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ISOLATION, SELECTION AND EVALUATION OF α-GLUCOSIDASE INHIBITORY ACTIVITY FROM ENDOPHYTIC *STREPTOMYCES* sp. ISOLATED FROM *CITRUS MYRTIFOLIA* CULTIVAR IN HOA BINH, VIETNAM

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SUMMARY

Cao Phong orange (*Citrus sinensis* Lour) was grown in Cao Phong district, Hoa Binh province. *Citrus sinensis* is one of the Vietnam's most valued commercial fruits. In this study, 30 microorganim strains were isolated from the samples of root, stem, and leaf of Cao Phong orange. The ratio of endophytic actinomycetes was found in different parts of the plant: root, stem, and leaf was 37%, 40%, and 23%, respectively. An isolate HBC6−2 was selected because of the highest α−glucosidase inhibition activity among six different strains. Morphological, physiological, and biochemical characterization showed that HBC6−2 strain belongs to the *Streptomyces* sp. The 16S rRNA sequence of HBC6−2 indicated 99% identity to the corresponding sequence of *Streptomyces costaricanus* thus the strain was designated as *S. costaricanus* EBL.HB6, and the gene was registered on GenBank with the code MT 453944.1.

Streptomyces costaricanus EBL.HB6 was able to produce melanin yelow pigment, and its aerial and substrate mycelia have brown and yellow-grey pigment on ISP2, ISP3, ISP4, and ISP5 cultivating media, respectively. The suitable pH range was from 5−10 and temperature from 15−40°C and exhibited salt tolerance up to 3% and utilized the carbon sources such as fructose, xylose, arabinose, cellulose, and rhamnose. In the following investigation, *S. costaricanus* EBL.HB6 displayed the highest α-glucosidase inhibitory activity by 68.98% among *Streptomyces* sp. Strains.

Keywords: *Citrus sinensis* Lour, *Streptomyces costaricanus* EBL.HB6, α−glucosidase inhibitors

INTRODUCTION

The Actinomycetes sp. belongs to a group of endophytic microorganisms that can produce a myriad of inhibitors against pathogenic microorganisms (Lee *et al.,* 2008). Therefore, several studies have investigated its role as a bio−control factor including promoting plant growth, reducing the risk of the infectious pathogen, and enhancing the viability of plants under different conditions (Strobel *et al.,* 2004). Actinomycetes took a major part in the

population of root microorganisms so they easily transmit to the plant and become an endophytic body. The population includes both *Streptomyces* and non− *Streptomyces* appear in plant tissues (Shutsrirung *et al.,* 2013).

Accordingly, Actinomycetes accounted for more than 50% of soil−borne mircroorganisms which are *Microbispora*, *Micromonospora*, *Nocardioide*, *Nocardia*, and *Streptosporangium* (Qin *et al.,* 2009). In 2016, Tan and his colleagues isolated 619 Actinomycetes from different species of tomato and all of them belonged to the genus of *Streptomyces* (Tan *et al.,* 2006). From 36 medicinal plants, Taechowisan isolated 360 species belonged to 4 different genera (*Streptomyces, Microbispora, Nocardia, Micromonospora*) of Actinomyces (Taechowisan *et al.,* 2005). Briefly, *Streptomyces* has a strong adaptability and growth among Actinomycetes.

A large number of publications in microbial compounds have reported that 45% of substances originated from Actinomycetes, 38% were from mushrooms, and 17% were from bacteria. Actinomycetes are one of the most important microorganisms due to the majority of their secondary metabolites including enzymes, antibiotics, antifungals go to industrial, agricultural and pharmaceutical markets (Sharma *et al.,* 2014). Most of these biologically active substances were discovered from terrestrial *Actinomycetes* which are recognized as a reliable resource for the production of antibiotics and new pharmaceutical compounds. The *Actinoplanes* sp. and *Streptomyces* sp. are the most popular species that capable of producing α−glucosidase inhibitors (AGIs) such as acarbose of *Actinoplanes* (Wang *et al.,* 2011), lipiarmycin of *Actinoplanes* (Parenti, Coronelli, 1979), validamycin of *Streptomyces* (Iwasa *et al.,* 1970), and other aminoglycosides, anthracyclin, glycopeptide, β−lactam, macrolides, nucleoside, peptide, polyene, polyester, polyketide, actinomycin and tetracycline (Chen *et al.,* 2016).

