The Exploration of Ketosynthase Gene on Endophytic Bacterial Root of *Vetiveria zizanioides* L.

Any Fitriani*, Any Aryani, Hasbi Yusuf, Yani Permatasari

***Abstract*— The exploration of ketosynthase gene has been conducted on 17 isolates of endophytic bacterial root of *Vetiveria zizanioides* L. Ketosynthase gene was detected by two pairs of degenerate oligonucleotide primers, that is DKF-DKR and HGLF-HGLR. Meanwhile, another partial-sequence analysis was conducted on 16S rRNA gene that was amplified by 63F and 1387R primers. Partial sequence of ketosynthase gene was obtained by sequencing the result of the 400 and 700 bp amplicon in size. The result of DNA amplification showed that there were only five species of endophytic bacteria detected to have ketosynthase gene. Through the analysis of 16S rRNA gene, the species information of the bacteria was obtained, i.e. *Lysinibacillus sphaericus* (Isolate A), *Pantoea sp* (Isolate H), *Bacillus sp* (Isolate K), *Acinetobacter sp* (Isolate M) and *Pseudomonas aeruginosa* (Isolate O). Through bioinformatics and phylogenetic study, it was proven that isolate H, M, and O are included in proteobacteria group with ketosynthase gene type I, while isolate A (*Lysinibacillus sphaericus)* and isolate K (*Bacillus* sp*)* belong to *Firmicutes* bacteria group with ketosynthase gene type II. The separation of the branches on bacterial family tree demonstrated the evolution of ketosynthase genes in the bacteria itself.**

Index Term* — Endophytic Bacteria, Ketosynthase, 16S rRNA, *Vetiveria zizanioides

I. Introduction

There is an ongoing need for novel drugs as they are highly effective in the treatment of cancer, drug resistant bacteria, fungal infections, emerging viruses and parasitic protozoan infections. Historically, natural products have provided the basis for the majority of new drugs, and the bioactive properties of a wide variety of flora are reflected in their continued roles in the traditional healthcare of many cultures. The successful use of plants in traditional medicine and modern natural products research has meant a renewed interest in exploiting various aspects of the underlying bioactivities [1].

In general, endophytic bacteria exist in lower population densities than rhizospheric bacteria or bacterial pathogens. It has not been resolved whether plants gain more benefits from endophytic bacterium than those from rhizospheric bacterium. There are more advantages for bacteria to become endophytic than rhizospheric. It is not clear which population of microorganisms (endophytic bacteria or rhizospheric bacteria) that promotes plant growth [2]. Potential properties of endophytic microbes have been studied for various purposes; one of which is to produce bioactive compounds as plant protection agents usually contained in a tissue system, such as leaves, stems, or roots of plants. These microbes are able to produce mycotoxins, enzymes and antibiotics. The active materials obtained from endophytic microorganisms are considered to have the same capabilities with the active materials produced by the plant as the host [3].

Endophytic bacteria are able to prevent the deleterious effects of certain pathogenic organisms. The beneficial effects of bacterial endophytes on their host plant appear to occur through similar mechanisms as described on rhizosphere-associated bacteria. Several genera of bacteria are well known for their diverse range of secondary metabolite products including antibiotics, anticancer compounds, volatile organic compounds, and antifungal, antiviral, insecticidal and immunosuppressant agents. While a wide range of biologically active compounds have been isolated from endophytic organisms, they still remain a relatively untapped source of novel natural products [4].

Low molecular weight of secondary endophytic metabolites demonstrates a high degree of structural diversity with the largest and most important groups of compounds including the polyketides, amino-acid-derived compound, and terpenes. Genetically, methods have been used to screen biosynthetic pathways involved in secondary metabolism. Genetic screening for natural microbial product genes has largely focused on the detection of the polyketide and non-ribosomal peptide synthesis pathways [1]. Polyketides was produced by most fungi, plants, bacteria and aquatic organisms. Superfamily from a wide range of structural products of active compounds has been widely found in the pharmaceutical applications, such as rapamycin (immunosuppressant), erythromycin (antibiotic), lovastatin (anti-cholesterol drugs), and β epothilone (anticancer).

