Makara Journal of Science 16/1 (2012) 39-45

# The Genetic Diversity of Endophytic and Phyllosphere Bacteria from Several Indonesian Herbal Plants

Yogiara, Susan Soka[*)], Stella Magdalena, and Devi Rachelia

Faculty of Biotechnology, Universitas Katolik Atmajaya, Jakarta 12930, Indonesia

[*)]*E-mail: susan.soka@atmajaya.ac.id*

## Abstract

Herbal plants have been believed by Indonesians to be an alternative medicine to treat illnesses. The bioactive compounds in the plant can be derived from secondary metabolites or from endophytic and phyllosphere bacteria which coexist within medicinal plants. A total of 18 endophytic bacteria and 32 phyllosphere bacteria were isolated from the herbal plants of *Citrus* sp., *Pluchea indica*, *Curcuma longa*, *Nothopanax scuttelarium*, *Piper crocatum*, and *Andrographis paniculata*. About 72% of endophytic bacteria isolates have proteolytic activity and about 11% have lipolytic activity. On the other hand, about 59% of phyllosphere bacteria isolates have proteolytic activity and about 19% have lipolytic activity. Phylogenetic diversity analysis was conducted by using the amplified ribosomal DNA restriction analysis (ARDRA) method and the sequence of 16S rDNA was digested with endonuclease restriction enzymes: MspI, RsaI, and Sau961. The diversity of endophytic and phyllosphere bacterium from the samples of herbal plants was high. Bacteria isolated from the same herbal plant does not always have a close genetic relationship except for the bacteria isolated from the *P. indica* plant which showed a close genetic relationship with each other.

## Abstrak

**Keragaman Genetik Bakteri Endofit dan Filosfer dari beberapa Tanaman Obat Indonesia**. Tanaman obat dipercaya oleh masyarakat Indonesia memiliki berbagai khasiat untuk menyembuhkan penyakit. Senyawa aktif yang ada pada tanaman dapat berasal dari metabolit sekunder atau dari bakteri endofit dan filosfer yang hidup berasosiasi dengan tanaman obat. Sebanyak 18 isolat bakteri endofit dan 32 isolat bakteri filosfer telah dimurnikan dari tanaman *Citrus* sp., *Pluchea indica*, *Curcuma longa*, *Nothopanax scuttelarium*, *Piper crocatum*, dan *Andrographis paniculata*. Sebanyak 72% isolat bakteri endofit memiliki aktivitas proteolitik dan sebanyak 11% memiliki aktivitas lipolitik. Bakteri filosfer yang memiliki aktivitas proteolitik sebanyak 59% dan sebanyak 19% memiliki aktivitas lipolitik. Analisis keragaman bakteri tersebut dilakukan dengan teknik *amplified ribosomal* DNA *restriction analysis* (ARDRA) dan digesti gen penyandi 16S rRNA dengan menggunakan enzim restriksi endonuklease *Msp*I, *Rsa*I, dan *Sau*961. Keragaman bakteri endofit dan filosfer pada beberapa sampel tanaman obat cukup tinggi. Bakteri yang diisolasi dari tanaman obat yang sama tidak selalu memiliki kekerabatan genetik yang dekat. Sementara itu, bakteri asal tanaman *P. indica* memiliki kekerabatan yang cukup dekat satu sama lain.

*Keywords: endophytic bacteria, herbal plants, lipolytic, phyllosphere bacteria, proteolytic*

## 1. Introduction

The World Health Organization (WHO) recorded that around 75–80% of the world's population uses herbal plants for medical cures due to its socially acceptance in the community, better tolerated by the human body, and have fewer side-effects [1]. Herbal plants are used to treat several ailments such as combating bacteria, diabetes, fungi, hypercholesterol, inflammation, tumors, cardiovascular diseases, as well as functioning as an anti depressant for the central nervous system, sitotoxin, diuretic, *et cetera* [2]. Herbal plants in Indonesia are used to treat various illnesses. Indonesia has high biodiversity of herbal plants. Approximately 10% of the existing plants are believed to have medicinal benefits [3]. Some herbal plants commonly used by Indonesians are the citrus fruit plant (*Citrus sp.*), *beluntas*-Indian March Fleabane (or *Pluchea indica*), turmeric (*Curcuma longa*), *mangkokan*-Fabian Aralia or *Nothopanax scuttelarium*), red betel (*Piper crocatum*), and *sambiloto*-King of bitters (*Andrographis paniculata*).