Several studies have discovered new biologically active substances and endophytic Actinomycetes isolating from plant tissue. Due to the significant potential, these endophytic Actinomycetes strains have drawn numerous interests and projects over the world, especially in America, Japan, China, Korea, and India. The ratio of discovering pharmaceutical compounds from endophytic Actinomycetes is higher than soil−borne and plant Actinomycetes. A new antibiotic, naphthomycin−K, was first discovered from endophytic *Streptomyces* sp. from *Maytenus Hookeri*−a medicinal plant that has effects against cancer. 5,7−Dimethoxy−4−phenylcoumari and 5,7−dimethoxy−4−p−methoxylphenylcoumarin strongly inhibited cancer growth which is frequently isolated from different plant species. Recently, they are also found in endophytic *S. aureofaciens* CMUAc130 (Qin *et al.,* 2010).

Methyllelaiophylin of *S. melanosporofaciens* inhibited α−glucosidase with IC_{50} at 10 μM (Lee *et al.,* 2011). Kaur (2016) isolated several antibiotics from endogenous *Streptomyces, Micromonospora, Microbiospora, Nocardia* on the leaves of the neem tree (*Azadirachta indica* A. Juss.) (Kaur 2016). A new active substance was extracted from *Streptomyces* sp. OUCMDZ−3434 isolating from algae samples. The new skeleton of wailupemycins H (1) and I (2) was assembled with 2 cores 6 (2−phenylnaphthalene−1−yl) pyrane−2−1 and 1 bond with CH_2. The substance 1 and 2 are new α−glucosidase inhibitors with Ki/IC_{50} were 16.8/19.7 and 6.0/8.3 M, respectively (Chen *et al.,* 2016). Wei et al. (2017) isolated 24 compounds from the culture broth of *S. xanthophaeus* that were numbered from 1−24, and their chemical formula was elucidated by NMR. The authors have identified 3 compounds including genistein, gliricidin, and daidzein which inhibited α−glucosidase *in vitro* with IC_{50} were 36.1 μM, 47.4 μM, and 174.2 μM, respectively. The results showed the inhibitory activity was higher than that of acarbose (Wei *et al.,* 2017).

In this study, we isolated endogenous actinobacteria from orange trees originated from Hoa Binh, Viet Nam to find new species that capable of producing α–glucosidase inhibitors for further research.

MATERIALS AND METHODS

Materials

Root (R), stem (C), and leaf (L) samples were obtained from ten years–old Cao Phong orange trees grown in the mountainous villages of Cao Phong, Hoa Binh province, Vietnam. The samples HB1, HB2, HB3 were collected from Dong Phong at coordinate position 20°42'43'' north, 105°19'35'' east; the samples HB4-HB7 were collected from Cao Phong at 20°42'42'' north, 105°19'34'' east; the samples HB8-HB10 were collected from Hoa Binh city at coordinate position 20°48'46'' north, 105°20'20'' east.

Chemical reagent

p–Nitrophenyl–α–D–glucopyranoside (*p*NPG), α–glucosidase, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KNO_3, $MgSO_4.H_2O$, $FeSO_4.7H_2O$, NaCl, peptone, glycerol, glucose, yeast extract, and malt extract were purchased from Merck. Humic acid, nystatin, and nalidixic acid, and asparagine were purchased from Sigma. Maltose, ethanol, ethyl acetate, methanol, formic acid, and H_2SO_4 were from China. All other chemicals are analytical grade, otherwise stated.

