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ARTICLE

Isolation, identification, and antibacterial assay for rhizobacteria associated with the root-mint plant *in vitro*

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ABSTRACT

The rhizosphere of medicinal plants is a rich source of bacteria that do not harm the host plant, mostly with antibacterial properties. In this study, 6-strains of bacteria were isolated from the rhizosphere of *Mentha spicata*. We tested these strains for antibacterial activity against *Ralstonia solanacearum* YY06. We identified the strain with the highest antibacterial activity by 16S rRNA gene sequencing and examined its enzymatic activity. Among the six isolated strains, strain NMA4 demonstrated the strongest antibacterial activity against strain YY06, with an inhibition zone of 33.87 mm. Molecular identification, supported by phylogenetic tree analysis, revealed that strain NMA4 shares 99% identity with *Paenibacillus ottowii*, the first recorded for this species in Iraq. The results further indicated that strain NMA4 secretes amylase, chitinase, and protease enzymes, which accounts for its antagonistic ability to suppress bacterial plant pathogens.

Keywords: *Paenibacillus ottowii; First Record; Antibacterial; Phylogenetic Tree; Activity Enzyme*

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INTRODUCTION

The tomato (*Solanum lycopersicum* L.) is a staple vegetable worldwide, serving as a food source for the majority of the global population (Hubab *et al*., 2025). Tomato fruits are a source of various bioactive compounds, such as carotenoids, particularly lycopene, which are associated with antioxidant effects (Wu *et al*., 2022; Janani *et al*., 2025). Tomato Bacterial Wilt (TBW), caused by Ralstonia solanacearum, can cause disease in all cultivars of tomato in the world (Seleim *et al*., 2014; Phiri *et al*., 2024). The plant is managed through the use of resistant cultivars and systemic bactericides. Chemical management of the pathogen with the use of pesticides is expensive, pollutes the ecosystem, and is potentially mischievous to animals and being-humans, and their duplication utilize encourage the emergence of bactericides-resistant bacteria (Jiang *et al*., 2025). Suppressing plant pathogens by applying bacteria that are harmless to plants offers an alternative strategy, yielding highly significant results that contribute, in a sustainable manner, to reducing the severity of plant diseases (Udayashankar *et al*., 2011). Rhizobacteria lives in the soil and colonize the root system without causing any adverse effect on the host, and the connection with the host is nutritional exchange (Duan *et al*., 2013; Egamberdieva *et al*., 2017). Rhizo-microorganisms were secluded of various medicinal plants (Qu *et al*., 2024; Patil *et al*., 2024). Rhizobacteria can be isolated from healthy plant parts such as roots of crops, and herbs and trees (Hussein *et al*., 2024). Rhizophytic bacterial are key substantial function in

disease suppression, support plant evolution and environmental stress management (Chinnaswamy *et al*., 2018).

Certain species of rhizospheric bacteria suppress plant pathogens by leveraging their natural survival strategies, which include antibiosis and competition for nutrients, and indirectly by inducing plant resistance reaction (Jiao *et al*., 2021). The important role for Rhizobacteria in antagonistic activity through excreting cell wall hydrolyses such as chitinases, amylases, proteases and lipases that can lyse pathogenic bacteria cell (Kelbessa *et al*., 2022). Overall it improves plant health, promotes growth, rate the rise of availability-nutrient and absorption, and plant defenses against adverse conditions and stressors. It has been reported that Rhizobacteria could enhance seedling emergence and increase plant evolution (Grady *et al*., 2016; Yuan *et al*., 2025a). This study aimed to: (1) isolate rhizobacteria from the root zone of a medicinal plant (mint); (2) investigate the effect of these isolates on *Ralstonia solanacearum* causes wilt disease in tomatoes; (3) perform molecular identification of the most inhibitory rhizobacteria isolate; and (4) examine potential antagonistic mechanisms under laboratory conditions..

