

Research Article

Exploration of indigenous free nitrogen-fixing bacteria from rhizosphere of *Vigna radiata* for agricultural land treatment

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Abstract: Nitrogen is the most abundant element in the atmosphere, however, most often deficient in agricultural lands. This research was an exploratory to get indigenous non-symbiotic nitrogen-fixing bacteria. Soil samples were collected from rhizosphere of green beans. This study was aimed to determine the bacterial population of the three regions; screening, isolation and selection of free nitrogen-fixing bacteria. Antagonism and pathogenicity tests were performed to observe its potential for a biofertilizer product. The highest number of free nitrogen-fixing bacteria was found from forest soil sample of 2.5×10^{11} CFU/ml. Screening and isolation process has obtained 10 free nitrogen-fixing isolates. Then was selected into 4 isolates namely SNF4, SNF5, SNF7 and SNF8 according to the ammonia production test qualitatively. When an antagonism activity performed, there was no inhibition zone each other. The pathogenicity test did not show the pathogenic symptom. This study also showed that bacterial isolates obtained significantly affected the germination growth of green beans compared to controls. Possibility, bacteria of this type produced growth hormone for a plant. Strain SNF8 has shown the highest ammonium production then was selected for 16S rRNA identification. Similarity test of genome sequence of strain SNF8 had 99% similarity with *Bacillus cereus*.

Keywords: *biofertilizer, indigenous bacteria, nitrogen-fixing bacteria, non-symbiotic*

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Introduction

Nitrogen (N₂) is one of the macro elements needed by plants in large quantities and absorbed by plants in the form of NH₄⁺ and NO₃⁻ ions. It is also nutrients that get much attention for crop production. This is because the amount of N₂ in the soil is small, but plants need it in large quantities. In addition, loss of N₂ is also quite large in the soil due to gaseous loss by soil microbial activity and reactions in the soil, washing caused by deforested land, and harvest (Leghari et al., 2016). The role of N₂ for plants is very large, so the availability is needed by farmers. In general, farmers apply N₂ using chemical fertilizers. Most of the agricultural land in

Indonesia has been transformed into critical land due to pollution from industrial waste, as well as the use of inorganic or chemical fertilizers that are much, continuously to make soil nutrients decrease. Esteban et al. (2010) explained that inorganic fertilizers are fertilizer made by factories by concocting chemicals and having high nutrient content. While the weakness of chemical fertilizers is that they only have macronutrients. Excessive use of chemical fertilizers can damage the soil if it is not balanced with manure or compost. Excessive administration of chemical fertilizers can cause the plants to die, causing soil fertility to decrease, destroying the lives of microorganisms, damaging

soil structure, and causing environmental pollution. Along with the increasing public awareness of environmental damage and the emergence of various diseases caused by excessive use of chemicals on agricultural land, then one alternative to overcome this problem is the use of biofertilizer.

Biofertilizers are often referred to as fertilizers that contain living or latent microorganisms that activate biological process for making fertilizers or forming an unavailable elemental being available to plants. The activity of these microorganisms affect soil ecosystems and produce additives for plant growth (Husen et al., 2007). However, the species and quantity of microorganisms in biofertilizer products are varied depending on inoculants and raw materials used to produce biofertilizer. Biofertilizers are able to restore soil fertility and maintain ecosystem of beneficial microorganisms on the ground. However, there are concerns about the use of biofertilizers because microbial inoculants when applied, often the results were not as expected (Bhardwaj et al., 2014). The use of microbial technology in agriculture is expected to improve microbial function in the application area, in addition to improving various crop production systems.