Isolation of endophytic actinobacterial

Plant samples were surface–decontaminated as described by Stutsrirung (2013) (Shutsrirung *et al.*, 2013). The materials were then homogenized with roughly 25 mL of sterile water in a mortar. The liquid was collected and spread on the screening humic acid medium containing (g/L): K_2HPO_4 20; KNO_3 20; $CaCO_3$ 0.2; $FeSO_4.7H_2O$ 0.1; NaCl 20; $MgSO_4.H_2O$ 0.5; humic acid 10; agar 18; pH 7.0. Nystatin and nalidixic acid were added to the medium at the final concentration of 100 mg/L and 10 mg/L, respectively, to prevent the growth of bacteria and fungus. The inoculator plates were incubated at 28°C. Typical actinomyces colonies were selected from 15–60 days and isolated by consecutive streaking on ISP2 medium containing (g/L): yeast extract 40; glucose 10.0; malt extract 4; agar 18; pH 7.0.

Identification of endophytic actinobacterial by biological characteristics

Study of biological characteristics by the method of ISP (1974) and Bergey's classification. The color of aerial and substrate mycelia, melanin, and soluble extracellular–pigment were accessed by Shirling and Gottlieb (1996) (Shirling, Gottlieb 1966) followed by the color table of Tresner and Backus.

The actinobacteria morphology, aerial and substrate mycelia, and soluble pigments were studied on the ISP agar media: yeast extract–tryptone (ISP1), yeast extract–malt extract (ISP2), oatmeal (ISP3), inorganic salts–starch (ISP4), glycerol–asparagine (ISP5), peptone yeast extract–iron (ISP6), and tyrosine–asparagine (ISP7). Melanin pigment was identified on ISP1, ISP6, and ISP7 media. The assimilation of carbon was identified on the ISP9 medium. The color group was identified by the color of actinomyces: White (W), Gray (Gy), Red (R), Yellow (Y), Green (Gn) (Momomura 1974; Shirling and Gottlieb 1966; Tresner et al. 1961). The morphology of sporophore and spore surface structure of *Actinobacteria* was observed under microscope JSM–5000 at Material Institute of Vietnam Academy of Science and Technology.

Physiological characteristics (effect of temperature, pH, and NaCl tolerance)

Suitable growth temperature, pH and salinity tolerances of the isolated strains were dertermined on the Bennet medium. The strain was incubated on a Bennet agar plate at the temperature range of 10–60°C. Their growth was observed after 5-7 h. Actinobacteria was cultured in Bennet medium at a pH range of 2-12 with a shaking speed of 150 rpm at 28°C. The samples were evaluated after 7 days of culture.

The strains were cultured in Bennet medium

was supplemented with NaCl 0-7% with shaking speed 150 rpm at 28°C. The samples were evaluated after 7 days of culture.

DNA isolation, identification of the chosen strain

The isolated strain was identified by the 16S rDNA sequencing method. Genomic DNA isolation was used to extract DNA from a potent AGI strain, as described before (Quyen *et al.*, 2007). The isolated DNA was amplified by PCR. The conserved gene of 16S rDNA was amplified by using 9F (5′–AGAGTTTGATCCTGGCTC–3′) as forward primer and 926R (5′–CCGTCAATTCCTTTGAGTT–3′) as reverse primer. The amplified gene was sequenced on ABI PRISM 3100 Avant Genetic Analyzer. Sequence alignments were constructed and analyzed using the program MegAlign DNAStar.

Inhibition assay of α–glucosidase enzyme

Assays were carried out in a 96–wells microplate system, according to Koh's protocol (2018) (Koh *et al.*, 2018) with slight modifications. Briefly, 100 µl of 1–2 U/mL α–glucosidase enzyme (Sigma), diluted in 0.1 M sodium phosphate buffer (PB) were mixed with 10 µL fermentation broth (control sample was replaced by the culture medium) and 40 µL of PB solution, and then was subjected to pre–incubation in 5 min at 30°C Continously, 100 µL of 4–nitrophenyl–α–D–glucopyranoside (Sigma) at 0.1 M in PB solution was added as substrate after pre–incubation. Next, the samples were incubated at 30°C for 10 min to allow α–glucosidase to react with 4–NPG and produce 4–nitrophenol. After the incubation, the formation of 4–nitrophenol in each well was measured by the intensity of absorbance at 405 nm and α–glucosidase enzyme inhibition was calculated using.

$$\% \text{ Inhibition} = \frac{\Delta A_C - \Delta A_S}{\Delta A_C} \text{ x } 100$$

ΔA_C : The change in measurement of OD value before and after incubation for 5 min of control.