Biosynthesis polyketides were made by large multimodular enzyme complexes, i.e. polyketide synthase (PKSs) [5]. Polyketide chain elongation requires a three-part compound core domain of the polyketide synthase, the acyltransferase domain (AT), acyl carrier protein (ACP) and ketosynthase domain (KS) [1]. Type I PKSs contain, within a

Co. Author: Any Fitriani is with Laboratory of Biotechnology, Department of Biology Education, Indonesia University of Education, Dr. Setiabudhi 229 Bandung 40154, Indonesia (email: anyfitriani@upi.edu).
Any Aryani is now with Laboratory of Biotechnology, Department of Biology Education, Indonesia University of Education, Dr. Setiabudhi 229 Bandung 40154, Indonesia (email: any_aryani@yahoo.com).
Hasbi Yusuf and Yani Permatasari are with Laboratory of Biotechnology, Department of Biology Education , Indonesia University of Education, Dr. Setiabudhi 229 Bandung 40154, Indonesia (email: hasbidakwatuna@gmail.com).

multifunctional polypeptide, all of enzymatic activities necessary for one cycle of β-keto chain elongating and processing, and may be either modular (mostly in bacteria) or iterative (in fungi). In modular PKSs, each polypeptide includes one or multiple modules, and each module is responsible for one round of condensation and β-keto chain processing. Each catalytic domain of modular type I PKSs is used only once during the biosynthetic process [6]. In recent years, ketosynthase is known as one of the superfamilies of enzymes associated with biosynthetic complex of secondary metabolites in prokaryotic, fungi and plants. Ketosynthase enzyme has been known to produce various products, such as antibiotics and other products needed for medical and industrial purposes in a large scale. Ketosynthase domain required for the condensation of an extender unit to the growing polyketide chain during polyketide biosynthesis occurs. Therefore, the ketosynthase's relationship is to determine the production of diverse structure metabolites [7].

Based on numerous studies, it is known that the superfamily of enzymes ketosynthase can be synthesized by a number of endophytic bacteria, especially on an aromatic plant tissue, so one of them indicates that it exists in *Vetiveria zizanioides* (vetiver) plant. It has much information about the pharmacology of *V. zizanioides*, especially the ability of essential oils. A research on *V. zizanioides* in India shows that the compounds, contained in the roots of *V. zizanioides*, have biological properties as an antifungal, antioxidant, and antibacterial agents [8]. The location of essential oil production in the roots is in the first membrane of outer cortex endoderm. The close relationship between the production of aromatic oils and those endophytic bacteria resulted in a hypothesis is that there is direct involvement of endophytic bacteria in the production of essential oils in *Vetiveria* [9]. Based on this condition, it is important to know the diversity and availability of ketosynthase enzyme from the endophytic bacteria, and then perform an exploration of *ketosynthase* gene on the endophytic bacterial root of *V. zizanioides*. The results of this analysis are expected to indicate that the type of ketosynthase from one group of bacteria is different from others. This evolutionary analysis is very important to gain a better understanding about the diversity of production systems and the biosynthesis of bioactive polyketides. The result can be used for making new drugs, antibiotics and other medical uses in the future [7].

II. MATERIALS AND METHODS

A. Sterilization of Tools and Media

The tools are washed and dried first. Then, the tools are wrapped with wrapping paper. After that, the tools are sterilized in autoclave for 15-20 minutes at 121° C with the pressure of 1.5 atm. Luria Bertani (LB) agar and LB broth, which have been used for culturing bacteria, are also sterilized for 15 minutes.

B. Cultivation of Bacteria

All tools and materials are stored in the laminar air flow with the first UV irradiated for 15 minutes. Then a total of 17 bacterial isolates are grown or sub-cultured in LB media in order to obtain pure cultures of each endophytic bacterial root species of *V. zizanioides*. Each bacterial isolate is incubated at room temperature for 2-4 days.

C. Bacterial Culture Preparation

Seventeen isolates of bacteria that have been previously grown in LB agar is recultured in liquid media of LB Broth. Each bacterial culture should be placed in a 1.5 ml sterilized micro centrifuge tube, and then centrifuged for 5 minutes at 7000 rpm. Supernatant is discarded until the only form left in the tube is bacterial cell sediment (pellet), and then resuspended in 200 µl of TE (Tris EDTA) buffer.

D. DNA Extraction

Extraction of total DNA (genome) in each sample of bacteria was done by using a commercial kit, Fermentas Genomic DNA Purification Kit (Lithuania) with the working procedures referring to the protocol of manufacturing employment in the kit. There are several modifications to the extraction process steps, such as the length of incubation and centrifugation.

Determination of the concentration of extracted DNA is done by measuring the absorbance of DNA on a spectrophotometer with a wavelength of 260 nm. The purity of DNA is also calculated by calculating the absorbance ratio of 260 and 280 nm [10].

