ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA FROM *PANAX VIETNAMENSIS* HA ET GRUSHV.

Ho Le Han*

The University of Danang - University of Science and Technology, Vietnam

*Corresponding author: hlhan@dut.udn.vn

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Abstract - This study aimed to isolate and characterize endophytic bacteria from *Panax vietnamensis* Ha et Grushv., a rare medicinal ginseng species endemic to Vietnam. Several bacterial strains were isolated from root, stem, and leaf tissues, among which strain HS10, identified as *Burkholderia* sp., exhibited notable plant growth-promoting traits including indole-3-acetic acid (IAA) production, proteinase activity, and strong growth. Phylogenetic analysis based on 16S rRNA gene sequences confirmed the taxonomic placement of HS10 within the *Burkholderia* genus. IAA production was evaluated over a 5-day period with varying concentrations of L-tryptophan, revealing optimal production (46.61±5 µg/mL) on the fourth day at 0.1–0.15% tryptophan. Additionally, antibiotic susceptibility testing showed that HS10 was highly sensitive to cefadroxil and tetracycline, while resistant to cefpodoxime. These findings contribute to the understanding of the microbial diversity associated with *P. vietnamensis* and support the potential application of endophytic *Burkholderia* strains as biofertilizers to enhance sustainable ginseng cultivation medium.

Key words - *Panax vietnamensis* Ha et Grushv.; *Burkholderia*; IAA production; phylogenetic analysis

1. Introduction

Endophytic bacteria are microorganisms that live within plant tissues without causing harm. These bacteria form symbiotic relationships with their host plants, enhancing growth, nutrient uptake, and stress tolerance. Found in roots, stems, leaves, and seeds, endophytes contribute to sustainable agriculture by fixing nitrogen, producing phytohormones, and solubilizing essential minerals. They also help plants resist drought, salinity, and pathogens by producing bioactive compounds and modulating defense mechanisms. Due to these benefits, endophytes are increasingly used in developing eco-friendly biofertilizers and biopesticides, reducing reliance on chemical inputs. Moreover, some endophytic bacteria have potential in bioremediation and pharmaceutical applications, as they produce antimicrobial and anticancer compounds, making them promising tools in both agriculture and medicine [1, 2].

Panax vietnamensis Ha et Grushv., or Vietnamese ginseng, is a rare medicinal plant endemic to Vietnam. It contains a unique profile of saponins, including ginsenosides and majonosides, particularly majonoside-R2, which exhibit strong adaptogenic, antioxidant, and neuroprotective effects. Compared to Korean and American ginseng, *P. vietnamensis Ha et Grushv.* has distinctive pharmacological properties and has long been used in traditional medicine to boost immunity, reduce fatigue, and improve overall vitality [3]. However, due to overharvesting and habitat loss, its conservation has become a concern. Recent research focuses on its cultivation and the biotechnological potential of its bioactive compounds. As demand for natural health products grows, *P. vietnamensis* Ha et Grushv. continues to attract attention for its therapeutic potential and importance in preserving plant-based medicinal resources [4].

Endophytic microbiology play an important role for the host plants, however, until now, there have not been many studies about the relationship between endophytic bacteria and *Panax vietnamensis* Ha et Grushv [5]. A study about the endophytic bacteria in *Panax vietnamensis* Ha et Grushv was conducted, and Nguyen *et al* isolated 45 strains from rhizobium, petioles and leaves [5]. Nguyen *et al* chose 27 isolates which were positive with β-glucosidase assay for further researches on biotransformating of ginsenoside compounds. Recently, a study has been conducted to find out about the effect of endophytes and the growth of Ngoc Linh gingseng [6]. The authors isolated a novel bacterium *Pseudomonas* sp HS6-2 producing plants' hormone auxin that could boost the growth of *Panax vietnamensis* Ha et Grushv.

