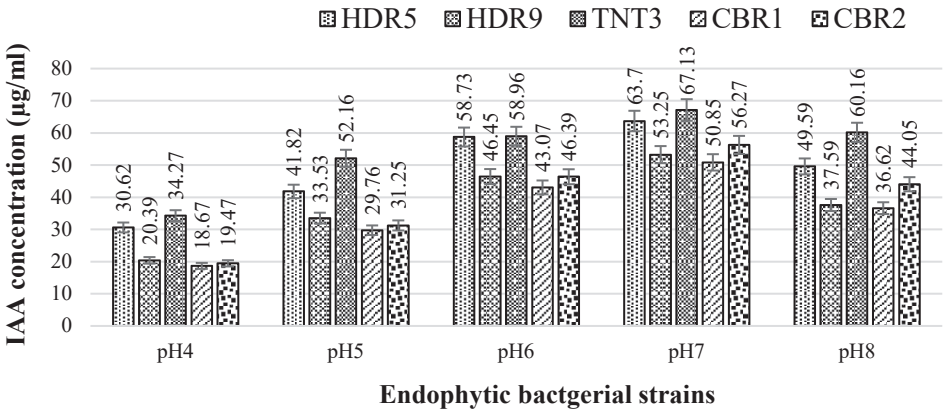


Fig. 2. Effect of pH on the ability to produce IAA

pH also influences membrane permeability and metabolic activity in the cell, affecting bacterial growth, development, and their capacity to produce IAA [12]. Thus, the pH value is a crucial environmental factor to consider when evaluating the potential for IAA production. Five bacterial strains (TNT3, HDR5, HDR9, CBR1, CBR2) were cultivated in liquid NA media supplemented with 100 mg/l L-tryptophan [13] at varied pH levels: 4, 5, 6, 7, 8, at 30°C in the dark, shaking at 200 rpm. The amount of IAA generated in the culture solution was measured using the Salkowski colorimetric technique at 530 nm after 72 hours (Fig. 2). At pH 7.0, all five bacterial strains generated the highest amount of IAA. The IAA content produced by different strains were: HDR5 (63.70 µg/ml), HDR9 (53.25 µg/ml), TNT3 (67.13 µg/ml), CBR1 (50.85 µg/ml), and CBR2 (56.27 µg/ml). At pH 4.0, the bacterial strains grew slowly and produced the least amount of IAA. The concentration of produced IAA increased gradually as the pH of the culture medium increased, with the highest value reported at pH 7.0. However, after peaking at pH 7.0, the concentration of IAA produced by the bacterial strains rapidly declined. According to Lebrazi et al. [15], the maximal IAA production by Rhizobium sp. at pH 6.5 is 135µg/ml. When studying endogenous bacteria from Brachiaria grass, Mutai et al. [14] also concluded that endophytic bacteria have a high ability to produce IAA at neutral pH.

3.4 Effect of temperature on the ability to produce IAA

Endophytic bacteria thrive in the environment provided by plant tissue. Continuous temperature fluctuations might create stress and modify IAA production [16, 17].

Therefore, examining the effect of temperature is critical to determine the appropriate temperature threshold for endophytic bacteria producing IAA. Selected bacterial strains were cultured in liquid NA media supplemented with 100 mg/l L-tryptophan at 30°C, 35°C, 40°C, 45°C, and 50°C in the dark, while shaking at 200 rpm. The amount of IAA generated in the culture solution was measured using a colorimetric technique after 72 hours (Fig. 3).

