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MULTIFUNCTIONAL MUTANTS OF *Azospirillum* sp. WITH ENHANCED CAPABILITY OF SOLUBILIZING PHOSPHORUS, FIXING NITROGEN, AND PRODUCING INDOLE ACETIC ACID

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ABSTRACT

Azospirillum sp. have long been known as biofertilizer for plant growth because of its capability to produce phytohormones and fix nitrogen from the atmosphere. Multifunctional *Azospirillum* strain Aj Bandung 6.4.1.2 isolated in 2009 from cauliflower (*Brassica oleracea*) rhizosphere in Lembang, Bandung, West Java, was capable of fixing nitrogen, solubilizing tricalcium-phosphate, and producing phytohormone indole acetic acid (IAA). The study aimed to modify the multifunctions of *Azospirillum* sp. for better capability of fixing N_2, solubilizing P, and producing IAA using ethyl methanesulfonate and 1-methyl-3-nitro-1-nitrosoguanidine (EMS) mutagen. The study was conducted at Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) in 2010. The results showed that this strain was genetically mutagenized using EMS for better performance in solubilizing P, fixing N_2 (nitrogenase activity), and producing phytohormone (IAA). The optimum concentration and the length of incubation time for the process have been determined. Nine selected mutants with increasing capability to solubilize P (determined by clear-zone formation on Pikovskaya's medium) have been characterized for nitrogenase activities and IAA production compared to wild type Aj Bandung 6.4.1.2. The effect of mutagenesis on IAA production and nitrogenase activities varied among the mutans. Two mutants, AzM 3.7.1.16 and AzM 1.7.2.12, showed superiority in the production of IAA, while two mutants, AzM 1.5.1.14 and AzM 3.7.1.15, were superior in nitrogenase activities. The EMS mutagenesis of *Azospirillum* sp. showed enhanced dissolving capability of unsoluble phosphate (tricalciumphosphate) and increased IAA production and nitrogenase activity.

[***Keywords***: *Azospirillum*, mutants, phosphorus, solubilization, nitrogen fixation, IAA]

INTRODUCTION

Plant growth-promoting bacteria (PGPB) or plant growth-promoting rhizobacteria (PGPR) were reported to stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or abiotic plant stresses without conferring pathogenicity. Enhancing PGPB attractive functions for agriculture application as biofertilizers, biopesticides or plant phytohormones promises better crop growth and yield.

Nitrogen (N) is one of the most abundant elements on earth. However, it is also the most critical limiting element for plant growth due to its low availability in the soil and because nitrate, the most common form of N fertilizer, is highly soluble in the soil solution and can be easily lost by leaching or bacterial denitrification (Vance 2001). This element is the most limiting nutrient for crop yields. After N, phosphorus (P) is also the major plant growth limiting nutrient. Many soil types (acidic, calcareous soils) throughout the world are P-deficient because P is mostly fixed by cations such as Ca^{2+} and Mg^{2+} in high pH soils and Al^{3+} and Fe^{3+} in acid soils.

Chemical fertilizers have been playing significant roles in modern agriculture, but excessive use has led to reduction in soil fertility and environmental degradation (Lal *et al.* 1989; Iordache and Borza 2010). Moreover, in India the use of chemical fertilizers has reached the theoretical maximum use beyond which there will be no further increase in yields (Ahmed 1995). N fertilizers are expensive inputs in agriculture costing more than US$45 billion per year globally (Ladha and Reddy 2003). In Indonesia, urea is the most commonly used N fertilizer. The production of chemical P fertilizers is an energy intensive process requiring energy worth US$4 billion per annum to meet the global need (Goldstein *et al.* 1993). This situation is further confounded by the fact that almost 75-90% of added P fertilizer is precipitated by Fe, Al, and Ca complexes present in soils (Vig and Dev 1984).

Many soil bacteria are reported to solubilize insoluble phosphates through various processes. A few reports have also indicated the P-solubilizing activity of some nitrogen fixers. In many reports, *Azospirillum* is considered as the most important rhizobacterial genus for improvement of plant growth or crop yield worldwide (Gunarto *et al*. 1999; Bashan *et al*. 2004;

Lestari *et al.* 2007). This group of bacteria initially interested researchers because of their capability to fix atmospheric nitrogen under microaerophilic conditions and colonize internal tissues of gramineous plants, like endophytic rhizobacteria. Based on these factors, a number of inoculant companies experimented with agronomical application of liquid or solid formulation of *Azospirillum* sp. in seeds of gramineous plants such as wheat or maize, and the technique was slowly introduced into agriculture on a small scale (Bashan and Holguin 1997).

