Int. J. Pharm. Sci. Rev. Res., 36(1), January – February 2016; Article No. 21, Pages: 124-128 ISSN 0976 – 044X

Research Article

Detection of Nonribosomal Peptide Synthetase (NRPS) Genes on Bacterial Endophytes from *Vetiveria zizanioides* L. and *Ageratum conyzoides* L.

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Accepted on: 21-11-2015; **Finalized on:** 31-12-2015.

ABSTRACT

Study to analyze nonribosomal peptide synthetase (NRPS) genes diversity on bacterial endophytes isolates from roots of *Vetiveriazizanioides* and *Ageratum conyzoides* has been conducted. The aim of the research is to study the presence and diversity of NRPS genes on nine bacterial endophytes from root of medicinal plant. The bacterial DNA chromosomes has been isolated and amplified by Polymerase Chain Reaction (PCR) methods using primer which targeting adenilation domain of NRPS genes. The amplified segments were sequenced and analyzed *in silico* with bioinformatics methods. The 700 bp length DNA segment have been amplified on seven bacterial endophytes isolates of plant root. Bioinformatics analysis of the DNA sequence showed that all of the amplicon were coding NRPS enzymes that contain conservative domains of Adenylate Forming Domain (AFD) superfamily. Sequence motif that determine NRPS substrates detected from three sequences of bacterial endophytes. Phylogenetic studies indicates that NRPS genes from *V. zizanioides* endophytes were grouping with NRPS from Gammaproteobacteria group, otherwise NRPS genes from *A. conyzoides* were grouping with NRPS genes from Firmicutes Group. Study indicates that NRPS genes of bacterial endophytes from each host plants evolved separately.

Keywords: Nonribosomal Peptide Synthetase, Bacterial Endophytes, *Vetiveria zizanioides*, *Ageratum conyzoides*.

INTRODUCTION

As a country with tropical climate, Indonesia has a high diversity of plant. Many plant species have ethnobotanical values because the presence of bioactive compounds in tissue of plants. Various bioactive compounds in the plants has been extracted, identified and examined its effectiveness in dealing with the disease and its causes, such usage is new trends of bioactive compound utilizations in the development of drugs[1]. Bioactive compounds in plants has not only synthesized by the plant itself. Some endophytic microorganisms that live in plants produce various types of active compounds that accumulate in plant tissues[2]. The compounds synthesized by the endophytic bacteria has many functions, such as antibacterial, antifungal, and phytohormones. Various types of bioactive compounds have been isolated from endophytic microorganisms in plants. However, the diversity of bioactive compounds that can be produced by microorganisms is very high and many active compounds remain unexplored[3].

Exploration of the active compound can be done by studying the biosynthetic pathway of the compound produced by microorganisms. Polyketides, nonribosomal peptide, and their hybrid is a group of active compounds with antimicrobes activity. Multimodular enzyme complex such as Polyketide Synthase (PKS) and Nonribosomal Peptide Synthetase (NRPS) are group of enzymes involved in the synthesis of polyketides and nonribosomal peptides[4]. Many molecular studies focused on finding the gene that expresses the NRPS and PKS enzymes, the evolution, and the diversity of the enzymes. The study also predicts active compounds that can be synthesized by the enzymes. Both of these genes have a great potential for the development of next generation antibiotic compounds[5].

Based on several studies, endophytic bacteria which have secondary metabolites with antibacterial activity was detected having NRPS enzymes complex[6,7,8]. Medicinal plants such as *Vetiveriazizanioides* and *Ageratumconyzoides* are a potential habitat for endophytic bacteria that colonize plant root tissues. Endophytic bacteria in many medicinal plants have genes that express detectable group NRPS and PKS enzymes[9]. Bacterial endophytes from roots of *V. zizanioides* and *A. conyzoides*, notably with antibacterial activities is predicted to have NRPS genes. To study the genetic potential of endophytic bacteria on both plant roots, detection of biosynthetic genes need to be active compounds, in particular NRPS genes by molecular methods.

MATERIALS AND METHODS

Isolates Preparation

Nine bacterial endophytes isolatesfrom cryo medium was grown in Luria Bertani (LB) medium. Five isolates (code M,O,H,A,K) is bacterial endophytes from *V. zizanioides* root and four isolates (code B14, B15, I13, I14) is bacterial endophytes from *A. conyzoides* root.