This study investigated the microbiome of *P. vietnamensis* Ha et Grushv. and isolated endophytic bacteria. Some endophytic bacteria with special characteristics, such as natural polymer degradation and auxin production, would be studied further. Among these endophytic bacteria, an endophytic bacterium HS-10, which promoted the plant's growth, was isolated from the roots of *P. vietnamensis* Ha et Grushv. In this study, an isolated strain HS-10, belonged to the genus *Burkholderia*, was determined the ability for producing indole-3-acetic acid (IAA) and antibiotics susceptibility, aiming to biofertilizer for Ngoc Linh gingseng.

2. Materials and methods

2.1. Samples collection and culture

2.1.1. Samples collection and endophytic bacteria isolation

Panax vietnamensis Ha et Grushv. samples, 1-year-old gingseng plants (Figure 1), were collected from two provinces of Vietnam, including Quang Nam (15°03'29.9"N 107°59'40.3"E) and Kontum (14°58'26.3"N 107°55'12.3"E) stored at 4° C during transportation[6]. To obtain endophytic microbes, the samples were sterilized using the method with a slight

modification. The samples were washed carefully under tap water to remove surface dirt and other particles. Each plant was separated into three tissues (root, stem, leaf). Surface sterilization of the tissue samples was done stepwise by immersing in 70% ethanol for 30 s, 2% sodium hypochlorite (NaOCl) for 10 min, $Na_2S_2O_3$ 2% for 1 min, and 70% ethanol for 30 s, followed by three rinses in sterile distilled water to remove the surface sterilization agent. After surface sterilization, the samples were kept between 2 layers of the sterilized tissue papers for proper drying. All the procedures were done in the clean bench. After the final washing, the water was streaked on to tryptic soy agar (TSA) plates for checking the sterilization of surface of samples [7].

After sterilization, these samples were crushed and homogenized with phosphate base solution. Then they were diluted serially and streaked on to starch media (yeast extract 0.05%, peptone 0.05%, glucose 0.05%, soluble starch 0.05%, agar 2%, pH 7), skim-milk media (yeast extract 0.05%, peptone 0.05%, glucose 0.05%, skim-milk 0.1%, agar 2%, pH 7), and lipid-degradation media (yeast extract 0.05%, peptone 0.05%, glucose 0.05%, tween 80 0.1%, agar 2%, pH 7), TSA, Nutrient agar (NA) and Luria Bertani (LB).

Figure 1. *Panax vietnamensis Ha et Grushv*

2.1.2. Bacterial isolation and culture

These primary plates were incubated at 25°C for one week and then observed regularly. The colonies from these plates were picked up and transferred onto the TSA. The isolates were preserved in 20% glycerol at -80°C

2.2. Phylogenetic analysis

The genomics DNA of isolated strains from *Panax vietnamensis* Ha et Grushv. were extracted from cells grown in agar by a NucleoSpin Microbial DNA kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The 16S rRNA genes were amplified with bacterial bacterial universal primers and sequenced with Sanger's sequencing method at DNA sequencing company limited (viet nam). Pairwise sequence similarity values of the 16S rRNA gene sequence were obtained through the EzBioCloud server (http://www.ezbiocloud.net). All sequences of the related strains retrieved from EzBioCloud were aligned and edited with the BioEdit software (version 7.2.5). Phylogenetic trees were constructed using the neighbor-joining (NJ) methods using the program Molecular Evolutionary Genetics Analysis (MEGA 7.0). Tree topologies were evaluated by a bootstrap analysis after 1000 replications[8].

2.3. Growth curve determination

With the aim of preliminarily assessing the bioactivity of the isolated bacteria, their growth curve needs to be established. Sterilized liquid TSB medium is prepared in 100 mL Erlenmeyer flasks. The isolated strain was initially cultivated to reach an optical density at 600 nm (OD600) of 1.03 ± 0.04, then inoculated into the prepared TSB medium at a concentration of 0.25% (v/v). The growth and development of HS-10 are monitored over time by measuring OD600 every hour. The collected data are used to plot the growth curve. Each experiment is repeated three times [6].

