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Postprint: Culturable Bacterial Diversity and Biological Activity of Acanthus ebracteatus

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Abstract

Acanthus ebracteatus is a rare true mangrove plant inhabiting mangrove ecosystems with considerable medicinal value. This study aimed to investigate the diversity of culturable endophytic and rhizosphere bacteria associated with A. ebracteatus, and to explore potential novel species and strains with special biological activities. Using seven different culture media, culturable bacteria from various plant tissues and rhizosphere soil were isolated via traditional dilution plating. The community structure and diversity characteristics of endophytic and rhizosphere bacteria were analyzed based on 16S rRNA gene sequences, and the antimicrobial activity of culturable bacteria was evaluated using plate confrontation assays against plant pathogens and spread predation activity tests. The results demonstrated that: (1) Based on 16S rRNA gene sequence analysis, a total of 144 culturable bacterial strains were isolated from the roots, stems, leaves, flowers, and rhizosphere soil of A. ebracteatus. These bacteria belonged to 66 species, 37 genera, 26 families, and 18 orders, with Bacillus and Streptomyces being the dominant genera, accounting for 15.1% and 13.6% of the bacterial species, respectively. (2) Antagonistic tests against multiple plant pathogens revealed that 29 bacterial strains exhibited antagonistic activity against plant pathogens, with 10 strains displaying broad-spectrum antimicrobial activity. Streptomyces strains demonstrated the strongest antagonistic effects, and strain Y129 was identified as a potential novel species. (3) Predation activity tests indicated that five bacterial strains exhibited predatory activity against Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA), and Escherichia coli. Pseudomonas strains showed the strongest predatory activity, with strain Y90 being a potential novel species. In conclusion, the mangrove plant Acanthus ebracteatus and its rhizosphere soil harbor abundant bacterial germplasm resources with diverse biological activities, which can serve as a source of biocontrol agents and medicinal bacteria. These findings also provide a foundation for improving the medicinal efficacy and cultivation

of the mangrove plant Acanthus ebracteatus.

Full Text

Diversity and Biological Activity of Culturable Bacteria from *Acanthus ebracteatus*

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Abstract

Acanthus ebracteatus is a rare true mangrove plant species in the mangrove ecosystem with significant medicinal value. This study investigated the diversity of culturable endophytic and rhizosphere bacteria from *A. ebracteatus* to discover potential novel species and strains with special biological activities. Using seven different culture media, traditional dilution plating was employed to isolate culturable bacteria from various plant tissues and rhizosphere soil. Based on 16S rRNA gene sequencing, the community structure and diversity of endophytic and rhizosphere bacteria were analyzed. Antimicrobial activity was evaluated through plate confrontation assays against plant pathogens and lawn predation activity tests. The results showed: (1) Based on 16S rRNA gene sequence analysis, a total of 144 culturable bacterial strains were isolated from roots, stems, leaves, flowers, and rhizosphere soil, belonging to 66 species, 37 genera, 26 families, and 18 orders. *Bacillus* and *Streptomyces* were the dominant genera, accounting for 15.1% and 13.6% of the bacterial species, respectively. (2) Antagonistic activity tests against multiple plant pathogens revealed

29 strains with antagonistic activity, including 10 strains with broad-spectrum antimicrobial activity. *Streptomyces* strains showed the strongest antagonistic effects, with strain Y129 identified as a potential novel species. (3) Predation activity tests identified five strains capable of preying on *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), and *Escherichia coli*, with *Pseudomonas* strains showing the strongest predatory activity, and strain Y90 identified as a potential novel species. In conclusion, the mangrove plant *A. ebracteatus* and its rhizosphere soil harbor rich bacterial germplasm resources with diverse biological activities, representing a valuable source of biocontrol and medicinal bacteria. These findings also provide a foundation for improving the medicinal efficacy and cultivation of *A. ebracteatus*.

Keywords: *Acanthus ebracteatus*, culturable bacteria, plant pathogens, predation activity

Introduction

Acanthus ebracteatus is a rare and endangered true mangrove plant belonging to the family Acanthaceae and genus *Acanthus*, primarily distributed in the intertidal zones of mangrove forests in Hainan, Guangdong, and Guangxi, China (China Flora Compilation Committee, 2004). Its fruits possess detoxifying and anti-swelling properties, leaf extracts exhibit neuroprotective effects, and roots are used in treating hepatitis B (Chen et al., 2019; Editorial Committee of Chinese Materia Medica, 1999). Over the past decade, overexploitation of mangrove forests has led to reduced species diversity, with nearly one-third of mangrove plant species now endangered, including *A. ebracteatus*. Huang et al. (2020) investigated the distribution and population characteristics of this species in Guangxi, finding fewer than 2,000 individuals existing only on the southwestern side of Jiangshan Peninsula in Fangchenggang City and in the midstream tidal flats of the Huangzhu River.