Several herbal plants have active compounds that are believed to be influenced by the coexistence of microbes within this plant. These microbes can produce active compounds with the potential to act as medicine [4]. Microbes that coexist with the plant can live on the surface of the plant or in the plant's system. The habitat above ground where microbes grow is called the phyllosphere and microbes that grow on the plant's surface are called epiphytes [5]. Microbes that grow in the plant's system are called endophytics [6]. Endophytics are symbiotic and exist in the plant's system without causing it any harm [4]. Nutrients needed by the phyllosphere microbe to grow, for instance carbohydrates, organic acid, and amino acid, come from the plant [7].

Several studies have reported that microbes which coexist with plants can produce secondary metabolites that are beneficial to treat ailments like tumors, bacteria, fungi, and compounds to regulate plant growth [4]. Guo *et al.* reported that endophytic microbes can also act as bio-controls in reducing toxic compounds found in nature [8]. Zhao *et al.* found that one microbe example which had a bioactive compound was Actinomycetes. Actinomycetes, is an endophytic that is effective as a broad spectrum antimicrobial [9].

Gayathri *et al.* stated that endophytic bacteria isolated from the swamp mangrove plant could produce the enzymes protease, inulase, and invertase [10]. Carrim *et al*. carried out research on the endophytic bacteria that was isolated from the *Jacaranda decurrens Cham*-trumpet creeper family plant and found that this bacteria produce amilase, esterase, and lipase enzymes [11]. Moreover, phyllosphere bacteria produce amilase, lipase, and protease enzymes [12].

Data on the diversity of the phyllosphere and endophytic bacteria from Indonesia, in particular those associated with herbal plants, is still scarce. Apart from that, the potential of these bacteria has not yet been fully studied. So, this study aims to gather data on the diversity of phylogenetic isolates of phyllosphere and endophytic bacteria from several Indonesian herbal plants by using the amplified ribosomal DNA restriction analysis technique (ARDRA). Moreover, the proteolytic and lipolytic activity of endophytic and phyllosphere bacteria were also characterized in this study.

## 2. Methods

**Bacterial Isolates.** The isolated bacteria used in this research came from the collection of Microbiology Laboratory, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta. Bacteria were isolated from the *Citrus plant sp., Pluchea indica, C. longa, N. scuttelarium* Merr., *P. crocatum,* and *A. paniculata*.

**Bacterial DNA genome extraction.** The extraction of the genomes was done according to Moore *et al.* by adjusting the volume of the solution used [13]. As much as 1.5 ml bacteria culture was transferred to a microtube, then centrifuged at a speed of 10000 x g for 10 minutes. The suspended cell pellets used 1X TE, pH 8.00 with final volume of 400 µl. Then, 25 µl lysozyme (50 mg/ml) was added to the sample and incubated at 37 °C for 1 hour. A total of 100 µl 10% SDS was further added to the sample and the tube was flipped up and down. Afterwards, 10 µl of Proteinase-K with a concentration of 10 mg/ml was added to the sample and incubated at 37 °C for 1 hour.

A total of 100 µL 5 M. NaCl and 100 µl 10% CTAB was added to each sample. The tubes were flipped and incubated at 65 °C for 30 minutes. Then 500 µl of the solution comprising Fenol : Chloroform : Isoamilalcohol (25:24:1) was added to the sample and centrifuged for 10 minutes at a speed of 10000 x g. The liquid formed phase was transferred to a new microtube and 500 µl of Chloroform : Isoamilalcohol (24:1) was added to the mixture then shaked and centrifuged at the same speed as before.

