MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.11, No.1, March 2017, p 18-22

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.11.1.3

# Studies for IAA (Indole-3-Acetic Acid) Production by Isolates H6 with Nitric Acid Mutation

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Nitric acid mutations are known could be used for strain improvement. This research aimed to study Indole-3-Acetic Acid (IAA) production by nitric acid mutan were compared with wild type. Mutation were conducted with some different treatment time such as 0, 30, 60, 90, and 120 min subsequently it were measured for IAA production. Isolate H6 as wild type isolates were also molecularly identified. The wild strain exhibited 53.83 µg $mL^{-1}$ of IAA while the nitric acid mutan within a range 77.39 µg $mL^{-1}$ to 95.70 µg $mL^{-1}$. Isolates H6.60 exhibited the highest IAA production which 39.87 µg $mL^{-1}$ higher were compared with wild-type. Based on 16S rRNA gene analysis, isolate H6 had similarity with *Lysobacter* sp. ES2-22.

Key words: IAA production, mutation, nitric acid

Mutasi asam nitrat diketahui dapat digunakan untuk perbaikan galur. Tujuan dari penelitian ini adalah untuk mempelajari produksi Indole-3-Asam Asetat (IAA) yang dihasilkan oleh mutan asam nitrat dibandingkan dengan tipe liarnya. Mutasi dilakukan dengan beberapa perbedaan perlakuan waktu yaitu 0, 30, 60, 90, dan 120 menit kemudian dilakukan pengukuran produksi IAA. Isolat H6 sebagai isolat tipe liar juga diidentifikasi secara molekuler. Tipe liar dapat menghasilkan 53,83 µg $mL^{-1}$ IAA, sedangkan mutan asam nitrat menghasilkan IAA dalam kisaran 77,39 µg $mL^{-1}$ sampai 95,70 µg $mL^{-1}$. Isolat H6.60 menghasilkan produksi IAA tertinggi yaitu 39,87 µg $mL^{-1}$ lebih tinggi dibandingkan dengan tipe liar. Berdasarkan identifikasi gen 16S rRNA isolat H6 mempunyai kemiripan dengan *Lysobacter* sp. ES2-22.

Kata kunci: asam nitrat, mutasi, produksi IAA

Plant growth promoting rhizobacteria (PGPR) are a group of microorganisms which inhabiting around or on the root surface. They are known for plant growth promotion and development,both directly or indirectly by producing and secreting of various regulatory chemicals in the vicinity of rhizosphere (Ahemad and Kibret 2014). PGPR could be used as inoculants of biofertilizers in agriculture (Daman *et al.* 2016). The genera of PGPR include *Azoarcus, Azobacter, Azorhizobium, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Chryseobacterium, Frankia, Gluconacetobacter, Herbaspirillum, Mycobacterium, Paenibacillus, Phyllobacterium, Pseudomonas, Rhizobium, Sinorhizobium, Sphingomonas*, and *Streptomyces* (Vejan *et al*. 2016). In addition, genus *Acetobacter, Enterobcter, Actinobacteria, Brevibacillus, Lysinibacillus, Terribacillus* and *Jeotgalibacillus* also included PGPR (Kundan *et al*. 2015).

The mechanisms of PGPR include hormonal regulation and nutritional balance, resistance induction for against plant pathogens, solubilizing nutrients for easy uptake by plants also synergistic and antagonistic interactions with microorganisms within the rhizosphere and beyond in bulk soil, which indirectly boosts plant growth rate (Vejan *et al.* 2016). Production of indole-3-acetic acid (IAA) hormone are widespread in a major property of rhizosphere bacteria that could stimulate and facilitate the plant growth (Glick and Pattern 1996; Damam *et al.* 2016). IAA function is to promote the growth by several ways in plant. IAA are known promote growth of auxiliary bud and bud formation, help in the apical dominance, stimulate lateral and adventitious root development and growth. Besides development, IAA were also play crucial role in leaf and flower abscission (Kundan *et al*. 2015).