E. Amplification of DNA

The process of DNA amplification is performed on MasterCycler Personal machine (Eppendorf, Germany). Primer pairs and conditions refer to Moffit and Neilan (2002) with slight modifications on the annealing temperature and duration of Polymerase Chain Reaction (PCR) process. The amplification of ketosynthase gene uses two pairs of degenerate oligonucleotide primers; DKF-DKR and HGLF-HGLR. The amplification is then performed according to the following profile; 5 minutes at 95◦C for pre denaturation and 30 cycles of 1 minutes denaturation at 95◦C, annealing, 1 minute at 51◦C for DKF/DKR and 47◦C for HGLF/HGLR, 1 minute for extension at 72◦C, and 7 minutes at 72◦C. In addition, the amplification of 16S rRNA gene is also performed based on the method described by [11].

F. Electrophoresis

Each of the amplified DNAs is detected through the process of electrophoresis by using a BIO-RAD Mini Sub Cell GT (CA, USA) on 2% agarose gel in 0.5x TBE buffer. Electrophoresis method is based on the method of [10].

G. Sequencing DNA

The process of DNA sequencing is conducted by using BigDye Applied Biosystem sequencer engine model 3730 at Macrogen inc., Seoul, South Korea.

H. Bioinformatics Data Analysis

The identification of endophytic bacteria isolate to the species or subspecies taxa is made through the sequence analysis of 16S rRNA gene, whereas the phylogenetics of ketosynthase genes were analyzed by using bioinformatics

methods. Sequencing results are compared with ketosynthase gene in the database of GeneBank NCBI (National Center for Biotechnology Information) at http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi. Protein sequence alignment of PKS (polyketide synthase) uses a program of multiple-sequence alignment tool from Clustal X software and MEGA software (version 5) to analyze phylogenetic endophytic bacteria through the phylogenetic tree produced.

III. RESULTS

A. Amplification and Electrophoresis

The result shows that only five species of bacterial isolate are detected to have ketosynthase gene from the amplification by two primer pairs of degenerate oligonucleotide (Table 1). The product of this ketosynthase gene amplification is visualized by using electrophoresis. From the results of DNA amplification using degenerate oligonucleotide primers (DKF-DKR), we find that only three bacterial isolates are detected to have ketosynthase genes, i.e. sample H with 400 bp DNA in size, and also sample M and O with 700 bp DNA in size. Visualization of the DNA amplification result using the degenerate heterocyst glycolipid primer (HGLF-HGLR) shows that only sample A, K, and O are successfully amplified. The size of the gene in each amplification is 400 bp in sample A and K and 700 bp in sample O (Fig 1 & 2). 16S rRNA gene amplification is also conducted on 17 samples of DNA (total genome) of endophytic bacteria roots of *V. zizanioides*. The amplification of 16S rRNA gene uses primers 63F and 1387R. The product of this 16S rRNA gene amplification is approximately 1300 bp (Fig 3).

B. Bioinformatics Data Analysis

Through the sequence analysis of 16S rRNA gene and the GeneBank BLAST program, there are some species of bacteria detected to have ketosynthase gene. These bacteria are *Lysinibacillus sphaericus* (Isolate A), *Pantoea sp* (Isolate H), *Bacillus sp* (Isolate K), *Acinetobacter sp* (Isolate M), and *Pseudomonas aeruginosa* (Isolate O). The closeness of kinship between these bacteria with other bacteria species are analyzed by phylogenetic tree of bacteria based on 16S rRNA gene (Fig. 4). Through phylogenetic studies based on the ketosynthase genes, as seen in the branches of the phylogenetic tree, bacteria H (*Pantoea sp*), M (*Acinetobacter sp*), and O (*Pseudomonas aeruginosa*) belong to Proteobacteria group that has close kinship with a group of bacteria with type I ketosynthase genes, whereas bacteria A (*Lysinibacillus sphaericus*) and K (*Bacillus sp*) belong to Firmicutes bacteria group that produces type II ketosynthase (Fig 5 & 6).

IV. DISCUSSION

The amplification of ketosynthase (KS) gene fragments is known to be used for homologous hybridization probe that can significantly facilitate the cloning of antibiotic biosynthesis. In addition, phylogenetic analysis of bacteria based on the fragment of KS can show the evolution of bacterial species that synthesize them or only ketosynthase molecular evolution of antibiotic biosynthetic genes. The amplification of ketosynthase gene used two pairs of degenerate oligonucleotide primers, DKF-DKR and the heterocyst glycolipid, HGLF-HGLR. The difference between both primer pairs is the purpose of amplification of the gene itself. Degenerate oligonucleotide DKF-DKR is used to detect gene Polyketide Synthase (PKS) type I in particular Ketosynthase domain of an organism, so that the main function is only to determine the distribution of type I ketosynthase genes of an organism in general. Primer is designed from alignment of the Ketosynthase cluster in PKS genes previously known from a number of bacteria, including Cyanobacteria and Mycobacteria [7]. While the heterocyst glycolipid primer (HGLF & HGLR) is used to determine the level of diversity in the type of ketosynthase from any organism, so that the result of gene amplification using this primer can show the type of ketosynthase diversity in each organism analyzed [7].

