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Isolation, Characterization of Endophytic Bacteria from *Citrus aurantifolia* Swingle Leaves and Testing of Antifungal Activity towards *Fusarium oxysporum*

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ABSTRACT

The endophytic bacteria has variety of roles, includes roles in plant protection. This study aimed to isolate and characterize endophytic bacteria from Citrus aurantifolia Swingle leaves, and tested the antifungal activity towards Fusarium oxysporum. Isolation of endophytic bacteria was done using spread plate method. Test of antifungal activity was carried out by growing each of endophytic bacteria in Tryptic Soy Broth medium for 24 hours cultivation at temperature of 27 ^{o}C and agitation of 120 rpm, further test was using agar diffusion method. Characterization of endophytic bacteria was done through observation of colony morphology, Gram staining and analysis of 16S rRNA. Results showed that 4 endophytic bacteria has been isolated. The 16S rRNA analysis indicated bacteria isolates obtained consecutively were Bacillus cereus RNS-01, Pantoea agglomerans ZFJ-15, Bacilus subtilis 55C1-1 dan Bacillus pumilus SH-B11. Isolates CA1 and CA2 does not has antifungal activity towards Fusarium oxysporum, while CA3 and CA4 showed antifungal activity.

Keywords: endophytic bacteria, *Citrus aurantifolia* Swingle, antifungal, *Fusarium oxysporum*.

INTRODUCTION

Previous study showed that endophytic bacteria has important role in plant protection [1,2], increasing plant growth [3], coped with environmental stress [4], increasing phytoremediation process [5,6] and produces compound that can be useful in industry [7,8,9].

The ability of endophytic bacteria in plant protection showed that the bacteria is able to produces bioactive compound. Those bioactive compound can be used as biopesticide. Utilization of biopesticide can be an effort to controlling pest and diseases in plants. One kind of disease that often attacks plant is wilt disease of tomato and banana caused by *Fusarium oxysporum*. From this study it is expected that isolates of endophytic bacteria isolates found can produces bioactive compound that can be used for wilt disease conter measures.

The selected plant as source of isolates is *Citrus aurantifolia* Swingle. These plant known to has antimicrobes activity towards bacteria and fungi [10,11,12]. The approach used in the selection of these plant are chemical compound contents (alkaloid contents) and ethnbotany approach (this plant commonly used as antimicrobes in traditional medicine). Beside that, this plant selected due to antifungal activity of endophytic bacteria towards *Fusarium oxysporum* in *Citrus aurantifolia* was poorly studied. The purposes of this study are to isolate and

characterize endophytic bacteria, and to examine antifungal activity towards *Fusarium oxysporum* from several medicinal plants.

MATERIALS AND METHODS

Plant Collection

Plants were collected from Lakuk, Simpang Haru Regency, Padang City. Just healthy plants that were collected. Methods for collecting plants refers to de Melo *et. al*. (2009) which has been modified [1]. The plant leaves that will be collected was cut using sterilized knife, then it washed with sterile distilled water. Sterilized plant leaves then put into plastic bag and placed in a cooler box (temperature maintaned to + 10^{o}C).

Sterilization of Plants Organ Surfaces

Collected leaves was cut into 1 cm^{2}. This cut pieces then decontaminated by ethanol 70% for 1 minute, hipochloride natrium 2% for 6 minutes, ethanol 70% for 30 seconds to get rid of hipochloride natrium and lastly the pieces was washed with sterile distilled water [13].

natrium hipoklorida 2% selama 6 menit, etanol 70% selama 30 detik untuk menghilangkan

Isolation, Purification and Gram staining of Endophytic Bacteria

Isolation technique refer to modificated de Melo *et. al*. [1]. Sterile plant leaves then crushed with sterile mortar and pestle, then it put into physiological solution NaCl 0.85% and homogenated. After the solution became homogen, it was inoculated into a petri dish containing TSA medium added benomil fungicide as much as 1 • L mL^{-1} with spread plate method [13]. It was incubated at 27^{0}C temperature for 1 – 3 x 24 hours. Bacterial colony that grown then observed for its morphologycal differences (colony form, color, edge shape and elevation) and purified. The isolates that already pured then brought to Gram staining method refers to Harley-Prescott[14].

Anttifungal Activity Test towards *Fusarium oxysporum*

Antagonistic test was done refer to the method used by Melliawati *et. al*. [15]. Fungi *Fusarium oxysporum* was obtained from Laboratory of Phytophatology, Agriculture Faculty, Andalas University. Each endophytic bacteria was grown in tryptic soy broth medium for 24 hours at 27^{o}C temperature and 120 rpm agitation. *Fusarium oxysporum* then inoculated inside PDA medium in a petri dish. Then, a piece of sterile filter paper with 0.5 cm diameter which has been given endophytic bacteria suspension as much as 10 µL was pasted. Petri dish was incubated at 30°C temperature for 1-3 x 24 hours until growth can be observed as a clear circle around the paper piece. Those circle is a signal of existence bioactive compound which produced by endophytic bacteria to protect themself towards attacks or growth of *Fusarium oxysporum*.