The newly formed liquid phase was transferred to a microtube, then isopropanol absolute cold solution with final volume of 0.6 x the volume of the transferred solution was added. After that, it was incubated for 1 hour at -20 °C, before the solution was centrifuged for 10 minutes at a speed of 10 000 x g. Pellets produced were washed with 700 µl ethanol 70% and centrifuged at a high speed. Ethanol was discarded and the pellets were air-dried for 15-30 minutes. After that the pellets were resuspended using 50 of µL of TE buffer. Gel agarose 1% was used to verify the isolated DNA and it was run for 30 minutes with a potential difference of 100 V.

**Amplification of a 16S-rDNA.** Amplification was conducted by using the PCR technique. The mixture for each PCR amplification gene 16S rRNA reaction consisted of 12.5 µL GoTaq Green Master Mix (Promega®, USA), primer 63f (5-CAGGCCTAACACATGCAAGTC-3') and primer 1387r (5'-GGGCGGWGTGTACAAGGC-3') each 1 µl (10 pmol/µl) [14], DNA template 1 µL, and $ddH_2O$ 9.5 µl. The procedure for PCR 16s rDNA was pre-denaturated at 94 °C for 5 minutes, followed by 30 cycles : denaturation at 94 °C, for 30 seconds; annealing at 55 °C for 30 seconds; and extention at 72 °C for 1 minute. The final extention was at 72 °C for 20 minutes. PCR results were kept in -4 °C for further use.

**Endonuclease Restriction Reaction**. The PCR product of 16S rDNA was digested with the restriction enzyme Sau961, MspI, and RsaI. A total of 7 µl PCR product was digested with 2 U restriction enzyme Sau961, MspI, and RsaI (New England Biolabs, Beverly, Ma) [15], with a final volume of 20 µl for each reaction. Results

of the digestion was run with electrophoresis gel agarose with a concentration of agarosa 2% in *buffer* electrophoresis 1X TAE pH 8.00 for 1 hour with a potential difference of 80V.

**Construction of Phylogenetic Tree.** To determine the genetic affinity between one isolate and another isolate, the pattern of DNA produced from the digested PCR products was converted into binary data. This data was further input into TREECON program for Windows ver. 1.3b [16]. The neighbour-joining method was used to construct a phylogenetic tree.

**Characterization of bacterial lipolytic and proteolytic activities**. To characterize the proteolytic activity of the bacteria, the media containing skimmed milk gel was used. The bacteria was further incubated at 30 °C overnight. Meanwhile, to characterize the lipolytic activity of bacteria, the bacteria was cultivated in a Rhodamine B media [17] and incubated at 30 °C overnight.

## 3. Results and Discussion

The PCR-amplified product of 16S rDNA isolated from phyllosphere and endophytic bacteria were digested to analyse their genetic diversity. The results of the digestion were converted into binary data then respective phylogenetic tree were generated.

The phylogenetic tree of endophytic bacteria (Figure 1) indicated a quite high genetic diversity because the isolates had different ARDRA patterns. The bacteria isolated from beluntas and sambiloto plant were in the same *cluster*. However, the bacteria from the citrus fruit plant, turmeric, and red betel werein the different *cluster*.

In general, the endophytic bacteria life-cycle occurs in the plant's cells [18]. This bacteria can colonize the plant's system without causing an infection or any negative effect on the plant. The endophytic bacterium commonly founded was from the genus *Cellulomonas, Clavibacter, Curtobacterium, Pseudomonas,* and *Microbacterium* [18].

Endophytic bacteria can be found in all plant species [8]. The endophytic bacteria that was successfully isolated only came from the plants of the beluntas, citrus fruit, turmeric, red betel, and sambiloto. Whereas, no endophytic bacteria was obtained from the mangkokan plant. The endophytic bacteria successfully isolated from the sambiloto and red betel plants consisted of only one isolate.

Most endophytic bacteria isolated from the beluntas plant and the bacteria from the red betel plant (SmerF1) were in the same *cluster*. The existence of isolates in one cluster shows that these isolates share a close genetic relationship to each other. The bacteria from other beluntas plants, BelF6 and BelF7, have a a close genetic relationshipwith the endophytic bacteria isolated from the citrus fruit plant and turmeric. The phyllosphere bacteria isolated from the same herbal plant tended not to group in the same *cluster*. However, it could be seen that the bacteria isolated from the same plant had a closer genetic relationship than the bacteria isolated from different herbal plants.

