

# Identification of bacterial species from healthy wood of *Aquilaria crassna* using colony PCR

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**Abstract.** Agarwood is one of the most expensive, sacred and valuable woods used in the biotechnology industry. This natural raw material is in high demand as a commodity and can be used to manufacture a variety of products. Isolating a microbe from its environment is crucial because several bacterial strains can produce novel compounds for biotechnological applications. Exploration of bacterial strains, such as bacterial endophytes, has been associated with bioactive compounds of the agarwood plant. The purpose of this study was to isolate and identify bacteria from Agarwood trees in the Kaeng Hang Mao Sub-district of Chanthaburi Province, Thailand by using molecular biology techniques. A colony PCR technique without prior DNA extraction for rapid and simple detection of bacteria was performed. After the DNA sequence analysis, the results showed that most populations in the normal layer are belonged to *Pantoea dispersa* (99%). Through analysis of phylogenetic tree by neighbor-joining method, the sequences of the *16s rRNA* gene were analyzed to understand evolutionary relationships of bacteria found in agarwood. In conclusion, good amplification of the *16s rRNA* sequence (1.5 kb) was detected with the specificity of the target sequences of the *16s rRNA*. In addition, the isolated bacterial strains are the most common species in agarwood, however, there has been no previous research on isolating these bacterial strains in *Aquilaria crassna*.

**Keywords.** *Aquilaria crassna*, Agarwood, Bacterial endophytes, Colony PCR, *16s rRNA*

## 1 Introduction

*Aquilaria crassna* is an agarwood producing tree of the family Thymalaeaceae [1,2]. This plant is found primarily in China, Bangladesh, India, and Thailand [3]. When aquilaria trees are wounded or stressed, their wood turns from white to yellow, brown, black, and eventually greasy black and spreads out. Furthermore, the resinous substances accumulated in the wood surrounding the wound can be secreted by the wood. It must, however, be left for several years until completely turning to black or brown color [4,5]. Many countries, including Egypt, China, Greece, and India, used agarwood essential oil or fragrant resins for medicinal therapy, incense in religious rites, perfumes, and ornamental materials [6,7].

Many bacteria are ubiquitous; that is, they are present everywhere. They are also highly adaptable to a variety of environmental conditions [8]. A previous report presented endophytic bacteria as playing a crucial role in the production of pharmaceutical compounds present in plants [9]. The wood was also used to decorate boxes, belts and clothing articles and its usage has recently

expanded to include new products such as detergent and shampoo [10]. There is also evidence that it has tonic and therapeutic properties [11,12]. According to recent research, endophytic bacteria have been discovered to be abundant among the resident microflora of the healthy inner tissues of numerous plant species [13]. Because the function of these bacteria is unknown, the isolation and identification of bacterial strains in these groups may aid in understanding the relationship between microbes and plants, particularly aquilaria species that produce agarwood, as well as the discovery of novel bacterial species.

In 2005, molecular approaches based on DNA gene markers were widely used to identify the genetic variation of plant species in agarwood producing tree species [14,15]. In the present study, we presented the results of the isolation and identification of major bacterial strains associated with the non-resinous part of the agarwood plant. This study provided the results of the isolation and identification of major bacterial strains associated with the non-resinous part of the agarwood plant. Furthermore, this study was the first investigation to identify bacteria strains that could be employed as an alternative bio-

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resource for the future production of bioactive compounds.

## 2 Materials and methods

### 2.1 Bacterial isolation from healthy wood sample

The wood samples (*Aquilaria crassna* Pierre ex Lec) were collected in the Kaeng Hang Maeo Sub-district of Chanthaburi Province (Figure 1). They were pulverized into fine powder. The powder (0.3 g) was soaked in 10 ml distilled water before being incubated at 37°C for 48 h. One milliliter of suitable dilutions of the suspension was used to inoculate a set of three Petri dishes, each containing 15mL Nutrient Agar (NA) medium (HIMEDIA®, India). Plates were then incubated at 37°C for 24 h.



