ISOLATION, IDENTIFICATION, AND BIOLOGICAL CHARACTERIZATION OF CHITINASE-PRODUCING ACTINOMYCES FROM YOK DON NATIONAL PARK

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ABSTRACT

Developing new biofertilizers has been an area of great interest in modern agriculture. This study focused on actinobacteria possessing high chitinase activity, biofilm formation, plant growth promotion, and antifungal activity. Two chitinolytic actinomyces strains, YSS-3.3 and YWS-5.1, were isolated from soil and chitin flake samples collected from Yok Don National Park in the Central Highlands, Vietnam. These strains exhibited high specific activities of chitinases and formed biofilms. 16S rRNA gene analysis revealed that both strains had the closest evolutionary relationship to *Luteimicrobium album* with 99.41% sequence similarity and 100% query cover. Plate assays showed that the actinomyces had cellulolytic and amylolytic activities; insoluble zinc, potassium, and phosphate solubilization; and produced siderophores. UV-HPLC analysis revealed that the bacteria secreted phytohormones (IAA, GA3, and zeatin) into their cultures. Dual culture assay indicated that the bacteria possessed antifungal activity against *Fusarium oxysporum*. These analyses indicated that the isolated actinomyces are good candidates for further studies concerning crop production. These strains are also potent resources for further research on their genome sequences and systems of chitinases, cellulases, and amylases.

Keywords: Luteimicrobium album, chitinase activity, biofilm formation, plant-growth promotion, biocontrol.

Phân lập, nhận diện và đặc tính sinh học của xạ khuẩn sinh enzyme chitinase ở vườn quốc gia Yok Đôn

TÓM TẮT

Việc tạo ra phân bón sinh học mới là lĩnh vực rất được quan tâm trong nông nghiệp hiện đại. Nghiên cứu này tập trung vào xạ khuẩn có hoạt tính chitinase cao, tạo màng sinh học, thúc đẩy sinh trưởng và kháng nấm bệnh hại cây trồng. Trong nghiên cứu này, hai chủng xạ khuẩn YSS-3.3 và YWS-5.1 được phân lập từ các mẫu đất và giá thể chitin thu tại vườn quốc gia Yok Đôn ở Tây Nguyên. Các chủng này có hoạt tính chitinase cao và tạo màng sinh học. Phân tích trình tự gen 16S rRNA cho thấy chúng có quan hệ di truyền gần gũi nhất với loài *Luteimicrobium album*, với độ tương đồng trình tự 99,41% và độ phủ trình tự 100%. Phương pháp nuôi cấy trên đĩa thạch chỉ ra rằng các chủng xạ khuẩn sở hữu hoạt tính thủy phân cellulose và tinh bột; hòa tan kẽm, kali và phosphate khó tan; và tổng hợp siderophores. Phân tích bằng UV-HPLC cho thấy các chủng này cũng sản xuất hormone tăng trưởng cây trồng như IAA, GA3 và zeatin. Kỹ thuật nuôi cấy kép chỉ ra rằng các các chủng xạ khuẩn có hoạt tính đối kháng nấm *Fusarium oxysporum*. Từ những cơ sở trên, *L. album* YSS-3.3 và *L. album* YWS-5.1 là các ứng cử viên tốt cho các nghiên cứu tiếp theo liên quan đến ứng dụng trong nông nghiệp. Các chủng này cũng là nguồn nguyên liệu tiềm năng để thực hiện các nghiên cứu sâu về trình tự bộ gen, hệ thống chitinase, cellulase và amylase của chúng.

Từ khóa: Luteimicrobium album, hoạt tính chitinase, màng sinh học, thúc đẩy sinh trưởng cây trồng, kiểm soát sinh học.

1. INTRODUCTION

Chitin, the second most common biopolymer in nature after cellulose, is an insoluble polymer of β-(1-4)-N-acetyl-D-glucosamine (GlcNAc). It is the primary portion of cell walls in fungi, crustacean shells, insect exoskeletons, and other invertebrates. Chitinases (EC 3.2.1.14) are glycosyl hydrolases that hydrolyze chitin polymers

into small oligosaccharides, primarily *N,N'*-diacetylchitobiose.