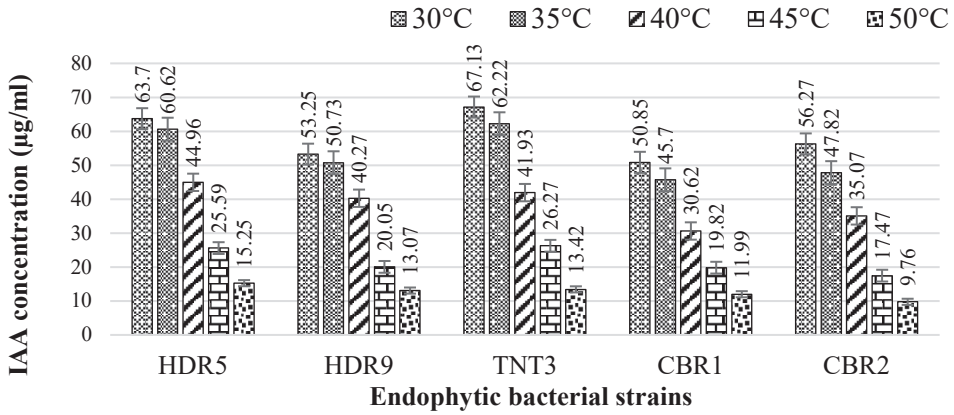


Fig. 3. Effect of temperature on the ability to produce IAA

At 30°C, strain TNT3 produced the highest amount of IAA, with a concentration of 67.13µg/ml. At 30°C, the IAA content produced by strains HDR5, HDR9, CBR1, and CBR2 was 63.70µg/ml, 53.25µg/ml, 50.85µg/ml, and 56.27µg/ml, respectively. When the experimental strains of bacteria were grown at temperatures ranging from 35 to 50°C, the level of IAA generated by the five endogenous bacterial strains was drastically reduced, reaching only 9.76 to 15.25µg/ml at 50°C. Most IAA-producing bacteria inhabit mesophilic conditions and cannot produce enzymes that are active in high-temperature environments [18]. According to Patil et al. [19], the optimal temperature for Acetobacter diazotrophicus L1 to biosynthesize IAA was 30°C. Apine and Jadhav [13] concluded that strain Pantoea agglomerans PVM produced the highest IAA biosynthesis at pH 7.0, temperature 30°C, and a supplemented tryptophan concentration of 0.1%.

3.5 Effect of Trp concentration on the ability to produce IAA

Tryptophan (Trp) is an important amino acid required for the biosynthesis of the plant hormone auxin, specifically in the Indole-3-Acetic Acid (IAA) pathway. Trp acts as both a precursor and a regulator of IAA production [20, 21]. This study aims to investigate the effect of Trp concentration on the IAA production ability of endogenous bacterial strains TNT3, HDR5, HDR9, CBR1, and CBR2. These strains were cultured in liquid NA media supplemented with L-tryptophan at concentrations of 50 mg/l, 100 mg/l, 150 mg/l, and 200 mg/l. The cultures were incubated in the dark at 30°C, pH 7.0, and shaken at 200 rpm.

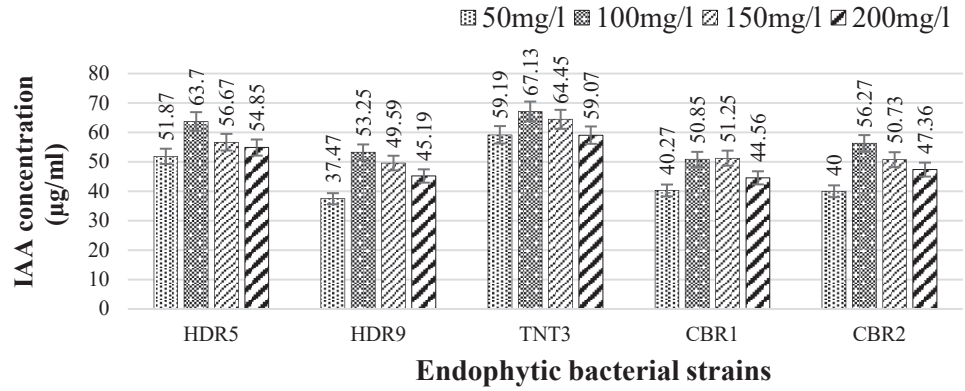


Fig. 4. Effect of L-Tryptophan on the ability to produce IAA

When L-Trp was added to the medium, the ability of the strains to produce IAA increased, reaching its maximum value at a Trp concentration of 100 mg/l (Fig. 3). However, when the Trp concentration was further increased to 150 mg/l, IAA biosynthesis not only failed to increase but actually decreased. This could be due to excessive Trp inhibiting IAA synthesis during bacterial growth. Trp, as an inducible precursor, can inhibit the IAA biosynthetic pathway and bacterial growth, leading to a significant decrease in IAA production [22]. Therefore, a Trp concentration of 100 mg/l is most suitable for IAA biosynthesis. However, the amount of exogenous Trp required for endogenous Trp conversion varies among bacterial strains, resulting in fluctuations in IAA biosynthesis [23]. In a study by Bhutani et al. [24], the highest IAA production was observed in MBN3 at an L-Trp concentration of 500µg/ml in 72 hours, followed by MJHN1 at 500µg/ml in 24 hours and MJHN10 at 300µg/min in 72 hours.

