

**ISOLATION AND PROPERTIES OF ENDOPHYTIC BACTERIA
AND ACTINOMYCETES OF *Catharanthus roseus* (L) G. Don GROWN
IN NHA TRANG, VIETNAM**

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ABSTRACT

Endophytic microbes of medicinal plants are known to involve the synthesis of several bioactive compounds, which promote plant growth and resistance to diseases. In order to investigate endophytes in the important medicinal plant *Catharanthus roseus*, 16 endophytic microorganism strains were isolated from root samples of *C. roseus* var. *roseus* and *C. roseus* var. *ocellatus* naturally growing in coastal areas of Nha Trang, Vietnam. Based on morphological characteristics and 16S rDNA gene marker sequences, four actinomycetes of *Streptomyces* and *Microbiospora* genera and twelve bacterial strains belonging to *Bacillus*, *Enterobacter*, *Pseudomonas*, *Paneniabacillus* and *Rhizobium* genera were identified. Analysis of the extracellular enzyme activities of actinomycetes strains indicated that *Streptomyces* strains produced proteases, cellulases, xylanases and amylases and *Microbiospora* strains exhibited protease and cellulase activities. The results demonstrated the diversity of endophytes in the roots of *C. roseus* plants and their potential extracellular enzyme activities for further application.

Keywords: *Catharanthus roseus*, endophytic bacteria, actinomycetes, extracellular enzyme.

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INTRODUCTION

Endophytes are microorganisms that reside in healthy plant tissues for at least a part of their life without causing any disease symptoms in the host plants (Petrini, 1991). They are diverse and have been found in a large number of plant species. In recent decades, this group of microorganisms has been intensively studied because of their contribution in plant development. Numerous researches have indicated that endophytic microorganisms play important roles in disease resistance, growth regulation and stress tolerance of plants (Glick, 2014; Zhao et al., 2016). In addition, they are known as a rich source of bioactive metabolites (Tan & Zou, 2001; Joseph et al., 2011). There are up to 23,000 bioactive secondary metabolites isolated from endophytic microorganisms (Sharma et al., 2014).

Catharanthus roseus (L) G. Don belongs to the *Catharanthus* genus, Apocynaceae family and widely grows in several coastal areas from Quang Ninh to Kien Giang province, Vietnam. The plant species has been considered as an important bioactive alkaloids source with more than 130 terpenoid indole alkaloids (TIAs), including vinblastine and vincristine used as powerful antitumor drugs (Sottomayor & Ros Barceló, 2005; Moudi et al., 2013). Vinblastine and vincristine were also reported to be produced by endophytic microorganisms such as *Fusarium oxysporum*, *Talaromyces radicus* and *Eutypella* spp-CrP14 (Kumar et al., 2013; Palem et al., 2016; Kuriakose et al., 2016). Recently, the other *C. roseus* endophytic bacteria *Microbacterium* sp. was found to produce vindoline (a precursor of vinblastine and vincristine) (Anjum & Chandra, 2019). Additionally, endophytic microbes of *C. roseus* as well as other medicinal plants can synthesize various hydrolytic enzymes such as pectinases, xylanases, cellulases, and proteases in helping them to penetrate into the tissues of plants. According to Kafur & Khan (2011), 65% of endophytic actinobacteria isolated from *C. roseus* leaves had anti-microbial activity. Ethyl acetate extract of endophytic

actinomycetes living in roots of *C. roseus* exhibited strong antioxidant activity as well as α -glucosidase inhibition activity (Jasmine & Agastian, 2013).

Therefore, in the present study endophytic bacterial communities in roots of two *C. roseus* varieties naturally grown on the coastal areas of Nha Trang, Khanh Hoa was isolated and explored for possible bioactivities. The ability to produce valuable extracellular enzymes of isolated actinomycetes is also evaluated for further application.