ΔA_S : The change in measurement of OD value before and after incubation for 5 min of the sample.

Fermentation

Six strains of *Streptomyces* sp. were selected. They were cultivated at 28°C with shaking (200 rpm) in a 100 mL flask with 30 mL of the ISP2 medium, pH7. To determine α–glucosidase inhibitory activity, the extracellular extracts were taken from the culturing medium and the α–glucosidase inhibitory activity tests were performed after 144 h. All the tests were conducted in triplicate.

Statistical analysis

All measurements were carried out in triplicate. For averages of experiments, the means were reported.

RESULTS AND DISCUSSION

Isolation of endophytic Actinomycetes from Cao Phong orange trees

30 colonies of endophytic Actinomycetes strains were isolated from the sample of root, stem, and leaf of Cao Phong orange trees (Table 1). The number of accumulating bodies fluctuated $0–10^3$ CFU/g. The ratio of endogenous actinobacteria was found in different parts of the plant: root, stem, and leaf was 37%, 40%, and 23%, respectively (Figure 1A).

In comparison with the same microorganism isolating in Panxi plateau, China, Zhao (2005) isolated 560 strains from 26 medicinal plants which gave the distribution of actinobacteria in root, stem, and leaf was 58.2%, 27.8%, and 14%, respectively (Zhao *et al.*, 2005). Verma (2009) and Zin (2010) reported that the majority of endogenous actinobacteria were isolated from the root rather than different parts of the plant. In another study, Thirty five actinobacteria strains include 21 strains (60% was from root), 9 strains (25.7% was from the stem), and 5 strains (14.3% was from leaf) was isolated from root, stem, and leaf of *Oryza sativa.* The *Streptomyces* genus dominate the actinobacteria population (Verma *et al.*, 2009; Zin *et al.*, 2010).

Table 1. The number and composition of Actinomycetes in the samples of Cao phong oranges tree collected in Hoa Binh province, Vietnam.

Order	Sample	The number of colonies (CFU/g)	Number of isolated Actinomycetes/ sample
1	HBC1	23	1
2	HBR1	170	1
3	HBL1	50	2
4	HBR2	2500	1
5	HBC2	2430	2
6	HBL2	30	2
7	HBC3	6	1
8	HBL3	50	2
9	HBR3	0	0
10	HBL4	0	0
11	HBC4	7	2
12	HBR4	46	2
13	HBL5	0	0
14	HBR5	50	1
15	HBC5	46	1
16	HBC6	50	1
17	HBL6	2500	1
18	HBR6	2430	1
19	HBC7	30	1
20	HBL7	0	0
21	HBR7	28	1
22	HBC8	50	1
23	HBL8	0	0
24	HBR8	210	1
25	HBC9	7	1
26	HBL9	0	0
27	HBR9	170	2
28	HBC10	50	1
29	HBL10	0	0
30	HBR10	16	1
Total strains			**30**

Note: The sample was isolated from different part of tree: Root (R), Branch (C), Leave (L). The number of isolating colonies was numbered from 1 to 20.