The results of electrophoresis DNA show that H, M, and O are successfully amplified by employing degenerate oligonucleotide primer. This means that among the 17 roots of *V. zizanioides* endophytic bacteria, only three isolates of bacteria are detected to have type I ketosynthase genes; those are H, M, and O isolates. The size of the gene is 400 bp in isolate H and 700 bp in isolate M and O. The result correlates with previous research that the ketosynthase gene has size of about 700 bp [7]. The difference of gene sizes of all three amplicons depends on their ketosynthase domain or type of bacteria. DNA samples amplified by heterocyst glycolipid primer are different from previous primer (DKF and DKR), except for O which is successfully amplified by both primer pairs. This is possible due to the differences in diversity of ketosynthase region or the differences owned by bacteria.

Phylogenetic tree reveals that the three bacteria are detected to have ketosynthase genes by degenerate oligonucleotide primers (DKF) which are included into groups of bacteria that have been clearly known to have type I ketosynthase genes (Figure 5). This is reinforced by the separation of bacterial groups with other genes (*hydrolase*) as an out-group with a group of bacteria that have the *ketosynthase* genes. In the phylogenetic tree, it can be seen that *Pantoea* sp. bacteria (Isolate H) is closely related to *Streptomyces coelicolor* bacteria previously known to have type I *ketosynthase* genes. It can be concluded that isolate H bacteria *(Pantoea sp*) has *ketosynthase* gene that is similar to *Streptomyces coelicolor*. Meanwhile, two other bacteria, i.e. isolate M (*Acinetobacter sp*) and isolate O (*Pseudomonas aeruginosa*) bacteria, which are separate from isolate H, form a new branch of the close kinship.

The amplification process using heterocyst glycolipid primers produces phylogenetic tree showing that isolate O bacteria (*Pseudomonas aeruginosa*) belong to a group of bacteria that have type I *ketosynthase* genes, while isolate K (*Bacillus sp*) and isolate A (*Lysinibacillus sphaericus*) bacteria belong to a group of bacteria with type II *ketosynthase* genes (Fig 6). However, the phylogenetic tree also shows that any bacteria have been identified separately from other bacterial groups. Based on [12], ketosynthase fragments may indicate the evolution of bacterial species, so unequal phylogenetic shows a distinct evolution of aromatic polyketides of each bacterium. It is clear that the separation of the bacterial groups shows the evolution of each ketosynthase

gene on each bacterial species itself. Further exploration and study of ketosynthase genes in various organisms is an interesting topic in recent research. Each of these findings can be used as future discovery of antibiotics. [13] reported that 17 isolates of *Streptomyces* include to type 2 of ketosynthase and devided into 6 clades. Among 17 isolates, 5 isolates have the potential ability to produce new type polyketide.

V. CONSLUSION

There are five species detected to have ketosynthase gene from the amplification by two primer pairs of degenerate oligonucleotide, i.e. DKF/DKR and HGLF/HGLR. Three isolates of bacteria are detected to have type I ketosynthase genes; those are H (*Pantoea* sp.), M (*Acinetobacter* sp.) , and O (*Pseudomonas aeruginosa*) isolates. Meanwhile the amplification process using heterocyst glycolipid primers showed that isolate O bacteria (*P. aeruginosa*) belong to a group of bacteria that have type I *ketosynthase* genes, while isolate K (*Bacillus sp*) and isolate A (*Lysinibacillus sphaericus*) bacteria belong to a group of bacteria with type II *ketosynthase* genes.

ACKNOWLEDGMENT

Research Grant of Kelompok Bidang Keilmuan (KBK) of Indonesia University of Education has facilitated this study and is gratefully acknowledged.