Endophytic bacteria colonize plant tissues either partially or entirely and are closely associated with plant growth, development, environmental adaptation, stress resistance, and even medicinal activity. Some endophytic bacteria produce active compounds identical or similar to those of their host plants (Lin, 2009), making medicinal plant endophytes a valuable source of antibiotics and biopesticides. In recent years, researchers have shown great interest in exploring the biological activities of bacteria from mangrove habitats, including antithrombotic (Li et al., 2020), anti-aging (Li et al., 2020), antagonism against plant pathogens (Li et al., 2021; Yan et al., 2018), antitumor (Liang et al., 2006), heavy metal tolerance (De La Rosa-Acosta et al., 2015), and polycyclic aromatic hydrocarbon degradation (Guo et al., 2005). Thus, culturable endophytic and rhizosphere bacteria from mangrove plants not only exhibit rich diversity but also represent an ideal resource library for medicinal and biocontrol agents.

Clinically, multidrug-resistant bacterial infections pose a serious threat to human health, characterized by broad resistance spectra and high toxicity, with antibiotic abuse exacerbating treatment difficulties (Chen et al., 2010; Wang et al., 2020). Consequently, discovering novel medicinal resources is crucial. Predatory bacteria can obtain energy and nutrients by "devouring"other bacteria under nutrient-deficient or competitive conditions (Kadouri et al., 2013; Negus et al., 2017). Arend et al. found that *Herpetosiphon* strain CA052B could prey on ten clinically relevant pathogens including *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *S. aureus*. Paul et al. demonstrated that *Myxococcus xanthus* employs multifactorial mechanisms to kill and degrade diverse prey. Gupta and Monnappa et al. showed that some predatory bacteria are obligate bacterial predators that do not harm mammalian cell lines (Gupta et al., 2016; Monnappa et al., 2016). Safety of predatory bacteria in vivo has been validated in mice, rabbits, guinea pigs, and chickens (J M et al., 1977; Atterbury et al., 2011; Shatzkes et al., 2017), indicating their potential as "live antibiotics."

In agriculture, epidemics caused by harmful microorganisms such as *Fusarium oxysporum* f. sp. *cubense* (banana wilt) and *Colletotrichum gloeosporioides* (mango anthracnose) significantly impact crop production and economic development. Current control measures include cultivation management, breeding resistant varieties, agricultural practices, chemical control, physical control, and biological control, with chemical control being predominant. However, widespread chemical pesticide use has damaged agricultural ecosystems (Zhan et al., 2005; Xu et al., 2007) and led to antimicrobial resistance, necessitating new control strategies (Huang et al., 2021). Biological control is widely considered the most promising approach due to its low toxicity and minimal environmental impact (Zhang et al., 2010; Wang et al., 2017).

This study used *A. ebracteatus* collected from Hainan mangrove forests as research material. Traditional dilution plating was employed to isolate culturable bacteria from plant tissues and rhizosphere soil, with activity tested against multiple plant pathogens and human pathogenic bacteria. The study aimed to address: (1) community structure and diversity of culturable endophytic and rhizosphere bacteria from *A. ebracteatus*; (2) different biological activities of culturable bacteria; and (3) preliminary identification of antimicrobial capabilities and functional enzyme activities of active strains to obtain strains with potential medicinal value, laying a foundation for conservation breeding and novel biomedical development of *A. ebracteatus*.

Materials and Methods

Sample Collection

Acanthus ebracteatus samples were collected from the Dongzhai Harbor Mangrove Conservation Center in Haikou City (11°34' 47" E, 19°57' 1" N). Roots, stems, leaves, flowers, and rhizosphere soil (10 cm depth) were collected separately, sealed in bags, stored at 4°C, and transported to the laboratory for pretreatment.

Indicator Strains

Plant pathogens: *Fusarium oxysporum* f. sp. *cubense* race 1 (Tr1) and race 4 (Tr4), *Colletotrichum musae*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, and *Botryosphaeria dothidea* were provided by Dr. Li Qili from Guangxi Academy of Agricultural Sciences.