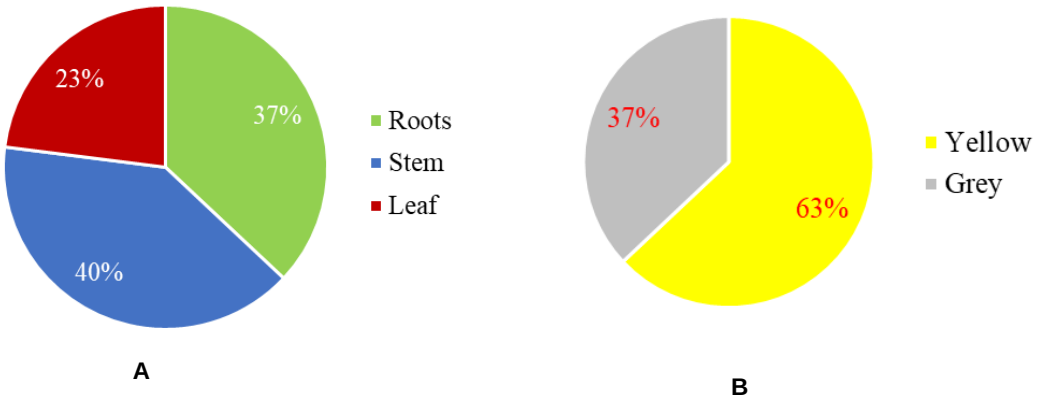


Figure 1. The percentage of endophytic Actinomycetes isolated from the root, stem, and leaf of Cao Phong-Hoa Binh orange trees (A). The diversity in color distribution of endophytic Actinomycetes obtained from Cao Phong-Hoa Binh orange trees (B).

Similarly, Gangwar's group reported that root is the recipient of water and nutrient for plants, and also the habitat for actinobacteria. Therefore, endogenous bacteria isolating from roots was more diverse than the one isolating from leaf (Gangwar *et al.*, 2014). In 2013, Shutsrirung's team isolated 252 actinobacteria strains from mandarin trees at 7 gardens in Thailand. Among them, 3 gardens were fertilized with minimal amount supplementing with organic fertilizer and natural pest control. In these 3 gardens, the number of isolating actinobacteria is more diverse, the highest number was recorded in the root (44.6%), stem (32.7%), and leaf (22.8%). Another 4 gardens were fertilized with frequently used method (high proportion of chemical fertilizers and utilization of synthetic pesticides), there are only 2 actinobacteria strains were isolated which belonged to *Streptomyces* sp. (95.4%). Generally, endogenous bacteria isolating from 7 gardens belonged to *Streptomyces* (85.3%) (Shutsrirung *et al.*, 2013).

The diversity of isolated actinobacteria

The diversity of isolating actinobacteria was evaluated by color and cultural characteristics. The culture conditions were carried out on some basic media such as ISP1, 2, 3, 4, 5, 6 và 7 and classified in table 2. In the orange tree of Cao Phong province, the yellow one accounted for the major part (63%), next is the grey one (37%) (Figure 1B). On the ISP2 medium, the morphology of the colony, aerial, and substrate mycelia are variable. Most of the colonies of these strains have a diameter of 1–3 mm, powder shape, insoluble, unevenly spread, convex or concave surfaces with white, yellow, grey, brown, pink, and green (Table 2; Figure 2). Our results are in agreement with previous studies. Straight, twisted, hooked, heavily branched spore chains, and long spore chains were characteristic of the *Streptomyces* sp. (Arifuzzaman *et al.*, 2010).

Physiological characteristics and the assimilation of carbon source of endogenous actinobacteria

The physiological characteristics of endogenous bacilli groups have been investigated to find out the optimal culture conditions such as temperature, pH, and salt tolerance. Most of the isolating actinobacteria have a growth temperature range of 15–40°C, preferably at 25–30°C, and a pH of 5–10. Thus, most of isolating endogenous bacteria were thermophilic strains. They can grow in a light salt concentration lower than 3–5% (Table 3). In other words, endogenous bacilli do not require any special cultural conditions so they have a high potential in producing bioactive substances.

Table 2. Biological characteristics of endophytic bacteria on Cao phong orange trees.

Color	Representative strain	Strain	Aerial mycelia	Substrate mycelia	Spore chain	Melanin pigment
Yellow	HBC5–1	HBC5–1 HBR4–1 HBL1–2 HBL6–1	Yellow orange turn brown	Brown olive yellow	Spore chains with hook–like shape, symmetrically branched	Yellow
	HBR5–1	HBR5–1 HBL2–2 HBC2–2 HBR2–2	Yellow–orange to olive-yellow with greenish	Yellow–brown	Long, straight spore chain	Yellow orange
	HBR9–6	HBR9–6 HBC1–1 HBR6–1 HBC9–1 HBL3–1	Olive brown gold	Yellow–orange brown	Short, straight spore chain	Light brown
	HBC3–2	HBC3–2 HBL1–1 HBR7–1 HBR8–1 HBR4–2 HBL3–2	Yellow–brown	Deep orange yellow	Long, straight spore chain	Light brown to red dark brown
Grey	HBR1–2	HBR10–2 HBC6–1 HBC8–1 HBC4–3	Beige yellow to golden gray	Golden brown to dark brown	Spiral spores containing 10–50 spores	Brown
	HBC6–2	HBC6–2 HBL2–1 HBR1–6 HBC10–1 HBC2–1 HBR9–5 HBC7–1	Gray brown	Yellow–brown	Spiral spores containing 10–50 spores	Yellow