MATERIALS AND METHODS

Isolation of endophytic bacteria and actinobacteria from root samples of *Catharanthus roseus*

C. roseus var. *roseus* (purple flower) and *C. roseus* var. *ocellatus* (white petals with red stamens) plants were collected from coastal areas of Nha Trang, Khanh Hoa province, Vietnam. Whole plants were transported to the laboratory (Department of Biological Resources, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology) for microorganism isolations within 48 hours. Endophytic bacteria strains were isolated using the method described by Shutsrirung et al. (2013) with minor modifications. Root samples were washed under running tap water to remove soil particles. They were cut into small pieces (~1 cm long) and then surface sterilized in 70% ethanol for 1 min, 0.1% Tween20 for 10 min and 1% NaOCl for 1 min. After washing twice with sterile water, the clean root pieces were crushed using an autoclaved pestle and mortar and then transferred in a flask containing 8 mL of sterilized distilled water. The flask was shaken at 120 rpm for 15 min. The solution (100 μ L) was spread on humic acid agar medium (pH 7.0) supplemented with 100 mg/l nystatin to inhibit fungal growth. The plates were incubated at 37 °C for 1–7 days. Endophytes were preliminary classified based on morphological characteristics; and then transferred to MPA medium (pH 7.0) for bacteria and Bennett medium (pH 7.0) for

actinobacteria (actinomycetes). All isolates were kept in 20% (v/v) glycerol and stored at -80 °C at the Department of Biological Resources, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology.

The endophytic isolated strains were primarily identified based on morphological characteristics (colony appearance and color) and growth rate on species-specific culture media. For actinomycetes strains, the lamellae specimens were prepared in the Department of Biological Resources, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology and spore morphology was observed under the scanning electron microscope (FESEM S4800, Hitachi, Japan) in the Laboratory of Magnetism and Superconductivity, Institute of Materials Science, Vietnam Academy of Science and Technology under the operating guideline of the manufacturer. Scanning electron microscope of spore chains and spores was performed at 3,000 X–5,000 X and 30,000 X–50,000 X magnification, respectively.

Total DNA extraction

Bacteria and actinomycetes strains were cultured in 50 mL liquid MPA and Bennett media, respectively, at 37 °C for 1–3 days. About 2 mL of culture broth was taken and centrifuged at 10,000 rpm for 5 min. The pellet was suspended in 0.5 mL of 5 M EDTA, pH 8.0. In the next step, 50 µL of 4 mg/mL lysozyme stock was added and incubated overnight at 37 °C. After that, 50 µL of 20% SDS and 50 µL of proteinase K (4 mg/mL) were added to the mixture and incubated at 55 °C for an hour. DNA was extracted by chloroform: isoamyl alcohol (24:1) solution. The supernatant layer was taken after centrifuging at 13,000 rpm for 15 min, 4 °C. This step was repeated 3 times. DNA was precipitated using twice the volume of isopropanol. The DNA pellet was washed with 70% ethanol, dried and dissolved in sterile deionized water (dH₂O, free-DNase and RNase). DNA quality was tested on 0.8% agarose gel electrophoresis.

Analysis of 16S rRNA gene marker sequences

The fragments of the 16S ribosome (rRNA) gene of all strains were amplified using primer pair 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAACC-3'). PCR mixture contained 10 µL of 2X Intron master mix, 1 µL of each primer 10 pmol/µL, 6 µL of dH₂O and 2 µL of DNA template. The reaction was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with the following cycle: initial denaturation at 94 °C for 5 min; 30 cycles at 94 °C for 1 min, 55 °C for 1 min 30s and 72 °C for 2 min and a final extension at 72 °C for 10 min. PCR products with the expected size of 1500 bp were checked by 0.8% agarose gel electrophoresis, purified using MEGA quick-spin™ Plus Fragment DNA Purification Kit (iNtRON) and sequenced by 1st Base (Singapore). The 16S rRNA gene sequences were compared with referred sequences available in GenBank NCBI. Genetic similarity and phylogenetic trees were constructed using the MEGA6 program. All strains were further grouped into their taxa based on sequences of 16S rRNA gene marker. The accession numbers of referred sequences are shown in Table 1.

Evaluation of extracellular enzyme activities of endophytic actinomycetes

Extracellular enzyme activities of actinomycetes were tested on indicator media. After 7 days on Bennett medium pH 7.0 at 37 °C, isolates were cultured on Czapek medium supplemented with 1% different dissolved substrates, including casein, carboxymethyl-cellulose (CMC), cellulose, starch, and xylan for the assessment of protease, cellulase, amylase, and xylanase activity, respectively. Actinomycetes growing on Bennett plate were taken using culture rods, then placed on substrate containing plates and incubated at 37 °C for 5 days. Casein hydrolysis was evaluated based on the formation of a clear zone around colonies after covering the plate

with 10% acid trichloacetic for 30 min. The cellulase, amylase and xylanase activities were detected by flooding the plates with Lugol dye. The diameter (D, mm) of degradation zones was used to assess enzyme activities. D values ≥ 25 , ≥ 20 , ≥ 15 and ≤ 10 mm were considered as very strong, strong, medium and weak enzyme production, respectively (Hawar, 2022). The experiments were performed in triplicate and data were analyzed using Microsoft Excel 2010 software.