Actinobacteria are gram-positive bacteria that are widely distributed in both terrestrial and aquatic ecosystems. Actinobacteria secrete various extracellular enzymes involved in degrading organic compounds and biopolymers. They play an important role in carbon cycling, especially regarding the hydrolysis of fungal cell walls and insect cuticles. Hence, these bacteria have been applied in crop production (Lacombe-Harvey *et al.*, 2018). Chitinase genes from a wide range of bacteria, including actinobacteria, have been identified and expressed, and their biological properties have been characterized in detail. Among these, numerous studies have shown bacterial chitinases and chitinolytic bacteria play a significant role in the control of phytopathogens by digesting chitin in fungal cell walls and nematode eggshells (Tsujibo *et al.*, 2000; Itoh *et al.*, 2003; Kawase *et al.*, 2004; Kawase *et al.*, 2006; Pentekhina *et al.*, 2020; Tran *et al.*, 2022a, b). In addition to chitinases, chitinolytic bacteria also produce extracellular lytic enzymes, substances that suppress phytopathogenic fungi and parasitic nematodes, and substances that exhibit plant growth-promoting traits (Lee *et al.,* 2014; Gu *et al.,* 2017; Trinh *et al.,* 2019). Therefore, chitinolytic bacteria are frequently used in agricultural production for biological control and as plant growth-promoting agents.

Vietnam is the world's largest black pepper and second-largest coffee producer. The Central Highlands region is the center of the nation's coffee and black pepper production. Currently, farmers in this area typically utilize chemical fertilizers to increase the yields of these products. According to various reports, chemical fertilizers decrease soil fertility and microorganisms, are more resistant to the environment, and pollute groundwater. For example, nitrogen fertilizers break down in the soil, convert into nitrates that are soluble in water, and easily pass through the soil. They can remain in that position for many years and can cause problems such as surface and groundwater pollution (Lin *et al.*, 2019). Our most recent research has shown that chitinolytic bacteria are effective agents in the production of coffee and black pepper in the Central Highlands. However, these bacteria were isolated from paddy soils in this area, and as such, they are known bacteria that have been extensively studied and widely utilized in agricultural cultivation, including *Bacillus subtilis*, *B. velezensis*, and *B. cereus* (Nguyen *et al.,* 2021a, b). Hence, to develop a new biofertilizer, in this study, chitinolytic actinomyces from Yok Don National Park in the Central Highlands were isolated, identified, and characterized.

2. MATERIALS AND METHODS

2.1. Sampling, isolation, and screening of chitinolytic actinobacteria

Sampling, isolation, and screening of chitinolytic actinobacteria were conducted as described by Tran *et al.* (2018c) with some modifications. Briefly, five soil samples (150-200g each) were collected at various places in Yok Don National Park, Dak Lak Province. At the same time, four nylon nets containing 10 g each of chitin flakes were immersed in the Serepok River water at different sites and recovered after 7 days of immersion. The soil and chitin flake samples (0.5g each) were inoculated into 100ml of synthetic medium (g/l: 0.85g KH_2PO_4, 5g $(NH_4)_2SO_4$, 0.5g $MgSO_4$, 0.15g K_2HPO_4, 0.1g $CaCl_2$, 0.1g NaCl) containing 0.2% chitin flakes for 2 days at 30°C and 150rpm. After that, 0.2g of chitin flakes from each culture were transferred into fresh synthetic medium. This inoculation was repeated four times to enrich the strong chitin-degrading bacteria. Finally, the chitinolytic actinobacteria were isolated and purified on synthetic agar medium containing 0.2% colloidal chitin as the sole carbon source. Strains that grew fast and formed large halo zones around their colonies on the colloidal chitin agar were chosen for the next examinations.

2.2. Measurement of specific activity of chitinases and protein content

Single colonies of the two selected actinomyces strains were respectively grown in the synthetic medium containing 0.2% colloidal chitin for 3 days (30°C, 150rpm). Bacterial cells were separated by centrifugation (9,000rpm, 5 min, 4°C), and the supernatant was dialyzed against 20mm sodium phosphate buffer (pH 6.0) overnight at 4°C. The dialyzed protein solutions were used for measuring the protein content and specific activity of the chitinases, as described previously (Tran *et al.,* 2018c). Briefly, a reaction mixture (600µl) containing 0.1% colloidal chitin and 300µl of crude enzyme in 20 mM sodium phosphate buffer (pH 6.0) was incubated for 25 min at 37°C, and the amount of reducing sugars released was measured. The protein concentration was measured using the Bradford method with bovine serum albumin as the standard.