The different strains of endogenous actinobacteria were cultured in ISP9 media supplementing with sugar 1% for investigation. The positive control was the medium supplementing with glucose, the negative control was the mineral medium without sugar. 9 different sources of sugar glucose, D–mannitol, D–sucrose, D–fructose, D–xylose, L–arabinose, D–cellulose, D–rhamnose, D–raffinose were used to identified actinobacteria. The results showed 6 strains can utilize various sources of sugar. Besides, most of the strains can grow on a medium supplementing with L–arabinose, a popular carbon source in the plant (Table 4). A previous study showed that endogenous actinobacteria already existed in plant tissues so they assimilate the plant carbon source (Romero et al. 2014). From the collected data, 6 strains of actinobacteria belonged to *Streptomyces* sp.

So, based on morphological, physiological, and biochemical characterization showed that HBC6–2 strain has the closest to taxonomic characteristics with Stomyces costaricanus (Table 5).

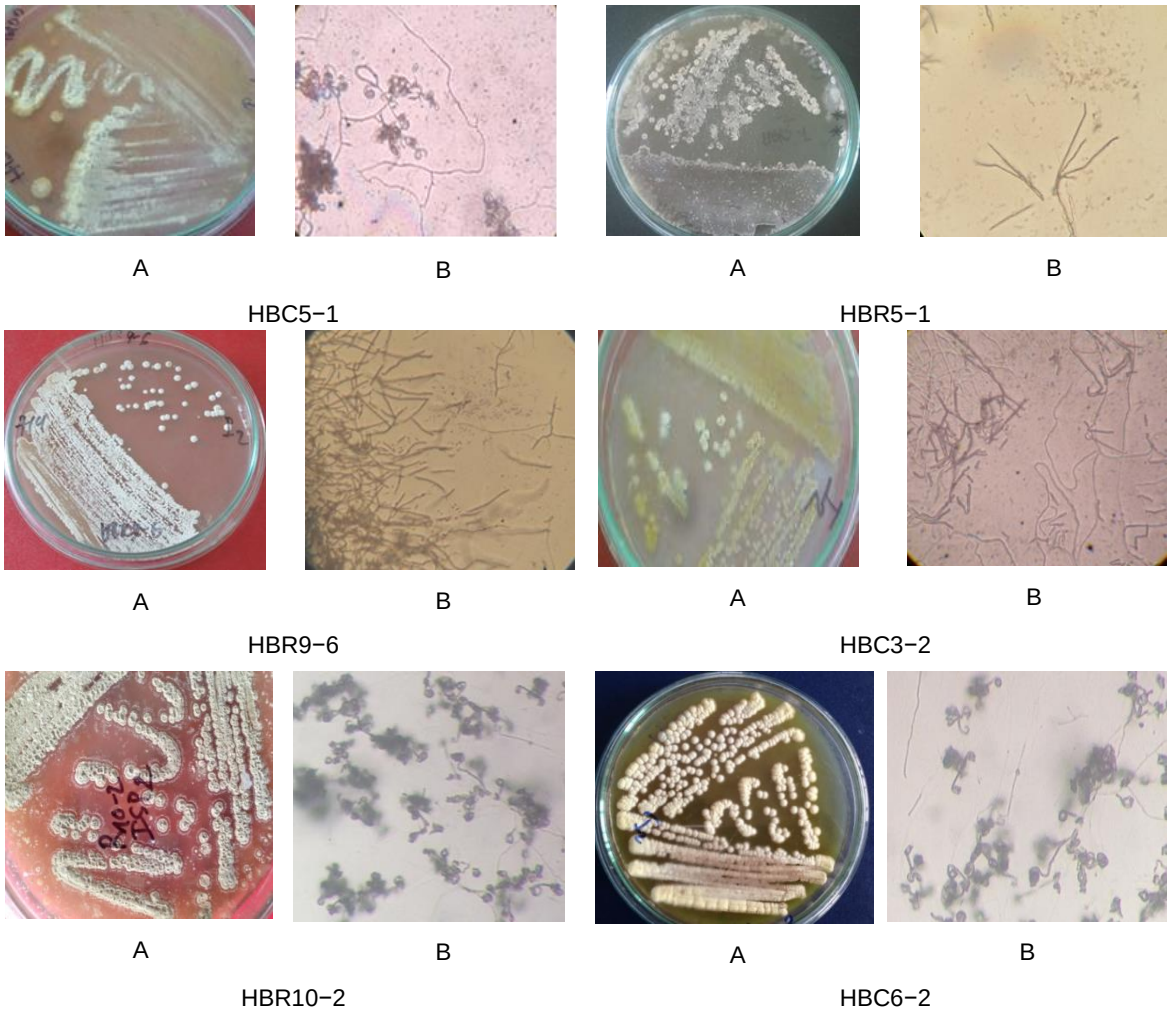


Figure 2. Colonies (A) and aerial mycelia and spore chains (B) of 6 endophytic Actinomycetes isolated in Cao Phong orange trees.

Table 3. Growth conditions of endophytic actinomycetes.

Order	Representative strain	Temperature	pH	Salt degree (%)
1	HBC3-2	15- 40	5- 11	0-5
2	HBC5-1	15- 42	5- 9	0-4
3	HBC6-2	15- 40	5 -10	0-3
4	HBR5-1	15- 40	5- 10	0-5
5	HBR9-6	20- 42	5- 11	0-5
6	HBR10-2	15- 40	5- 10	0- 5