**Fig. 1.** Characteristics of ground wood for bacterial isolation

### 2.2 Colony PCR

The PCR reaction mixtures were carried out in a volume of 25 µl with 2.5U of Pfu DNA polymerase (Biotech rabbit, Germany), 2.5 µl of 10X reaction buffer, 200µM of each dNTP, 1.5mM MgCl<sub>2</sub>, 25 pmole of each primer and were adjusted with distilled water. Primer forward (5'AGA GTT TGA TCM TGG CTC AG3') and reverse (5'TAC GGY TAC CTT GTT ACG ACT T3') to 16S rRNA PCR amplification were used for bacterial identification. A single colony of cream whitish-colored colonies was selected as the DNA template for PCR. The PCR reaction was carried out using a DNA Engine thermal cycler (BIORAD) with an initial denaturation step of 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 30 sec, and an additional extension step at 72°C for 10 min. This process used distilled water as a negative control to check for contamination. The PCR reaction components and conditions are shown in Table 1 and Table 2.

**Table 1.** PCR reaction components and the volumes for PCR amplification

PCR component	Volume (µl)
Distilled water	19.0
10X PCR buffer	2.5
1.5 mM MgCl <sub>2</sub>	1.5
200 µM dNTPs	0.5
2.5 pmol/ µl forward primer	0.5
2.5 pmol/ µl reverse primer	0.5
2.5 U/ µl Pfu DNA polymerase	0.5
Total volume	25.0

**Table 2.** Condition for colony PCR amplification

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	3 min	1
Denaturataion	95	30 sec	} 35
Annealing	50	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	1

### 2.3 Detection of PCR products and sequencing

The presence of PCR products was investigated using 2% agarose gel electrophoresis. PCR yields were estimated by comparing them to a low DNA mass ladder (Promega, USA). The post PCR purification step was then carried out using a QIAquick PCR purification Kit (QIAGEN, USA). The purified PCR product was sequenced with the ABI PRISM™ BigDye Terminator Cycle Sequencing Kit. The amplification was carried out in a DNA Engine thermal cycler (BIORAD) under the following conditions: 25 cycles of 96°C for 10 sec, 55°C for 5 sec and 70°C for 1 min. The DNA was recovered by ethanol precipitation after amplification and the dye-labeled DNA was analyzed using an Applied Biosystems 377 sequencer (Perkin-Elmer, Norwalk, CT, USA).

## 2.4 Data analysis using Bioinformatics tools

After obtaining the nucleotide sequences of the *16s rRNA* gene, they were checked by using the nucleotide blast database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All alignment illustrations were created using the ClustalW program [16] and the GeneDoc software was used to display the output [17]. MEGA 7 was used to create the phylogenetic trees using the Neighbor-Joining method and Bootstrapping analysis [18]. The data has been bootstrapped 1,000 times.

## 3 Results and discussion

### 3.1 Characteristics of colonies obtained by isolating bacteria from wood

The selected isolates underwent presumptive phenotypic identification based on bacterial morphology after gram staining, colony morphology and the ability to grow on NA media and culture conditions. The results showed that the isolated microorganism displayed cream whitish-colored colonies, round, raised, smooth on each plate shown in Figure 2. From the gram staining, it was found gram-negative rod (data not shown). This pattern is similar to that seen in previous research [9, 13].

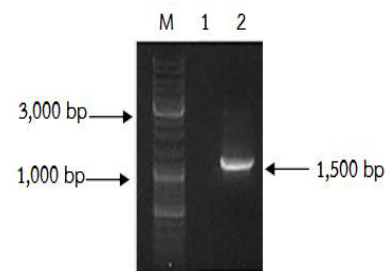


**Fig. 2.** The colony morphology of bacteria in wood cultures grew on NA media

### 3.2 Colony PCR for detection of the *16s rRNA* gene

In this study, colony PCR was used as the universal primers. The PCR products were about 1,500 base pairs long, which was the expected size. As shown in Figure 3, the universal primers produced the amplified fragment of the expected size on the gel when compared with the 100 bp DNA marker. The *16S rRNA* gene is universal in bacteria commonly due to its conservation across bacteria and is popularly used to establish the evolution of bacteria through the approximately 1,500 base pair (bp) locus of this gene [19]. According to the results, *16S rRNA* sequence analysis found isolates that were more accurate than traditional methods [20]. In addition, the *16S rRNA* gene sequences were also generated between groups of known bacteria and the novel isolates of related bacteria