Human pathogens: *Staphylococcus aureus* ATCC6538 and methicillin-resistant *S. aureus* ATCC43300 were obtained from the American Type Culture Collection (ATCC); *Escherichia coli* CMCC(B)44102 was obtained from the National Center for Medical Culture Collections (CMCC).

Culture Media

(1) Isolation Media

- **AGG**: Soluble starch 10 g, glucose 1 g, glycerol 5 mL, composite salt solution 10 mL, agar 15 g, deionized water 1,000 mL
- **ISP7**: Glycerol 15 mL, L-tyrosine 0.5 g, L-asparagine 1 g, composite salt solution 10 mL, agar 15 g, deionized water 1,000 mL, pH 7.2-7.4
- **ISP3**: Soluble oat powder 20 g, composite salt solution 10 mL, agar 15 g, deionized water 1,000 mL
- **M7**: Yeast extract 5 g, L-asparagine 1 g, glycerol 10 mL, composite salt solution 10 mL, agar 15 g, deionized water 1,000 mL, pH 7.2-7.4
- **M5**: Trehalose 5 g, proline 1 g, composite salt solution 10 mL, agar 15 g, vitamin solution 1 mL, deionized water 1,000 mL, pH 7.2-7.4
- **Humic acid medium**: Humic acid 1 g, $CaCO_3$ 0.02 g, Na_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, KCl 1.7 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 15 g, deionized water 1,000 mL, pH 7.2-7.4
- **M10**: Starch 10 g, hydrolyzed casein 0.5 g, composite salt solution 10 mL, agar 15 g, deionized water 1,000 mL

Composite salt solution: KNO_3 1 g, NaCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, K_2HPO_4 0.5 g, NH_4NO_3 0.1 g, $FeSO_4$ 0.01 g, $MnCl_2 \cdot H_2O$ 0.001 g, $ZnSO_4 \cdot 7H_2O$ 0.001 g, deionized water 10 mL (Note: K_2HPO_4 solution must be prepared separately and added after autoclaving)

Inhibitors: Potassium dichromate and cycloheximide solutions were prepared separately, filtered through 0.22 m sterile filters, and added to media at final concentrations of 25 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$, respectively.

Modified ISP2 medium: Yeast extract 2 g, malt extract 2 g, anhydrous glucose 2 g, deionized water 1,000 mL.

(2) Indicator Strain Media

- **LB medium**: Tryptone 10 g, yeast extract 5 g, NaCl 10 g, deionized water 1,000 mL, pH 7.2-7.4

- **PDA medium**: Potato 200 g, glucose 20 g, agar 15 g, deionized water 1,000 mL

(3) Predation Activity Test Medium

- **TPM medium**: 1 mL 1 $\text{mol} \cdot \text{L}^{-1}$ Tris-HCl solution, 1 mL 0.8 $\text{mol} \cdot \text{L}^{-1}$ $MgSO_4 \cdot 7H_2O$ solution, pH adjusted to 7.6 with 1 $\text{mol} \cdot \text{L}^{-1}$ KH_2PO_4 solution

(4) Enzyme Activity Screening Media

- **Esterase screening medium**: Tween 20, Tween 60, or Tween 80 as substrates (1% w/v), tryptone 1 g, deionized water 1,000 mL, agar 15 g
- **Urease screening medium**: 30% urea solution as substrate (2% v/v), tryptone 1 g, NaCl 5 g, glucose 1 g, KH_2PO_4 2 g, phenol red 0.012 g, agar 15 g, deionized water 1,000 mL, pH 6.8-6.9

Media for cellulase, chitinase, amylase, and protease screening were prepared according to literature methods (Song et al., 2018; Roberts & Selitrennikoff, 1988; Zhao et al., 2018).

Sample Pretreatment

(1) Plant tissues: Surface soil on *A. ebracteatus* tissues was rinsed with sterile water. Surface sterilization was performed following Li et al. (2017): sequential immersion in 5% sodium hypochlorite for 3 min, 0.2% Tween 20 for 3 min, and 75% ethanol for 3 min, with three sterile water rinses after each treatment. Sterilized tissues were air-dried, cut into 1 cm × 1 cm pieces with a sterile scalpel, ground thoroughly, and suspended in 1 mL sterile water to prepare stock solutions.

