Characterization of Antibiotics Producing Bacteria Isolated from *Citrus aurantifolia* Swingle

Akmal Djamaan[1], Syukria Ikhsan Zam[2], Syamsuardi[3], AnthoniAgustien[3], Dwisari Dilasamola[1], and Rika Sari Lalfari[4]

[1]Faculty of Pharmacy, University of Andalas, Padang, Indonesia
[2]Dept. of Agrotechnology, UIN Sultan Syarif Kasim, Pekanbaru, Indonesia
[3]Dept. of Biology, Faculty of Sciences, University of Andalas, Padang, Indonesia
[4]Dept. of Chemistry, Faculty of Sciences, University of Andalas, Padang, Indonesia

Author for correspondence: akmaldjamaan@phar.unand.ac.id

ABSTRACT

In an effort to find new antibiotics from plant endophytic bacteria, we have collected and characterized many bacteria from the medicinal plants of Sumatra, Indonesia. In this paper we reported, the characterization of antibiotics producing bacteria isolated from *Citrus aurantifolia* Swingle. The isolation of bacteria was used by spread plate technique and characterization for antibiotic-producing bacteria was done by 16S rRNA analysis. The results showed that from morphological colonies obtained 4 different bacteria. From 16S rRNA analysis observed the bacteria isolated were of *Bacillus cereus* RNS_01 (CA-01), *Pantoeaagglomerans*ZFJ-15 (CA-02), B*acillus subtilis*55C1-1 (CA-03) and *Bacillus pumilus* SH-B11 (CA-04). From antibiotic activity testing indicated that CA-01 isolate able to inhibited the growth of *Streptococcus mutans*, CA-02 isolate able to inhibited *Vibrio chloreae*, CA-03 isolate able to inhibited *Staphlococcusaureus*, *Salmonella thypii*and *Enterococcus faecalis*, whereas CA-04 isolate able to inhibited *Salmonella thypii* and *Salmonella thyposa*. The process of optimizing production using a biorector by fermentation and elucidation of the antibiotic structure produced is running out.

Keywords: characterization, antibiotic, bacteria, *Citrus aurantifolia* Swingle

INTRODUCTION

Antibiotics have been used since 1928 for disease preventive and treatment of infectious diseases. Nowadays, the use of antibiotics are increase along with the adding of new infectious disease cases and resistance increase of some bacteria that cause the infection against the antibiotics used. Most antibiotics that are commercially used are synthetic antibiotics that are prone to trigger resistance to pathogens, especially bacteria [1,2,3]. This situation led to the need for exploration to find a source of new and more potent natural antibiotics. One of the natural source antibioticsthat widely developed recently by various research institutions is the endophytic bacteria that live mutually in the host plant [4,5].

Several types of endophytic bacteria have been successfully isolated and are known to produces active compounds with antibiotic[6], antimalaria [7]and antifungal [8,9] properties. The ability of endophytic bacteria to produce these active compounds is a potential that can be developed to replace the process of discovery of active compounds by extracting plants, especially medicinal plants[10]. One disadvantage of obtaining an active compound from the plant is it requires longer time and more complex processes with smaller yield compared to extracting active compound from bacteria after a fermentation process has been done.

One of the plants that has antibiotic potential is the *Citrus aurantifolia* plant. Natural content of *Citrus aurantifolia* plant that it has secondary metabolite in the form of essential oil. It was reported by many researchers that the essential oil of lime leaves has antibacterial and antifungal properties [11,12]. In society, lime leaves was used to treat skin diseases, sore throat and sprue. Some study also reported that lime leaves used as anti-inflammatory and mouthwash, because the leaf is known also contains essential oils [13,14].

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MATERIAL AND METHODS

The sterile leaves of *Citrus aurantifolia* leaves were finely grounded using a sterile mortar and then inserted into a serial dilution of 0.85% NaCl solution and vortex. Each serial dilution was inoculated into TSA medium and benomil fungicide as many as 1 µL mL-1 was added with an antifungal spread plate method[18]. It was incubated at 27^0C for 1 to 3 x 24 hours. The colonies of grown bacteria were observed morphologically and purified. Pure bacteria isolates then pass through Gram staining [16].

The characterization of bacterial isolate by 16S rRNA method was done at the Laboratory of Industrial Microbiology of LIPI Biotechnology Research Center, Bogor, Indonesia. The base order was checked and edited using the Bioedit Sequence Alignment Editor (*http://www.mbio.ncsu.edu/BioEdit/bioedit.html*). The equality analysis was done by using the Basic Local Alignment Tool at the National Center For Biotechnology Information (*http://www.ncbi.nlm.nih.gov*). While the evolution analysis was done by using ClustalW2 Phylogenetic Tree (*www.ebi.ac.uk*).