2.3. Biofilm formation

Overnight cultures of the two isolated actinomyces (2µl each) were respectively inoculated into 96-well microtiter plates containing 200µl of Luria-Bertani (LB) medium (g/l: 5g yeast extract, 5g NaCl, 10g polypepton; pH 7.5) and incubated at 26°C without shaking. After 24 h of incubation, the biofilms were evaluated as described previously (Tran *et al.,* 2018c). Briefly, the medium was discarded, and the wells were rinsed with water three times. The cells bound to the wells were stained with 1.0% crystal violet for 2 min and rinsed three times with water. The dye was then solubilized with 33% acetic acid. Finally, biofilm formation was measured by examining the absorbance at 630 nm using a microplate absorbance reader (Bio-Rad, USA).

2.4. Amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene sequence

Preparation of the genomic DNA, amplification of the nearly full-length sequence of the 16S rRNA gene, sequencing of the amplified DNA, and phylogenetic analysis were performed as described previously (Tran *et al.,* 2018c), with some modifications. Briefly, each isolate was cultured in 5ml of LB medium for 16 h (30°C, 150rpm). The genomic DNA of each strain was isolated by boiling for 5 min and centrifugating (13,000rpm, 1 min, 4°C), and then used as a template for PCR amplification. A nearly full-length fragment of the 16S rRNA gene sequence of each isolate was amplified using the primers (27f-YM: 5′-AGAGTTTGATYMTGGCTCAG-3′ and 1492r: 5′-TACCTTGTTACGACTT-3′) and Phusion high-fidelity DNA polymerase (Thermo Scientific, USA) following the manufacturer's instructions. The reaction mixtures were incubated in a C1000 thermal cycler (Bio-Rad, USA) under the following programs: predenaturation at 98°C for 3 min; 40 cycles of denaturation at 98°C for 10s, annealing at 50°C for 20s, and extension at 72°C for 45s; and a final extension at 72°C for 5 min before cooling to 4°C. The amplified products were then separated by electrophoresis on 1.5% agarose gel. The target bands were cut out from the gel and purified using the Wizard SV Gel and Clean-Up Kit (Promega Co., USA). The purified products were sequenced by the 1st BASE company (Selangor, Malaysia). Finally, the nucleotide sequences obtained by sequencing were compared with those of the 16S rRNA genes available in the Genbank/DDBJ/EMBL databases to determine the taxonomic positions of the isolated strains. A phylogenetic tree was built by MEGA 6.0.

2.5. Extracellular hydrolytic enzymes and plant growth-promoting traits

Single colonies of each isolate were inoculated onto Dubos mineral salt agar plates (g/L: 0.5g $NaNO_3$, 1g K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.02g $FeSO_4 \cdot 7H_2O$, 0.2g KCl, and 20g agar; pH 7.5) containing 0.05% carboxymethyl cellulose. The plates were incubated at 30°C for 2 days and stained with 0.5% Congo red solution. The cellulolytic activity was indicated by halo zones formed around colonies. The cellulolytic activity index

was calculated based on the ratio of the halo zone diameter (mm) and the colony diameter (mm) (Eida *et al.,* 2012).

The amylolytic activity of each isolate was examined using a starch agar medium (g/l: 3g beef extract, 10g soluble starch, and 15g agar) as described previously (Cavite *et al.,* 2021). After 2 days of incubation at 30°C, each plate's surface was submerged with an iodine solution to detect the presence of starch. The amylolytic activity index was calculated based on the ratio of the halo zone diameter (mm) and the colony diameter (mm).