RESULTS AND DISCUSSION

Isolation of endophytic bacteria and actinomycetes from roots of *Catharanthus roseus*

C. roseus is a medicinal plant, commonly grown across our country, however studies of

their endophytic actinomycetes and bacteria have not been done. In this study, under the same growing conditions and using a similar isolation method, the number of endophytic bacteria isolated from the roots of the white flower plant is lower than from the purple flower plant. Five bacterial strains (PV1, PV2, PV3, PV4, PV5) and two actinomycetes (RX1, RX2) were isolated from root samples of purple flower *C. roseus* plants. For white flower *C. roseus* plant samples, two actinomycetes (WX1, WX2) and seven bacterial strains (WX1, WX2, WX3, WX4, WX5, WX6, WX8) were obtained. It is obviously that endophytic communities in plants vary depending on several factors including host plant varieties, kind of tissues, plant development stage, and environmental living conditions.

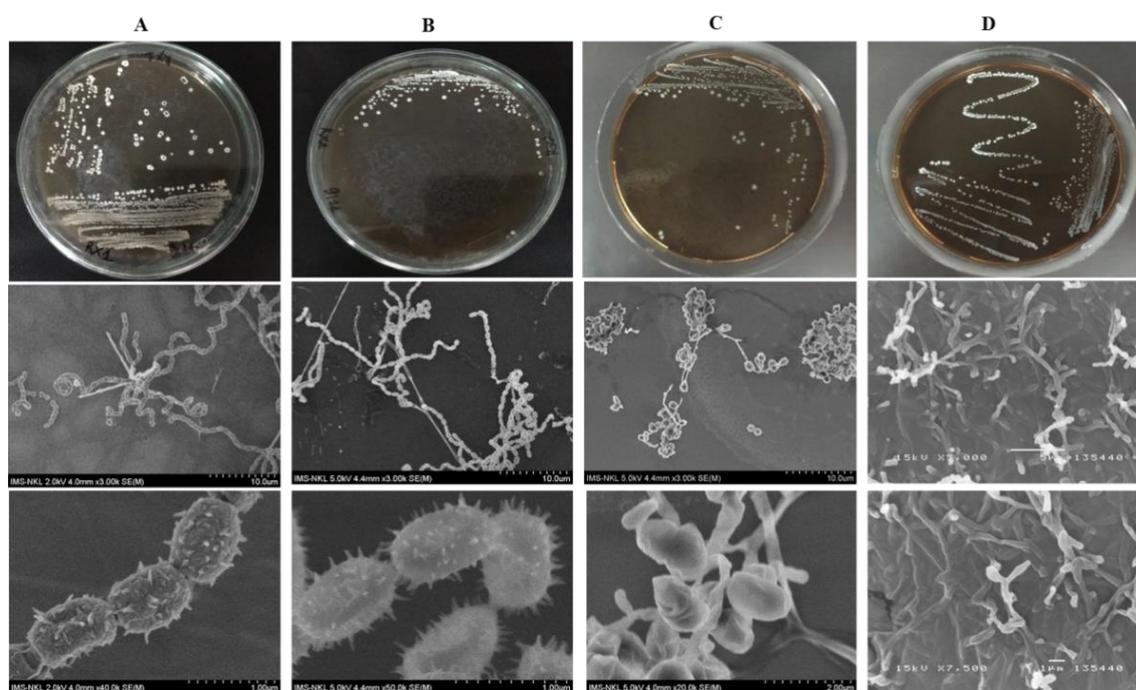


Figure 1. Morphological characteristics of four isolated actinomycetes cultured on Bennett medium after 7 days. (A) RX1, (B) RX2, (C) WX1, (D) WX2.

Scale bars (1–10 μm) are shown in the images

The morphological analysis showed that RX1 and RX2 isolates have the typical characteristics of the *Streptomyces* genus

with the spiny spore surface, long spiral spore chains (Fig. 1). Their aerial hyphae produce many spore chains (Figs. 1A, 1B).