Analysis of 16S rRNA Endophytic Bacteria

Analysis of 16S rRNA all bacteria isolates was done in Microbiology Industry Laboratory, Research Center of Biotechnology, LIPI. The base sequence was tested and edited using *BioEdit Sequence Alignment Editor* (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Similarity anaysis was done using the *Basic Local Alignment Tool* pada National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Evolution analysis was done by *ClustalW2 Phylogenetic Tree* (www.ebi.ac.uk).

RESULTS AND DISCUSSION

The endophytic bacteria that can be isolated in this study were 7 colony. Result of colony morphology observation showed that endophytic bacteria isolated consist of 4 difference species (Figure 1, Table 1). Based on Gram staining and miroscopic observation, those 4 endophytic bacteria has bacil cell form, 3 bacteria from Gram positive and 1 bacteria from Gram negative group (Table 1). Small amount of endophytic bacteria obtained allegedly influenced by sampling from just 1 location and organ used as isolates source just leaves [16]. According to Gao *et al.* amount of endophytic bacteria in a plant can be fluctuating [18].

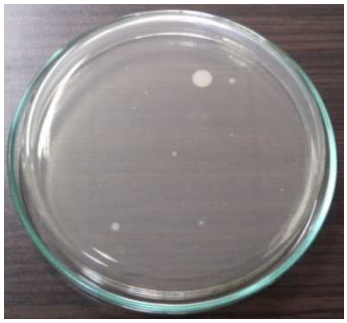

Figure1. Isolated endophytic bacteria from *Citrus aurantifolia* Swingle leaves

Table 1. Results of colony characteristic observation, Gram staining, cell form and antifungal activity of some endophytic isolated bacteria from *Citrus aurantifolia* Swingle leaves

No.	Isolate	Cell amount(CFU/gr)	Colony Characteristic				Gram Stain	Cell form	Antifungal Activity
			Form	Elevation	Margin	Pigmen			
1.	CA 1	3.0 x 10^2	Circular	Convex	Undulate	White	+	Bacil	-
2.	CA 2	1.0 x 10^2	Circular	Umbonate	Entire	White	-	Bacil	-
3.	CA 3	1.0 x 10^2	Circular	Convex	Entire	Cream	+	Bacil	+
4.	CA 4	2.0 x 10^2	Circular	Umbonate	Entire	Cream	+	Bacil	+

Explanation:
- : no antifungal activity showed
+ : antifungal activity showed

The result showed that some of isolates endophytic bacteria has antifungal activity towards *Fusarium oxysporum*, those are isolates coded with CA3 and CA4. Two another isolates, CA1 and CA2 did not showed any antifungal activity (Table 1, Figure 2). Endophytic bacteria ability in hampering *Fusarium oxysporum* growth can be seen from formation of inhibition zone (Figure 2). The formation of inhibition zone caused by endophytic bacteria ability to produced compound with antifungal activity towards the bacteria. Compound that produced usually same with the compound produced by host plant [19]. The result of Zohra *et al*. also observed that extract of *Citrus* spp. had antifungal activity towards *Fusarium oxysporum* [20].

Fragment of 16S rRNA was isolated anf amplified using PCR at Microbiology Industry Laboratory, Research Center of Biotechnology, LIPI. The size of fragment 16S rRNA that already amplified was 1500 bp (Figure 3). Fragment 16S rRNA then purified and sequenced using primer *forward* and *reverse* (9F dan 1541R), same with one used in PCR.BLAST analysis showed that isolates CA1 is *Bacillus cereus* RNS_01, CA2 is *Pantoea agglomerans* ZFJ-15, CA3 is *Bacilus subtilis* 55C1-1 and CA4 is *Bacillus pumilus* SH-B11 (Table 2). *Bacillus* and *Pantoea* are genera of endophytic bacteria that commonly found in plants, and there are many research about those genera [21, 22, 23]. *Bacillus subtilis* [24]and *Bacillus pumilus* [25]known to has ability to producing antifungi compound, one of them is towards *Fusarium oxysporum*. *Bacillus* is potential genera to be developed as biocontrol agent [26].