Zinc solubilization was evaluated by observing the halo zones formed around bacterial colonies when the isolates were grown on a mineral salt agar medium (g/l: 10 g D-glucose, 6.06 g Tris HCl, 4.68g NaCl, 1.49g KCl, 1.07g NH_4Cl, 0.43g Na_2SO_4, 0.2g $MgCl_2 \cdot 2H_2O$, 30mg $CaCl_2 \cdot 2H_2O$, and 15g agar) amended with 0.1% zinc oxide at 30°C for 3 days. The zinc solubilizing index was calculated based on the ratio of the halo zone diameter (mm) and the colony diameter (mm) (Gandhi *et al.,* 2016).

Potassium solubilization of isolates was examined by growing bacterial colonies on solid Aleksandrov agar medium (g/l: 5g glucose, 0.005g $MgSO_4 \cdot 7H_2O$, 0.1g $FeCl_3$, 2.0g $CaCO_3$, 3.0g potassium feldspar powder, 2.0g calcium phosphate, and 20g agar) at 30°C for 5 days. Then, the potassium solubilizing index was calculated based on the ratio of the halo zone diameter (mm) and the colony diameter (mm) (Sun *et al.,* 2020).

Phosphate solubilization was investigated based on the method described by Janati *et al.* (2022). Bacterial colonies were grown on the National Botanical Research Institute's phosphate growth medium (g/l: 10g glucose, 5g $Ca_3(PO4)_2$, 5g $MgCl_2 \cdot 6H_2O$, 0.25g $MgSO_4 \cdot 7H_2O$, 0.2g KCl, 0.1g $(NH_4)_2SO_4$, and 15 g agar) for 2 days at 30°C. The phosphate solubilizing index was calculated based on the ratio of the halo zone diameter (mm) and the colony diameter (mm).

Siderophore production by the isolates was tested following a modified version of the method described by Louden *et al.* (2011). Bacterial colonies were grown on Chrome Azurol S agar medium for 3 days at 30°C. The siderophore-producing index was calculated based on the ratio of the halo zone diameter (mm) and the colony diameter (mm).

The production and measurement of IAA, GA3, and zeatin followed the procedure described by Sharma *et al.* (2014) with modifications. Single colonies of chitinolytic actinomyces were respectively grown in LB medium containing tryptophan (5mm) at 30°C and 150 rpm for 5 days under dark conditions. The cultures were then centrifuged (14,000rpm, 4°C, 15 min), and the supernatants were used to measure phytohormones using a high-performance liquid chromatography system (Thermo Scientific, USA).

2.6. Antagonistic activity

The antagonistic activity of the isolated actinomyces was evaluated by measuring the inhibition of the growth of phytopathogenic *Fusarium oxysporum* F.TTN 02 on *potato dextrose* agar plates using the dual-culture plate assay, as described by Tran *et al.* (2018c).

3. RESULTS AND DISCUSSION

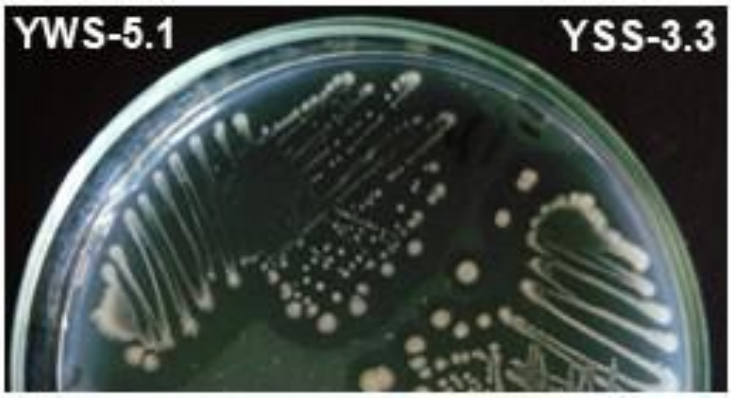
3.1. Isolation of chitinolytic actinomyces

In this study, more than 8,600 chitinolytic isolates formed halo zones around their colonies on the synthetic agar plates containing colloidal chitin. Two strains (YSS-3.3 and YWS-5.1), which formed larger halo zones and grew faster than the others, were picked for the next examinations (Figure 1). According to an assessment based on Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005), the colony morphology of these strains when grown on the synthetic agar plates containing colloidal chitin was round, smooth, white, pulvinate, and rhizoid.