The P-solubilizing capability of different strains of *A. halopraeferans* has been reported (Seshadri *et al.* 2000). Other strains, *A. brasilense* (Cd and 8-I) and *A. lipoferum* JA4 have been reported capable of producing *in vitro* gluconic acid and solubilizing P from low solubility sources when grown on sparingly soluble calcium phosphate medium and their usual fructose carbon source is amended with glucose. Clearing halos were detected on solid medium plates with calcium phosphate (Rodriguez *et al.* 2004), indicating that the strains are capable of solubilizing P from tricalciumphosphate.

Genetic improvement of bacterial strains could be achieved by mutagenesis. Ethyl methanesulfonate and 1-methyl-3-nitro-1-nitrosoguanidine (EMS) have been reported as an effective mutagen for various microorganisms (Del Galloa *et al.* 1987; Tripura *et al.* 2007) and plants (Persello-Cartieaux *et al.* 2001). Significant improvement in the symbiotic nitrogen fixation was observed using azide resistant mutants of *Rhizobium leguminosarum* (Ram *et al.* 1978) and *Azospirillum* (Zaied *et al.* 2009). Azide is an inhibitor of the terminal segment of the electron transport chain and has been reported to have several effects on the growth of bacterial cells. The aim of this study was to modify the multifunctions of *Azospirillum* sp. for better capability of fixing N_2, solubilizing P, and producing IAA using EMS mutagen.

MATERIALS AND METHODS

The study was conducted at the Laboratory of Molecular Biology of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor, West Java, in 2010.

Bacterial Strain and Growth Condition

Wild type *Azospirillum* strain Aj Bandung 6.4.1.2 was used in this study. The strain was isolated from cauliflower (*Brassica oleracea*) rhizosphere at Microbiology Laboratorium of ICABIOGRAD in 2009. This strain has multifunctions, including the ability for solubilizing P, fixing N_2 and producing auxin phytohormon. Isolation method was described in Fred and Waksman (1928).

Bacterial strain was grown routinely on Okon medium containing (per 500 ml) 3 g K_2HPO_4, 2 g KH_2PO_4, 2.5 g DL-malic acid, 1.5 g NaOH, 0.25 g yeast extract, 2.5 ml $MgSO_4.7H_2O$ 2%, 2.5 ml NaCl 1%, 2.5 ml $CaCl_2$ 0.2%, 2.5 ml $FeCl_3.6H_2O$ 0.17%, 2.5 ml $Na_2MoO_4.2H_2O$ 0.02%, and 10 g bacto agar at pH 6.8. The growth required temperature of 29°C for 1-2 days of incubation.

Mutagenesis Experiment

Optimization of mutagenesis process was carried out using various concentrations of EMS, i.e. 0, 0.5, 1.0, 1.5, and 2.0% (v/v). Cells were centrifuged at 10,000 rpm, and then washed three times using buffer A containing 600 mM K_2HPO_4, 33 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, and 1.7 mM sodium citrate at pH 7.3. The cells were then treated with EMS and incubated for 15, 30, 45, 60, and 90 minutes at room temperature. After the treatment, cells were washed with the same buffer and resuspended into half volume of buffer A. The treated cells were then diluted up to 10^{-4}-10^{-7} and spread on Pikovskaya's agar medium. The plates were incubated at 29°C for 6 days. The optimum concentration of EMS and the optimum length of incubation time were recorded.

Mutant Selection

Strain was treated with optimum condition of EMS and then cultured on Pikovskaya's medium containing tricalciumphosphate (per liter medium contains 10 g glukosa, 5 g $Ca_5(PO_4)_3OH$, 0.2 g NaCl, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 2.5 mg $MnSO_4.H_2O$, 2.5 mg $FeSO_4.7H_2O$, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$ and 15 g bacto agar at pH 6.8). Strain was incubated at 29°C for 6 days and selected based on the clear-zones formed on the media.

P Index for Phosphate Solubilization

The capability of the strains for dissolving tricalciumphosphate on the media was observed based on clear-zone performed around the colony on the Pikovskaya's medium. Solubilization index was measured based on the ratio of total diameter (clear-zone + colony) and colony diameter (El-Azouni 2008).