The phylogenetic tree of the phyllosphere bacteria that was sucessfully isolated from the plants of the citrus fruit, mangkokan, beluntas, sambiloto, and red betel was shown in Figure 2. Bacteria isolated from the same plant was in a different *cluster*. They produced two bacteria (Jer11 and Bel6) in the same branch.

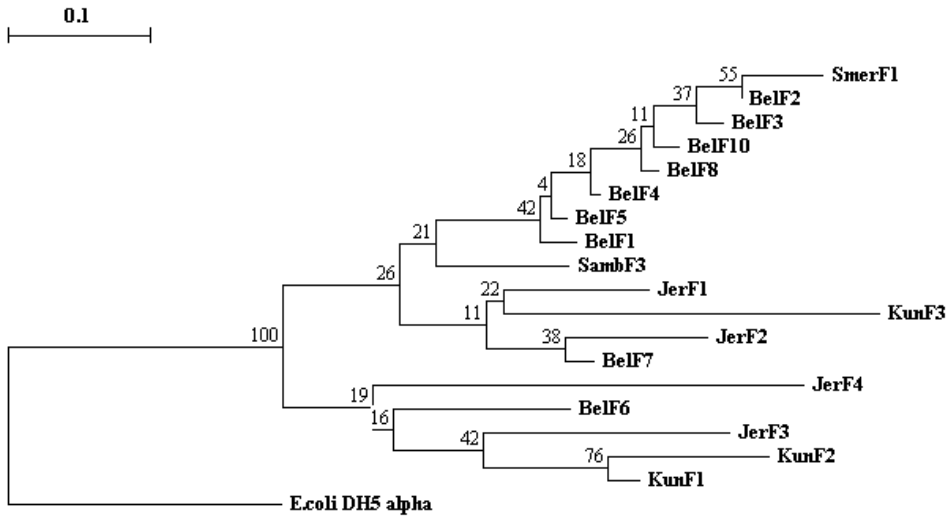


**Figure 1**. **Phylogenetic Tree of Endophytic Bacteria from the Plants of the Beluntas (Bel), Orange (Jer), Kunyit (Kun), Sambiloto (Samb), Andred Sirih (Smer)**