2.4. Detection of plant growth-promoting characteristics in vitro

2.4.1. Indole acetic acid (IAA) detection

IAA, one of the most physiologically active auxins, is a common product of L- tryptophan metabolism produced by several microorganisms including Plant Growth Promoting Rhizobacteria (PGPR). Each of isolated strain was cultivated in TSB with supplementary compound L-tryptophan. After 24h cultivation, the broth culture was centrifuged, and the supernatant was transferred to Eppendorf tubes. Then, the solution of Salkowsky's reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$ solution) was added and mixed by pipetting. The tubes were kept in the dark for 30 min before measuring the optical density [9].

2.4.2. Proteinase detection

Single colonies of endophytic bacteria were spot inoculated on a skim-milk media, and then the zones of proteolysis around the colonies were observed [10].

2.5. Measurement of IAA production

The ability of the isolated strains to biosynthesize IAA was analyzed using the colorimetric Salkowsky method. Each bacterial strain was cultured in TSA medium as previously described. L-tryptophan was added as an IAA precursor. Test tubes (15 mL) containing 5 mL of modified TSA medium were prepared, supplemented with L-tryptophan (T) (0.1% v/v) (Merck, purity ≥98%), with one set of control tubes lacking tryptophan supplementation. A single bacterial colony from a TSA Petri dish was inoculated into each tube in triplicate (n = 3), and three additional tubes were used as the control medium. The tubes were incubated at 30 ± 2°C for 96 hours.

After incubation, the cultures were centrifuged at 4000 rpm for 10 minutes to separate the cells from the supernatant. The supernatant was then mixed with Salkowski reagent at a ratio of 1:2 and incubated in the dark for 30 minutes. The appearance of a pink-orange color indicated IAA production, while an orange color suggested the presence of other indole compounds. The absorbance was measured at 530 nm using a UV-Vis spectrophotometer (LABMED, USA). For quantification, a calibration curve was constructed using a standard IAA solution with the following equation:

$$y = 0.01x + 0.01, R^2 = 0.997,$$

where, x is the IAA concentration in µg/mL and y is the absorbance at 530 nm [6].

2.6. Antibiotic Sensitivity Assay of the Isolated strain

The assay for antibiotic sensitivity was conducted similarly to the antibacterial activity test, except that the isolated bacteria were spread directly onto TSA plates. The antibiotics used in this study included cefadroxil, tetracycline, ampicillin, amoxicillin, cefpodoxime, and cefdinir, each prepared at concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, and 1000 µg/mL. The antibiotics were added into wells on the agar plates with a volume of 80 µL per well.

3. Results and discussion

3.1. Isolation and identification

The TSA plates for checking the sterilization had not any colony after incubation, therefore, the sterilization of surface of samples killed all of the microbiology.

After incubation, some endophytic bacteria were isolated and purified. Some isolates have special characteristics such as protein degradation, lipid degradation, starch degradation, and auxin production which were purified and analyzed further (Figure 2).

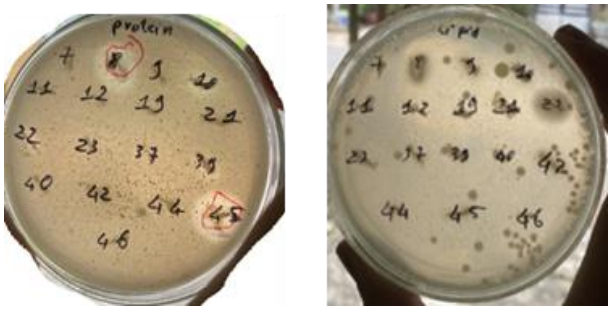

Figure 2. *The ability of protein and lipid degradation of isolated strain. The number 8, 10, and 45 related to PN8, HS10 and PN45 respectively*