(2) Rhizosphere soil: Soil samples were spread evenly in ceramic evaporating dishes and heat-shocked at 90°C for 30 min. One gram of treated soil was suspended in 9 mL sterile water to prepare stock solutions.

Strain Isolation and Pure Culture

Stock solutions of plant tissues and rhizosphere soil were diluted to 10^{-3} and 10^{-4} with sterile water. Aliquots (0.2 mL) of diluted suspensions were spread-plated on seven different isolation media and incubated at 28°C for 7-28 days. Colony morphology and numbers were recorded, and distinct colonies were numbered and streaked onto modified ISP2 medium for purification. Pure strains were maintained on ISP2 slants at 4°C and as 30% (v/v) glycerol stocks at -80°C.

16S rRNA Phylogenetic Analysis and Strain Identification

DNA was extracted using the Chelex-100 method (Zhou et al., 2010). A 0.1 mL aliquot of sterilized 20% Chelex-100 resin solution was mixed with a small amount of pure culture cells using a sterile toothpick. The mixture was vortexed, heated at 100°C for 10 min, and centrifuged to pellet the Chelex-100 particles. The supernatant was used as PCR template. Primers 27F (5' -AGAGTTTGATCCTGGCTCAG-3') and 1522R (5' -AAGGAGGTGATCCAGCCGCA-3') were used for amplification following Walsh et al. (1991). PCR conditions: initial denaturation at 95°C for 8 min, followed by 32 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 90 s. Amplicons were verified by 1% agarose gel electrophoresis, purified, and cloned into pGM-18T vector using *E. coli* Trans10 competent cells. Clones were screened using M13F (5' -GTTTTCCCAGTCACGA-3') and M13R (5' -CAGGAAACAGCTATGA-3') primers. Positive clones were sequenced by Shanghai Sangon Biotech. Sequences were analyzed using BioEdit and DNAstar software, compared against the EZbiocloud database (https://www.ezbiocloud.net/), and phylogenetic trees were constructed using MEGA 10.0 with Neighbor-Joining and Maximum-Likelihood methods based on 1,000 bootstrap replicates (Tamura et al., 2011).

Antagonistic Activity Against Plant Pathogenic Fungi

Plate confrontation assays were performed against six indicator plant pathogens. Four culturable bacterial strains were inoculated by spot-inoculation 2 cm from the edge of PDA plates. Fungal pathogen discs (5 mm diameter) were placed at the center. PDA plates inoculated only with pathogens served as controls. All treatments were performed in triplicate and incubated at 28°C. Growth and inhibition were recorded, and inhibition rates were calculated:

Inhibition rate = (Control colony radius - Treatment colony radius) / Control colony radius × 100%

Predation Activity Test

Staphylococcus aureus, MRSA, and *E. coli* were cultured in LB broth at 37°C, 180 rpm for 24 h. Cells were harvested by centrifugation, washed three times with TPM broth, and resuspended to 10^6 $\text{CFU} \cdot \text{mL}^{-1}$. Aliquots (0.3 mL) were spread on TPM agar plates. Test strains were spot-inoculated on predator-containing plates, with predator-free TPM plates as controls. All experiments were performed in triplicate and incubated at 28°C for 7 days. Predation zones were measured.

Enzyme Activity Identification

Positive strains from antagonistic and predation assays were screened for enzyme activities. Strain discs (5 mm diameter) were inoculated onto various enzyme screening media and incubated at 28°C for 2-7 days. Presence of clear zones indicated positive enzyme activity.

Results

Diversity of Endophytic and Rhizosphere Bacteria from *A. ebracteatus*

A total of 144 bacterial strains were isolated from *A. ebracteatus* samples collected from Dongzhai Harbor Mangrove Conservation Center. Based on 16S rRNA gene sequencing of selected strains, 66 bacterial species were identified, belonging to 37 genera, 26 families, and 18 orders (Table 1). Plant tissues yielded 46 species (17 orders, 19 families, 28 genera), while rhizosphere soil yielded 37 species (14 orders, 19 families, 21 genera). Phylogenetic analysis revealed that *Bacillus* and *Pseudomonas* were the dominant genera in both endophytic and rhizosphere bacterial communities, with *Streptomyces* also being dominant in roots and rhizosphere soil (Figs. 1 and 2).