Free (non-symbiotic) nitrogen fixation is carried out by free-living microorganisms, such as *Enterobacteriaceae*, *Bacillus*, *Azotobacter*, *Azospirillum*, and *Herbaspirillum* which have been shown to be able to fix nitrogen (McGill and Cole, 1981). In addition, *Azotobacter* is a nitrogen fixation bacterium that is capable of producing substances that promote the growth of gibberellins and cytokines to stimulate plant growth (Subba-Rao, 1995). Understanding of the rhizosphere nice and microbial community interactions will assist development of inoculants across farming systems with potentially greater consistency in performance and survival, especially the use of indigenous microorganisms (Torsvik and Øvreås, 2002). The use of indigenous microorganisms is more sustainable than the introduction of microorganisms when applied as a biological fertilizer or for bioremediation (Nuraini et al., 2015; Arfarita et al., 2016; Arfarita et al., 2017).

Materials and Methods

The initial step of exploration of non-symbiotic nitrogen-fixing bacteria was to screen on specific media for the soil collected around rhizosphere of green bean plants from three locations. The isolation and selection were carried out based on differences bacterial morphology and ability to produce ammonium qualitatively. Four bacteria

were then selected and studied for their potential as a bacterial consortium by antagonism test and pathogenicity test on green bean sprouts. For potential applications in the field and its safety, four bacteria were identified by DNA using 16S rRNA.

Soil samples

Soil samples were collected from rhizosphere of green bean plants in three areas in Malang, East Java, namely the experimental garden of Kendal Payak (KP), Jembe Gede (JG) and also Junrejo Forest (JF). Green bean plants were removed with the roots carefully, top of the plant were cut, while the roots and soil were put in a plastic bag, stored in a cooling box and then taken to the laboratory and stored at 4-8°C to isolate the bacteria immediately.

Soil bacteria population

Estimation of bacterial population was carried out by taken 10 grams of sample soil and dissolved in 90 ml of 0.85% NaCl solution, then made a series of dilutions to 10^{-10} . Started from 10^{-2} to 10^{-10} dilutions, 1 ml of diluted solution was taken, then total population colony was calculated by counting colony which grows based on Standard Plate Count (SPC).

Screening and isolation of non-symbiotic fixing bacteria

Screening and Isolation of non-symbiotic N_2 -fixing bacteria were carried out using N-free semisolid malate media. The ingredients for making N-free semi-solid malate media were K_2HPO_4 0.5 g, $FeCl_3 \cdot 6H_2O$ 0.015 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, DL-Malic Acid 5 g, KOH 4.8 g, Bromothymol Blue 0.1% 10 ml, Agar 3 g, distilled water 1000 ml and pH adjusted to 6.8. To obtain non-symbiotic N-fixing bacterium, a soil sample was taken 10 g and dissolved into 90 ml of 0.85% NaCl solution, then shaken for 30 minutes. After leaving 5 minutes, then 1 ml was taken to make dilution series up to 10^{-7} .

Qualitative test of ammonium production

Qualitative tests of ammonia production were carried out using the Tetra Kit. Before used, the Tetra Kit was shaken on reagent bottle. Then clean measuring tube twice with distilled water. The measuring tube was then filled with 5 ml of bacterial culture. In the first reagent add 14 drops then close tightly and shake. Then re-open the second reagent measuring tube, add 7 drops, and add the third reagent of 7 drops. Observe colour changes, by comparing no more than the maximum period of 20 minutes with the standard Tetra Kit bar. Place the tube on white paper,

compare the colour in a top position looking down. The colour changes that occurred were observed and compared with standard Tetra Kit bar.

Antagonism test

The four isolates selected were refreshed with a streak technique from pure culture to a petri dish containing TSA medium (Trypticase Soy Agar) and incubated for 24 hours at room temperature. The association test results between the consortium isolates showed negative test results if they did not form an inhibition zone when the isolates were cultured simultaneously. Thus the isolates used in this consortium are not antagonistic to each other, so they can be cultured and used together in biofertilizer formulation.