3.2. 16S rRNA gene analyses

The analysis of 16S rRNA gene sequences from the EzBioCloud server revealed that the phylogenetic tree of these isolates (Figure 3). These isolates including HS10 belongs to the genus *Burkholderia*, HS6-2 and PN45 belong to the genus *Pseudomonas*, PN8 and HS5 belong to the genus *Stenotrophomonas* and *Bacillus* respectively (Figure 3a). In the phylogenetic trees (Figure 3), all reference strains are type strains with 16S rRNA accession number in GenBank. Among them, the strain HS10 showed some special characteristics such as protein, lipid degradation and auxin production. The phylogenetic trees based on 16S rRNA gene sequences showed monophyletic clustering of strain HS10 with reference strains. *Pontimicrobium aquaticum* CAU 1491^T was used as an outgroup for studying the phylogenetic analysis of the strain. The outgroup plays a crucial role in rooting the phylogenetic tree, which is inferred the direction of evolutionary change [11].

The 16S rRNA analysis confirmed the taxonomic placement of HS10 within the *Burkholderia* genus. *Burkholderia* species plays a beneficial role in plants by promoting growth through nitrogen fixation, phosphate solubilization, and IAA production. The strains also exhibit biocontrol activity and degrade soil pollutants, supporting plant health. These traits make *Burkholderia* valuable for sustainable agriculture and microbial biofertilizer development [12].

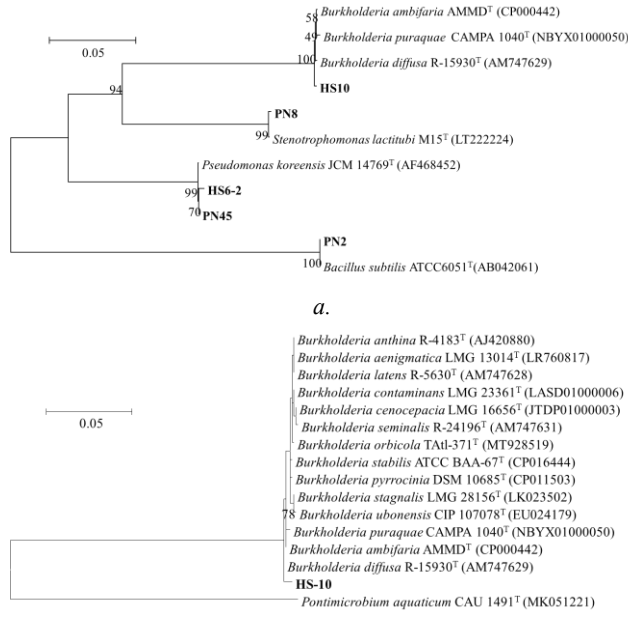

Figure 3. *Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship of isolates and closely related strains*

a. Five special isolates, showing natural polymer degradation, identified 16S rRNA. b. The novel strain Burkholderia HS-10 and closely relative species in the genus Burkholderia and Pontimicrobium aquaticum CAU 1491^T was used as an outgroup. Bar 0.05 substitutions per nucleotide position.

3.3. Growth curve determination

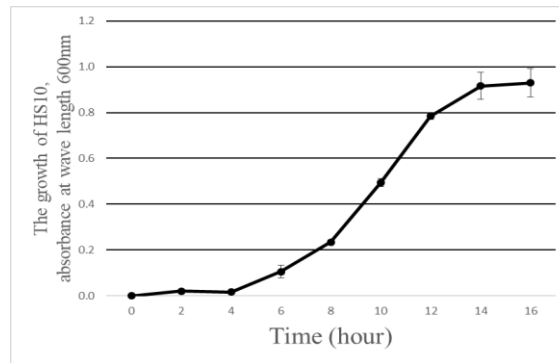

Figure 4. *Growth curve of HS10 in TSB*

The growth curve of HS10 was investigated, and the result was shown in Figure 4. It can be observed that the endophytic bacterial strain isolated from the *Panax vietnamensis* Ha et Grushv. exhibited strong growth, reaching the exponential phase after 6 hours. Strain HS10 demonstrated rapid growth, surpassing the lag phase after 4 hours and quickly reaching the stationary phase after 14 hours of cultivation in TSB medium. The growth curve will help to identify the stationary phase of the isolated strain and the secondary metabolites are produced in this phase