Table 1 Diversity distribution of endophytic and rhizosphere bacteria from *Acanthus ebracteatus*

Order	Family	Genus	Number of Species
Bacillales	Bacillaceae	*Bacillus*	
Brevibacteriales	Brevibacteriaceae	*Brevibacterium*	
		Fictibacillus	
		Metabacillus	
		Neobacillus	
Burkholderiales	Comamonadaceae	*Comamonas*	
		Diaphorobacter	
Demequinales	Demequinaceae	*Demequina*	
Enterobacterales	Enterobacteriaceae	*Klebsiella*	
	Erwiniaceae	*Erwinia*	
		Delftia	
		Pantoea	
		Rosenbergiella	
	Morganellaceae	*Providencia*	
	Yersiniaceae	*Serratia*	
Lysobacterales	Lysobacteraceae	*Stenotrophomonas*	
Microbacteriales	Microbacteriaceae	*Agromyces*	
		Xanthomonas	
		Microbacterium	
Micrococcales	Micrococcaceae	*Micrococcus*	
Micromonosporales	Micromonosporaceae	*Micromonospora*	
Mycobacteriales	Gordoniaceae	*Gordonia*	
	Mycobacteriaceae	*Mycolicibacterium*	
	Nocardiaceae	*Nocardia*	
	Nocardioidaceae	*Nocardioides*	
Pseudomonadales	Moraxellaceae	*Acinetobacter*	
		Moraxella	

Order	Family	Genus	Number of Species
	Pseudomonadaceae	*Pseudomonas*	
	Acuticoccaceae	*Acuticoccus*	
	Aurantimonadaceae	*Jiella*	
Rhizobiales	Rhizobiaceae	*Martelella*	
		Neorhizobium	
Sneathiellales	Ferrovibrio_f	*Ferrovibrio*	
Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	
Streptomycetales	Streptomycetaceae	*Streptomyces*	
Streptosporangiales	Thermomonosporaceae	*Actinomadura*	
Pseudonocardiales	Pseudonocardiaceae	*Pseudonocardia*	

Fig. 1 Neighbor-Joining phylogenetic tree of endophytic bacteria based on 16S rRNA gene sequences

Fig. 2 Neighbor-Joining phylogenetic tree of rhizosphere soil bacteria based on 16S rRNA gene sequences

Analysis of bacterial distribution across different tissues (Fig. 3) showed that rhizosphere soil yielded the highest number of bacterial taxa (21 genera, 37 species), followed by roots, while flowers contained the fewest endophytic bacteria (8 genera, 9 species). Plant tissues and rhizosphere soil shared 12 genera: *Acinetobacter*, *Acuticoccus*, *Bacillus*, *Gordonia*, *Klebsiella*, *Microbacterium*, *Micromonospora*, *Mycolicibacterium*, *Novosphingobium*, *Pseudomonas*, *Pseudonocardia*, and *Streptomyces*.

Regarding tissue-specific distribution, rhizosphere soil uniquely contained *Brevibacterium*, *Diaphorobacter*, *Fictibacillus*, *Jiella*, *Metabacillus*, *Nocardia*, *Nocardioides*, *Rosenbergiella*, and *Serratia*. Plant tissues uniquely harbored endophytes belonging to *Actinomadura*, *Agromyces*, *Comamonas*, *Delftia*, *Demequina*, *Erwinia*, *Ferrovibrio*, *Martelella*, *Micrococcus*, *Moraxella*, *Neobacillus*, *Neorhizobium*, *Pantoea*, *Providencia*, *Stenotrophomonas*, and *Xanthomonas*.

Fig. 3 Number of bacteria isolated from different plant tissues and rhizosphere soil

Fig. 4 Venn diagram of genus-level classification of endophytic and rhizosphere bacteria from *Acanthus ebracteatus*

Bioactivity Testing of Strains

Plate confrontation assays identified 29 strains capable of inhibiting at least one plant pathogen, with an overall positive rate of 43.9%. Ten strains showed broad-spectrum activity against four or more plant pathogens. Predation activity tests using oligotrophic medium identified five strains capable of preying on all three human indicator pathogens.