Pathogenicity test

Pathogenicity test was conducted using green bean sprout grown on Yoshida medium. Culture stock of Yoshida medium was prepared by mixing 1000 mL solution consisting of NaH_2PO_4 40.3 g, NH_4NO_3 80 g, CaCl_2 88.6 g, FeCl_3 7.7 g, K_2SO_4 71.4 g, MnCl_2 1.5 g, MgSO_4 32.4 g, H_3BO_3 0.93 g, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ 0.074 g, ZnSO_4 0.035 g, and CuSO_4 0.031 g. Those chemicals were dissolved into 4 litres of distilled water and heated until the ingredients were mixed well. The Yoshida solution was filled in a test tube and cover the surface of Yoshida's solution with cotton. Before being sterilized, the test tubes were covered with cotton. The Yoshida Solution was sterilized using an autoclave at a temperature of 121°C for 15 minutes. Green bean seeds were chosen which had a growth of more than 80%. The surface of selected green beans were sterilized, by soaked in 46% alcohol for 1 minute, transferred into sterile distilled water three times each for 10 minutes, transferred to 0.2% HgCl_2 for 2 minutes, dipped into the sterile distilled water six times and soak in sterile distilled water for 5 hours. Green bean seeds were sown in a petri dish containing sterile wet cotton and let stand 2-4 days at room temperature until the stems and roots grow along ± 1 cm. Aseptically, 1-2 sprouts were cultivated into test tubes containing sterile Yoshida media. Input the inoculation of 1 ml of pure NFB culture into the tube and incubated for 10 days in the growth room which were indirectly sun-exposed. In the same way, used controls which were grown without inoculation of NFB culture.

Bacterial identification

Determination of bacterial species is needed, especially for applications in the field for security on its use. Bacteria that have the highest ammonium production activity then was identified

by amplification of 16S rRNA Gene and determined its sequence. Firstly, DNA isolate was extracted, and its concentration was measured, then amplified 16S rRNA using PCR. PCR was conducted by mixing the chromosomal DNA with a master mix (containing all dNTP's, buffer, BSA, primer) then initiated by the addition of Taq polymerase. After amplification, PCR products were tested with 1% agarose gel electrophoresis then purified using EXO SAP for sequence reaction. Purified PCR products were sent to DNA Centre Facility for sequencing. Sequencing results then are submitted into the GenBank online.

Results and Discussion

Total population of soil bacteria

The total population of soil bacteria were estimated by dilution series and spreading of $10 \mu\text{l}$ a dilution on nutrient agar. The results showed that there were differences in the number of populations of non-symbiotic nitrogen-fixing bacteria. Table 1 shows the population of non-symbiotic nitrogen-fixing bacteria isolated from the three regions. Forest land samples (FS) was the largest population of 2.5×10^{11} . SK and SJ soil samples have a lower number of bacterial populations, which were 7.8×10^8 and 3.7×10^8 respectively.

Table 1. Total population of bacteria observed on Nutrient Agar (NA) plate

Soil Samples	The average of NFB bacteria population and other bacteria
Forest of Junrejo (FS)	2.5×10^{11}
Sample of Kendalpayak (SK)	7.8×10^8
Sample of Jambegede (SJ)	3.7×10^8

The highest total population was FS samples due to high organic matter content (Table 2) so that it could support the life of microorganisms in the soil. Although soil is a complex habitat for microorganisms, soil organic matter usually composes only 5% of the total weight of the soil. This small number plays an important role in determining soil fertility which also directly affects the development and growth of soil microbes and plants. Decomposition of organic matter in the soil cannot be separated from the activity of soil bacteria. Decomposer bacteria are the largest group consumer of simple carbon compounds, such as root exudates and crop

residues. Bacteria convert energy into soil organic matter into a form that is beneficial to other organisms. Decomposer bacteria have an

important role, especially in the immobilization of nutrients and can prevent nitrogen loss from root areas (Handayanto and Hairiah, 2007).