as a survival mechanism in response to nutrient depletion, or other stresses. Many industrially important compounds such as antibiotics, alkaloids, and flavonoids are produced during this stage.

3.4. Detection of plant growth-promoting characteristics in vitro

3.4.1. Indole acetic acid (IAA) detection

These isolated strains were determined IAA production, only HS6-2 and HS10 showed the strong ability for IAA production, the other strains did not have any ability for IAA production. The incubated broth culture changed to red color after cultivating these strains in TSB for 24h.

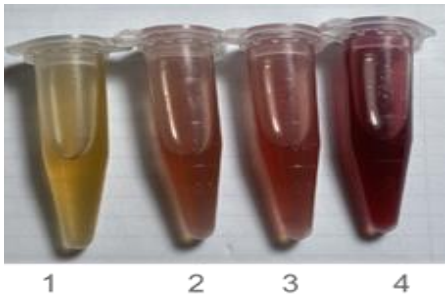

Figure 5. *IAA detection in endophytic bacteria*

1. Negative control, 2. HS10, 3. HS6-2, 4. Positive control

3.4.2. Proteinase detection

Some isolates showed the halo zones on the skim-milk agar, which revealed the protein degradation. These isolates PN8, PN45 and HS10 demonstrate the proteinase production. Among them, PN45 has the largest halo zones (Figure 2).

3.5. Measurement of IAA production

The influence of cultivation duration and tryptophan supplementation on IAA (Indole-3-acetic acid) production was examined using tryptic soy broth (TSB) medium supplemented with different concentrations of tryptophan (0%, 0.05%, 0.1%, and 0.15%). IAA concentrations were measured over a 5-day cultivation period (Figure 6).

It is clearly from the Fig. 6 IAA production increased significantly with both extended cultivation time and higher tryptophan concentrations. On the first day, IAA levels remained low across all treatments (from 5–10 µg/mL), indicating limited metabolic activity during early growth. The results were consistent with the growth curve of HS10 (Figure 4). Then, a sharp increase in IAA production was observed on the second day, especially in the presence of 0.05% and 0.1% tryptophan, with the highest concentration (43 µg/mL) recorded in the 0.05% treatment group.

Interestingly, IAA levels appeared to plateau or slightly decline on the third day, possibly due to nutrient depletion or metabolic feedback inhibition. However, a second peak in IAA biosynthesis was recorded on the fourth day, where the cultures supplemented with 0.1% and 0.15% tryptophan yielded the highest IAA concentrations (45–50 µg/mL). On the fifth day, IAA levels stabilized or slightly decreased, suggesting that prolonged cultivation beyond four days does not further enhance IAA production.

These results demonstrate that both tryptophan concentration and incubation period play crucial roles in optimizing IAA synthesis. The optimal condition for maximum IAA production was observed at 0.1–0.15% tryptophan concentration after 4 days of cultivation. Furthermore, the findings are consistent with previous reports on IAA production by plant PGPR [13, 14]. Li *et al*. isolated phosphate-solubilizing bacteria (PSBs) from the peach rhizosphere and assessed their IAA production using a single tryptophan concentration (0.5 g/L or 0.05%). Among the tested strains, WPD85 produced up to 45 µg/mL IAA after 5 days of incubation, a value comparable to that achieved by HS10 in this study. However, the lack of a time-course or dose-response analysis in Li *et al*.'s work limits direct optimization. In contrast, the results clearly identify on the fourth day and 0.1–0.15% tryptophan as optimal conditions for IAA biosynthesis, offering a practical framework for microbial inoculant formulation [13]. Similarly, Thakur *et al*. evaluated 42 PGPR strains isolated from tea rhizosphere soils and reported a broader IAA production range (2–85 µg/mL) [14].