The WX1 isolate produces white, powdery colonies and filamentous edges; double spores with smooth surface (Fig. 1C). The WX2 isolate grows more slowly than other isolates. Colonies of WX2 were only 1–1.5 mm in diameter, ivory-white to pale yellow in color and contained moist surfaces during the first 10 days of the growth stage (Fig. 1D).

Colonies of the bacterial isolates differ in their shapes, sizes, color, and texture (Fig. 2). The colonies have sizes ranging from 0.25 to 1.5 mm, ivory white or light brown in color, most having a smooth surface; except PV2 and WV1 have a rough surface with jagged edges. In addition, WV4 isolates has the ability to produce yellow pigments on MPA medium.

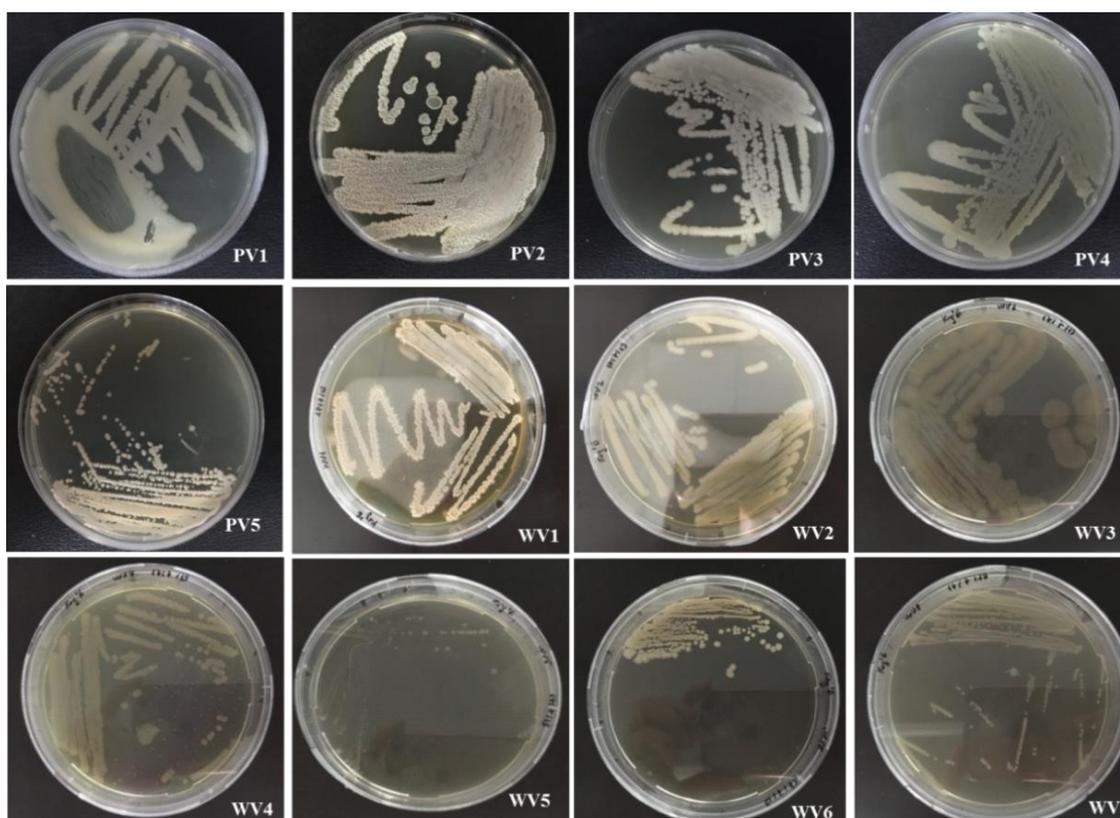


Figure 2. Colonies of 12 endophytic bacteria strains cultured on an MPA medium after 16 hours. PV1- PV5: strains isolated from root samples of the *Catharanthus roseus* purple flower plant. WV1-WV6, WV8: strains isolated from the *Catharanthus roseus* white flower plant

Analysis of 16S rRNA gene marker sequences of isolated strains

Phylogenetic analyses based on sequence polymorphism of 16S rRNA gene fragments and referred taxa (Fig. 3) showed that the isolated strains belonged to 6 families (Bacillaceae, Penibacillaceae, Streptosporangiaceae, Rhizobiaceae, Enterobacteriaceae and Pseudomonadaceae) and

7 genera (*Bacillus*, *Paenibacillus*, *Microbispora*, *Streptomyces*, *Rhizobium*, *Enterobacter* and *Pseudomonas*). Most bacterial strains belong to the *Bacillus* genus (PV1–PV5, WV1, WV3, WV6), the remaining strains belong to *Enterobacter* (WV2), *Pseudomonas* (WV4), *Paenibacillus* (WV5) or *Rhizobium* genera (WV8). Four actinomycetes strains belonged to *Streptomyces* (RX1, RX2) and *Microbispora* genera (WX1, WX2).