Table 2. Chemical analysis of soil samples of three locations from Malang-East Java

Code	Organic-C (%)	Total-N (%)	C/N	Organic Material (%)	P-Bray I (mg/kg)
Sample FS	1.33	0.19	7	2.31	4.67
Sample SJ	1.12	0.11	10	1.94	10.56
Sample SK	1.31	0.15	9	2.26	27.44

Screening, isolation and selection of nitrogen-fixing bacteria (NFB)

Screening and bacterial isolation were carried out using the spread plate method on N-free semisolid malate medium. Of the 10 strain isolated, 4 isolates were selected namely SNF4, SNF5, SNF7 and SNF 8. First, the selection of four isolates was based on differences in colony morphology. In addition, microscopic cell forms and gram types of bacterial isolates were observed. The differences in cell morphology and staining can be seen in Figure 1. Bacterial staining aims to facilitate bacterial observation with a microscope, to see the shape of the cell, outer structure and structure in bacteria such as cell walls. This will be known for the physical and chemical properties that are typical of bacteria, as well as increasing the contrast of microorganisms with their surroundings.

Qualitative test of ammonium production

Total ammonium production from 10 Nitrogen-fixing bacterial isolates was carried out using the *Tetra Visocolour Alpha Ammonium Detection Kit*. This method is to get the range of ability of bacterial isolates to produce ammonia with high concentration. The observation was done by looking at changes in the culture medium. Colour changes in the media due to the presence of NH₃ which was alkaline, causing the blue bromothymol indicator to turn blue. Then change the colour of culture compared to the colour of the standard solution or colour of the stock solution. Qualitative test of ammonium production was carried out on 10 isolates namely SNF1, SNF2, SNF3, SNF4, SNF5, SNF6, SNF7, SNF8 SNF9 and SNF10 (Table 3). The results showed that isolate SNF4, SNF5, SNF7 and SNF8 produced colour changed to dark green. Of the four selected isolates, the highest content of ammonium (NH₄⁺) was 25.0 mg/l.

Antagonism test

The four best nitrogen-fixing isolates, namely SNF4, SNF5, SNF7, and SNF8 were

simultaneously cultured on nutrient agar plates. Each showed a negative result because it did not form an inhibitory zone (Figure 2). In a complex environment that contains various kinds of organisms, the metabolic activity of an organism will affect its environment. Microorganisms like other organisms in complex environments are always in contact with their biotic and abiotic environment. Very few microorganisms that live in nature can live individually. Antagonism test was done to determine its potential as consortium bacteria, especially in a biofertilizer product.

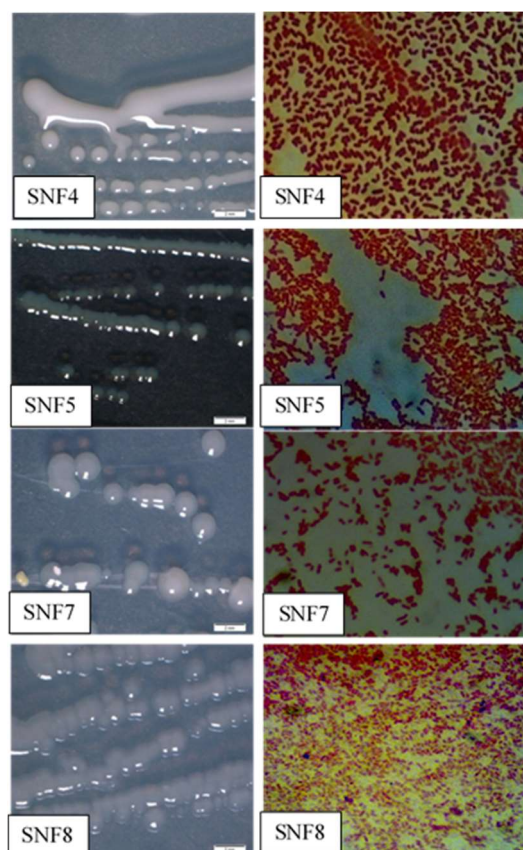


Figure 1. Colony morphology of four selected isolates 2-days old (left). Morphology of bacterial cells after gram staining and observed under a microscope (right)