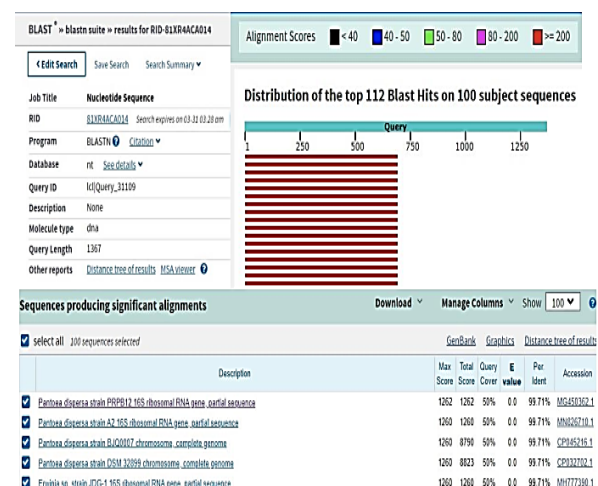
to study the evolutionary relationships of living organisms [21].



**Fig. 3.** Agarose gel electrophoresis of direct colony PCR using the universal primers: Lane M 100bp ladder, Lane 1 negative control, Lane 2 indicates PCR product of *Pantoea dispersa*

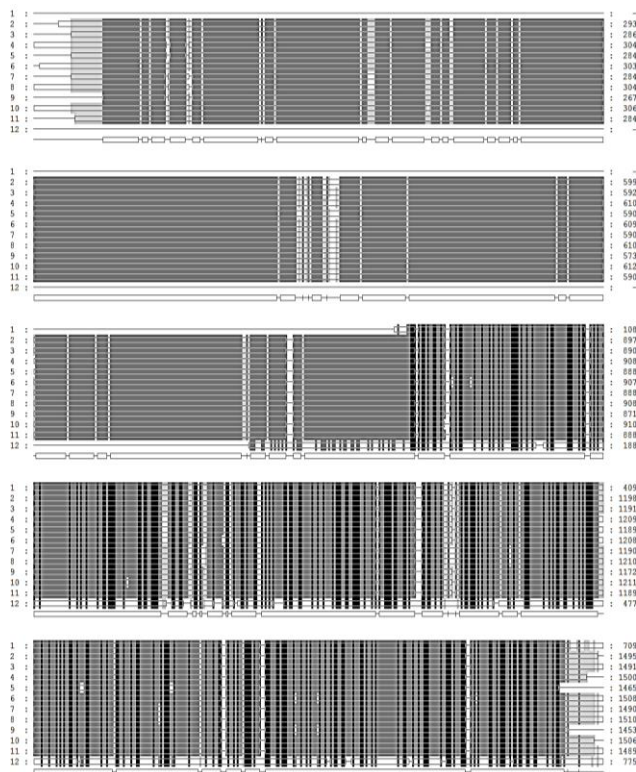
### 3.3 Sequence analysis of the *16S rRNA* gene and Phylogenetic analysis

After obtaining the sequences from this study, they were compared with the bacterial species recorded in the NCBI database (Figure 4). A single query sequence of query ID 31109 found their best match with the species belonging to the Enterobacteriaceae family. It was found that *Pantoea dispersa* had the highest identity at 99%. In addition, the sequences of all bacterial strains were aligned with species recorded in the database (No.2-No.12). From the result of multiple sequence alignment, the polymorphic nucleotides were examined using the Clustal W program [16] and the GeneDoc program [17]. It is composed of *Pantoea cyripedii* (NR\_041973.1), *Pantoea cyripedii* strain LMG 2657 (NR\_118394.1), *Enterobacter kobei* (LT5478211), *Kluyvera cryocrescens* (NR\_114108.1), *Pantoea stewartii* (NR\_104928.1), *Erwinia billingiae* (NR\_118431.1), *Erwinia billingiae* strain Billing E63 (NR\_104932.1), *Pantoea coffeiphila* (KJ427829.1), *Pantoea dispersa* strain DSM 30073 (NR\_116797.1), *Pantoea dispersa* (AB907780.1) and *Aedes albopictus* (DQ397921.1). The results of multiple sequence alignment were presented in Figure 5.