Isolation of Chromosomal DNA

Bacterial endophytes were cultured for 18 hours in LB broth medium. DNA isolation according to Wilson[10]. A total of 1.5 ml culture was transferred into a micro tube and centrifuged for 2 min at 10,000 rpm to obtain ±100

mL pellets of bacterial cells. The supernatant was discarded, and pellet resuspended in 567 mL TE. Furthermore, 10% SDS detergent was added as much as 30 mL and 3 mL proteinase K total. The mixture was incubated for 1 hour at 37° C. After incubation, added 100 mL of 5 M NaCl, and 80 mL CTAB solution. Samples were incubated at 65° C for 10 minutes. Chloroformisoamyl alcohol (24: 1) added and centrifuged at 15,000 rpm for 5 minutes. Upper phase was transferred into a new micro tube and added absolute ethanol and centrifugated. The supernatant discarded and the DNA pellet washed with 70% ethanol. The pellets were dried in an oven, and resuspended using 50 mL of TE. The quality and quantity of DNA was measured by spectrophotometric method and electrophoresis[11].

DNA Amplification

DNA Amplification is done by PCR using Dreamtaqgreen kit (Fermentas, Lithuania). The final concentration of PCR solutionare 1xDreamtaq Green MasterMix, 0.5 µM forward primer, 0.5µM reverse primer. The amount of DNA sample used was 200 ng. Primers used in the amplification is primer pair A3F(GCSTACSYSATSTACACSTCSGG) – A7R (SASGTCVCCSGTSCGGTAS)[12]. PCR conditions used are initial denaturation at 95° C for 5 minutes, 35 cycles of 95° C for 30 seconds, annealing at 51.5° C for 1 min, elongation at 72° C for 1 minute 30 seconds, and final elongation at 72° C for 10 minutes. DNA template was amplified using PCR machine (Eppendorf, Germany).

Sequencing of DNA

Amplicons sequenced in Macrogeninc. Korea with BigDye Applied Biosystems sequencer engine (BigdyeApplied Biosystem 3730XL Sequencer, USA). Sequencing carried out from two directions for each amplicon.

Bioinformatics Analysis

Each amplicons analyzed for homology with BLASTX software. Phylogenetic tree of NRPS gene sequences generated with MEGA 5.0. In this study, conservative domain detected from sequence by Conserved Domain Search (CDS) software[13]. In addition, the sequence analyzed *in silico* to predict substrate with NRPS predictor[14].

RESULTS AND DISCUSSION

NRPS Genes Amplification

DNA segments with ±700bp length have been successfully amplified from chromosomal DNA template. Figure 1 shows that seven of nine isolates of endophytic bacteria from medicinal plants contain NRPS genes. Bacterial isolates with positive NRPS genes is isolates M, O, H, I13, I14, B14, and B15. Meanwhile, two isolates that do not have the NRPS gene is a bacterial isolates A and K, which is bacterial endophytes of *V. zizanioides*.

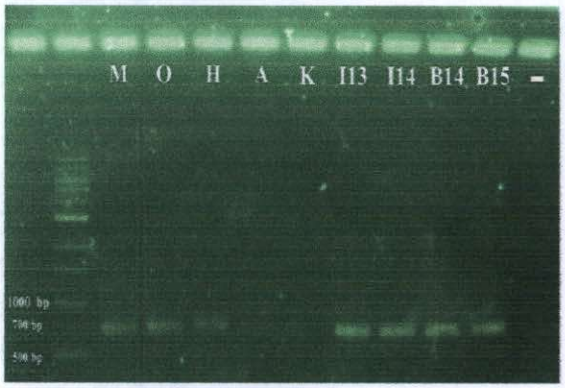

Figure 1: Electroforegrams of NRPS genes amplification of bacterial endophytes from *V. zizanioides* (Isolates M, O, H, A, and K) and *A.conyzoides* (Isolates I13, I14, B14, and B15) with negative control (-). Symbols L for 1 kb NEB ladder used in this study. Picture shows the amplicon size are ±700 bp.

Segment of 700 bp corresponding to the length of the target, which is between motif A3 and A7 located in adenylation domains. Isolates with negative NRPS gene clusters not indicates bacterial endophytes with lack of bioactive compounds produced. Some endophytic bacteria capable of synthesizing antibacterial compounds which synthesized by PKS I and PKS II enzymes complex[15].