Table 3. Qualitative test of ammonium production of isolated bacteria

No	Code of bacterial strain	Colour Change	Estimation of Ammonium Production (mg/L)
1	SNF1	Light Green	5.0
2	SNF2	Light Green	5.0
3	SNF3	Light Green	5.0
4	SNF4	Dark Green	25.0
5	SNF5	Dark Green	25.0
6	SNF6	Light Green	5.0
7	SNF7	Dark Green	25.0
8	SNF8	Dark Green	25.0
9	SNF9	Light Green	5.0
10	SNF10	Light Green	5.0



Figure 2. Antagonistic Test of 4 isolates, a two-day incubation, an arrow which shows the edge of the streak has not been fused (left). A four-day incubation, an arrow which shows no antagonism or the end of the streak has fused (right)

Pathogenicity test

The four nitrogen-fixing bacteria, strain SNF4, SNF5, SNF7, SNF8 were then tested for their pathogenicity on green bean sprouts because young plants are very susceptible to soil-borne diseases. The selection was carried out by growing N-binding bacterial isolates in Nutrient Broth (NB) media. Green bean seeds were germinated first until they reach a size of ± 1 cm. Then pure cultures of N-fastening bacterial isolates were inoculated around the roots of green bean sprouts grown in a test tube. Pathogenic isolates will show decaying, yellowing, brown lesions on the stems or stunted growth. From the results of visual observations (Figure 3), each bacterial isolate showed no symptoms of pathogens or plant sprouts showed no symptoms of a disease and were not inhibited by growth compared to controls. BNT test results for each growth variable are presented in Table 4. The results of variance analysis of plant length, total root length, and fresh weight showed a significant effect on the treatment tested of 7-days old of

green bean sprout after bacterial inoculation. The nitrogen-fixing bacteria isolates significantly affected green bean germination. These types of bacteria were probably producing growth hormones. This was marked by increasing plant length, total root length, and fresh weight compared to controls. The largest value is found in SNF8 treatment. This is likely because these isolates have the ability to provide more available nitrogen compared to other bacterial isolates. The element nitrogen (N_2) plays a role for growth, especially the increase in plant length, total root length, and fresh weight to help plants grow well.



Figure 3. The pathogenic properties of 4 bacterial isolates on 7-day green bean plants in Yoshida media solution, which showed no pathogenicity symptoms

In this study, exploration of NFB potential as a biological fertilizer was also determined using 16S rRNA. Purified PCR products were submitted to DNA Centre Facility for sequencing then be uploaded into the GenBank online. Similarity test of genome sequence of strain SNF8 had 99% similarity with *Bacillus cereus*. The results of this study are consistent with West et al. (1985) that *B. cereus* has interactions with a number of microorganisms found in the rhizosphere. Whereas *Bacillus cereus* bacteria is one of the non-pathogenic agents in plants that have great potential to be used as biological controllers. These bacteria have specific hosts, are not harmful to natural enemies of pests and other non-target organisms (Finlay et al., 2000). Based on these references, We selected strain SNF8 for next future study. Naturally, non-symbiotic nitrogen fixation is carried out by microorganisms that live freely. It was reported that *Enterobacteriaceae*, *Bacillus*, *Azotobacter*, *Azospirillum*, and *Herbaspirillum* had been shown to be capable of N_2 fixation. In addition, *Azotobacter* is an N_2

fixation bacterium that is capable of producing substances that promote the growth of gibberellins and cytokines so as to stimulate root growth (Azcón and Barea, 1975). Free-living nitrogen-fixing bacteria were isolated from the rhizosphere of seven different plants namely in Chungbuk Province, Korea. Five isolates with nitrogenase activity above 150 nmol/h/mg protein were identified based on phenotypic and 16S rDNA

sequences analysis, and identified as *Stenotrophomonas maltophilia*, *Bacillus fusiformis* and *Pseudomonas fluorescens*, respectively. The ability of non-symbiotic (Park et al., 2005) N-fixing bacteria to bind nitrogen without the presence of the host and its ability to live in acidic conditions makes this group of bacteria have a high level of tolerance to the environment.