Table 4. The ability to assimilate sugar source of isolated endophytic actinomycetes.

Order	Strains	Positive control	D-manitol	D-sucrose	D- fructose	D-xylose	L-rabinose	D-cellulose	D-rhamnose	D-raffinose	Negative control
1	HBC3-2	+	++	+-	+	++	++	++	+-	+-	-
2	HBC5-1	+	++	+	++	-	+-	++	-	+-	-
3	HBC6-2	+	-	-	+	++	+	+-	+-	-	-
4	HBR5-1	+	+	+	+	+	+	++	+	+	-
5	HBR9-6	+	++	+-	+	++	+	+	+	+-	-
6	HBR10-2	+	+-	+-	++	+	++	+-	-	-	-

Note: Control (+) is the medium supplementing with glucose, ĐC (-)is the medium supplementing without sugar

Table 5. Comparison of taxonomic characteristics of actinomycete strains (Esnard *et al.,* 1995).

Characteristics	**HBC6-2**	***Streptomyces costaricanus***
1. Spore stem	Spiral	Spiral
2. Spore surface	Slippery	Slippery
3. Number of spores/strings	10-50	10-50
4. Color of aerial mycelia	Gray brown of ISP2, ISP3, ISP4, and ISP5	Gray brown of ISP2, ISP3, ISP4, and ISP5
5. Color of substrate mycelia	Yellow-brown of ISP2, ISP3, ISP4, and ISP5	Yellow-brown of ISP2, ISP3, ISP4, and ISP5
6. Solube pigment	Yellow of ISP2 and ISP5	Yellow of ISP2 and ISP5
7. Melanin pigment	-	-
8. Sugar source utility		
Glucose	+	+
Fructose	+	+
L-arabinose	-	-
D-mannitol	+	+
Saccarose	-	-
Xylose	+	+
Rhamnose	-	-
Raffinose	-	-

Screening of α–glucosidase inhibitory activity for *Streptomyces* sp. strains

The fermentation broths of 6 strains of *Streptomyces* sp. were examined the α–glucosidase activity. Among *Streptomyces* sp. strains, *Streptomyces* sp. HBC6–2 displayed the highest α–glucosidase inhibitory activity by 68.98% (Figure 3).