DNA Sequence Alignment

Table 1 shows the results of homology analysis of sequences using BLASTX. All of the seven sequences obtained have highest identity with amino acid sequence from NRPS genes in bacteria.

Sequence identity of M has the highest value (95%) with its homolog sequences, NRPS genes in *Pseudomonas putida*. Meanwhile, the identity of the sequence I13 has the lowest value (54%) with its homologs, NRPS genes of *Bacillus amyloliquefaciens*.

Table 1 shows that the sequences homologous to the *Bacillus* NRPS (B14, B15, I13 and I14) has lower identity value than sequence which homologs to *P. putida*.Low identity value (<70%) in NRPS from bacterial endopytes of *V. Zizanioides* shows the novelty of the genes[8].

Table 1: Homology analysis of endophyric bacteria

Sample	Long of base (bp)	Pair of BLASTX	Identity (%)	*E-value*
M	777	Non – ribosomal peptide synthetase [*Pseudomonas putida*]	95	2e-124
O	718	Non – ribosomal peptide synthetase [*Pseudomonas putida*]	88	9e-75
H	727	Non – ribosomal peptide synthetase [*Pseudomonas putida*]	72	4e-53
B14	747	Non – ribosomal peptide synthetase [Bacillus methylotrophicus]	63	3e-34
B15	715	Non – ribosomal peptide synthetase [Bacillus siamensis]	56	9e-23
I13	712	Non – ribosomal peptide synthetase [*Bacillus subtilis*]	54	8e-19
I14	742	Peptide synthetase [*Bacillus amyloliquefaciens*]	58	7e-52

Table 2: Specific domain detection with CDS for NRPS sequences of bacterial endophytes

No.	Sequences	Specific Domains	Interval	E-value
1.	M	Adenylation (A) domain of NRPS	24 – 245	9,97e-70
2.	O	Adenylation (A) domain of NRPS	43 – 233	3,54e-38
3.	H	Adenylation (A) domain of NRPS	45 – 196	8,62e-21
4.	B14	Adenylation (A) domain of NRPS	139 – 217	4,81e-12
5.	B15	Adenylation (A) domain of NRPS	29 – 104	1,09e-11
6.	I13	Adenylation (A) domain of NRPS	74 – 156	5,92e-09
7	I14	Adenylation (A) domain of NRPS	36 – 153	5,09e-20

Table 3: Amino acids substrate prediction with NRPS predictor

Sequences	Signature sequences	Specific motifs	Substrates predictions	Score	Precision
M	LASAFEGFIEQPDLHVGGELNTYGPTEATVVATT	EGEDHGTVV	val/leu/ileu/abu/iva	1,030	0,900
O	THXS-----RPXFHV GGELNTYGPTETT VMTTT	---XHGT VM-	val/leu/ileu/abu/iva	1,240	0,900
I14	TWQLFDGFVTSTIITXAGE-------TVS MAMS	DGTITA-SM-	phe/trp	0,166	0,671

Conservative Domain Analysis

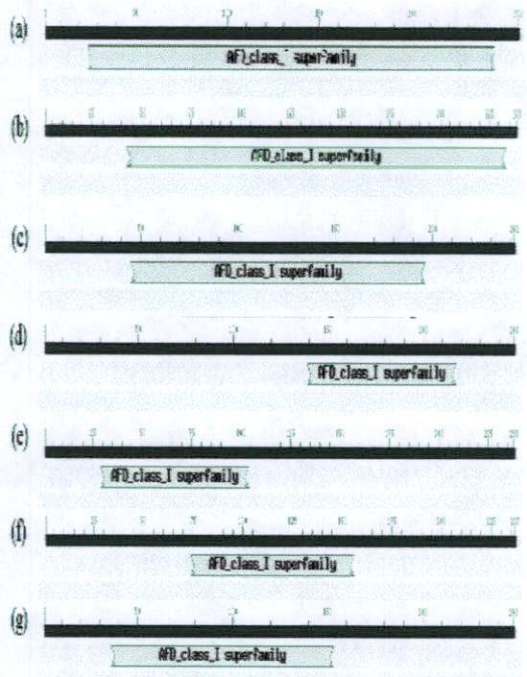

Figure 2: Results of CDS analyse for (a) sequences M (b) sequences O (c) sequences H (d) sequences B14 (e) sequences B15 (f) sequences I13 and (g) sequences I14

Conservative domain analysis results based on the database CDS is obtained in the form of a diagram in Figure 2. Seven sequences detected contain conservative domain of adenylate forming domain (AFD) superfamily. AFD Class 1 enzymes includes NRPS adenylation domain and fireflyes luciferase enzymes[13]. Table 2 shows the specific domain detected by CDS. All sequence contain adenylation domain of NRPS.