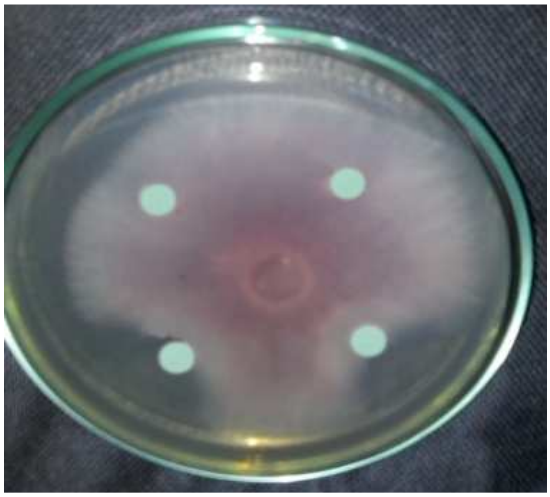

Figure 2. Antifungal activity of endophytic bacteria isolated from *Citrus aurantifolia* Swingle Leaves towards *Fusarium oxysporum*

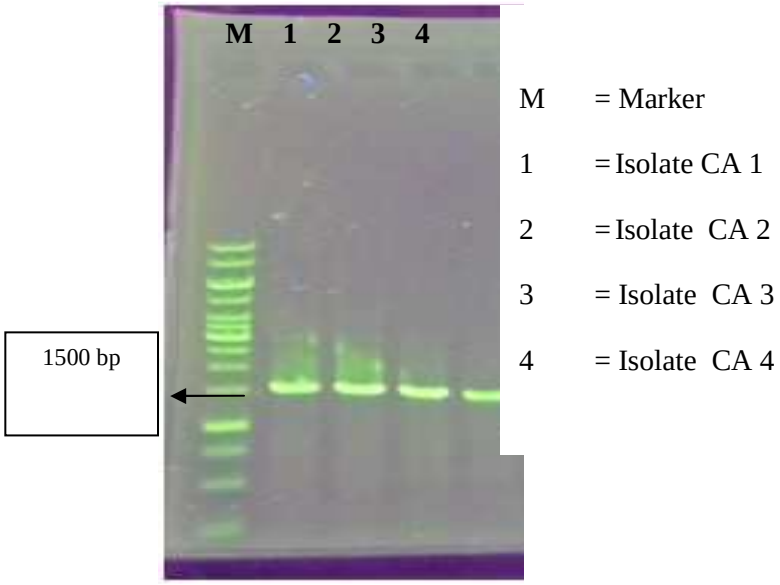

Figure 3. The result of PCR 16S rRNA purification of endophytic bacteria isolated from *Citrus aurantifolia* Swingle Leaves

Evolution analysis was done using *ClustalW2 Phylogenetic Tree* (www.ebi.ac.uk). In this analysis, the outgroup used is *Escerichia coli* K12. Analysis result showed that *Bacillus subtilis* 55C1-1 has close relationship with *Bacillus pumilus* SH-B11 (Figure 4). According to *Pairwise Sequence Alignment*analysis, both of those bacteria has similarity for 93.9%. *Bacillus* are dominant genera in this study, this condition allegedly because high existence of *Bacillus* genera in microhabitat of *Citrus aurantifolia* Swingle studied. Besides that it was also reported *Bacillus* has great ability to penetrate into plant tissues [27, 28, 29].

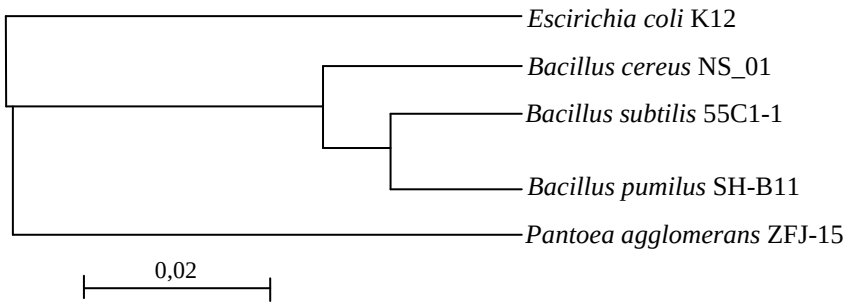

Figure 4. Phylogenetic tree of 16S sRNA sequence of endophytic bacteria

Table 2. Sequence analysis of 16S rRNA endophytic bacteria isolated from *Citrus aurantifolia* Swingle Leaves