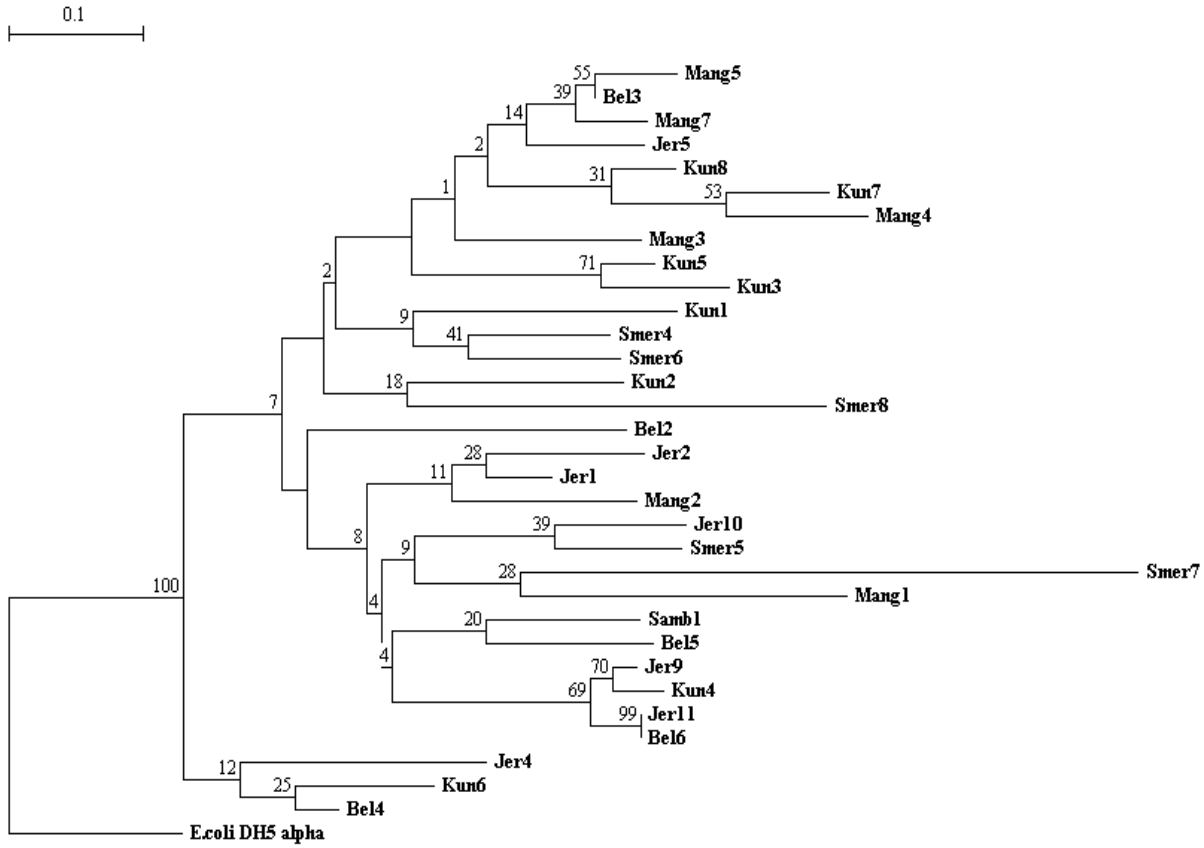


**Figure 2. Phylogenetic Tree of Phyllosphere Bacteria from the Beluntas Plant (Bel), Citrus Fruit (Jer), Turmeric (Kun), Mangkokan (Mang), Red Betel (Smer), and Sambiloto (Samb)**

From the phylogenetic tree of phyllosphere bacteria, it can be seen that each plant has various kinds of isolated bacteria. The diversity of the phyllosphere bacteria can be caused by fluctuation in physical conditions and by the nutrients on the plant's surface. The surface of the plant experiences rapid changes in temperature and humidity in response to the presence of dew and rain. Apart from that, the plant's surface limits nutrition for the bacteria colony [5] and high ultraviolet radiation [7]. Generally phyllosphere bacteria can withstand this kind of environmental stress [5].

In general, phyllosphere bacteria can also withstand high ultraviolet radiation on the leaf's surface. Several phyllosphere bacterium can produce pink or orange pigment or extracellular polysaccharide (EPS) to protect the bacteria from this radiation [6]. EPS can also protect the bacteria from the limitations of water and helps the cell to adhere to the surface of the leaves, EPS can also protect the bacteria from antibioticsand other obstructing compounds [6,19].

The existence of phyllosphere bacteria can also be affected by the bacteria that may, under certain conditions, have ice nucleus activity ($Ice^+$), for instance *Pseudomonas syringae*. This bacteria can cause frozen lesions on the leaves and causes water to freeze at -2 °C so that any other bacteria living in the plant dies. Anti microbes produced by several phyllosphere bacteria can also influence the diversity of phyllosphere bacteria [5].

Isolate Bel6 and Jer11 were in the same branch because they have the same digestion pattern with the same restriction enzyme. From the phyllosphere and endophytic bacteria, the phylogenetic tree showed that the endophytic BelF2 bacteria is also in the same branch as bacteria Bel6 and Jer11. The similarity of this pattern could be dued to the similarity of both isolated species. But some species of bacteria may have the same digestion pattern because the enzyme restriction site used was not specific [20].

A unique reduction pattern can be produced if it uses two or more endonuclease restriction enzymes. To distinguish the species three kinds of restriction enzymes were needed. The success of phylogenetic relations analyse using ten restriction enzymes is 76-100% [20].