IAA Analysis Using Spectrophotometer

One loop-full colony was inoculated into 9 ml liquid nitrogen-free semisolid (NfB) medium, and then 1 ml sterile L-tryptophan 2% was added. The solution was then incubated in the shaker incubator at room temperature for 24 hours. NfB medium (per liter) contains 5 g DL-malic acid, 4 g KOH, 0.5 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.01 g $MnSO_4.H_2O$, 0.05 g $FeSO_4.7H_2O$, 0.02 g NaCl, 0.01 g $CaCl_2$, 0.002 g $Na_2MoO_4.2H_2O$, 1 g NH_4Cl, and 0.05 g yeast extract at pH 6.8. Three ml of the culture was then transferred into sterile eppendorf tube and centrifuged at 4°C, 10,000 rpm for 10 minutes. To the 10 ml supernatant, 2 ml Salkowski reagent was added and incubated for 30 minutes under darkness. The amount of IAA produced was determined colorimetrically at 540 nm (Gordon and Weber 1951). Salkowski reagent (per lier) contains 20 ml $FeCl_3.6H_2O$, 400 ml H_2SO_4, and 580 ml distilled water.

Nitrogenase Analysis Using Acetylene Reduction Assay Method

Nitrogenase activity was performed based on the Acetylene Reduction Assay (ARA) method using gas chromatography. One loop-full colony was homogenized with 50 ml sterile water in the eppendorf tube by vortexing. The solution was then inoculated on semisolid NB medium on a test tube with cotton cap, and incubated at 30°C in the dark for 7-10 days until pelicels were formed. The cotton cap was then replaced with rubber stopper after pelicels were formed. The test tube was sealed with a rubber cap, then 2.0 ml acetylene was injected into the culture tube and incubated at 30°C in the dark. The volume of the gas-phase was 21 ml. The amount of acetylene produced from acetylene in the gas-phase was measured by using gas chromatography (Hitachi 263-30 fixed with a Porapak N column, Hitachi Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Optimization of Mutagenesis Process Using EMS

The effect of EMS as a mutagen on the *Azospirillum* cell viability was evaluated by applying various concentrations of EMS for 15 minutes of incubation time. The result showed that increase in EMS concentrations decreased cell viability. EMS concentration of 2% caused cells lost their capability to survive (Fig. 1).

The effect of incubation time and 15% EMS concentration showed that the longer the incubation time, the lower was the cells survival. Most cells lost their viability at 90 minutes of incubation time as shown in Figure 2.

From this result, EMS concentration of 1.5% and incubation at room temperature for 15 minutes were chosen for mutagenesis process. That condition also produced higher mutants with different P solubilizing capabilities (shown by varied clear-zones on Pikovskaya's medium).

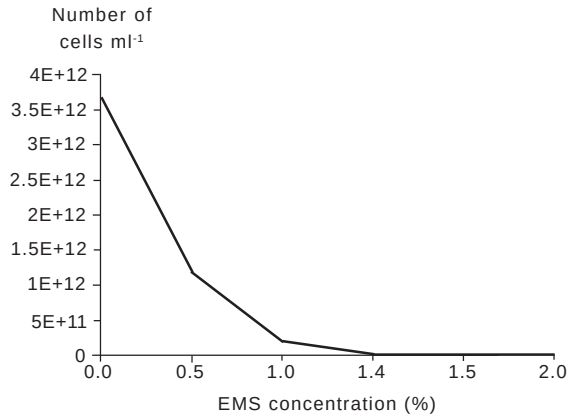

Fig. 1. Effect of ethyl methanesulfonate and 1-methyl-3-nitro-1-nitrosoguanidine (EMS) concentration on cell viability of *Azospirillum* strain Aj Bandung 6.4.1.2, Bogor, West Java, 2010. 4E+12, etc = $4x10^{12}$ cells ml^{-1}.

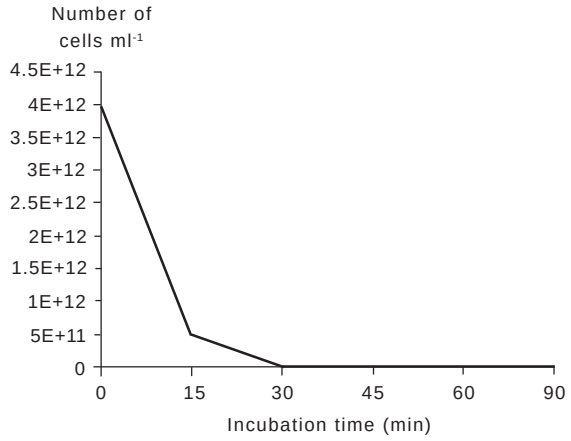

Fig. 2. Effect of ethyl methanesulfonate and 1-methyl-3-nitro-1-nitrosoguanidine (EMS) at 1.5% concentration and incubation time on cell viability of *Azospirllum* strain Aj Bandung 6.4.1.2, Bogor, West Java, 2010. 4.5E+12 = $4.5x10^{12}$ cells ml^{-1}.