Genus of PGPR could produce IAA by several pathway. Biosynthesis of IAA which are used by single bacterial strain sometimes containing more than one pathway (Patten and Glick 1996). The most widely pathway of IAA biosynthetic in bacteria is indole-3-pyruvate (IPA) and indole-3-acetamide (IAM) anabolic pathways. Those pathways used tryptophan as

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precursor. IPA pathway mainly conducted by plant growth promoting bacteria (PGPB), whereas the IAM pathway is present in phytopathogenic bacteria (Vega-Celedon *et al.* 2016). *Rhizobium* sp. could produce IAA through the IAM and IpyA pathway (Spaepen *et al.* 2007). The expression level of IAA are depending on biosynthesis pathway, location of genes involved, either on chromosomal or plasmid DNA, their regulatory sequences, and the presence of enzymes that could convert it to more active, free IAA into an inactive also conjugated form. (Patten and Glick 1996). *Pseudomonas* spp. were isolated from the rhizosphere of soybean plants in Cirebon, West Java has been known have ability to produce the IAA hormone IAA within a range 0.33 ppm to 23.04 ppm in medium culture were added of tryptophan. Those isolates could promote the growth of seeds in vitro assay (Wahyudi, Astuti and Giyanto 2011). Isolates of *Bacillus* sp. were derived from soybean rhizosphere in Cirebon, West Java also known had ability to produce IAA with different concentrations within a range 0.81 mg $mL^{-1}$ to 15.16 mg $mL^{-1}$ (Wahyudi *et al*. 2011).

Level of IAA concentration is regulated in plants. Bacterial rhizosphere have been known could modulate that IAA levels (Vega-Celedon *et al.* 2016). IAA hormone which produced by bacteria are known an efficient biofertilizer inoculants to promote the plant growth (Damam *et al.* 2016). Therefore required a technique to improve a potential bacterial strain that had ability for IAA production. That one way were conducted by mutation. Mutations are known as change in the nucleotide sequence of the genetic material, which could result in amino acid sequence modification of the protein encoded by the gene (Tanja van Mourik 2013). Nitric acid mutations are known could be used for strain improvement. Production of L-asparaginase from a marine fungus *Beauveriabassiana* SS18/14 which were obtained from nitric acid mutants had higher asparaginase production. The wild strain produced 6.32 IU $mL^{-1}$ of L-asparaginase activity while nitrous acid mutant UVF4-N-2 exhibited 10.44 IU $mL^{-1}$ enzyme activity. The strain improvement programme could increased L-asparaginase activity 1.65 times if compared to the wild strain (KamalaKumari *et al.* 2015). Therefore, this research aimed to improve of IAA production by H6 isolate with nitric acid mutation.

## MATERIALS AND METHODS

**Nitric Acid Mutation.** The suspension of parent strain was prepared by using acetate buffer pH 7.5. A total of 1 mL of culture suspension was centrifuged subsequently the pellets were subjected to nitrous acid (0.1M sodium nitrite in buffer of phosphate) treatment with different time such as 0, 30, 60, 90 and 120 min by incubating the mixture at 30 °C. After incubation, the suspension was centrifuged at 10.000 rpm. The pellets were washed twice with phosphate buffer (pH 7.0) and suspended in phosphate buffer. Samples were diluted and plated on to nutrient agar (NA) (23 g $L^{-1}$) medium. The plates were incubated at 28 °C for 24 h. (KamalaKumari *et al* 2015). Each of colony are grown subsequently calculated for number of colonies.

**Test of IAA Production.** Mutant isolates of each treatment and wild-type were inoculated into 10 mL NB medium supplemented with 0.2 mM tryptophan and incubated for 24 h on a shaker. A total of 2 mL bacterial culture mutant and wild-type H6 centrifuged at 10.000 g for 10 min the temperature of 4 ºC. IAA production was evaluated by the colorimetric method as described by Gordon and Weber (1951). The supernatant was taken and inserted into a test tube susequently added 2 mL of Salkowsky reagent (150 mL concentrated $H_2SO_4$, 250 mL of distilled water, and 7.5 mL of 0.5 M $FeCl_3$). Reagent was mixture with supernatant and incubated at room temperature in the dark condition for 30 min. Furthermore, an absorbance was measured using a spectrophotometer at a wavelength of 520 nm. Absorbance results are used to calculate the concentration of IAA with the equation obtained from the IAA standard curve.