3.2. Specific activity of chitinases and biofilm formation

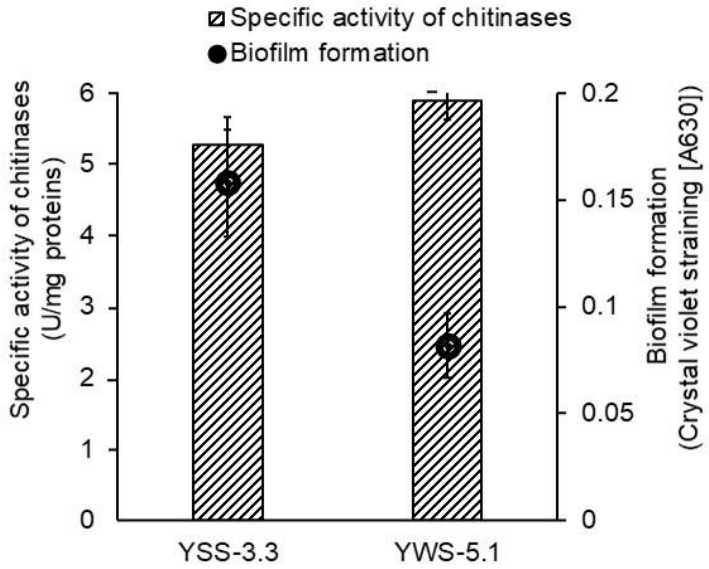
As shown in Figure 2, strains YSS-3.3 and YWS-5.1 exhibited high specific activities of

chitinases and formed biofilms at remarkable levels. Of the isolates, strain YWS-5.1 showed a higher specific activity of chitinases than strain YSS-3.3, although it had a lesser capacity to produce biofilms. The results of the specific activity of chitinases and biofilm formation of the chitinolytic isolates in this study were similar to those of chitinolytic bacteria isolated from a freshwater lake in Niigata, Japan (Tran *et al.,* 2018c). Microorganisms form biofilms by adhering to surfaces. Biofilm-forming bacteria can attach to fungal cell walls, develop cell density, and secrete secondary metabolites, such as antifungal substances. Additionally, metabolites may be coated by the biofilm, enhancing antagonistic activity against phytopathogenic fungi (Morikawa *et al.,* 2006; Seneviratne *et al.,* 2008). These analyses indicated that our isolates could have a role in crop production as biofilm-forming agents.



Note: Chitinolytic isolates were grown on synthetic agar plates containing 0.2% colloidal chitin as the sole carbon source at day 2 of incubation at 30°C. YSS-3.3, strain YSS-3.3; YWS-5.1, strain YWS-5.1.

Figure 1. Chitin-degrading zones by strains YSS-3.3 and YWS-5.1



Note: The specific activity of chitinases of each isolate in the culture supernatants was measured by a modified version of the Schales' procedure. Biofilm formation of the isolates formed in 96-well polystyrene microtiter plates containing LB medium was estimated by absorbance at 630 nm using a microtiter plate reader (Bio-Rad, USA). Data are the mean of triplicates and standard deviations. YSS-3.3, strain YSS-3.3; YWS-5.1, strain YWS-5.1.

Figure 2. The specific activity of chitinases and biofilm formation of isolated actinomyces

3.3. 16S rRNA gene amplification, sequencing, and phylogenetic analysis

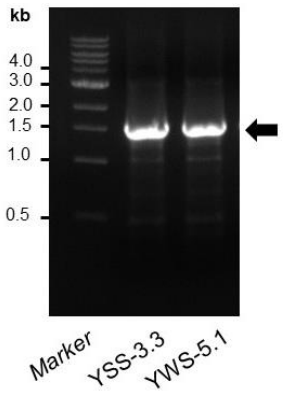
Figure 3 shows that the nearly full-length sequence (approximately 1500 bp) of the 16S rRNA gene of the two isolated strains was successfully amplified by PCR.