MATERIALS AND METHODS

Isolation of root-associated bacteria

Rhizobacteria were isolated from healthy roots of the mint plant (*Mentha spicata* L.). The samples were thoroughly rubbed in lab-water to separate the soil remnants associated with root hairs. Samples-root were divided into slight sections (~4 cm) and sterilized externally utilizing methanol 70% for 1 minute and then 1% sodium hypochlorite for 4 minutes, and then removed alcohol residue from the samples of root by sterile H_2O. Sterile sectioned root tissue (1 g) was smashed separately in 9 mL of NaCl solution (0.85%). A series of dilutions was performed on the suspension of crushed root tissue to achieve a dilution of 10^{-5}. 0.5 mL of each dilution was homogenized in 9-cm plates with 9.5 mL of Nutrient Agar medium consisting of 0.5, 0.3, and 1.5% peptone, extract-yeast, and agar, respectively. Plates of the trial were kept at 32 °C for 2 nights. Single bacterial colonies were picked and transferred to plates with fresh 9 mL of NA medium. 30% glycerol was utilized to store bacterial strain-isolated.

In vitro screening for antibacterial

Antibacterial effects of the isolated rhizobacteria from a medicinal plant were tested against a strain of *Ralstonia solanacearum* YY06 taken from the plant disease lab, College of Agriculture, University of Misan. The bacterial strain YY06 was identified as a causative agent of wilt in the Solanaceae family (Abd Alamer *et al*., 2020). It was investigated using the method of Soliman (2022) on an agar plate. All isolated rhizobacteria and bacterial strain YY06 were grown in nutrient medium (without agar) at 32 ºC for one night. 200 µl suspension of strain YY06 (10^8cfu/ml) was homogenized with 9.8 ml NA medium in 9-cm plates. After solidification, the medium was inoculated with 5µl suspension of rhizobacteria isolates (10^8cfu/ml), which were spotted on the medium at equal distances from each other and kept at 32 ºC for 2 nights. The efficiency of strains as antibacterial agents was assessed by the diameter of the inhibitory area about the any colonies-bacterial of the strains.

Bacterial root-associated identification

Rhizobacteria identification with high antibacterial activity was carried out utilizing16S rRNA gene sequencing. The NMA4 strain was culture for 12 h on NA medium and one single-colony was lock onto for “colony-PCR” utilization primers pair 27F (AGAGTTTGATCCTGGCTCAG) and 1492F (GGTTACCTTGTTACGACTT) (Diniz *et al*., 2025). PCR reactions were carried out utilizing the GoTaqR G2 Flexi DNA Polymerase (Promega) with colorless buffer following the manufacturer’s recommendations. Thermal cycling parameters were as follows: a denaturation step at 95 °C for 5 min followed by 30-cycles at 95 °C for 1 min, 55 °C for 30 s, and 72 ◦C for 90 s. Finally, an elongation step at 72 °C for 10 min. PCR amplicons were confirmed by gel-electrophoresis. The PCR product refined was sequenced by Sangon Biotech (Shanghai, China).

Tree-Phylogenetic assay

Sequences of nucleotide were analyzed, aligned, and compared using the BLASTn tool available in the National Center for Biotechnology Information (NCBI) database. Nine bacterial strains closely related to isolate NMA4 were selected from GenBank, along with *Geobacillus stearothermophilus* to serve as an out-group (Table 1). The sequences were compiled and aligned using alignment software. A tree-phylogenetic analysis was designed utilizing the software-MEGA (version 7.0.26) via the neighbor-joining method, with bootstrap support values reaching 10000 replicate. The sequence of strain NMA4 was recorded in the (NCBI) database.