Cross-measurement of inhibition zones was used to analyze the antimicrobial capacity of 10 strains with broad-spectrum antifungal activity and 5 predatory strains. As shown in Fig. 7, strains Y108, Y263, Y553, and Y130 exhibited significant activity, achieving >30% inhibition against all six plant pathogens. Strains Y134, Y133, and Y454 showed relatively weaker antagonistic activity. Regarding specific pathogen inhibition, Y108 strongly inhibited *Botryosphaeria dothidea* and *Colletotrichum musae*; Y553 strongly inhibited *F. oxysporum* f. sp. *cubense* race 4 and *B. dothidea*; Y130 and Y129 showed strong inhibition against *C. musae* and *C. gloeosporioides*, respectively. Among the five predatory strains, all showed varying predation activity against *S. aureus* and MRSA. Except for Y330, which showed weak predation against *E. coli*, the other strains showed no significant difference in predation activity against this bacterium. Strain Y90 exhibited the strongest predation against both MRSA and *S. aureus*, followed by Y522, while Y145 showed weaker activity against both *Staphylococcus* species.

Fig. 5 Inhibition zones of selected active strains against plant pathogenic fungi

Fig. 6 Inhibition zones of predatory active strains against indicator bacteria

Fig. 7 Antimicrobial activity analysis of 15 active strains

Based on 16S rRNA gene analysis (Table 2), broad-spectrum antifungal strains belonged to *Bacillus*, *Streptomyces*, *Actinomadura*, *Serratia*, *Pseudomonas*, and *Nocardia*, with strain Y129 identified as a potential novel species. Predatory strains were distributed among *Delftia*, *Diaphorobacter*, *Pseudomonas*, and *Stenotrophomonas*, with strain Y90 identified as a potential novel species. At the genus level, *Bacillus* and *Streptomyces* each accounted for 30% of antifungal strains, while *Pseudomonas* accounted for 40% of predatory strains. Both *Bacillus* and *Pseudomonas* contained strains with both antifungal and predatory activities. Active strains were primarily isolated from rhizosphere soil (60%), followed by roots (26.7%) and stems (13.3%).

Table 2 Identification and source distribution of active strains from *A. ebracteatus*

Strain	Reference Strain	Homologous Similarity (%)	Activity	Source
	Pseudomonas oleovorans subsp. *oleovorans*		Predation	Rhizosphere soil
	Diaphorobacter ruginosibacter		Predation	Rhizosphere soil
	Pseudomonas hibiscicola		Predation	Rhizosphere soil
	Delftia tsuruhatensis		Predation	Plant roots
	Stenotrophomonas indicatrix		Predation	Plant stem

Strain	Reference Strain	Homologous Similarity (%)	Activity	Source
	Bacillus tequilensis		Antifungal	Plant stem
	Bacillus siamensis		Antifungal	Plant root
	Streptomyces misionensis		Antifungal	Rhizosphere soil
	Streptomyces cellostaticus		Antifungal	Rhizosphere soil
	Serratia marcescens		Antifungal	Rhizosphere soil
	Bacillus stercoris		Antifungal	Rhizosphere soil
	Actinomadura maheshkhaliensis		Antifungal	Plant root
	Streptomyces rapamycinicus		Antifungal	Rhizosphere soil
	Pseudomonas atacamensis		Antifungal	Plant roots
	Nocardia africana		Antifungal	Rhizosphere soil

Enzyme Activity Analysis of Bioactive Strains

Fifteen active strains were tested for enzyme activities using different substrates (Table 3). All active strains exhibited at least three functional enzyme activities. *Streptomyces* strains Y130 and Y129, and *Actinomadura* strain Y134 showed the highest enzyme activity positive rates (75%). *Bacillus* strains Y553 and Y263, *Pseudomonas* strain Y523, *Delftia* strain Y330, *Serratia* strain Y319, and *Stenotrophomonas* strain Y145 also showed good enzyme activity (62.5% positive rate). Analysis of different enzyme activities revealed 15, 12, 8, 5, 5, and 3 strains positive for chitinase, urease, esterase, protease, cellulase, and amylase, respectively, corresponding to positive rates of 100%, 73.3%, 53.3%, 33.3%, 33.3%, and 20%.

Table 3 Enzyme activity identification results of active strains from *A. ebracteatus*

Strain	Urease	Esterase (Tween 60)	Esterase (Tween 20)	Esterase (Tween 80)	Protease	Amylase	Chitinase	Cellulase

Note: - indicates negative activity; + indicates positive activity.