The target products were then recovered and purified using the Wizard SV Gel and Clean-Up Kit (Promega, USA). After sequencing, nucleotide sequences (1350 bp each) of the 16S rRNA gene of strains YSS-3.3 and YWS-5.1 were obtained. Analysis using the Blastn program (https://blast.ncbi.nlm.nih.gov/Blast) showed that both sequences showed the highest identity (99.41%) (100% coverage) to that of *Luteimicrobium album* RI148-Li105 (GenBank accession NR_108122.1). Furthermore, a phylogenetic analysis (Figure 4) showed that our strains had the closest evolutionary relationship to *L. album* with a 93% bootstrap confidence percentage. These analyses implied that the isolated actinomyces belong to species *L. album*. The determined nucleotide sequences in this study were deposited in the DDBJ/GenBank/EMBL databases under accession numbers LC771089.1 and LC771090.1.

According to Hamada *et al.* (2012), *L. album* is a gram-positive bacterium that belongs to the genus *Luteimicrobium*, the family *Micrococcineae*, and the class *Actinobacteria*. To date, only two works regarding *L. album* have been documented. One of them described the taxonomy of the species (Hamada *et al.,* 2012), while the other revealed that *L. album* possesses a novel protein-asparaginase (Miwa *et al.,* 2019). However, research on the genome sequence, chitinase molecules, and plant growth promotion of this species have yet to be published. These analyses indicate that our isolated actinomyces have potential for subsequent characterizations concerning their secondary metabolites, genome sequences, and roles in crop production.

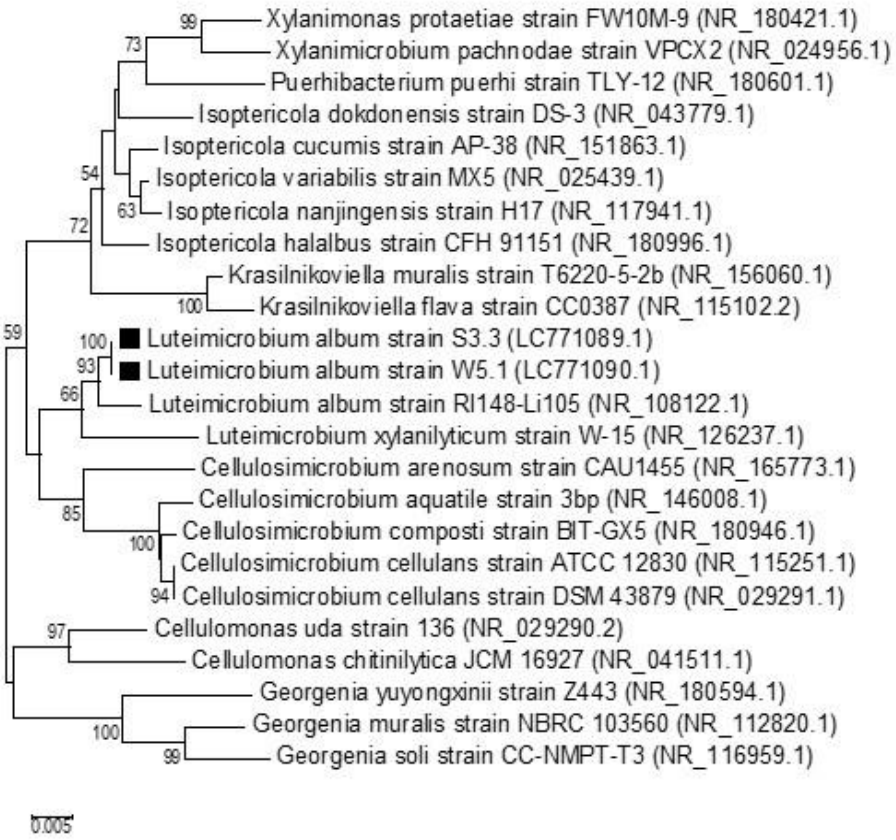
3.4. Extracellular hydrolytic enzymes and plant growth-promoting traits

Figure 5A reveals that *L. album* YSS-3.3 and *L. album* YWS-5.1 possess cellulolytic and amylolytic activities. Among them, both strains exhibited higher cellulolytic activity compared to their amylolytic activity. Cellulase-producing microorganisms play an important role in the hydrolysis of lignocellulosic polymers. In agriculture, these microorganisms can treat agricultural wastes containing cellulose and convert them into biofertilizers (Juturu and Wu, 2014). Starch molecules are too large to enter the bacterial cell membrane. Various bacteria produce amylases to split starch molecules into small fragments as nutrient sources, and therefore have potential applications for crop production (Paul *et al.,* 2021). Hence, our isolated actinomyces might be good candidates for later assessments concerning crop production. Further studies are necessary to identify, express the cellulase and amylase genes, and characterize the recombinant enzymes of these strains.