The test antibiotic activity was done towards testing bacteria obtained from UAAC Bacteria Culture Center, Biotechnology Laboratory, University of Andalas, Padang, Indonesia. Test bacteria that were used in this research are *Streptococcus mutans*ATCC 25175, *Staphlococcusaureus*ATCC 25923, *Eschericia coli*ATCC 25922, *Enterobacterfaecalis*ATCC 29212, *Micrococcus luteus*ATCC 10240, *Pseudomonas aeruginosa*ATCC 27853, *Bacillus subtilis*ATCC 6633, *Vibrio chloreae*INABA, *S.Epidermidis*ATCC 12228, *Salmonella thypii*, *Salmonella thyposa*NCTC 786 *Salmonella thypimurium*ATCC 14028 and Methicillin-Resistant *Staphylococcus aureus*(MRSA).

RESULTS AND DISCUSSION

After isolation and screening of endophytic bacteria from*Citrus aurantifolia* leaf, as much as seven bacterial colonies were detected to produce antibiotic compounds. The morphology of these seven bacteria turned out to be grouped into four colonies of different bacteria. Based on the results of characterization using Gram staining method and microscopic observation, it shown that the four isolated bacteria which all of them in the form of bacillus, three bacteria from Gram positive group and one Gram negative (Tab.1). The number of bacteria that grew in the medium is relatively small, this is due to the environmental factors and the organ taken in this research only the leaves of plant. It also known that the presence of endophytic bacteria in plants is influenced by the environmental factors and plant organs[16].

Table 1. Macroscopic and microscopic results of antibiotic-producing endophytic bacteria isolate from *Citrus aurantifolia* leaves.

Isolate Code	Macroscopic observation of the colony				Microscopic observation of the colony	
	Form	Color	Border	Elevasion	Gram	Cell form
CA 01	Circular	White	Undulate	Convex	Positif	Bacil
CA 02	Circular	White	Entire	Umbonate	Negative	Bacil
CA 03	Circular	Cream	Entire	Convex	Positif	Bacil
CA 04	Circular	Cream	Entire	Umbonate	Positif	Bacil

Table 1 shows that the macroscopic and microscopic characteristics of antibiotic-producing endophytic bacteria isolates from *Citrus aurantifolia* leaves are very diverse. This is shows that the presence of bacteria contained in leaf tissue is also relatively large and diverse as well. It was also shown that all isolated antibiotic-producing bacteria colony were in circular form, three endophytic bacterial isolates were Gram-positive. The bacteria were CA 01, CA 03, CA 04 whereas CA 03 Gram was negative with all bacil cell form. It is known that theoretically the colony's distinction from bacteria is a characteristic of a particular species [17,18].

Furthermore, Table 2 shows the results of antibiotic activity test from antibiotic-producing endophytic bacteria from *Citrus aurantifolia* leaf. It is demonstrated that endophytic bacterial isolate can inhibit the growth of some testing bacteria. The inhibitory power of each isolate bacteria is different, as CA1 isolate bacteria positive to be able to inhibit *Streptococcus mutans* bacteria, CA 02 isolate bacteria can inhibit *Vibriochloreae*bacteria, CA 03 can inhibit *E. faecalis* bacteria and CA 04 can inhibit *Salmonella thypii* and *Salmonella thyposa*bacteria. It is characterized by the activity of each isolates that have the same ability with its host plant. This ability is

antibacterial, because the host plant have secondary metabolite compound that is essential oil which have ability as antibacterial. It has been reported by previous researchers that *Citrus aurantifolia* plant in addition to benefits for health, it also containing essential oil with antibacterial and antifungal properties [12].

Table 2. The profile of antibiotic activity of the bacterial fermentation of endophytic bacteria which are isolated from *Citrus aurantifolia*leaves.

Isolate Code	Antibiotic activity against bacterial test												
	S.thypii	*S. thyposa* NCTC 786	*S. thypimurium*A	*E. coli* ATCC 25922	*E. faecalis* ATCC 29212	*S. mutan* ATCC 25175	*M. luteus* ATCC 10240	*V. chloreae*INA RA	*B. subtilis* ATCC 6633	*MRSA*	*P. aeruginosa*AT	*S. epidermidis* ATCC 12228	*S. aureus* ATCC 25923
CA 01	-	-	-	-	-	+	-	-	-	-	-	-	-
CA 02	-	-	-	-	-	-	-	+	-	-	-	-	-
CA 03	+	-	-	-	+	-	-	-	-	-	-	-	+
CA 04	+	+	-	-	-	-	-	-	-	-	-	-	-