In further analysis, endophytic, and phyllosphere bacteria were combined into one phylogenetic tree (Figure 3). The endophytic and phyllosphere bacteria

coming from the same plant tended not to group in the same *cluster*. It resulted in two phyllosphere bacterium and one endophytic bacteria in the same branch, that is the Bel 6, Jer11, and BelF2.

The phylogenetic tree of endophytic bacteria, phyllosphere bacteria, and the combination of both is not very different. In the three above mentioned trees the bacteria isolated from the same herbal plant did not group in the same *cluster*. This shows a degree of diversity in the isolated bacteria which was isolated from several Indonesian herbal plants.

The isolates were later tested for proteolytic and lipolytic activities (Table 1). From the 50 available bacterial isolates, the result revealed 32 isolates had proteolytic activity (64%) and 8 isolates had lipolytic activity (19%).

The proteolytic activity of the bacteria was indicated by the existence of a clear zone that formed on the skimmed milk gel. To characterize the lipolytic activity of the bacteria, the luminescent colony of these bacteria was observed under a ultraviolet light. The media used for the lipolytic activity test was olive oil and pink rhodamine B. Positive results revealed the existence of luminescent orange coloured fluorescene. Meanwhile, the bacteria that did not produce lipase accumulated the rhodamine B and the pink colony [17].

A total of 19 phyllosphere bacteria isolates (59%) produced a clear zone in the skim milk gel medium. A total of 6 isolates of the phyllosphere bacteria had also lipolytic activity (19%) More of these phyllosphere bacteria produced the protease enzyme than the lipase enzyme.

Results showed that 13 endophytic bacteria isolates (72%) produced the protease enzyme. A total of 2 endophytic bacteria isolates (11%) could glow under ultraviolet light. Likewise, with the phyllosphere bacteria, the endophytic bacteria produced more of the protease enzyme.

From these results it is clear that only a few bacterium isolates produce the lipase enzyme. There are more bacteria isolates producing protease enzymes. Based on