Table (1) Information of rhizobacteria used structure of Phylogenetic Tree

***Paenibacillus* spp.**	**Strain**	**Isolate Source**	**Geo-location**	**Sequence No.**
Rhizobacteria	NMA4	Iraq	Mint Root	submitted
P. chinjuensis	WN9	Korea	Soil	NR_025024.1
P. xylanisolvens	X11-1	Thailand	Soil	NR_112906.1
P. solanacearum	T16R-228	Korea	Tomato Root	NR_159229.1
P. cremeus	JC52	South Korea	Soil	MF446616.1
P. validus	JCM 9077	Japan	Soil	NR_040892.1
P. filicis	S4	China	soybean root	KR051056.1
P. mellifer	MBLB2552	South Korea	Honey bee	OL468736.1
P. timonensis	BioMol-2301032	France	Soil	AY323612.1
P. vulneris	CCUG 53270T	Germany	Soil	HE649498.1
P. peoriae	AFS098494	United States	Soybean Root	OP986885.1
P. ottowii	HBCM-B1270	Vietnam	Soil	PZ951194.1
Geobacillus stearothermophilus	IFO 12550	Japan		NR_040794.1

Enzymatic activity of rhizobacteria NMA4

Amylase activity

The using starch agar medium was utilized to assay of amylase activity of isolate NMA4 (Deb *et al*., 2013). A 5 µl suspension of fresh culture of isolate NMA4 grown on LB broth was dropped onto NA medium (0.5%-peptone, 0.3% extract-yeast, and 1.5%-agar), modified with 1% soluble starch. As a control, 5 µl of LB broth only was dropped onto starch agar medium. Test-plates were kept at 32 °C for 4 nights. The test-plates were tucked in 1% of liquid iodine for 6 min, and visible obvious zones about colonies indicated positive amylase-activity.

Chitinase activity

The ability of rhizobacteria NMA4 to produce chitinase was assayed on the chitin agar medium (Krithika and Chellaram, 2016). The 5 µl suspension of fresh culture was dropped in the center of plates containing 15 mL of NA medium supplemented with 2% colloidal chitin. As a control, 5 µl of LB broth only was dropped onto colloidal chitin media. Test-plates were kept at 32 °C for 2 nights. The colonies growing on the plates were soaked in a 0.1% Congo red solution for 30 minutes, after which the plates were rinsed with a 1% sodium chloride (NaCl) solution to remove the dye. The appearance of clear zones around a colony indicates that it produces the enzyme Chitinase.

Protease activity

The 5µl suspension of fresh culture of Rhizobacteria NM4 was dropped in the center of plates containing 15 mL of SMA medium [Milk-skim (15g), extract-yeast (0.5g), agar (9.5g), and H_20 (1L)]. As a control, 5 µl of LB broth only was dropped onto SMA medium (Xu and Kim, 2014). Test-plates were kept at 32 °C for 2 nights. The appearance of obvious regions about bacterial colonies stipulated protease production.

Analysis-Findings Statistically

Lab trials were conducted with 5-replicates, following a completely randomized design. Statistical analysis was performed using SAS software (version 9.4). Significant differences among means were assessed using the t-test at (0.05).

RESULTS AND DISCUSSION

Rhizobacteria strains isolated

A total of six rhizobacteria isolates were obtained from the roots of the mint plant. The bacterial isolates varied in colony morphology, color, distribution, and growth rate, indicating microbial diversity in the area surrounding the mint roots. We preliminarily identified two rhizobacteria isolates as *Pseudomonas* sp. (NMA1 and NMA2) and four as Bacillus sp. including as NMA3, NMA4, NMA5, and NMA6 (Figure 1). The rhizospheric zone of the mint plant serves as a nutrient-rich microenvironment that supports the growth of a bacterial community; specifically, species of *Bacillus* such as *subtilis*, *endophyticus*, and *thuringiensis* (Khanafari *et al*., 2012). Additionally, two species of the *Pseudomonas* sp. (*fluorescens* and *putida*) were isolated from mint roots and demonstrated activity in promoting plant growth and development (Cappellari *et al*., 2015).