**Fig. 4.** Graphical summary output of BLAST showing the homology coverage between query and the hits and description section in the BLAST report showing one-line summaries of sequences producing significant alignments

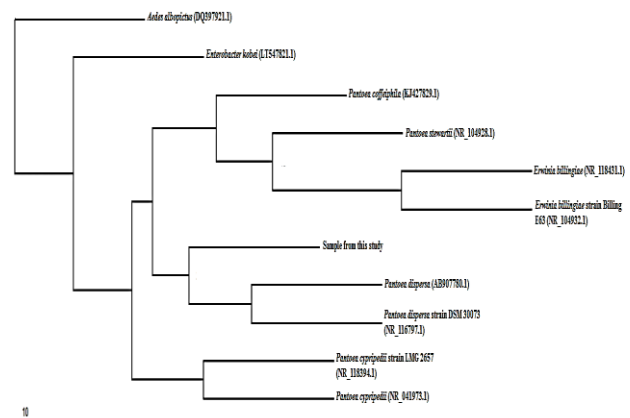




**Fig. 5.** Multiple sequence alignment of the 16S rRNA gene from 13 species including 1) sequences from this study. 2) *Pantoea cypripedii* (NR\_041973.1), 3) *Pantoea cypripedii* strain LMG 2657 (NR\_118394.1), 4) *Enterobacter kobei* (LT547821.1), 5) *Kluyvera cryocrescens* (NR\_114108.1), 6) *Pantoea stewartii* (NR\_104928.1), 7) *Erwinia billingiae* (NR\_118431.1), 8) *Erwinia billingiae* strain Billing E63 (NR\_104932.1), 9) *Pantoea coffeiphila* (KJ427829.1), 10) *Pantoea dispersa* strain DSM 30073 (NR\_116797.1), 11) *Pantoea dispersa* (AB907780.1), 12) *Aedes albopictus* (DQ397921.1). Black represents 100% identical nucleotide sequences and white has no DNA sequence similarity

Morphological identification becomes difficult because of the invisibility of some features followed by not well-developed specimens, which can even make the identification impossible. Therefore, molecular biology studies are necessary to support the morphological studies of bacteria isolated from natural sources. Phylogenetic trees were constructed based on the multiple sequence alignment of the 16S rRNA using neighbor-joining (NJ). In the phylogenetic trees, the laboratory sequences are checked according to the morphologically identified characteristic, while other species name is given for the sequences downloaded from NCBI GenBank. The evolutionary data was inferred using the Neighbor-Joining method. The percentage of replicating trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was analyzed to predict tree characteristics [18]. The phylogenetic tree (Figure 6) was established with the outgroup of *Aedes albopictus* (accession number DQ397921.1). Based on the sequence comparison and phylogenetic tree construction, the bacterial isolate from the healthy wood of *Aquilaria crassna* presents 99% homology with *Pantoea dispersa*,

a known Gram negative bacteria of the family Enterobacteriaceae. Genus *Pantoea* is isolated from Enterobacteriaceae in 1989 [22]. The genus *Pantoea* is classified into 20 different species named *Pantoea eucalyptii*, *Pantoea agglomerans*, *Pantoea vagans*, *Pantoea conspicua*, *Pantoea deleyi*, *Pantoea anthophila*, *Pantoea brenneri*, *Pantoea ananatis*, *Pantoea allii*, *Pantoea stewartii*, *Pantoea cypripedii*, *Pantoea calida*, *Pantoea gavinae*, *Pantoea dispersa*, *Pantoea septica*, *Pantoea wallisii*, *Pantoea eucrina*, *Pantoea rodasii*, *Pantoea rwandensis*, and *Pectobacterium carotovorum*. *Pantoea dispersa* is commonly isolated from plants, as evidenced by the study by *Erwinia* [22,23]. The genus *Pantoea* is a gram-negative, flagellated, nonencapsulated, nonspore, and capsule-forming ubiquitous *Brevibacterium cruentum* [24,25]. This genus is primarily associated to plant pathogens, with a few historical human diseases [25,26]. It was previously reported that this microorganism is useful as a biocontrol agent for black rot in sweet potatoes [27]. In addition, several members of the genus have been potentially found to enhance growth and yield, control plant pathogenic microbes, and increase abiotic tolerance in rice plants [28]. To our knowledge, this is the first report describing the isolation of bacterial species from healthy wood of *Aquilaria crassna* with rapid detection method.