Sequence M has interval range 24-245, shows that length of conservative amino acid domain detected on sequence M is 221 amino acids. This value is the highest, according to Figure 2 which shows the sequence M has the longest domain region. Shortest interval range value found on the sequence B15 (29-104), with E-value 1,09e-11. B15 sequence was detected 75 amino acids as conservative domain. In the sequence obtained, length of the conservative domain detected is relatively low. At[16], the whole genome sequence detects adenylation domain with ±400 amino acids length.

NRPS Substrate prediction

Adenylation domain contain binding pocket motifs, which determines the substrate for NRPS enzymes[17]. Table 3 shows the binding pocket motif detected in sequence of bacterial endophytes using NRPS predictor[14].

Software managed to detect the presence of the binding pocket motifs in three sequences, M, O, and I14 (Table 3). Sequences M has a specific motif EGEDHGTVV with a score of 1,030 and precision value of 0,900. While the sequence O has a specific motifs -XHGTVM- with a score of 1,240 and precision value of 0,900. Table 3 shows that the specific motive which owned by both sequence has same substrates predictions, amino acid group of nonpolar aliphatic side chains, particularly valine - leucine - isoleucine - aminobutyric acid - and isovalin. Both sequence have a similar pattern, with the alignment ---- HGTV-. Based on[9], NRPS sequence with the same substrate predictions, has similar motives as well. Isolate of I14 has a specific motif DGTITA-SM- with a score of 0.161 and precision value 0.671. That motifs predicts the substrate used is a group of amino acids with aromatic side chains, notably phenylalanine and tryptophan[14]. Sequence with detected binding pocket motifs (M, O, and I14) is a sequence with high interval range of conservative adenylation domain detected (Table 2).

Phylogenetic tree

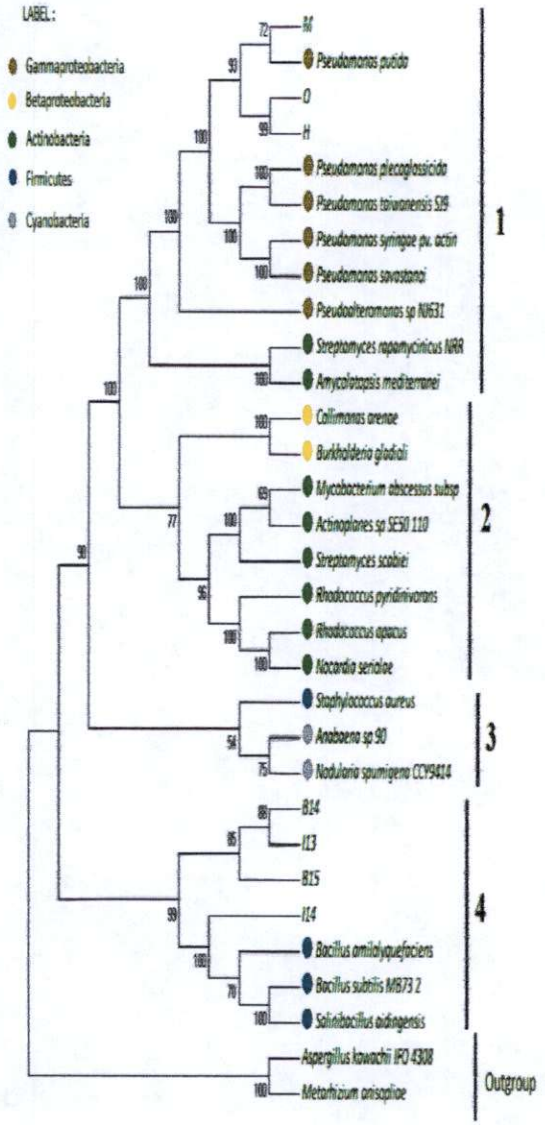

Figure 3: Phylogenetic tree of NRPS genes amino acid sequences NRPS generated by maximum parsimony methods with 500x bootstrap values. Colored circle represents the Classis of each taxa. Line with numbers 1 – 5 represents grouping based on phylogenetic branch.