3.6 Effect of incubation time on the ability to produce IAA

Incubation time refers to the duration required for bacterial growth, which varies depending on the bacterial species and environmental conditions. The incubation period significantly influences bacterial growth rate and the production of secondary metabolites [16]. In this experiment, the five bacterial strains were cultured on NA media supplemented with 100 mg/l L-tryptophan at 30°C, pH 7.0, and 200 rpm shaking. The IAA production was examined after 24, 48, 72, 96, and 120 hours of culture. Figure 5 illustrates the differences in total IAA production.

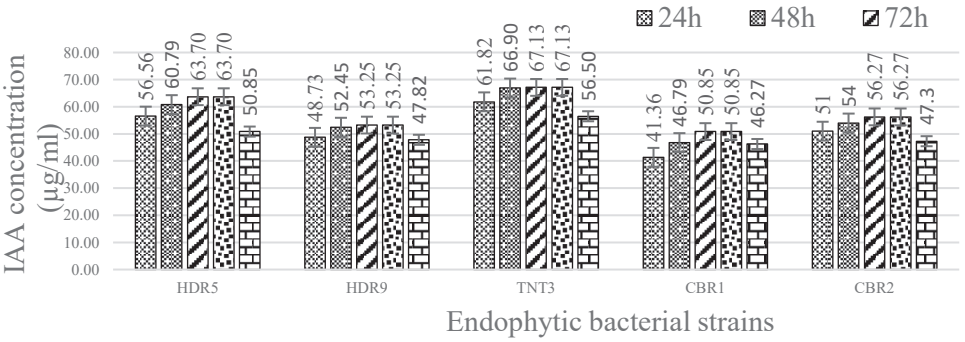


Fig. 5. Effect of incubation time on the ability to produce IAA

After 72 hours of incubation, all experimental bacterial strains reached their maximum IAA production. The concentration of IAA produced by strain TNT3 was 67.13µg/ml after 72 hours of culture. The other strains exhibited the following IAA contents: HDR5 63.70µg/ml, HDR9 53.25µg/ml, CBR1 50.85µg/ml, and CBR2 56.27µg/ml. While the bacterial strains continued to produce IAA beyond 72 hours, the content steadily decreased. This suggests that endogenous bacterial strains possess iac or iaa gene clusters, enabling them to utilize environmental IAA as a carbon source for cellular metabolism [25, 26]. Similar results were reported by Nalini & Rao [27] in their study on endophytic bacteria isolated from legumes. They found that the cb4 strain exhibited maximum IAA production of 55.8µg/ml at 72 hours, which declined after 96 hours of culture. However, previous research has shown that Bacillus siamensis exhibited peak IAA production after 96 hours of incubation [28].

3.7 Antagonistic Activity of the selected endogenous bacteria

The resistance capabilities of pathogenic bacterial strains were assessed using the approach described by Hadacek et al. [7]. Microorganism strains that can inhibit harmful bacteria form a halo around the agar well. Among the five selected endogenous bacterial strains, two exhibited antagonistic activity against harmful microorganisms. In this study, strain HDR5 demonstrated antagonistic activity against *Xanthomonas* sp. and *Xanthomonas axonopodis*. The antagonistic halo formed by HDR5 against *Xanthomonas axonopodis* measured 1.5 cm, while the halo diameter against Xanthomonas sp. was 1.8 cm. CBR2 exhibited a halo diameter of 0.7 cm (Fig. 6). These findings suggest the potential use of endogenous bacterial strains as biofertilizers to combat harmful bacteria in plants. Bacterial leaf blight, caused by the bacterium Xanthomonas, is a significant rice pest that reduces yield and product quality in rice-growing areas. Sessitsch et al. [28] evaluated the antagonistic capabilities of pathogenic fungi and bacteria against endophytic microorganisms obtained from field-grown potato plants. The results revealed that a significant percentage of endogenous bacterial strains had the ability to inhibit *Xanthomonas* sp.