REFERENCES

[1] K.I.Miller, C. Qing, D.M.Y. Sze, B.A. Neilan.
[2] "Investigation of the Biosynthetic Potential of Endophytes in Traditional Chinese Anticancer Herbs", *PLoS One*, vol. **7**, pp. e35953. 2012.
[3] M. Rosenblueth, M. Martinez-Romero. "Bacterial Endophytes and Their Interactions with Hosts", *Molecular Plant-Microbe Interactions*, vol. 19, pp. 827–837. 2006.
[4] G. Strobel, B. Daisy. "Bioprospecting for Microbial Endophytes and Their Natural Products". *Microbiology and Molecular Biology Reviews*, pp. 491–502. 2003.
[5] R.P. Ryan, K. Geermaine, A. Franks, D.J. Ryan, D.N. Dowling. "Bacterial endophytes: recent developments and applications". *FEMS Microbiology Letter*.,vol. 278, pp. 1–9. 2007.
[6] D.A. Hopwood, D.H. Sherman. "Molecular genetics of polyketides and its comparison to fatty acid biosynthesis". *Annual Review Genetics*, vol. 24, pp. 37–66. 1990.
[7] T.A. Castoe, T. Stephens, B.P. Noonan, C. Calestani. "The novel group of type I polyketide synthases (PKS) in animals and the complex phylogenomics of PKSs". *Genetics*, vol. 392, pp. 47–58. 2006.
[8] M.C. Moffitt, B.A. Neilan. "Evolutionary Affiliation Within the Superfamily of Ketosynthases Reflect Complex Pathway Associations". *Journal of Molecular Evolution*, vol. 56, pp. 446-457. 2002.
[9] M. Maffei,. "*Vetiveria* : The Genus of *Vetiveria*." Taylor and Francis group, New York. 2002.
[10] L.D. Giudice. "The Microbial Community of *Vetiver* Root is Necessary for Essential Oil Biosynthesis". *Proceedings of the 52nd Italian Society of Agricultural Genetics Annual Congress.* 978-88-900622-8-5. 2008.
[11] J.R. Marchesi, T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiom, W.G. Wade. "Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify Genes Coding for Bacterial 16S rRNA". *Applied and Environmental Microbiology*, vol. 64, pp. 795-799. 1998.
[12] J. Sambrook, D.W. Russel. "*Molecular Cloning-A Laboratory Manual*". Cold Spring Harbor Laboratory Press, New York. 2001. Chap. 12, pp.83-84.
[13] M. Metsa-Ketela, L. Halo, E. Munukka, J. Hakala. P. Mantsala, K. Ylihonko. Molecular Evolution of Aromatic Polyketides and Comparative Sequence Analysis of Polyketide Ketosynthase and 16S Ribosomal DNA Genes from Various *Streptomyces* Species. *Applied and Environmental Microbiology*, vol. 68, pp. 4472-4479. 2002.
[14] Qiu Liu, Changjian liu, Jicheng Yu, Jianfang Yan, Xiaohui Qi. 2012. Analysis of The ketosynthase Genes in Streptomyces and Its Implications for Preventing Reinvestigation of Polyketides with Bioactivities, *Journal of Agricultural Science*, vol. 4, No. 7, pp. 262-270.

Table I
Bacterial strains analyzed in this study by 16S rRNA PCR, degenerate ketosynthase PCR (DKF/DKR) and degenerate heterocyst glycolipid PCR (HGLF/HGLR)
(+) indicates positive PCR result (-) indicates negative PCR result

Sample	16S rRNA	DKF-DKR	HGLF-HGLR
Isolate A	+	-	+
Isolate B	+	-	-
Isolate C	+	-	-
Isolate D	+	-	-
Isolate E	+	-	-
Isolate F	+	-	-
Isolate G	+	-	-
Isolate H	+	+	-
Isolate I	+	-	-
Isolate J	+	-	-
Isolate K	+	-	+
Isolate L	+	-	-
Isolate M	+	+	-
Isolate N	+	-	-
Isolate O	+	+	+
Isolate P	+	-	-
Isolate Q	+	-	-

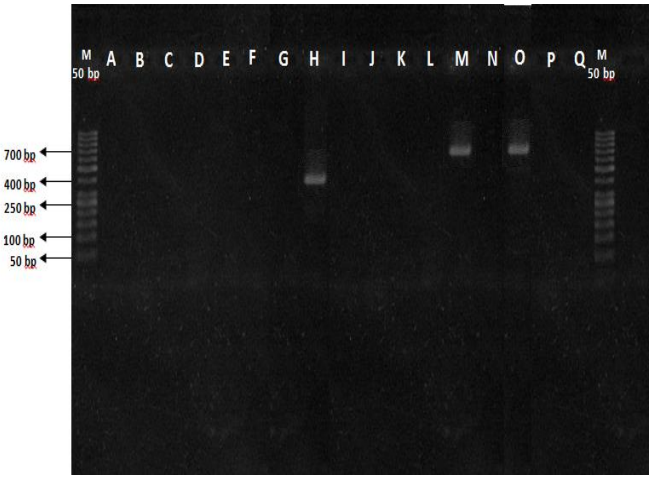

Fig. 1. The result of ketosynthase PCR. The DNA samples of endoophytic bacterial root amplified by *degenerate oligonucleotide ketosynthase* (DKF-DKR)
M 50 bp = DNA *Marker* 50 bp *Gen Ruller* (Fermentas)