The time- and dose-dependent approach employed in our study provides valuable insights for future applications. It also demonstrates that tryptophan serves as a limiting precursor up to 0.1%, beyond which IAA production does not significantly increase. Additionally, the decline in IAA levels after day 4 at higher tryptophan concentrations may suggest feedback inhibition, substrate exhaustion, or cellular stress responses, aligning with observations in other auxin-producing bacteria.

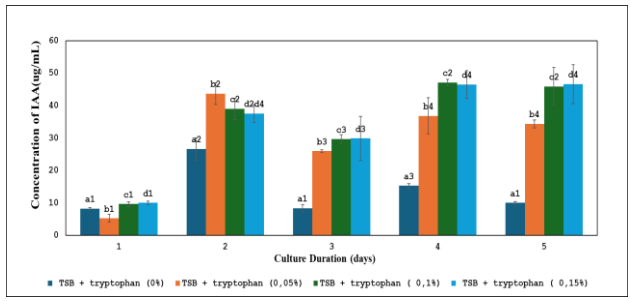

Figure 6. *Measurement of IAA production*

3.6. Antibiotic Sensitivity Assay of the Isolated Strain

In this study, the susceptibility to commonly used antibiotics was assessed. If the isolated strain HS10 is sensitive to common antibiotics, such as cefadroxil, tetracycline, ampicillin, amoxicillin, cefpodoxime, and cefdinir, this indicates low risk of antimicrobial resistance. The results were obtained across varying concentrations ranging from 25 µg/ml to 100 µg/ml of these antibiotics to evaluate the survival of the endophytic bacterial isolates HS10 in the presence of antibiotics, as shown in Table 1 and Figure 7. In the Figure 7, the concentration of antibiotics changed from 1 to 5, related to 1000 to 25(µg/ml). Table 1 presents the antibiotic susceptibility profile of strain HS10, as determined by the diameter of the inhibition zones (mm) formed on agar

plates at various antibiotic concentrations (25–1000 µg/mL). The results reveal that strain HS10 exhibits differential sensitivity to the tested antibiotics, with susceptibility increasing proportionally to the concentration in most cases.

Cefadroxil displayed the most effective inhibitory activity, producing inhibition zones as large as 36 ± 0.5 mm at 1000 µg/mL. Zone diameters increased steadily from 18 ± 0.5 mm at 25 µg/mL, indicating a dose-dependent response and consistent susceptibility. Tetracycline also showed strong efficacy, particularly at higher concentrations. Ampicillin exhibited moderate activity, with inhibition zones first appearing at 100 µg/mL (11 ± 0.5 mm) and increasing to 20 ± 0.5 mm at 1000 µg/mL. Similarly, amoxicillin showed no effect at lower concentrations, indicating delayed but measurable sensitivity.

Table 1. *The diameter (mm) of the inhibition zone of strain HS10*

antibiotics	Concentration of antibiotics (µg/ml)				
	25	50	100	250	1000
Cefadroxil	18 ± 0.5	21 ± 0.5	22 ± 1	29 ± 0.2	36± 0.5
Tetracycline	0	18 ± 1	21 ± 0.5	25 ± 1.5	35 ± 0.5
Ampicillin	0	0	0	0	20 ± 0.5
Amoxicillin	0	0	0	0	25±1
Cefpodoxim	0	0	0	0	0
Cefdinif	0	15±0.5	16 ± 0.5	18 ± 0.5	23 ± 1.2