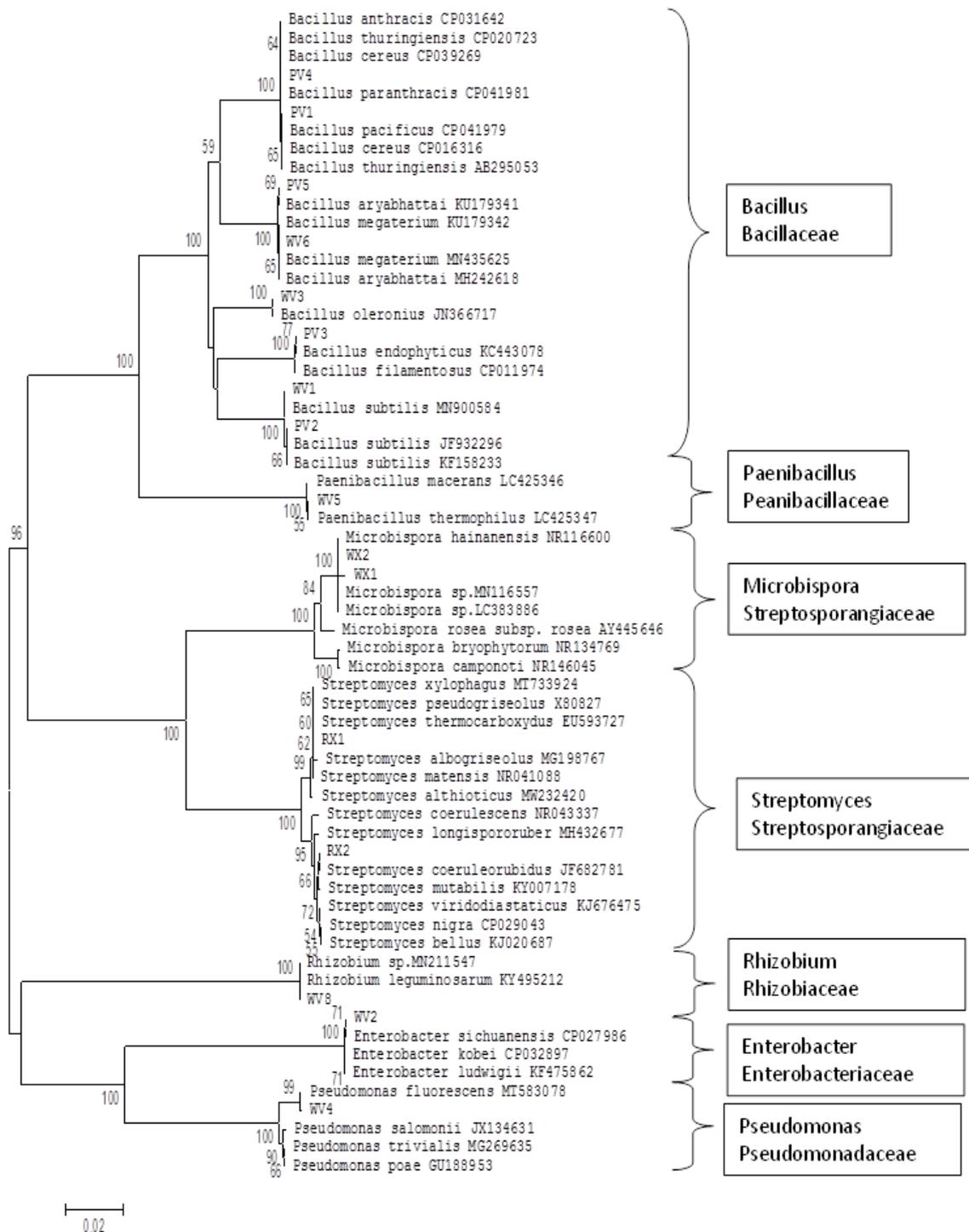


Figure 3. Phylogenetic Neighbor Joining tree reconstructed based on DNA polymorphism of 16S rDNA fragments of 16 endophytic strains and related referred taxa. Numbers after referred taxa are accession numbers from GenBank. Numbers next to nodes of clades are bootstrap values (%). Evolutionary analyses were conducted in MEGA6 (Tamura, 2013)