**Fig. 6.** Neighbor-joining phylogenetic tree based on the 16S rRNA showing the relative positions of the *Pantoea* isolates and other *Pantoea* type strains. Evolutionary analyses were conducted in the MEGA7 program and bootstrap values (based on 1,000 replicates). The bar represents nucleotide substitution rate unit of 10.

## 4 Conclusion

The shape of the colony and the content in agarwood samples indicated cream whitish-colored colonies and gram-negative rods based on observation and isolation. According to the NCBI database, *Pantoea dispersa* showed the highest identity at 99%, and was regarded as a candidate bacterial endophyte. Furthermore, we found that species-specific colony PCR can be used to identify *P.*

*dispersa* without the need for DNA extraction from a natural sample. In comparison to conventional methods, it produces results in less time, requires less effort and resources, and is less expensive, rendering it suitable for biotechnological application.

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## References

- [1] I.A. Gibson, The role of fungi in the origin of oleoresin deposits ( agaru ) in the wood of *Aquilaria agallocha* Roxb, Bano Biggyan Patrika, 6, (1977): 16–26
- [2] L. E. Hai, C. Y. Shyun, A. M. Yusoff, Early survival and growth in field trials of *Aquilaria malaccensis* (karas) and *Azadirachta excelsa* (sentang), Journal of Tropical Forest Science, 11, 4 (1999): 852–854
- [3] S. Kamonwannasit, N. Nantapong, P. Kumkrai, P. Luecha, S. Kupittayanant, N. Chudapongse, Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall, Annals of Clinical Microbiology and Antimicrobials, 12, 20 (2013): 12-20
- [4] D.T. Ahmaed, M. Mohammed, A.M. Masaad, S. N. Tajuddin, Investigation of agarwood compounds in *Aquilaria malaccensis* & *Aquilaria Rostrata* chipwood by using solid phase microextraction, Biomedical Journal of Scientific & Technical Research, 1, 6 (2017): 1609-1616
- [5] M. Gao, X. Han, Y. Sun, H. Chen, Y. Yang, Y. Liu, H. Meng, Z. Gao, Y. Xu, Z. Zhang and, J. Han, Overview of sesquiterpenes and chromones of agarwood originating from four main species of the genus *Aquilaria*, The Royal Society of Chemistry, 9, 8 (2019): 4113–4130
- [6] H. Chen, Y. Yang, J. Xue, J. Wei, ZZhang, Z., and, H. Chen, Comparison of compositions and antimicrobial activities of essential oils from chemically stimulated agarwood, wild agarwood and healthy *Aquilaria sinensis* (Lour.) Gilg trees, Molecules, 16, 6 (2011): 4884-4896
- [7] A. López-Sampson, T. Page, History of use and trade of agarwood, Economic botany, 72, 1 (2018):107-129
- [8] L. A. Ogilvie, P.R. Hirsch, Microbial ecology theory: current perspectives. Caister Academic Press, Norfolk, (2012): 71-85
- [9] S. J. Bhole, J. Preveen, K. I. Kandasamy, Isolation and identification of bacterial endophytes from pharmaceutical agarwood-producing *Aquilaria* species, Pharmacognosy Research, 5, 2 (2013): 134–137
- [10] K. Chakrabarty, A. Kumar, V. Menon, Trade in Agarwood, TRAFFIC India and WWF- India, New Delhi, (1994): 51pp
- [11] B. I. Henry, A dictionary of the economic products of the Malay Peninsula. Vol. I. Governments of Malaysia and Singapore, Ministry of Agriculture and Co-operative (1966), HD9016 M3B9
- [12] H. Okugawa, R. Ueda, K. Matsumoto, K. Kawanishi, A. Kato, Effects of agarwood extracts on the central nervous system in mice, Planta Medica, 59, 1 (1993):32-36
- [13] J.J. Tian, X.X. Gao, W.M. Zhang, L. Wang, L.H. Qu, Molecular identification of endophytic fungi from *Aquilaria sinensis* and artificial agarwood induced by pinholes- infusion technique, African journal of biotechnology, 12, 21 (2013): 3115-3131
- [14] M. Ito, K.I. Okimoto, T. Yagura, G. Honda, F. Kiuchi, Y. Shimada, Induction of sesquiterpenoid production by methyl jasmonate in *Aquilaria sinensis* cell suspension culture, Journal of Essential Oil Research, 17, 2 (2005): 175–180
- [15] H. Chhipa, N. Kaushik, Fungal and Bacterial diversity isolated from *Aquilaria malaccensis* tree and soil, induces agarospirol formation within 3 months after artificial infection, Frontiers in Microbiology, 8, (2017): 1286
- [16] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Research, 22, 22 (1994):4673–4680
- [17] K. B. Nicholas, H. B. Nicholas Jr, D. W. Deerfield, GeneDoc: analysis and visualization of genetic variation, EMBNEW News, 4, (1997): 1-4
- [18] S. Kumar, G. Stecher, K. Tamura, MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets, Molecular Biology and Evolution, 33, 7 (2016):1870–1874
- [19] A. Cheng, C.Y. Liu, H.Y. Tsai, M.S. Hsu, C.J. Yang, Y.T. Huang, C.H. Liao, P.R. Hsueh, Bacteremia caused by *Pantoea agglomerans* at a medical center in Taiwan, 2000-2010, Journal of Microbiology, Immunology and Infection, 46, 3 (2013):187–194
- [20] L. Benga, W. P. Bente, E. Engelhardt, K. Köhrer, C. Gougoula, M. Sager, 16S ribosomal DNA sequence-based identification of bacteria in laboratory rodents: a practical approach in laboratory animal bacteriology diagnostics, Laboratory Animals, 48, 4 (2014):305-312
- [21] P. P. Bosshard, R. Zbinden, S. Abels, B. Böttgerhaus, M. Altwegg, E.C. Böttger, 16S

- rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID- GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory, *Journal of Clinical Microbiology*, 44, 4 (2006):1359-1366
- [22] A. M. Walterson, J. Stavrinides, *Pantoea*: insights into a highly versatile and diverse genus within the Enterobacteriaceae, *FEMS Microbiology Reviews*, 39, 6 (2015): 968–984
- [23] C. Brady, I. Cleenwerck, S. Venter, M. Vancanneyt, J. Swings, T. Coutinho, Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA) , *Systematic and Applied Microbiology*, 31, (2008): 447-460
- [24] H. Schmid, S. Schubert, C. Weber, J.R. Bogner, Isolation of a *Pantoea dispersa*-like strain from a 71- year- old woman with acute myeloid leukemia and multiple myeloma, *Infection*, 31, 1 (2003): 66-67
- [25] N. Asai, Y. Koizumi, A. Yamada, D. Sakanashi, H. Watanabe, H. Kato, A. Shiota, M. Hagihara, H. Suematsu, Y. Yamagishi, H. Mikamo, *Pantoea dispersa* bacteremia in an immunocompetent patient: a case report and review of the literature, *Journal of Medical Case Reports*, 13, 1 (2019): 33
- [26] Y. Yang, H. Hu, C. Zhou, W. Zhang, Y. Yu, Q. Liu, T. Lu, Q. Zhang, Characteristics and accurate identification of *Pantoea dispersa* with a case of spontaneous rupture of hepatocellular carcinoma in China: A case report, *Medicine (Baltimore)*, 14, (2022): e28541
- [27] L. Jiang, J.C. Jeong, J.S. Lee, J.M. Park, J.W. Yang, M.H. Lee, S.H. Choi, C.Y. Kim, D.H. Kim, S.W. Kim, J. Lee. Potential of *Pantoea dispersa* as an effective biocontrol agent for black rot in sweet potato *Scientific Reports*, 9, (2019): 16354
- [28] F. Doni, N. S. M. Suhaimi, B. Irawan, Z. Mohohamed, M. S. Mispan, Associations of *Pantoea* with Rice Plants: As Friends or Foes?, *Agriculture*, 11, 12 (2021): 1278