***In vitro* antibacterial activity**

The six rhizobacteria isolated from mint plant roots were able to suppress the bacterial pathogen strain YY06 in vitro to different levels (Figure 1). Rhizobacterium *Bacillus* sp. of strain NMA4 displayed high inhibition against strain YY6 at 33.87 mm, with significant differences from other tested strains. Strain NMA5 of *Bacillus* sp. exhibited moderate inhibition against the pathogenic strain YY06, with an inhibition zone of 19.87 mm. *Bacillus* sp. of strains NMA6 and NMA4 showed notable inhibition, with zones of 15.18 mm and 14.03 mm, respectively. The smallest inhibition zones were observed for *Pseudomonas* sp. strains NMA2 and NMA1, measuring 7.93 mm and 5.17 mm, respectively. The rhizophytic bacterial are very important as biological control agents because they inhibit plant disease by many mechanisms. Directly are displayed such as antibiosis, enzymes activity and competition for nutrients and indirectly by induction of plant resistance reaction (Egamberdieva *et al*., 2017; Langendries and Goormachtig, 2021; Yuan *et al*., 2025b). Rhizobacteria are exhibit variation in their ability to inhibit plant pathogens, depending on the genre and the specific strain within a species. Out of 150 rhizobacteria strains, only 13 demonstrated an inhibitory potential against the growth of *R. solanacearum* (Kurabachew and Wydra, 2013).

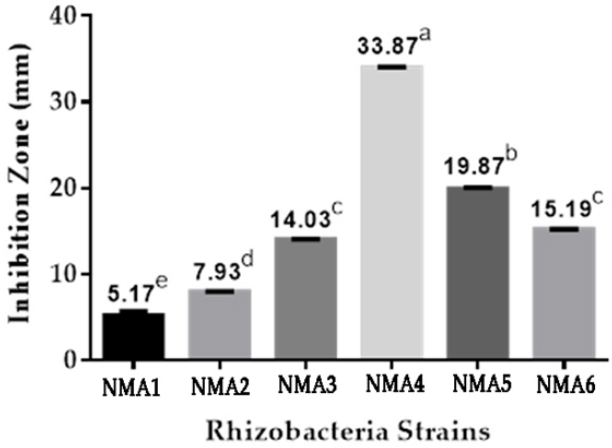


Figure (1) Rhizobacteria strains isolated from mint inhibited the *R. solanacearum* YY06

Identification of strain NMA4

The identity of the rhizobacteria strain NMA4 was verified through the amplification of the 16S rRNA gene region (Diniz *et al*., 2025). The amplification results yielded a sequence with a length of 1,552 bp. Upon analysis of the NMA4 isolate sequence via the nucleotide Blast analysis (BLASTn), it was determined that the NMA4 strain matched 99% with the bacterial *Paenibacillus ottowii* strain HBCM-B1270 which isolated from the soil in Vietnam, with the accession number PZ951194.1. Furthermore, the phylogenetic tree analysis yielded consistent results, confirming a 99% similarity between strain NMA4 and *P. ottowii* HBCM-B1270 and 87% with *P. peoriae* strain AFS098494 (Figure 2). The sequence of the isolate has been deposited in GenBank under the accession number QB188128.1. The species of *P. ottowii* had been isolated from rhizosphere of wheat and seed maze with highly antifungal fascicles in Brazil (Nishisaka *et al*., 2024; Diniz *et al*., 2025).