Table 1. Species identification of isolated endophytic strains based on the 16S rRNA markers in this study

Host plants	Isolates	Accession number (reference taxa) on GenBank NCBI	Species determination
Purple flower <i>Catharanthus roseus</i> plant	PV1	CP016316 (<i>Bacillus cereus</i>), CP041979 (<i>Bacillus thuringiensis</i>), AB295053 (<i>Bacillus pacificus</i>)	<i>Bacillus cereus sensu lato</i> (s.l.)
	PV2	JF932296 (<i>Bacillus subtilis</i>)	<i>Bacillus subtilis</i>
	PV3	KC443078 (<i>Bacillus endophyticus</i>)	<i>Bacillus endophyticus</i>
	PV4	CP020723 (<i>Bacillus cereus</i>), CP039269 (<i>Bacillus thuringiensis</i>), CP031642 (<i>Bacillus anthracis</i>), CP041981 (<i>Bacillus paranthracis</i>)	<i>Bacillus cereus sensu lato</i> (s.l.)
	PV5	KU179341 (<i>Bacillus aryabhatai</i>), KU179342 (<i>Bacillus megaterium</i> ; synonym: <i>Bacillus aryabhatai</i>)	<i>Bacillus aryabhatai</i>
	RX1	EU593727 (<i>Streptomyces thermocarboxydus</i>)	<i>Streptomyces thermocarboxydus</i>
	RX2	JF682781 (<i>Streptomyces coeruleorubidus</i>)	<i>Streptomyces coeruleorubidus</i>
White flower <i>Catharanthus roseus</i> plant	WV1	MN900584 (<i>Bacillus subtilis</i>)	<i>Bacillus subtilis</i>
	WV2	CP027986 (<i>Enterobacter sichuanensis</i>)	<i>Enterobacter sichuanensi</i>
	WV3	JN366717 (<i>Bacillus oleronius</i>)	<i>Bacillus oleronius</i>
	WV4	MT583078 (<i>Pseudomonas fluorescens</i>)	<i>Pseudomonas fluorescens</i>
	WV5	LC425346 (<i>Panenibacillus thermophilus</i>)	<i>Panenibacillus thermophilus</i>
	WV6	MN435625 (<i>Bacillus megaterium</i>), MH242618 (<i>Bacillus aryabhatai</i> , synonym: <i>Bacillus megaterium</i>)	<i>Bacillus aryabhatai</i>
	WV8	KY495212 (<i>Rhizobium leguminosarum</i>)	<i>Rhizobium leguminosarum</i>
	WX1	MN116557 (<i>Microbispora</i> sp.)	<i>Microbispora</i> sp.
	WX2	NR116600 (<i>Microbispora hainanensis</i>)	<i>Microbispora hainanensis</i>

Alignments between sequences of 16S rRNA gene fragments of isolated strains and reference taxa in each clade in Figure 3 showed a high homology level (100%). Fourteen bacterial strains were classified at the species level by the 16S rRNA gene marker and shown in Table 1. The PV1 and PV4 strains were identified as belonging to the *Bacillus cereus* group. This group, also known as *B. cereus sensu lato* (s.l.), is a species complex that contains numerous closely related lineages (Laura et al., 2022). In the case of actinomycetes, strain RX1 was classified as *Streptomyces thermocarboxydus*, RX2 as *Streptomyces coeruleorubidus*. This finding is

in accordance with their morphological characteristics typical to the *Streptomyces* genus. Two strains WX1 and WX2, which were isolated from the white flower plant were identified as *Microbispora* sp. and *Microbispora hainanensis*, respectively.

Microbispora and *Streptomyces* are the most popular actinobacteria genera found in plants. When isolating endophytic actinomycetes from fallen leaves of nine different plant species, Matsumoto et al. (1998) noticed that *Microbispora* accounted for 44% of isolated strains. Li et al. (2008) isolated 41 *Streptomyces* strains having anticancer and antimicrobial activities from

medicinal plants in the rainforest. Thao et al. (2016a, 2016b) isolated and characterized the endophytic *Streptomyces* sp. TQR12-4 from Citrus nobilis Cultivar Ham Yen of Vietnam and *Streptomyces parvulus* HNR3X4a from pomelo (*Citrus grandis* L. Dien), which displayed strong antimicrobial activities.