**Identification Based on 16S rRNA Gene Analysis and Phylogenetic Tree.** Isolates endophytic H6 derived from root nodule of green beans which were planted in Wonosari, Central Java. H6 isolates were molecularly identified based on 16S rRNA gene. One colony of isolates was taken with a sterile toothpick then inserted into eppendorf tube containing 100 mL $dH_2O$ subsequently it were vortex. A total of 1 mL suspension was used for the amplification with polymerase chain reaction (PCR) technique. PCR (TC 5000 TECHNE) of 16S rRNA gene by using universal primer 520 F (5'-GTGCCAGCAGCCGCGG-3') and 920 R (5'-GTCAATTCCTTTGAGTTT-3'). A total volume 50 µL of PCR were contains 1 µL DNA template, 2 µL of primer for each forward and reverse, 25 µL of 2X KAPA Taq Ready Mix (KAPA Biosystem) and $ddH_2O$ 20 µL. Amplification was performed for 30 cycles that include initial denaturation stage at a temperature 96 ºC for 5 min, denaturation at a temperature 96 ºC for 30 s, annealing a temperature 55 ºC for 30 s, extension at a temperature 72 ºC for

1 min, final extension at a temperature 72ºC for 7 min. The success of the PCR were checked by electrophoresis for 30 min with a voltage of 100V 500mA (BIO-RAD Mupid-exU-75577 Advance Japan). Amplicon DNA were purified and sequenced with two directions. Sequence analysis were conducted by comparing the sequences with GenBank database using Blastn in the National Center for Biotechnology Information (http://www.ncbi.nlm.nch.gov) to determine similarity of the sequence homology. Sequence of 16S rRNA gene were also analyzed for their phylogenetic tree using Mega5 and ClustalW software with neighboor joining method.

## RESULTS

The result showed that the colonies number of survivals from each treatment time with nitric acid mutation. The number of colonies decreased in the period of exposure 0-90 min afterwards the number of colonies increased in the time span to be exposed for 120 min (Table 1). Colonies are grown then measured for IAA production. The wild strain exhibited 53.83 µg $mL^{-1}$ of IAA while the nitric acid mutan within a range 77.39 µg $mL^{-1}$ to 95.70 µg $mL^{-1.}$ Isolates H6.60 exhibited the higgest IAA production which 39.87 µg $mL^{-1}$higher were compared with wild-type (Table 2).

H6 isolate without exsposure to mutagen or parent isolates were analyzed and compared for homology of 16S rRNA gene sequence. in Genbank using BlastN program. Based on 16S rRNA sequence analysis, isolate H6 as much as 92% sequence similarity belonged to *Lysobacter* sp. ES2-22 with accession number KJ878604.1. Furthermore, through phylogenetic tree known phylogenetic relationship with other PGPR isolates which sequence of base are known (Fig 1).

## DISCUSSION

The number of mutant colonies are grown in the time tratment of 0-90 min exposure had inversely proportion to the treatment time. More longer of exposure time, mutans growth are slightly, except in the treatment time of 120 min. It could happen due to differences in exposure times of nitric acid as mutation agent. Nitric acid is a very strong oxidizing agent. Reaction with nitric acid could produce nitric oxide ($N_2O$) that is identified by Frazier and Hage as a reproductive toxin (Young 2002). It toxin cause the death of the organism or mutation. In the treatment time of 120 min more colonies were grown. Those colonies might be had the high mutation probability more than other treatment time exposure previously.

The nitrosating agent are formed from nitric oxide autoxidation, it could cause DNA damage or deamination of DNA bases. The damage are followed with nitrosation of primary amine functions. The result of deamination are the formation of xanthine and uracil within G:C base pairs Caulfield, Wishnok and Tannenbaum 1998). Deamination are known occur in DNA, RNA, and their precursors via a hydrolytic and a nitrosative reaction. The generated deaminate products had mutagenic potential because of their structure similarity to natural bases, which in turn leads to fault nucleotide base pairing and disruption of cellular metabolism. Incorporation of deaminate precursors into the nucleic acid strand occured during nucleotide synthesis by DNA and RNA polymerases or base modification by DNA and or RNA editing enzymes (Kuraoka 2015).

Mutations treatment with nitric acid for 60 min produced mutants with the highest IAA production if compared with wild type. Based on the result, it proved that strain improvement with nitric acid as classical strain improvement became one of effective way. Classical strain improvement such as UV and X-ray irradiation or mutagenic chemicals such as nitrous acid, formic acid or hydrazine has been known for their history of success more than 50 years ago. *Bacillus subtilis* OUT 8103 which are mutated with classical methode by nitrosoguanidine (NTG) resulted mutan Arghx4, Arghx6, Arghx7 and Arghx13 that had shown high arginine production titers in the range of 1.2 mg $mL^{-1}$ up to 1.65 mg $mL^{-1}$. Out of the four colonies, Arghx 7 produced the highest levels of arginine until 1.62 mg $mL^{-1}$ (Rao *et al*. 2012).