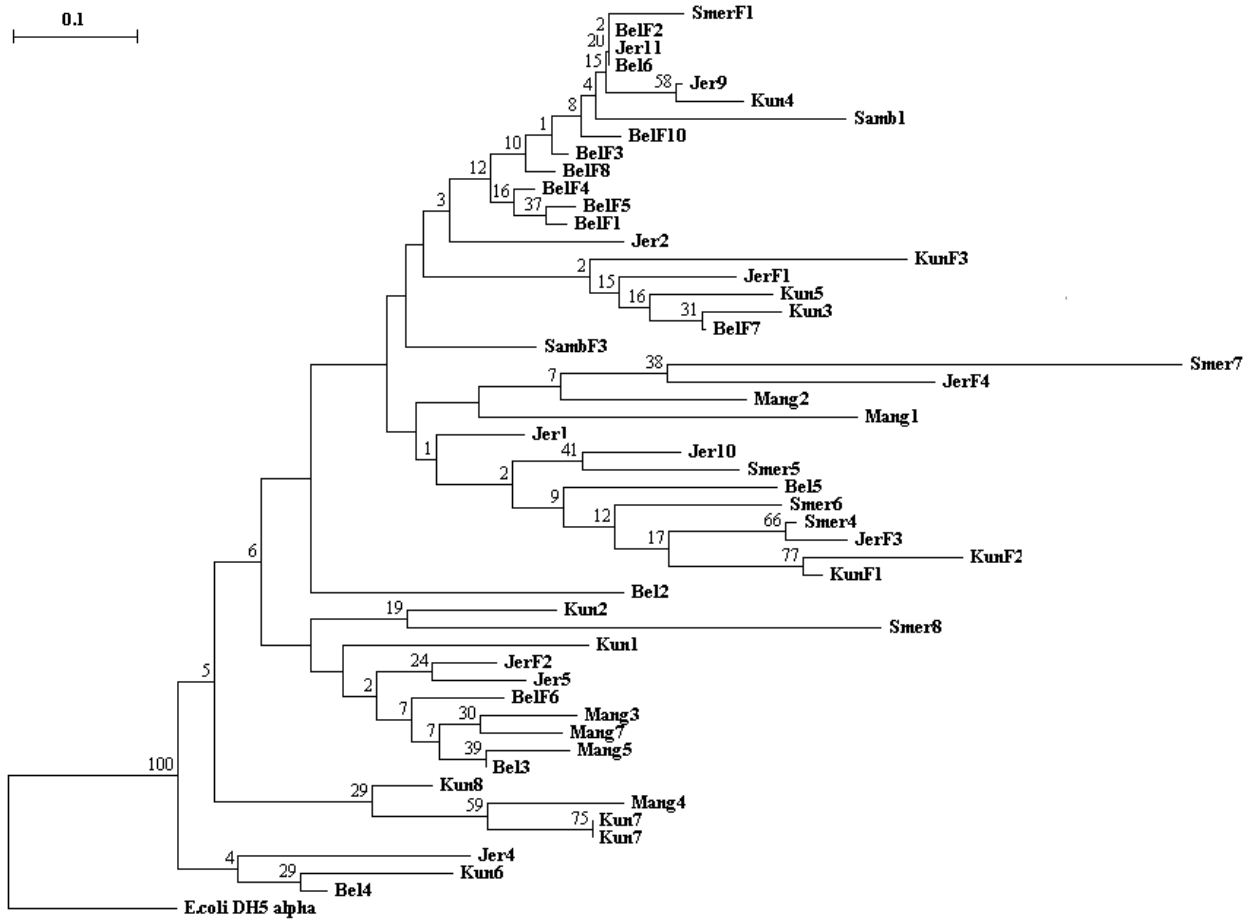


**Figure 3. The Phylogenetic Tree of Endophytic and Phyllosphere Bacteria from the Plants of the Beluntas (Bel), Citrus Fruit (Jer), Turmeric (Kun), Mangkokan (Mang), Red Betel (Smer), and Sambiloto (Samb)**

**Table 1. Results of the Proteolytic and Lipolytic Activity Test**

| Isolate Code | Phyllosphere Bacteria | | Isolate Code | Endophytic Bacteria | |
|---|---|---|---|---|---|
| | Protease | Lipase | | Protease | Lipase |
| Beluntas | | | | | |
| Bel2 | - | - | BelF1 | - | - |
| Bel4 | + | - | BelF2 | - | - |
| Bel5 | + | - | BelF3 | + | - |
| Bel6 | - | - | BelF4 | + | + |
| | | | BelF5 | + | - |
| | | | BelF6 | - | - |
| | | | BelF7 | + | - |
| | | | BelF8 | + | - |
| | | | BelF10 | - | - |
| Jeruk | | | | | |
| Jer1 | + | - | JerF1 | + | - |
| Jer2 | + | - | JerF2 | + | - |
| Jer4 | - | - | JerF3 | - | - |
| Jer4 | - | - | JerF4 | + | - |
| Jer5 | - | - | | | |
| Jer9 | - | - | | | |
| Jer10 | + | - | | | |
| Jer11 | + | - | | | |
| Kunyit | | | | | |
| Kun1 | + | - | KunF1 | + | - |
| Kun2 | + | - | KunF2 | + | - |
| Kun3 | + | + | KunF3 | + | - |
| Kun4 | + | - | | | |
| Kun5 | - | - | | | |
| Kun6 | + | + | | | |
| Kun7 | - | - | | | |
| Kun8 | - | - | | | |
| Mangkokan | | | | | |
| Mang1 | + | + | | | |
| Mang2 | + | - | | | |
| Mang3 | + | + | | | |
| Mang4 | - | - | | | |
| Mang5 | + | - | | | |
| Mang7 | - | + | | | |
| Sambiloto | | | | | |
| Samb1 | + | - | SambF3 | + | - |
| SirihMerah | | | | | |
| Smer3 | + | - | SmerF1 | + | + |
| Smer4 | - | - | | | |
| Smer5 | + | - | | | |
| Smer6 | - | - | | | |
| Smer7 | + | + | | | |
| Smer8 | - | - | | | |

theuis results, endophytic bacteria has higher proteolytic activity than phyllosphere bacteria. Whereas, phyllosphere bacteria has higher lipolytic activity. Previous studies also found the proteolytic and lipolytic activity from the endophytic and phyllosphere bacteria. The endophytic bacteria isolated from the mangrove plant yielded protease activity of around 58.3% [10].