Extracellular enzyme activities of isolated endophytic actinomycetes

Actinomycetes are known to produce an extensive range of bioactive compounds

including extracellular enzymes for degrading organic macromolecules such as protein, starch, chitin, cellulose, lignocellulose. In this study, the ability to produce proteases, cellulases, amylases and xylanases of four isolated actinomycetes was evaluated.

The result showed that the casein substrate was degraded by all isolates. It revealed that proteases were produced by all actinomycete isolates, in which RX1 had the strongest activity with the substrate degradation clear zone of 19 mm in diameter.

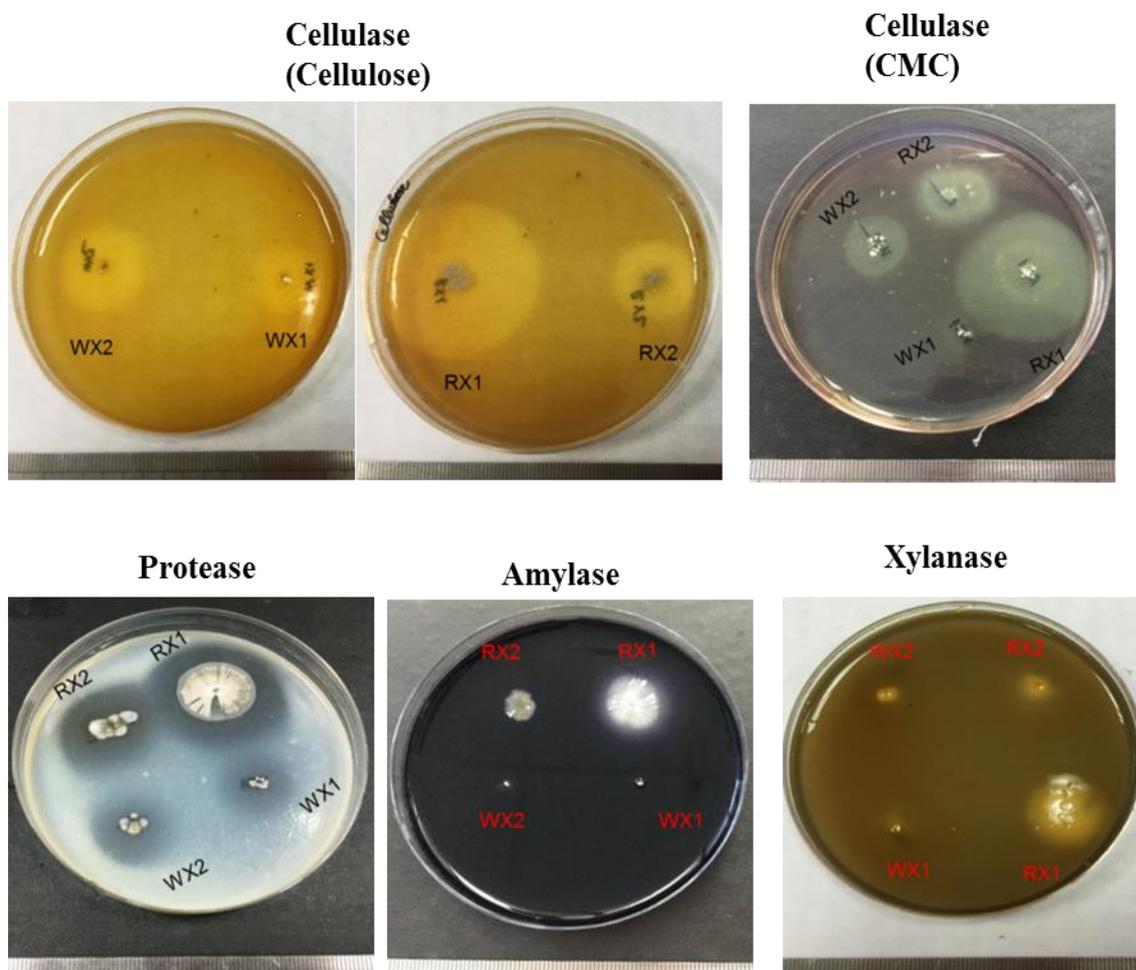


Figure 4. Extracellular enzyme activities of *Bacillus roseus* endophytic actinomycetes evaluation on agar plates. Clear zones/halos correspond to the degradation of substrates