No.	Isolate	Sequence 16S rRNA	Result of BLAST NCBI	Homology (%)
1.	CA1	AGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC CCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAA CCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGT CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAG TGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTG AATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGC GCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGT CATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGC GGTGAAATGCGTAGAGATATGGAGGAACACCAGGTGGCGAAGGCGACTTTTCTGGT CTGTAACTGAACACTGAAGGCCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGGGTTAGAGGGGTTTCCGCCCCTT AGTGCTGAAGTTAACGCATTAAGCACTCCCCCTGGGGAGTACGGCCCCAAGCCTGAA ACCTCAAAGAAATTGACCGGGGCCCCCAACAAGCGGGTGGAGCATGTGGTTTAATC CGAAGCAACGCGGAAGAACCTTACCCAGGTCTTGACATCCTCTGACAAACCCTAGA GGATAGGCCTCTCTCCTTGGGGACCAGAGGGCCAGGTGGGCCATGGTGGCGGTCACT CGTGGTGGTGAGATGTTGGGTTAATTCCGGCACCGAGGCCAACCTTTGTCCTTAGTG CCCACCATTAAGTTGGCCATCCTAAGGTGATGCCCGGGGCCAACCCGGAGGAAGGT GGGGAGGAGGTCAATTCTTCATCCCCCTTATGCCTTGGCTTCCCCACGTCTTCCAATG GACGGTCCAAAGAGCTCCAGGACCGGGAGGGGGAGTTATTTTCATAAACCCTTTTTC AGTTGGGATTTTGGGCTCCAATTGCCTTCCAGGAAGCGGGAATCCTTGGTAATCGGG GATCACCAGGCCGCGTGGAATAGGTTCCCGGCCCTGTTCCCCCCCCCCGGCCCCACC CGGGGATTTGGTACCACCGGAAGTGGGTGGGGTACCTTTTTGGAGCCAGCCGCCTAA GG	*Bacillus cereus* RNS_01 (KT380683.1)	94
2.	CA2	GCAGTCGAGCGGAGTTGACGGAAAGCTTGCTTTCCTGATACTTAGCGGCGGACGGGT GAGTAACACGTAGGCAACCTGCCCTCAAGCTTGGGACAACTACCGGAAACGGTAGC TAATACCGAATACTTGTTTTCTTCGCCTGAAGGAAACTGGAAAGACGGAGCAATCTG TCACTTGGGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA GGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTTCCGCAATGGGCGAAAGC CTGACGGAGCAATGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGC CAGGGAAGAACGCTTGGGAGAGTAACTGCTCCCAAGGTGACGGTACCTGAGAAGAA AGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGT CCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCATGTAAGTCTGGTGTTTAATC CCGGGGCTCAACCCCGGATCGCACTGGAAACTGCGTGACTTGAGTGCAGAAGAGGA GAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG GCGAAGGCGACTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCA AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGG GGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTAC GGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTA TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCAACTAAC GAGGCAGAGATGCGTTAGGTGCCCTTCGGGGAAAGTTGAAACAGGTGGTGCATGGT TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT ATATTTAGTTGCCAGCATTTCGGATGGGCACTCTAAATAGACTGCCGGTGACAAACC GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA CGTACTACAATGGCCGGTACAACGGGCAGTGAAGCCGCGAGGTGGAACCAATCCTA AAAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATT GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACAC CGCCCGTCACACCACGAGAGTTTATAACACCCGAAGTCGGTGGGGTAACCGCAAGG AGCCAGCCGCCGAAGGTGGGATAGAT	*Pantoea agglomerans* ZFJ-15 (EU931554.1)	99
3.	CA3	GCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAG TAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAA TACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCA CTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA CGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAAGCTCTGTTGTTTA GGGAAGAACCAAGTACCGTTCGGAATAGGGGCGGTACCTTGACCGGTACCTAACCC AGAAAGCCACGGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC GTTGTCCGGAAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGT GAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAG AAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC ACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTG GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA GTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTG GGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATC CTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGC	*Bacilus subtilis* 55C1-1 (JN366797.1)	99

		ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA CCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACA AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA CACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAAT CCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGG AATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC ACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTT TAGGAGCCAGCCGCCGAAGGTGGGACAGATGAT		
4.	CA4	GCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGT AAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGC ATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCA TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAG AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA TGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAAC TGCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGC AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG AAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACT GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA CGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCT AACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA GAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCG GGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCCTGTCCTGAGATGTTGGG TTAAGTCCCCCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCAC TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGC GAGACCGCAAGGTTTAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTG CAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTG AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACC CGAAGTCGGTGAGGTAACCTTTATGGAGCCAGCCGCCGAAG	*Bacillus pumilus* SH-B11 (CP010997.1)	99

CONCLUSION

From this study, 4 endophytic bacteria isolates were found. Isolates CA1 and CA2 did not has antifungal activity, while CA3 and CA4 showed antifungal activity towards *Fusarium oxysporum*. Result of analysis 16S rRNA endophytic bacteria consecutively were *Bacillus cereus* RNS_01, *Pantoea agglomerans* ZFJ-15, *Bacilus subtilis* 55C1-1 and *Bacillus pumilus* FI39.

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