Screening of Mutants Based on the Clear-Zones

Azospirillum strain Aj Bandung 6.4.1.2 was a mutant produced by using EMS based on the mutagenesis optimization as shown in Figure 2. Selection processes were carried out using Pikovskaya's medium containing tricalcium phosphate. Mutants with clearer and bigger zones were selected for further examination.

Mutants selected were then dissolved into sterile water. Amount of 20µl was then drop-cultured on a fresh sterile Pikovskaya's medium and incubated for 6 days at 29°C. The P index of clear-zone was determined according to the method developed by Seshadri *et al.* (2000) (Fig. 3).

Nine selected mutants (AzM 2.7.3.4, AzM 3.7.1.1.1, AzM.6.7.1.4, AzM 5.7.2.11, AzM 1.5.1.14, AzM 2.7.3.2, AzM 3.7.1.15, AzM 1.7.2.12, and AzM 3.7.1.14) were then characterized for P index compared to the wild type. All of the selected mutants had higher P index compared to the wild type Aj Bandung 6.4.1.2, while mutant AzM 3.7.1.14 had the highest P index of about 4 (Fig. 4).

The production of IAA was measured for all of the nine mutants compared to the wild type. Figure 5 showed that the production of IAA of the mutants varied. Mutant AzM 1.5.1.14 produced less IAA compared to the wild type (Aj Bandung 6.4.1.2), and other mutants were increasing with variation of concentration. The highest IAA productions were obtained from AzM 3.7.1.16 and AzM 1.7.2.12 (Fig. 5).

Nitrogenase activities for all of the selected mutants and the wild type were assayed based on the ARA method using gas chromatography. The results showed the varied activities. Two mutants, AzM 1.5.1.14 and AzM 3.7.1.15, produced higher IAA activities compared to others and the wild type (Fig. 6).

Interestingly, the two highest nitrogenase producers did not produce the highest IAA, nor did they have the highest P index. This result gives a promising application of improvement of *Azospirillum* properties. Mutants showing highest increase in enzyme activities, i.e. P solubilization and phytohormone production, can be used as inoculants for plant production.

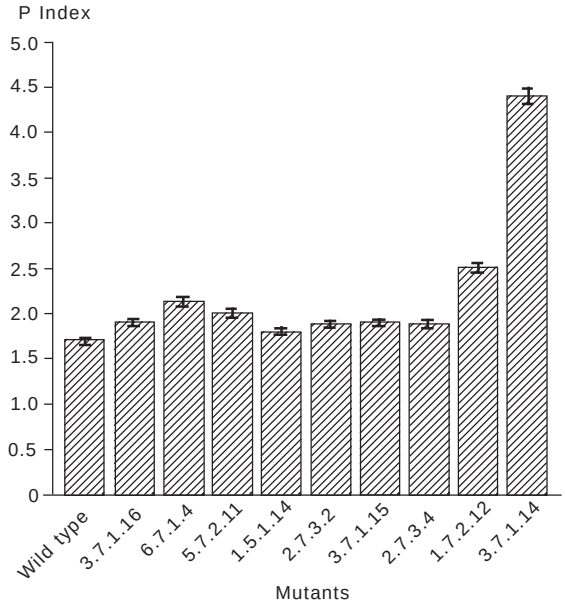

Fig. 4. Effect of ethyl methanesulfonate and 1-methyl-3-nitro-1-nitrosoguanidine (EMS) application on P index for several *Azospirillum* mutants, Bogor, West Java, 2010. Bars indicate standard deviation.