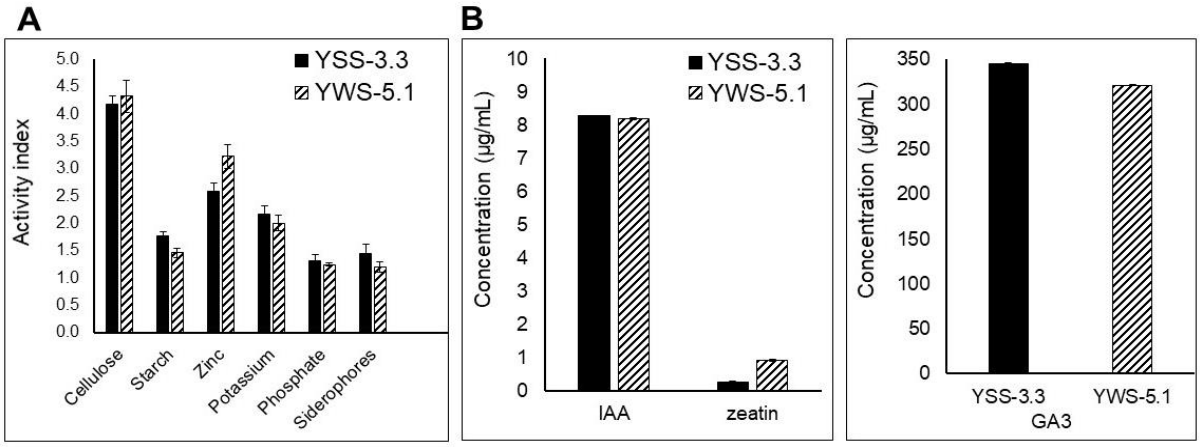
Note: The arrow indicates the target PCR product. kb, kilobase; YSS-3.3, strain YSS-3.3; YWS-5.1, strain YWS-5.1.

Figure 3. Amplification of the 16S rRNA gene of the chitinolytic isolates by PCR



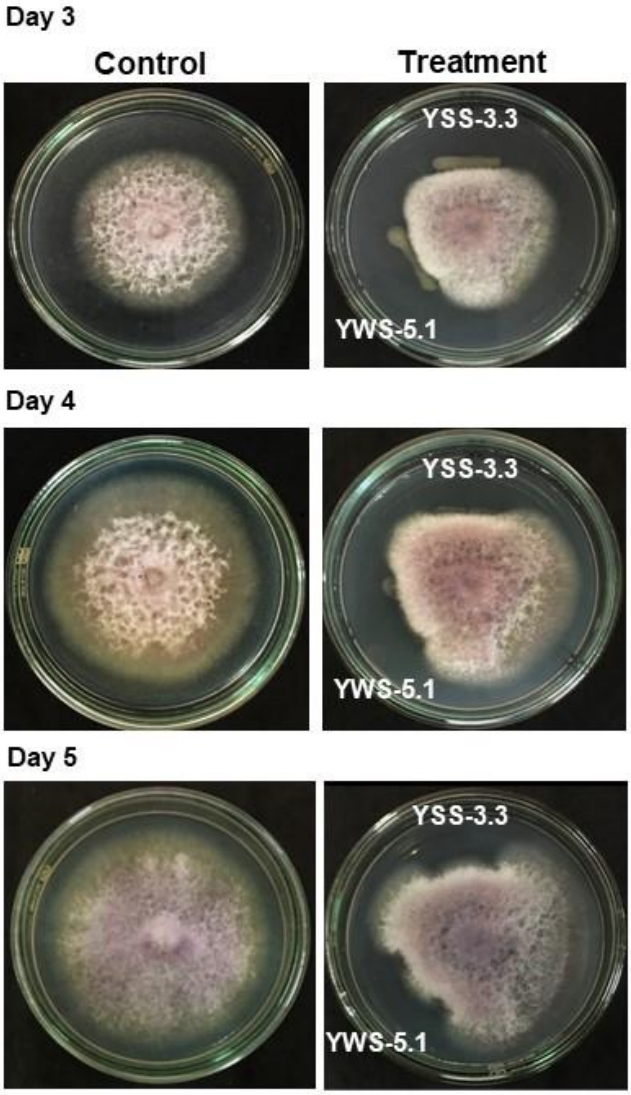
Note: Accession numbers for the 16S rRNA gene sequences used are given in parentheses after the species and strain names. The phylogenetic tree was built using MEGA 6.0. The numbers at the branches are bootstrap confidence percentages (%) based on 1000 resampled data sets, and only bootstrap values ≥50% are shown. Bar, 0.005 substitutions per nucleotide position.