Screening for extracellular cellulase production by microorganisms was done on agar plates containing carboxymethyl cellulose

(CMC) or cellulose as substrate. Four tested isolates were able to degrade cellulose substrate. Three strains (RX1, RX2, WX2)

showed the degradation of CMC substrate. Among them, RX1 exhibited the strongest cellulase activity against both types of tested substrates. Amylases (starch substrate) and xylanases (xylan substrate) were produced by RX1 and RX2 with moderate and weak

activities, respectively (Fig. 4, Table 2). Thus, under similar culture conditions, two endophytic actinomycetes (RX1 and RX2) of the *Streptomyces* genus could produce more extracellular bioactive enzymes than two strains (WX1 and WX2) of the *Microbispora* genus.

Table 2. Extracellular enzyme activities of isolated endophytic actinomycetes

Actinomycetes	Host plants	Diameter of enzyme degradation zone (mm)				
		Casein	Cellulose	CMC	Starch	Xylan
RX1	<i>Bacillus roseus</i> var. <i>roseus</i> (pink flower)	19 ± 0.88	37 ± 0.92	34 ± 0.87	15 ± 0.58	18 ± 0.62
RX2		18 ± 0.76	17 ± 0.53	17 ± 0.71	5 ± 0.42	5 ± 0.38
WX1	<i>Bacillus roseus</i> var. <i>ocellatus</i> (white flower)	15 ± 0.36	12 ± 0.27	0	0	0
WX2		17 ± 0.78	18 ± 0.34	16 ± 0.32	0	0

Streptomyces is the largest actinomycete genus belonging to the Streptomycetaceae family. Similar to our findings, many previous studies have indicated that endophytic *Streptomyces* spp. were accomplished by producing a range of valuable extracellular enzymes. When screening 160 *Streptomyces* strains, Wachinger et al. (1989) reported all of them were able to produce cellulases. In an examination of 39 *Streptomyces* strains, Writh and Ulrich (2002) indicated that 11 strains degraded 4 types of tested cellulose and 17 strains degraded CMC only. In addition to roles in promoting plant growth and controlling pathogens, Sousa et al. (2008) observed that *Streptomyces* species produced amylases, lipases and catalases. Among 45 endophytic actinomycetes isolated from several plant species, there were two (DR61 & DR69) of four strains isolated from *C. roseus*, that showed strong hemicellulase activity (Robl et al., 2019). Extracellular enzymes of *Streptomyces* have been applied in industry and agriculture such as decomposing toxic wastes, converting wastes into biofuels, replacing better substances used in the food industry, degrading lignin in paper production,... (Kumar et al., 2020). In the present study, it is revealed that endophytic *Streptomyces thermocarboxydus* RX1 and *Streptomyces coeruleorubidus* RX2 synthesized such valuable enzymes of proteases, cellulases, amylases and xylanases, especially strain RX1 exhibited a very strong

cellulase activity. Therefore, these strains could be the potential sources for the production of extracellular enzymes.

Two actinomycetes of the *Microbispora* genus, WX1 and WX2, isolated from *C. roseus* white flower plant also expressed cellulases and proteases activities at moderate levels, but not amylases and xylanases. This result is consistent with the study of Holtz et al. (1991), in which the xylanase activity of all *Microbispora* species isolated from silage and cattle manure was remarkable weaker compared to that of *Streptomyces* and *Actinomadura* genera. However, Eida et al. (2012) reported that six *Microbispora* species isolated from sawdust and coffee grounds had cellulase, xylanase, β -glucanase, mannanase and protease activities. Apart from producing less extracellular enzymes, the growth rate of two *Microbispora* strains was slower than *Streptomyces* under the same culture conditions. Further research is needed to clarify which are the most suitable growth conditions for the production of extracellular enzymes by *Microbispora* strains.

CONCLUSION

In the present study, four actinomycetes and twelve bacteria strains were isolated from two *C. roseus* varieties growing naturally in the coastal areas of Nha Trang, Vietnam. The analysis of extracellular enzyme activities of four actinomycetes indicated that they were

able to produce at least one type of proteases, cellulases, amylases, and xylanases. This is the first report about endophytic actinomycetes and bacteria isolated from *C. roseus* plants in Vietnam. These results have confirmed the diversity and potential of *C. roseus* endophytic microorganisms as sources for discovering valuable bioactive compounds.

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