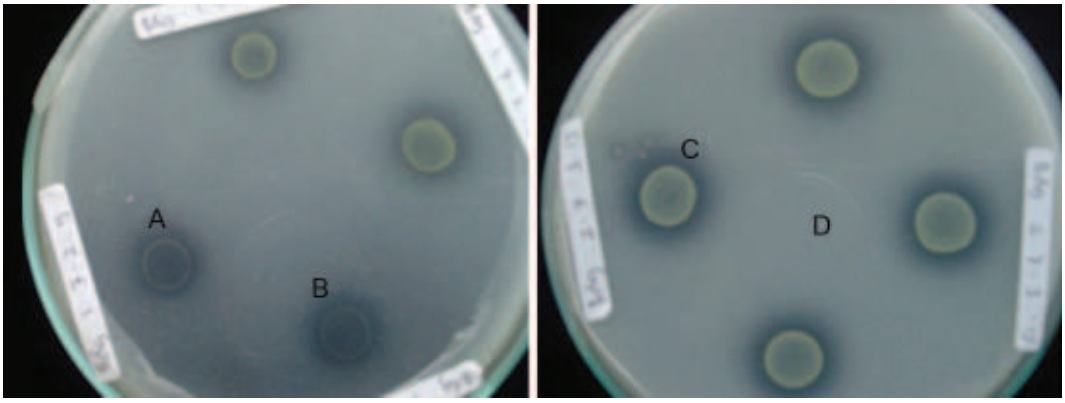

Fig. 3. Selection of *Azospirillum* mutants based on the clearer or wider diameters of clear-zone of the colonies. A and B = mutant colonies with wide diameter of clear zone, C = mutant colony with very clear zone, D = Pikovskaya's medium containing tricalciumphosphate showing white/milk color.

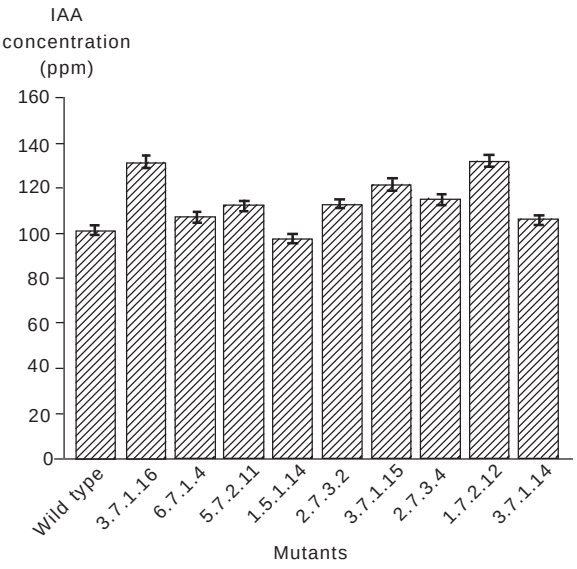

Fig. 5. Effect of ethyl methanesulfonate and 1-methyl-3-nitro-1-nitrosoguanidine (EMS) mutagen on IAA concentration for several *Azospirillum* mutants, Bogor, West Java, 2010. Bars indicate standard deviation.

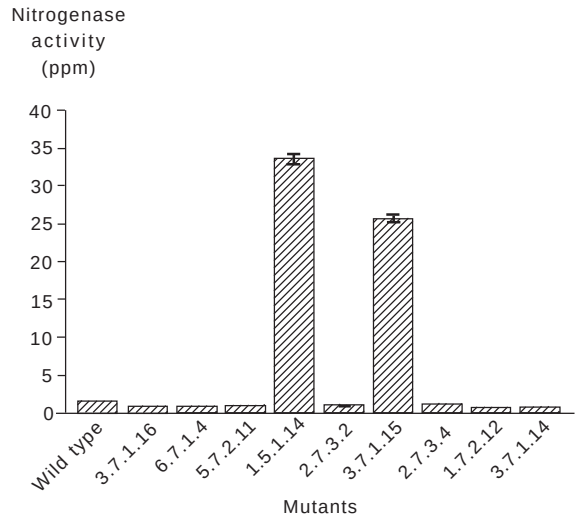

Fig. 6. Effect of ethyl methanesulfonate and 1-methyl-3-nitro-1-nitrosoguanidine (EMS) mutagen on nitrogenase activities of several *Azospirillum* mutants, Bogor, West Java, 2010. Bars indicate standard deviation.

CONCLUSION

Wild type *Azospirillum* with multifunctions of N-fixation, P solubilization, and phytohormone production have been isolated from rhizosphere. The three characters could be manipulated with mutagenesis using EMS at 1.5% concentration and 15 minutes incubation at room temperature. The mutation process increased enzyme activities for the three characters. Mutants with very high increase in enzymatic production, i.e. AjM 1.5.1.14 and AjM 3.7.1.14, can be used as inoculants for rice or other gramineous plants.

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