The ability of the inhibitory power of the endophytic bacteria isolate fermentation fluid to one of the test bacteria, *Streptococcus mutans*. The results of this experiment showed that each endophytic bacterial isolate produces antibiotic compounds that different capabilities towards the test bacteria used (Table 2). This allegedly caused by the differences in the active compound and molecular structure of the resulting antibiotic compounds. Concerning this condition, further research on the purification and determination of the molecular structure of each antibiotic compound produced is needed. In another study it was reported that the essential oils and antioxidant content of *Citrus aurantifolia* leaf with volatile oils concentrations of 20%, 10%, 5%, 1% were able to inhibited the growth of *Staphylococcus aures, Bacillus subtilis, Enterobacterfaecalis, Salmonella paratypii, E.coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Serratiamarcescens* [19]. It is also observed that endophytic bacterial isolates that have a large inhibitory ability was the CA 01 bacterial isolate that able to inhibit the *Streptococcus mutans*. This condition isshown by the clear zone formed. The clear zone was showed that the activity of CA 01 bacterial isolate has the content of bioactive compounds which is similar to the leaves of *citrus aurantifolia*.

The result of endophytic bacteria 16S rRNAmolecular sequence analysis is shown in Table 3. The evolutionary analysis was performed using Clustal W2 Phylogenetic Tree (*www.ebi.ac.uk*). In this analysis, the outgroup used was *Escherichia coli* K12. The results showed that *Bacillus subtilis* 55-C1-1 usually has very close genetic relationship with *Bacillus pumilus* SH-B11 (Figure 1). From the results of Pairwise Sequence Alignment analysis both bacteria have the similarity of 93.9%. In this study, most of the bacteria obtained is from the *Bacillus*genera, this is because *Bacillus* existence is very abundant in the environment *Citrus aurantifolia* and *Bacillus* bacteria have the ability to penetrate into plant tissue.

Table 3. Result of endophytic bacteria 16S rRNA sequence analysis isolated from *Citrus aurantifolia* leaves in this research.

Isolate Code	16S rRNA Sequence	BLAST NCBI Result	Homology (%)
CA 01	AGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAA CCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAA CATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATG GATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCA ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAA ACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGAC GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA CGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTG	*Bacillus cereus* RNS_01 (KT380683.1)	94

	GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG AAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCG GTGAAATGCGTAGAGATATGGAGGAACACCAGGTGGCGAAGGCGACTTTTCT GGTCTGTAACTGAACACTGAAGGCCGCGAAAGCGTGGGGAGCAAACAGGAT TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGGGTTAGAGGG GTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCCCCTGGGGAGT ACGGCCCCAAGCCTGAAACCTCAAAGAAATTGACCGGGGCCCCCAACAAGC GGGTGGAGCATGTGGTTTAATCCGAAGCAACGCGGAAGAACCTTACCCAGGT CTTGACATCCTCTGACAAACCCTAGAGGATAGGCCTCTCTCCTTGGGGACCA GAGGGCCAGGTGGGCCATGGTGGCGGTCACTCGTGGTGGTGAGATGTTGGGT TAATTCCGGCACCGAGGCCAACCTTTGTCCTTAGTGCCCACCATTAAGTTGGC CATCCTAAGGTGATGCCCGGGGCCAACCCGGAGGAAGGTGGGGAGGAGGTC AATTCTTCATCCCCCTTATGCCTTGGCTTCCCCACGTCTTCCAATGGACGGTC CAAAGAGCTCCAGGACCGGGAGGGGGAGTTATTTTCATAAACCCTTTTTCAG TTGGGATTTTGGGCTCCAATTGCCTTCCAGGAAGCGGGAATCCTTGGTAATCG GGGATCACCAGGCCGCGTGGAATAGGTTCCCGGCCCTGTTCCCCCCCCCCGG CCCCACCCGGGGATTTGGTACCACCGGAAGTGGGTGGGGTACCTTTTTGGAG CCAGCCGCCTAAGG		
CA 02	GCAGTCGAGCGGAGTTGACGGAAAGCTTGCTTTCCTGATACTTAGCGGCGGA CGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGCTTGGGACAACTACCGGA AACGGTAGCTAATACCGAATACTTGTTTTCTTCGCCTGAAGGAAACTGGAAA GACGGAGCAATCTGTCACTTGGGGATGGGCCTGCGGCGCATTAGCTAGTTGG TGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAT CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT AGGGAATCTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATGCCGCGTGAGT GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGCTTGGGA GAGTAACTGCTCCCAAGGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTA CGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATT GGGCGTAAAGCGCGCGCAGGCGGTCATGTAAGTCTGGTGTTTAATCCCGGGG CTCAACCCCGGATCGCACTGGAAACTGCGTGACTTGAGTGCAGAAGAGGAGA GTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAG TGGCGAAGGCGACTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGC TAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATT CCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGA CCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCT TACCAGGTCTTGACATCCAACTAACGAGGCAGAGATGCGTTAGGTGCCCTTC GGGGAAAGTTGAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATATTTAGTTGCCAGCATT TCGGATGGGCACTCTAAATAGACTGCCGGTGACAAACCGGAGGAAGGTGGG GATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACA ATGGCCGGTACAACGGGCAGTGAAGCCGCGAGGTGGAACCAATCCTAAAAA GCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAAT TGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTA CACACCGCCCGTCACACCACGAGAGTTTATAACACCCGAAGTCGGTGGGGTA ACCGCAAGGAGCCAGCCGCCGAAGGTGGGATAGAT	*Pantoeaagglomerans* ZFJ-15 (EU931554.1)	99
CA 03	GCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGG TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC GGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTG GCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAG GTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGG CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG AAGGTTTTCGGATCGTAAAAGCTCTGTTGTTTAGGGAAGAACCAAGTACCGT TCGGAATAGGGGCGGTACCTTGACCGGTACCTAACCCAGAAAGCCACGGGCT AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA ATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGC CCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGA AGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAG GAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCG AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA CGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACG CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGAC GTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTT GCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGA AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG TGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATC CCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAG CTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGG GCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG GTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGGGACAGATGAT	*Bacilussubtilis* 55C1-1 (JN366797.1)	99