Figure 4. Phylogenetic relationships among the isolated actinomyces in this study (filled rectangles) **and known actinomyces**



Note: YSS-3.3, L. album YSS-3.3; YWS-5.1, L. album YWS-5.1. Data are the mean of triplicates and standard deviations.

Figure 5. Extracellular hydrolytic enzymes and plant growth-promoting traits produced by the isolated actinomyces



Note: The hyphal growth of F. oxysporum F.TTN 02 on the PDA plates in the absence (Control) and presence (Treatment) of the isolated actinomyces on day 5 of incubation at 30°C. YSS-3.3, L. album YSS-3.3; YWS-5.1, L. album YWS-5.1. The experiments were performed in triplicate.

Figure 6. The antagonistic abilities of the isolated actinomyces against the hyphal growth of *Fusarium oxysporum* F.TTN 02 using the dual-culture assay

As shown in Figure 5A, the two isolated actinomyces can solubilize insoluble zinc, potassium, and phosphate sources at remarkable levels. These strains showed higher solubilizing activity against zinc than potassium and phosphate sources. Micronutrients, including zinc, potassium, and phosphate, have been reported to be essential for the growth and development of a wide range of crops. However, only 1% to 2% of these minerals are in soluble forms for plant uptake, the rest being bound with other minerals and hence in insoluble forms (Sun *et al.,* 2020; Othman *et al.,* 2022; Amri *et al.,* 2023). This analysis indicated that our isolates would be good candidates for treating insoluble zinc, potassium, and phosphate sources used in agricultural cultivation.

Siderophore production was examined by growing the chitinolytic actinomyces on CAS agar plates. The results showed that both strains produced siderophores (Figure 5A).

Siderophore production is one of the important characteristics of bacteria. Siderophore-producing bacteria can increase the availability of iron near roots to promote plant growth (Ghavami *et al.*, 2017). This analysis implied that our isolated actinomyces may have a positive effect on plant growth.

The two isolated actinomyces were examined for IAA, GA3, and zeatin production. As shown in Figure 5B, both strains secreted IAA, GA3, and zeatin into the culture supernatant. It has been reported that bacterial phytohormones (cytokinins, auxins, abscisic acid, jasmonic acid, gibberellins, and salicylic acid) are important in plant growth and development, and phytohormone-producing bacteria have been used for agricultural cultivation as biofertilizers (Kudoyarova *et al.*, 2015). Therefore, our isolated actinomyces may play a role for crop production as biofertilizers.

3.5. Antagonistic activity

Figure 6 shows that *L. album* YWS-5.1 and *L. album* YSS-3.3 inhibited the growth of the phytopathogenic fungus *F. oxysporum* F.TTN 02 by 30.6% and 31.9%, respectively, on day 5 of examination, indicating that our isolated actinomyces possess antifungal activities.

4. CONCLUSIONS

L. album YSS-3.3 and *L. album* YWS-5.1 have great potential for future evaluation and development as biofertilizers. These bacteria possess good bio-properties for plant growth, including high chitinase activity and biofilm production; cellulolytic and amylolytic activities; insoluble zinc, potassium, and phosphate solubilization; siderophores; phytohormones (IAA, GA3, and zeatin); and antifungal activity. Further studies are necessary to characterize these actinomyces in the greenhouse and field in terms of their genome sequences, and cellulase, amylase, and chitinase molecules.

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