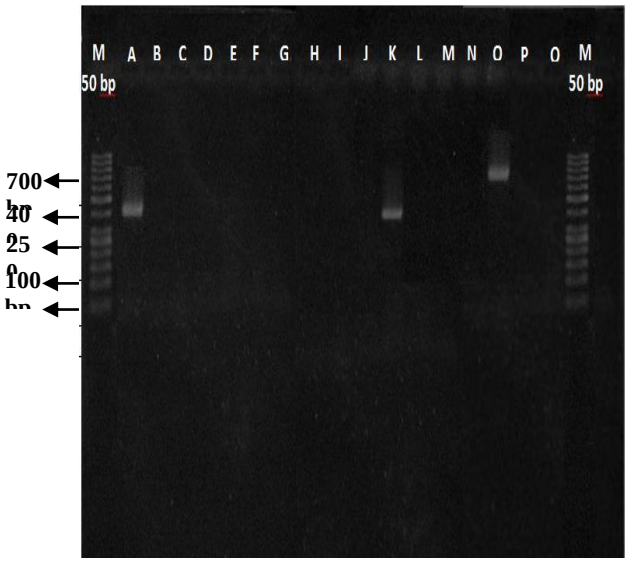

Fig. 2. The result of ketosynthase PCR. The DNA samples of endophytic bacterial root amplified by *degenerate oligonucleotide heterocyst glycolipid* (HGLF-HGLR)
M 50 bp = DNA *Marker* 50 bp *Gen Ruller* (Fermentas)

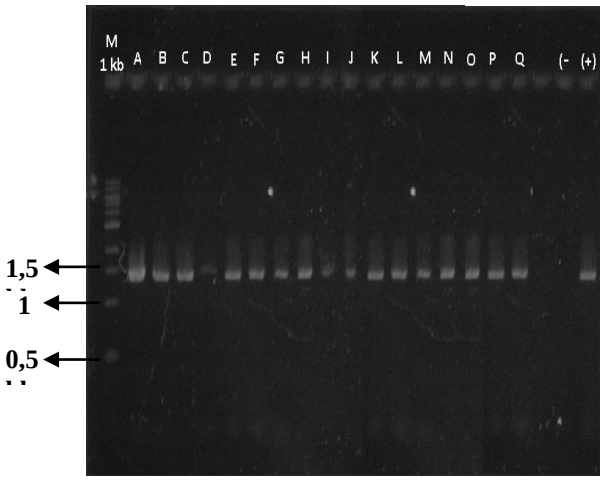

Fig. 3. The result of *16S rRNA* gene amplification
(+) = Positive Control PCR, (-) = Negative Control PCR.