Phylogenetic tree of NRPS sequences have been generated with maximum parsimony method (Figure 3). Phylogenetic tree shows that NRPS sequence of M, O, H, and *P. putida* grouping with NRPS genes from other *Pseudomonas* genus (*P. plecoglossida*, *P. taiwanensis*, *P. syringae* and *P. savastanoi*) with bootstrap values of 100. The group consisting NRPS genes from *Pseudomonas* has the closest relatives *Pseudoalteromonas* NRPS genes, with bootstrap values of 100. Both genus is the members of Gammaproteobacteria. NRPS genes sequence M, O, and H can be considered similar to NRPS gene of Gammaproteobacteria group.

NRPS genes phylogenetic tree depicting the sequence B15, B14, I13 and I14 have the same ancestor gene with NRPS genes from genus *Bacillus* and *Salinibacillus* because it arises from the same branching point. *Bacillus* and *Salinibacillus* is a member of Firmicutes taxa, indicate that NRPS sequences of isolates B14, B15, I13 and I14 are related to Firmicutes. Genus Bacillus is a member of Firmicutes with very diverse metabolites, functioning as antimicrobial and cytotoxic compound. Genetic relationship between NRPS genes of sequence B14, B15, I13 and I14 with Firmicutes illustrates the potential of bioactive compounds diversity synthesized by its NRPS enzymes[8].

Figure 3 shows the phylogenetic tree have a tendency to form a large group with same taxa. Group 1 has a predominantly members of Gammaproteobacteria, group 2 members dominated by Actinobacteria taxa, Cyanobacteria members dominated group 3, and group 4 is dominated by members of Firmicutes. Outgroup sequence of fungal NRPS separate from the other group from the initial branch. Grouping showed a tendency of related organisms has NRPS sequences with high similarity.

Research conducted by[8] also showed NRPS gene clustered in similar patterns on generated phylogenetic tree, which NRPS adenylation domains from endophytic bacteria tend to be clustered in accordance with taxa at the Classis level. Based[4], the diversity of NRPS enzymes arise because the process of gradual change in the composition of DNA at the level of domain, module, as well as genes that alter the amino acid sequence and thus changes the structure and function of NRPS enzymes on different groups of taxa.

The phylogenetic tree shows the NRPS sequence of *Staphylococcusaureus* separated from the group Firmicutes group and clustered with NRPS from Cyanobacteria. *S. aureus* actively enriching its genome by horizontal gene transfer, the presence of genes that are similar to organisms with distant taxa is commonly found[17]. Based[18], endophytic bacteria that live in the same community on host plants perform horizontal gene transfer of NRPS genes. There is also a possibility that NRPS genes from endophytic bacteria analyzed in this study develop through a process of horizontal gene transfer from closest genes relatives in phylogenetic tree, such as horizontal gene transfer between isolates M, O, and H with members Gammaproteobacteria or B14, B15, I13 and I14 with members of Firmicutes.

Phylogenetic tree showed that NRPS from endophytic bacteria used in this study clustered based on their host plants. Isolates M, O, and H which isendophytic bacteria of *V. zizanioides* has NRPS sequences clustered in group 1 (Gammaproteobacteria), meanwhile NRPS from endophytic bacteria of *A. conyzoides* (I13, I14, B14, B15) are clustered in group 4 (Firmicutes). This indicates that the NRPS gene on endophytic bacteria from each plant hosts evolved independently. Roots of plants have different microhabitat conditions, so that the community of endophytic bacteria which can grow on the roots were

varied. Endophytic bacteria with habitat limited to specific host plants tend to be isolated from other bacterial endophytes populations that live in other host plants. Microhabitats isolation limits the gene flow to the other bacterial communities that live on others host plants (Preston, 1998).

CONCLUSION

NRPS genes successfully amplified in seven isolates of endophytic bacteria of *V. zizanioides* and *A. conyzoides* roots. Analysis showed that all of the sequence obtained contain adenylation domains that belong to the superfamily AFD I.

Based on phylogenetic studies, NRPS genes of bacterial endophytes obtained from *V. zizanioides* has the closest relatives with NRPS genes from Gamma proteobacteria while NRPS gene from bacterial endophytes of *A. Conyzoides* closely related with NRPS from Firmicutes group.

Acknowledgement: The author would like to thank the management of Indonesia University of Education for their support and encouragement.

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Source of Support: Nil, **Conflict of Interest:** None.