CA 04	GCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG CCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCC TTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGAT GGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA GTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCT CTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGCACCTTGACGGTA CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTT CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC TGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTG TAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCC CTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCA AGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTG ACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAATGACAGGTGGTGCA TGGTTGTCGTCAGCTCCTGTCCTGAGATGTTGGGTTAAGTCCCCCAACGAGCG CAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCC GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTAT GACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACC GCAAGGTTTAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCA ACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT GCAACACCCGAAGTCGGTGAGGTAACCTTTATGGAGCCAGCCGCCGAAG	*Bacillus pumilus*SH-B11 (CP010997.1)	99

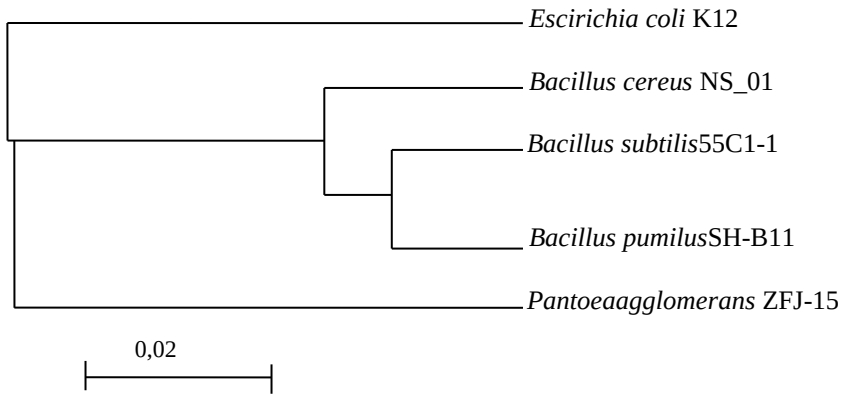

Figure 1. The phylogenetic tree of 16S rRNA sequence of isolated antibiotic-producing endophytic bacteria from from *Citrus aurantifolia* leaves in this research.

CONCLUSIONS

It can be concluded that as much as four endophytic bacterial isolates that could potentially produce antibiotic compounds was obtained. First, isolates with CA01 code has fermentation liquids with inhibitory activity against *Streptococcus mutans* bacteria. Second, isolate with code CA02 was able to inhibit the growth of*Vibriochloreae* bacteria. Third, isolate with the CA03 code was able to inhibit bacterial growth, *Salmonella thypii* and *E. faecalis*. Fourth, isolate with CA04 code has inhibitory activity on *Salmonella thypii* and *Salmonella thyposa* bacteria. Base on the the molecular characterization, four endophytic bacteria that potentially producing the antibiotic were *Bacillus cereus* RNS_01 (code CA01), *Pantoeaagglomerans* ZFJ-15 (Code CA 02), *Bacillus subtilis* 55C1-1 (Code CA 03) and*Bacillus pumilus* SH-B11 (Code CA 04).

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