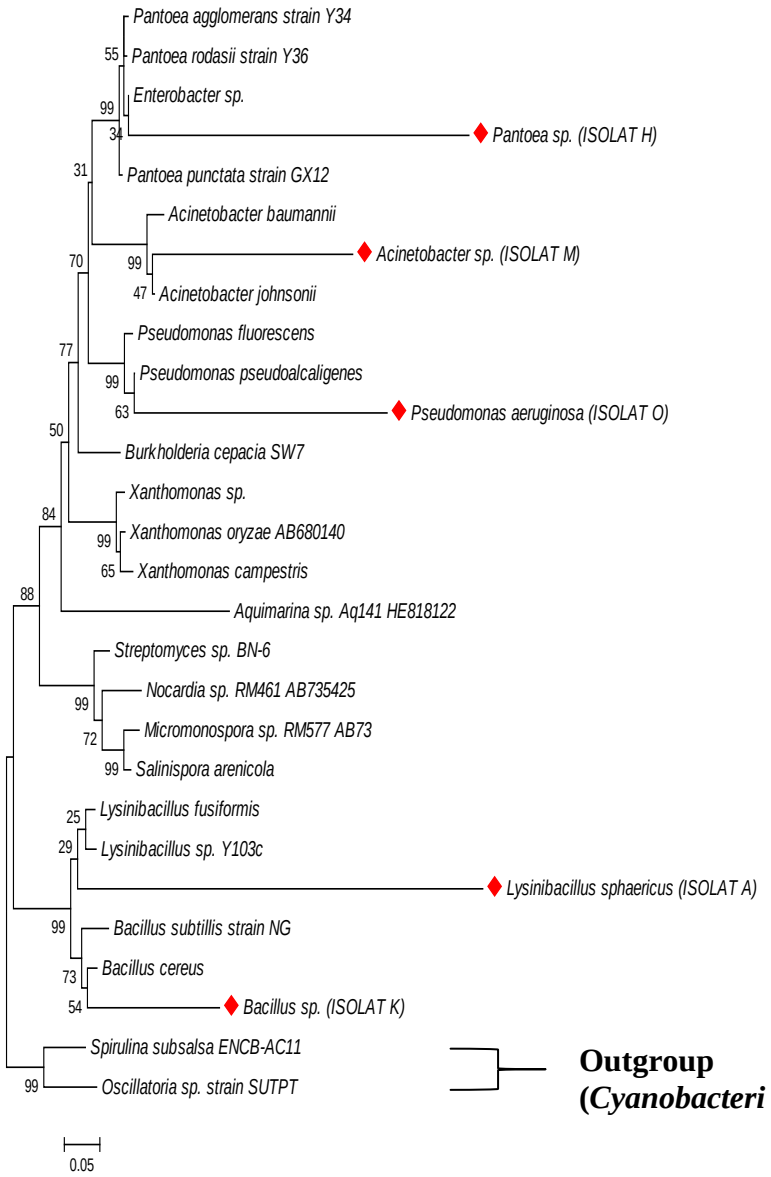

Fig. 4. Phylogenetic analysis of *Vetiveria zizanioides* L. root endophytic. Sequences obtained during this *16S rRNA* gene study are given in red symbol. Other sequences are obtained from GenBank; Sequences are aligned using the MEGA v.5 and Clustal X programs