Table 4. Average of the growth of green bean sprouts

Treatments	Average		
	Long Sprouts (cm)	Total length of Roots (cm)	Fresh weights (g)
Control	14.33 a	37.42 a	0.96 a
SNF4	14.50 a	38.73 a	0.66 a
SNF5	16.60 ab	51.15 ab	0.96 b
SNF7	17.54 b	54.82 b	0.97 b
SNF8	18.65 b	60.38 b	0.98 b
F. Table 5%	Significant	Significant	Significant
LSD	2.15	12.48	0.15

According to Roper and Gupta (2018), non-symbiotic nitrogen-fixing bacteria can contribute around 10 to 15 kg N/ha/year, depending on the availability of carbon sources. Bacteria that live freely and have the ability to fix molecular nitrogen can be divided into obligate aerobic organisms, facultative aerobes, and anaerobes. Obligatory aerobic bacteria are included in the genera *Azotobacter*, *Beijerinckia*, *Derxia*, *Archromobacter*, *Mycobacterium*, *Arthrobacter* and *Bacillus*. Facultative anaerobic bacteria include genera of *Aerobacter*, *Klebsiella* and *Pseudomonas*. Anaerobic nitrogen-fixing bacteria are represented by genera *Clostridium*, *Chlorobium*, *Chromatium*, *Rhodomicrobium*, *Rhodopseudomonas*, *Rhodospirillum*, *Desulfovibrio* and *Methano bacterium*. In some of these genera, nitrogen fixation occurs in photoautotrophs which is shown by the presence of photosynthetic pigments in their cells such as the well-known *Rhodopseudomonas* genus. While the genus *Desulfovibrio* fixes nitrogen in the process of reducing sulfate.

As a living material, microbes need time to adapt to environmental conditions that support their lives (Fernandez et al., 2014). Biofertilizer application is expected to enrich the beneficial microbe type and amount, but its success is still determined by the quality of biological fertilizers and ability to grow microbes in biological fertilizers in new environmental conditions (Prihastuti and Harsono, 2012). Many researchers argue that failure in the use of rhizobia inoculants is due to the highly competitive power of

indigenous rhizobia populations than those introduced. Therefore, the use of indigenous bacteria needs to be considered in biofertilizer application.

Conclusion

There were differences in the number of population in each soil sample observed, namely Kendalpayak (SK) of 7.8×10^8 CFU/ml, Jambe Gede (SJ) of 3.7×10^8 CFU/ml, and Forest Junrejo (FS) of 2.5×10^{11} CFU/ml. The highest total population was due to high organic matter content so that it could support the life of microorganisms in the soil. Of the 10 isolates from screening results, 4 isolates were selected, namely SNF4, SNF5, SNF7, and SNF8. The results showed that isolates SNF4, SNF5, SNF7 and SNF8 produced colour changes to dark green. They have a high content of ammonium was 25.0 mg/L. Four of these isolates were not antagonistic because they did not form a zone of inhibition when cultured simultaneously. They were also not pathogenic in the test plants. Determination using 16S rRNA showed that strain of SNF8 belonged to *Bacillus cereus*. *Bacillus cereus* bacteria is one of the non-pathogenic agents in plants, has specific hosts, not harmful to natural enemies of pests and other non-target so that it has great potential to be used as biofertilizer. The selected bacteria will then be formulated on a biofertilizer product, tested their survival on the field and their role to increase crop production.

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