Discussion

The unique mangrove habitat inevitably fosters distinctive and abundant microbial diversity, with microorganisms serving as the primary drivers of material cycling and energy flow in mangrove ecosystems (Cao et al., 2008). This study investigated the diversity of endophytic bacteria from four plant tissues (roots, stems, leaves, flowers) and rhizosphere soil of *A. ebracteatus* based on 16S rRNA gene sequencing. A total of 66 culturable bacterial species were isolated, distributed across 18 orders, 26 families, and 37 genera. Rhizosphere soil exhibited greater bacterial diversity than plant tissues, followed by roots, while flowers showed the lowest endophytic bacterial diversity. These results are consistent with previous studies on endophytic bacteria from various mangrove plants in Guangxi and Hainan (Li et al., 2020; Li et al., 2021), likely because mangrove soils are rich in organic matter from leaf litter and root exudates, providing abundant energy sources for microbial growth (Zhuang & Lin, 1993). Furthermore, endophytic and rhizosphere bacterial communities showed similar composition, with *Bacillus* and *Pseudomonas* as dominant genera, and both contained bacteria from Actinobacteria, Firmicutes, Gammaproteobacteria, and Alphaproteobacteria. However, community structure also showed specificity, with 11 unique genera from plant tissues and 9 unique genera from rhizosphere soil, confirming that endophytic community structure varies with plant tissue and exhibits diversity, ubiquity, and specificity.

Studies have shown that some endophytic bacteria from medicinal plants can produce physiologically active compounds identical or similar to their hosts, making medicinal plant endophytes a resource reservoir for antimicrobial, antipest, antiviral, and anticancer substances (Xiao et al., 2011). The mangrove habitat features typical marine environmental characteristics. To adapt to high salinity, low pressure, humidity, and organic matter accumulation, mangrove microbial groups have evolved unique metabolic pathways and biological activities, showing potential for developing novel marine pharmaceuticals (Cao et al., 2008; Lu et al., 2021). This study screened multiple bioactive strains from culturable endophytic and rhizosphere bacteria of *A. ebracteatus*. In terms of diversity, 10 broad-spectrum antifungal strains were obtained, belonging to *Bacillus*, *Streptomyces*, *Actinomadura*, *Serratia*, *Pseudomonas*, and *Nocardia*. Five predatory strains against human pathogens were obtained from *Delftia*, *Diaphorobacter*, *Pseudomonas*, and *Stenotrophomonas*. Active strains showed varying bioactivities, with *Streptomyces* exhibiting the strongest antifungal activity and *Pseudomonas* showing the strongest predatory activity. Regarding novelty, *Streptomyces* strain Y129 and *Pseudomonas* strain Y90 were identified as potential novel species. Studies indicate that endophytic bacteria living in plant tissues throughout their life cycle can protect plants from viruses and pathogens and promote plant growth by producing bioactive secondary metabolites and functional enzymes (Saikkonen et al., 2004; Afzal et al., 2014; Fan et al., 2020). Therefore, discovering potentially novel species with good bioactivity provides biological material for developing new harmless biomedical agents.

Analysis of active strain sources revealed that most were isolated from rhizosphere soil and plant roots, consistent with results from Chen et al. (2006) who screened active strains from rhizosphere soil, roots, leaves, and fruits of 18 mangrove and 4 semi-mangrove species in Hainan. Liu and Hong (2006) and Strobel (2003) demonstrated that root and rhizosphere bacteria can inhibit plant pathogen growth by producing bioactive secondary metabolites and siderophores, making rhizosphere bacteria an ideal source of medicinal strains. Enzyme activity tests revealed that active strains generally have the potential to secrete multiple functional enzymes, particularly showing significant activity for esterase, protease, and cellulase. This may be because mangroves are intermittently inundated by tides, accumulating large amounts of marine animal and plant residues and leaf litter, providing abundant organic nutrients such as metabolites, cellulose, and proteins (Zhao et al., 2018). These hydrolases may be related to bacterial predatory activity, as predatory bacteria can inhibit pathogens not only by producing active compounds but also by secreting various lytic enzymes (Lambert et al., 2006, 2008; Keane & Berleman, 2015). For example, *Myxococcus xanthus* uses antibiotics like myxovirescin, hydrolases, and protease MepA in coordination with outer membrane vesicles to lyse prey cells, producing various secondary metabolites and lytic enzymes during predation that can serve as sources of novel bioactive compounds (Keane & Berleman, 2015). Therefore, discovering strains with multiple functional enzyme activities provides theoretical basis and guidance for targeted development and utilization of medicinal microbial resources.

In summary, the medicinal mangrove plant *A. ebracteatus* from Dongzhai Harbor Mangrove Conservation Center in Hainan harbors rich diversity of culturable endophytic bacteria with multiple biological activities, representing ideal material for future research on novel biomedical agents.

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