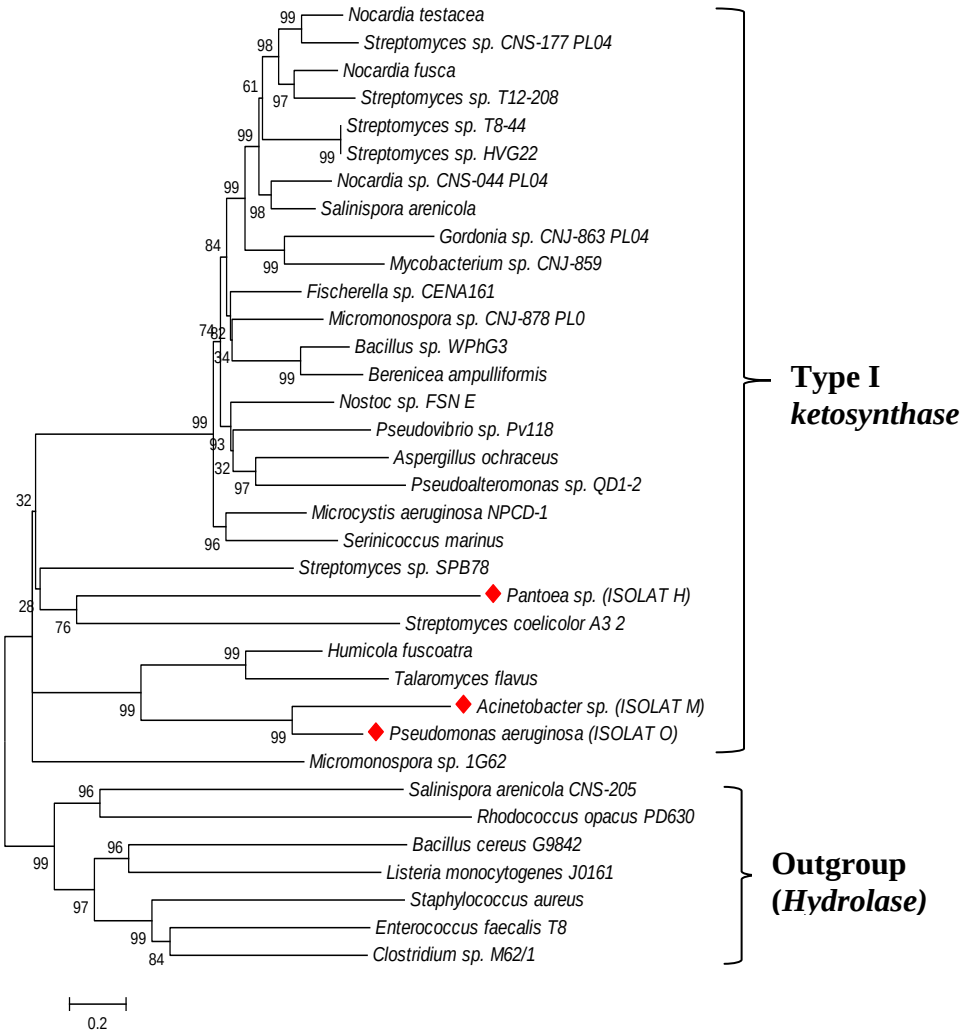

Fig. 5. Phylogenetic analysis of type I ketosynthase domains. Sequences obtained during this study by using degenerate primers are given in red symbol. Other sequences are obtained from GenBank; Sequences are aligned using the MEGA v.5 and Clustal X programs.

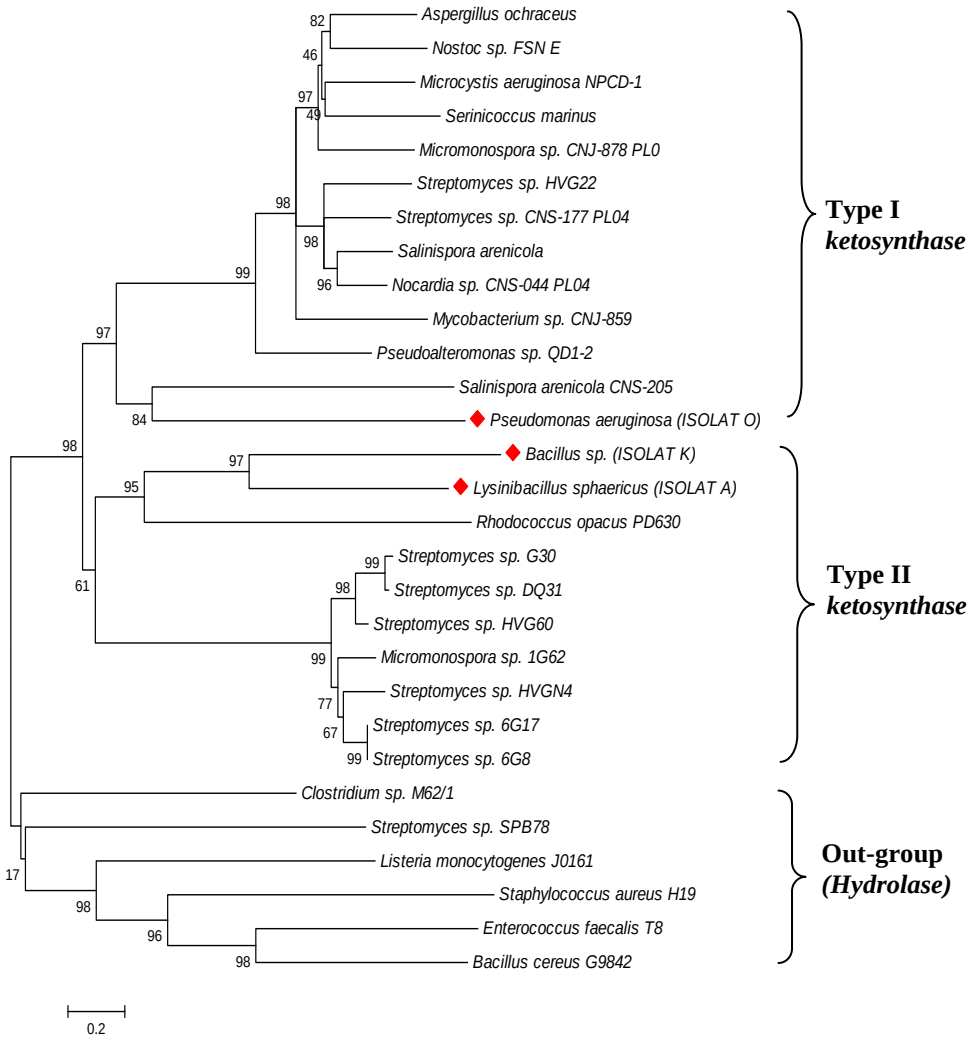

Fig. 6. Phylogenetic analysis of heterocyst glycolipid biosynthetic ketosynthase regions with respect to a diverse range of ketosynthase domains, including type I and type II. Sequences obtained during this study by using degenerate primers are given in red symbol. Other sequences are obtained from GenBank; Sequences are aligned using the MEGA v.5 and Clustal X programs.