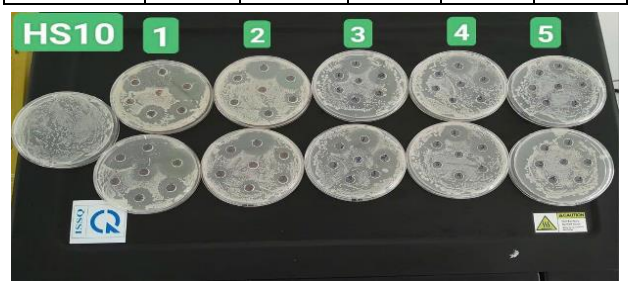

Figure 7. *The antibiotics sensitivity of HS10*
From 1 to 5: the concentration of antibiotics reduced from 1000 to 25 (µg/ml)

The antibiotics were chosen for sensitivity test in this study, represent a broad spectrum of β-lactam and tetracycline groups, commonly used in both clinical and agricultural settings [15]. The usage of antibiotics has several purposes: assessing biosafety for agricultural use and monitoring potential for antibiotic resistance transfer. As regards of the purpose: assessing biosafety for agricultural use. These antibiotics are among the most frequently detected in soils due to overuse in livestock and human medicine. The isolates HS10 is sensitive to some tested antibiotics such as cefadroxil and tetracycline, intermediate sensitivity to ampicillin, amoxicillin, and cefdinif, and resistance to cefpodoxim; the antibiotics assay helps determine whether the strain HS10 could survive in environments where these compounds are present. In addition, about the monitoring potential for antibiotic resistance transfer: Cefadroxil, cefpodoxime, and cefdinir are cephalosporins - broad-spectrum antibiotics often used in medicine. Ampicillin and amoxicillin are penicillin antibiotics, widely used in veterinary and crop protection. Tetracycline is still heavily applied in aquaculture, livestock, and agriculture [15, 16].

Overall, strain HS10 demonstrates high susceptibility to Cefadroxil and Tetracycline, intermediate sensitivity to Ampicillin, Amoxicillin, and Cefdinif, and resistance to Cefpodoxim. These results not only reflect the adaptive antibiotic response of HS10 but also highlight potential treatment options or selective agents in microbial formulation studies. Furthermore, the result of antibiotics assay demonstrated the strain HS10 is biosafety approval, especially for use in open environments like agricultural soil.

The biosafety concerns of *Burkholderia* sp HS10 are highly relevant due to the genus Burkholderia being known for some species that are opportunistic pathogens. Some strains such as *B.pseudomallei* and *B.mallei* are responsible for melioidosis and glanders, both of them are serious infections [15]. However, other *Burkholderia* species can provide valuable agricultural benefits, such as promoting plant growth through processes like nitrogen fixation and auxin production, the potential risks of pathogenicity in certain strains need careful consideration for biofertilizer applications [17]. Therefore, HS10 could be proceeded for whole-genome sequencing and it will be screened and detected for pathogenic and toxic genes before the isolates could be applied as biofertilizer. Specifically, *Burkholderia* sp. HS10 was isolated as an endophytic bacterium from *Panax vietnamensis* Ha et Grushv, and while it does demonstrate beneficial traits, including auxin production and degradation of organic compounds, the biosafety of this strain is a concern due to its affiliation with the *Burkholderia* genus, which includes both beneficial and harmful species [1].

4. In conclusion

The microbiome of *Panax vietnamensis* Ha et Grushv. was investigated and elucidated, and some endophytic bacteria were isolated and analyzed. Among them, an endophytic bacteria *Burkholderia* sp HS10 showed some special characteristics with IAA production, and protein degradation, isolated from *P.vietnamensis*. This study contributed to elucidate the microbiome of *Panax vietnamensis* Ha et Grushv. and it not only confirms the IAA-producing capacity of isolates HS6-2 and HS10 but also highlights the importance of optimizing culture conditions to maximize output. Compared to published literature, our results offer a more controlled and detailed optimization strategy, which is critical for the development of microbial bio-fertilizers aimed at enhancing plant growth through phytohormone modulation.

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