Abdulkhair (2018) isolated 55 strains of marine *Actinomycetes*, of which, only 7 strains were found to have α–glucosidase inhibitory activity (Abdulkhair et al. 2018). Ganesan (2011) identified 41 bacteria strains that exhibited glucosidase inhibitory activity from 181 isolated strains of marine actinobacteria (Ganesan et al. 2011). The study by Cansigno and his colleagues

also showed that seaweeds from *Veracruz* capable of producing α–amylase and AGIs (Landa-Cansigno et al. 2020).

The strain *Streptomyces* sp. HBC6–2 produced melalin yellow pigment, non–fragmenting substrate mycelia (hypha diameter around 10 µm) and formed spiral spores containing 10–50 spores. The color of the aerial and substrate mycelia of *Streptomyces* sp. HBC6–2 was gray brown and yellow brown (Figure 4A, Figure 2A,). 16S rRNA sequence of *Streptomyces* sp. HBC6–2 was identified. BLAST results of 16S rRNA from HBC6–2 strain indicated 99.69% identity with 16S rDNA from *Streptomyces costaricanus* MR7 (KY753206), 99.59% to *Streptomyces costaricanus* MJM5482 (FJ799179) (Figure 4B). It was registered on GenBank with the code MT 453944.1.

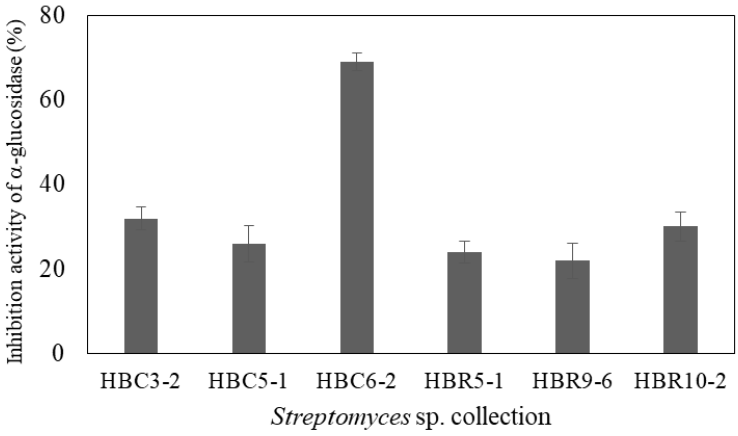
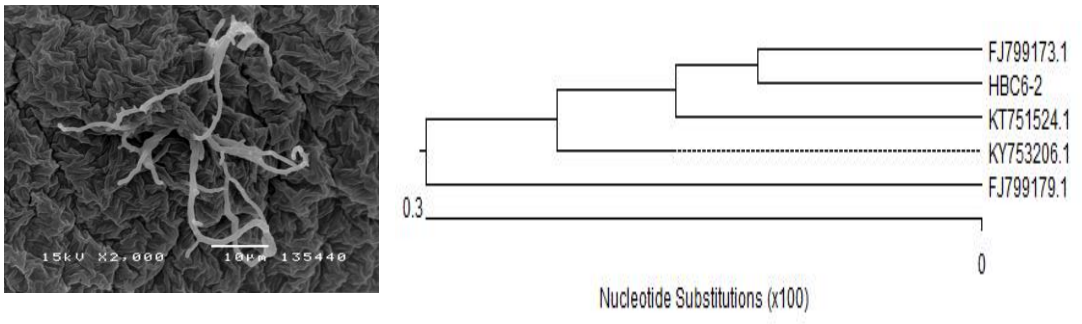


Figure 3. Screening of α−glucosidase inhibitory activity from the fermentation broth of 6 *Streptomyces* sp. strains



A B

Figure 4. (A) Substrate mycelia (magnification ×2000) and (B) Phylogenic relationships of *Streptomyces* sp. HBC6–2 with other *Streptomyces costaricanus* basing on 16S rDNA sequence

CONCLUSION

We selected 30 microorganims strains that were isolated from the samples of root, stem, and leaf of Cao Phong orange. The isolating HBC6–2 was selected for its high production of α–glucosidase inhibitor from 6 different *Streptomyces* strains and was registered on

GenBank with the code MT 453944.1. The α−glucosidase inhibition activity of *Streptomyces* sp. HBC6−2 exhibited the highest point at 68.98%.

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