The endophytic bacteria isolated from the *Jacaranda decurrens Cham*. plant yielded a total of 60% isolates with proteolytic activity and 40% with lipolytic activity [11]. Meanwhile, the phyllosphere bacteria isolated from the mangrove plant in Orissa Coast yielded isolates with protease enzyme activity totalling 57.14% and isolates with lipase enzyme activity of 3.26% [12].

## 4. Conclusions

Bacteria isolated from the herbal plants of beluntas, citrus fruit, turmeric, mangkokan, sambiloto, and red betel have quite high diversity. The total number of endophytic bacteria that was successfully isolated was less than the phyllosphere bacteria. Bacteria from one herbal plant tended to have a closer phylogenetic relationship than with bacterium from another herbal plant. The number of bacterium with proteolytic activity was more than the bacteria with lipolytic activity both in the endophytic and phyllosphere bacteria.

## Acknowledgements

This research was fully funded by the Research Centre of Atma Jaya Catholic University, Indonesia.

## References

[1] P. Jayaraj, Int. J. Green Pharm. 4 (2010) 10.
[2] D.S. Fabricant, N.R. Fransworth, Environ. Health Perspecti. 109 (2001) 69.
[3] K.I. Miller, S.D. Ingrey, A. Alvin, M.Y.D. Sze, B.D. Roufogalis, B.A. Neilan, Microbiol. Aust. 31 (2010) 13.
[4] S. Kumala, E.B. Siswanto, Microbiol. Indones. 1 (2007) 145.
[5] S.E. Lindow, M.T. Brandl, Appl. Environ. Microbiol. 69 (2003) 1875.
[6] J.M. Whipps, P. Hand, D. Pink, G.D. Bending, J. Appl. Microbiol. 105 (2008) 1744.
[7] C.H. Yang, D.E. Crowley, J. Borneman, N.T. Keen, PNAS 98 (2000) 3889.
[8] B. Guo, Y. Wang, X. Sun, K. Tang, Appl. Biochem. Microbiol. 44 (2008) 136.
[9] K. Zhao, P. Penttinen, T. Guan, J. Xiao, Q. Chen, J. Xu, K. Linström, L. Zhang, X. Zhang, G.A. Strobel, Curr. Microbiol. 62 (2011) 182. doi:10.1007/s00284-010-9685-3.
[10] S. Gayathri, D. Saravanan, M. Radhakrishna, R. Balagurunathan, K. Kathiresan, Indian J. Biotechnol. 9 (2010) 397.
[11] A.J.I. Carrim, E.C. Barbosa, J.D.G. Vieira, Brazil Arch. Biol. Technol. 49 (2006) 353.
[12] N. Gupta, S. Das, U.C. Basak, Mal. J. Microbiol. 3 (2007) 15.
[13] E. Moore, A. Arnscheidt, A. Kruger, C. Strompl, M. Mau, Mol. Microbiol. Ecol. Man. 2 (2004) 1.
[14] J.R. Marchesi, T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiom, W.G. Wade, Appl. Environ. Micriobiol. 64 (1998) 795.
[15] C.L. Moyer, J.M. Tjedje, F.C. Doblos, D.M. Karl, Appl. Environ. Microbiol. 62 (1996) 2501.
[16] Y. van de Peer, R. de Wachter, Conput. Applic. Biosci. 10 (1994) 569.
[17] G. Kouker, K.E. Jaeger, Appl. Environ. Microbiol. 53 (1987) 211.
[18] R.P. Ryan, K. Germaine, A. Franks, D.J. Ryan, D.N. Dowling, FEMS Microbiol. 278 (2007) 1.
[19] G.A. Beattie, S.E. Lindow, Phytopathology 89 (1999) 353.
[20] M.Y. Sklarz, R. Angel, O. Gillor, M.I.M Soares, Antonie van Leeuwenhoek 96 (2009) 659.