Based on analysis of 16S rRNA gene, H6 isolates were closely related to the species *Lysobacter* sp. ES2-22. *Lysobacter* are used in this study proved could produce IAA hormone. Their potency which could produce IAA hormone supporting their role as plant growth promoter. Other studies showed that *Lysobacter* also had another potency as plant growth promoting rhizobacteria (PGPR) beside of produce IAA. *Lysobacter enzymogenes* are known could produce antibiotics and some enzyme activities which made it became attractive candidates for use in biological control of plant diseases and of nematodes (Hayward *et al.* 2009). *L. capsici* TM5405 and *L. enzymogenes* TM2502 had proved for their ability as consistent disease suppression of a broad range two

Table 1 Effect of nitric acid for number of colonies after exposure

| Exposure time (min) | Number of colonies ($mL^{-1}$) |
|---|---|
| 0 | $8.10^7$ |
| 30 | $5.10^7$ |
| 60 | $2.10^7$ |
| 90 | $4.10^5$ |
| 120 | $10.10^6$ |

Table 2 IAA production of isolate mutan H6

| Mutan H6 | IAA production (µg $mL^{-1}$) |
|---|---|
| H6.0 | 93.89 |
| H6.30 | 89.99 |
| H6.60 | 95.70 |
| H6.90 | 77.39 |
| H6.120 | 84.24 |
| Wild type | 53.83 |

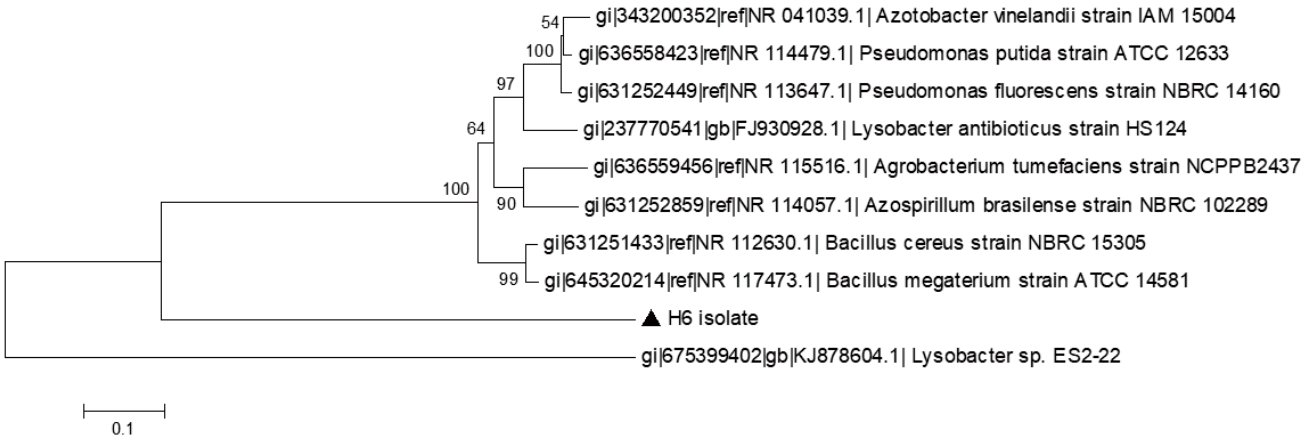


Fig 1 Phylogenetic tree based on 16S rRNA gene of H6 isolate compared with 16S rRNA gene of other plant growth promotion species. Scale showed that distance evolution on the branch length, while the numbers on the branches indicate bootstrap values.

important soilborne diseases in worldwide such as root rot diseases caused by *Rhizoctonia solani* AG-8 and caused by *Gaeumannomyces graminis* var. *tritici* with produce extracellular metabolite, such as sidherophores and protease. *Lysobacter* sp. strains also have broad antifungal and antibacterial activities againts *Pythium ultimum*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Botrytis cinerea*, *R. solani*, *Botryosphaeria dothidea,* and *Bacillus subtilis which* produced xanthobaccins A, B and C (Wang *et al.* 2014).

Therefore, their phylogenetic relationship are known through the phylogenetic tree. H6 isolates showed the fit results between BlastN with the phylogenetic tree. Bootstrap value of 100% on the phylogenetic tree directly justify of BlastN results showing that isolates H6 included in *Lysobacter* sp. ES2-22. *Lysobacter* isolates have closely genetic relationship with PGPR group that has been previously known. This is indicated by bootstrap values on each branch of the phylogenetic tree.

## ACKNOWLEDGMENT

Authors thank and appreciate to all staff of Plant Symbiotic Microbes Laboratory for the supports are

given to carry out this research.

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