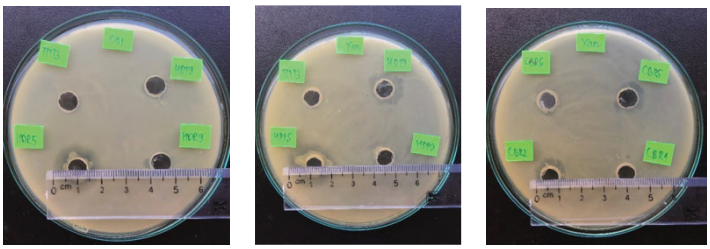
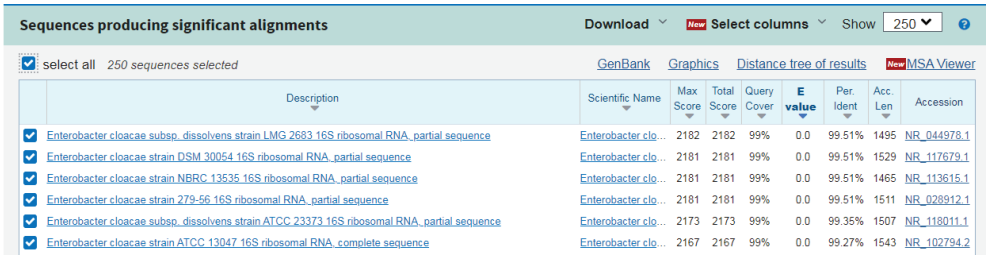


Fig. 6. Antagonistic activity of endogenous bacteria

3.8 DNA Isolation and Sequencing

Among the five endogenous bacterial strains studied, strain HDR5 showed the highest IAA production and resistance to pathogenic pathogens. The PCR results using primers 27F and 1495R were satisfactory, with all samples producing bands of approximately 1269 bp in size. The 16S rRNA nucleotide sequencing was performed on strain HDR5, and the sequence was identified using the BLAST program (Fig. 7).



Sequences producing significant alignments — Download — Select columns — Show 250

select all 250 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter cloacae subsp. dissolvens strain LMG 2683 16S ribosomal RNA, partial sequence	Enterobacter clo...	2182	2182	99%	0.0	99.51%	1495	NR_044978.1
Enterobacter cloacae strain DSM 30054 16S ribosomal RNA, partial sequence	Enterobacter clo...	2181	2181	99%	0.0	99.51%	1529	NR_117679.1
Enterobacter cloacae strain NBRC 13535 16S ribosomal RNA, partial sequence	Enterobacter clo...	2181	2181	99%	0.0	99.51%	1465	NR_113615.1
Enterobacter cloacae strain 279-56 16S ribosomal RNA, partial sequence	Enterobacter clo...	2181	2181	99%	0.0	99.51%	1511	NR_028912.1
Enterobacter cloacae subsp. dissolvens strain ATCC 23373 16S ribosomal RNA, partial sequence	Enterobacter clo...	2173	2173	99%	0.0	99.35%	1507	NR_118011.1
Enterobacter cloacae strain ATCC 13047 16S ribosomal RNA, complete sequence	Enterobacter clo...	2167	2167	99%	0.0	99.27%	1543	NR_102794.2

Fig. 7. Comparison of 16S rRNA nucleotide sequencing of HDR5 strain using the BLAST program

The results of comparing the 16S rRNA nucleotide sequencing of HDR5 strain using the BLAST program revealed that the HDR5 strain is closely related to strains of the genus Enterobacter, with 99% similarity (Fig. 7).

4 Conclusion

From samples of elephant grass collected from Hai Duong province, Trung Khanh district, Cao Bang province, and Pho Yen town, Thai Nguyen province, we isolated five endophytic bacterial strains with the highest potential for IAA production. These strains exhibited the highest IAA concentrations when incubated under optimal conditions, including a pH of 7, 100 mg/l L-tryptophan, and 30°C on liquid NA media. Additionally, all strains showed antagonistic activity against Xanthomonas sp. The comparison of 16S rRNA nucleotide sequencing of strain HDR5 using the BLAST program indicated its affiliation with the genus Enterobacter. After conducting safety evaluations, these endophytic bacterial strains can be suitably used for bio-preparation production.

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