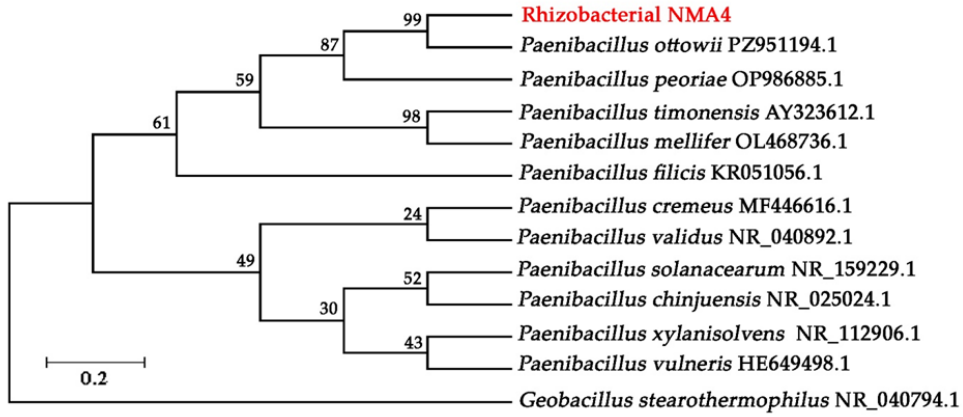


Figure (2) Phylogenetic tree displays the % matches of strain NMA4 with 11 species of the *Paenibacillus* (based on the 16S gene), showing a 99% match with the species *P. ottowii*.

Hydrolytic enzymes released by strain NMA4

The objective of the present test was to investigate some putative traits associated with strain NMA4 that are antagonistic to the bacterium YY06. Three extracellular hydrolytic enzymes were discovered that may be associated with antibacterial activity (Figure 3). Remarkably, strain NMA4 showed amylase, chitinase, and protease activity by producing clear zones around the bacterial colonies on soluble starch agar, colloidal chitin agar, and skimmed milk agar, compared with control plates. The rhizobacteria are very important as biological control agents because they inhibit plant disease by many mechanisms, directly or indirectly. Directly has through enzyme activity (Dobrzyński and Naziębło, 2024; Mohammad *et al*., 2024). One of the mechanisms most commonly used by rhizobacteria for inhibiting the pathogen is by releasing hydrolytic enzymes such as lipases, chitinases, amylases, proteases, and cellulases (Seo *et al*., 2016; Guta *et al*., 2024).

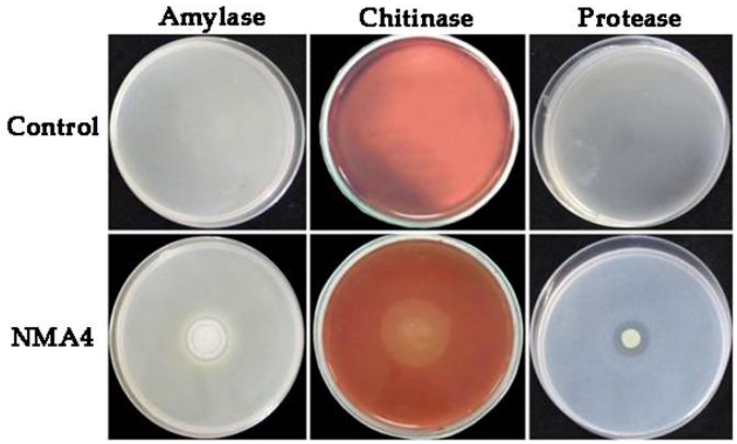


Figure (3) Hydrolysis of soluble starch by Amylase, colloidal chitin by chitinase, and casein degradation by a protease by the *P. ottowii* strain NMA4.

CONCLUSIONS

Our results indicate that the rhizosphere of mint roots is enriched with beneficial rhizobacteria. The strain NMA4 demonstrated a high level of inhibition of *Ralstonia solanacearum* in LB medium *in vitro*. Amplification and analysis of the 16S rRNA gene revealed that strain NMA4 shares 99% identity with *Paenibacillus ottowii*, a finding that marks the first recorded occurrence of this species in Iraq. Furthermore, strain NMA4 was found to secrete amylase, chitinase, and protease enzymes, which may play a significant role in countering pathogenic bacteria. Our findings pave the way for more in-depth research into this species and the investigation of its potential for managing plant diseases in field applications.

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