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16s rRNA Sequence Analysis and Ammonium Excretion Ability of Nitrogen Fixing Bacteria Isolated from Mineral Acid Soil

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Abstract

Nitrogen fixing bacteria defined as bacteria which is capable to transform free nitrogen molecules into ammonium v (PCR). Nitrogenase activity of these selected isolates was measured using Acetylene Reduction Assay (ARA). The ability of these selected isolates in ammonium excretion was qualitatively and quantitavely measured using Nessler reagent and spectrophotometry method respectively. Taxonomic position of the selected bacteria were determined based on their 16S rRNA sequence analysis. Genetic diversity analysis of these 15 isolates of nitrogen fixing bacteria yield eight selected bacteria for subsequent analysis. Sequence of nifH gene from all of these selected bacteria were successfully amplified. Nitrogenase assay of these selected bacteria revealed 6 isolates with high nitrogen fixation capasity namely GMA3, GMA5, GMA6, GMA9, GMA12 AND GMA 13. Ammonium excretion analysis revealed 4 isolates which have remarkable ability of producing high level of ammonium namely GMA1, GMA3, GMA6, and GMA9. The 16S rRNA sequence analysis shown that isolates GMA3, GMA5, GMA11 and GMA12 had a close relationship with *Brevibacillus formosus* strain DSM 9885T, *Flexibacter canadensis* strain ISSDS-428, *Rhizobium tropici* strain rif 200849, and *Azotobacter tropicalis* strain RBS. Respectively, isolate GMA1 and GMA13 had a close relationship with *Sthenotropphomonas sp*. Strain MFC-C, while isolate GMA6 and GMA9 had a close relationship to *Azotobacter vinelandii* strain ISSDS-428.

Key words : nitrogen fixing bacteria, ammonium excretion, identification

Introduction

Nitrogen-fixing bacteria (NFB) are bacteria that capable to transform the free N2 gas in the atsmosphere into ammonium via electron reduction and N2 protonation using nitrogenase complex enzyme (Zahran, 1999). Ammonium produced via this mechanism will be assimilated by cell to produce organic nitrogen compounds such as protein, amino acids and nucleic acid, (Reitzer, 1996). After the cell is lysed, which is caused by death of the cell, the organic nitrogen compunds will be released into the environment and subsequently utilized by other organisms such as plant. This phenomenon is the basic of biofertilizer by which the NFB is used as the nitrogen-supplying agent for the plant.

One among difficulties challenged in the application of the free living NFB as biofertilizer is the low quantity of nitrogen compound could be released to the environment, hence its contribution in nitrogen supply becomes relatively low, (Dobbelaere *et al*, 2003). This fact occures since the nitrogen that have been fixated is used by the bacteria itself and only small amount of the nitrogen will be released to the environment after the cell lysed and decomposized (Boussiba, 1991; Bali *et al*., 1992). Moreover,

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ammonium accumulation as the result of nitrogen fixation will prohibit both synthesis and activity of nitrogenase enzyme complex in the cell, resulted the nitrogen fixation to stop (Colnaghi *et al.*, 1997).

Some differences of nitrogen-fixing regulation mechanism found in several nitrogen-fixing bacteria strains, in which the ammonium produced by nitrogen fixation will be excreted via a simple diffusion mechanism (Kleiner, 1982). This fact reason in two important consequences in the application of nitrogen-fixing bacteria as biofertilizer, which are 1) Ammonium excretion caused of the non-cumulative ammonium within the cell so the nitrogen fixation will occured continously, 2) Ammonium that excreted from the bacteria is possibly utilized directly by plant (Colnaghi *et al.*, 1997). This research was aimed to obtain and identified nitrogen-fixing bacteria that are capable to excrete a high concentration of ammonium.

Materials and Methods

Nitrogen fixing bacteria isolates

Azotobacter vinelandii DSM 2289, *Azospirillum brasilense* DSM 1224 and 15 nitrogen fixing bacteria isolates encoded as GMA1, GMA2, GMA3, GMA41, GMA42, GMA5, GMA6, GMA7, GMA8, GMA9, GMA92, GMA10, GMA11, GMA12, and GMA13 used in this research, obtained from collection of Soil Microbiology Laboratorium Gadjah Mada University.

16S rRNA gene amplification

Isolated bacterial DNA (50 ng/m1) were amplified using forward primer 27f (5'-AGAGTTTGATC[A/C]TGGCTCAG-3') and reverse primer 1492r (5'-TACGG[A/T/C]TACCTTGTTACGACTT-3') with concentration of each is 25 pmol/m1. PCR cycle used are as following : 95^{0}C for 1 min, 30 cycles (95^{0}C for 1 min, 50^{0}C for 1 min, and 72^{0}C for 1,5 min) and 72^{0}C for 10 min to lengthen the final products. Electrophoresis was performed to PCR product in 0,8% agarose gel, with 100 Voltage for 30 min.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Result of 16S rRNA gene purification with DNA gel Kit Spin Prep™ were then separated with *Hae*III enzyme. DNA phragmen produced were electrophorised in 2% agarose gel with 100 voltage for 25 min using electrophoresis buffer solution TAE 0,5X. Differences of DNA polimorphism pattern were analysed using NTSYSpc-2.02i software with *Unweight Pair Group with Mathematical Average* (UPGMA method).

Gen nifH amplification

Isolated DNA were amplified using *nifH-univ for A site* (5'-GCIWTITAYGGNAARGGNGG-3') primer and *nifH-univ for rev-site* (5'-GCRTAIABNGCCATCATYTC-3') primer with 25 pmol/m1 concentration for each. PCR cycle occured in early denaturation condition in temperature of 95^{0}C for 5 min and followed by 35 cycles consisting of denaturation (94^{0}C for 30 min, primary attachment (56^{0}C for 30 min) and polimerisation (72^{0}C for 1 min). After the final cycle, polimerisation continued undergoing in temperature of 72^{0}C for 10 min.

Nitrogenase activity test

Test of nitrogenase activity was undertaken with *Acetylene Reduction Assay* (ARA) method (Turner and Gibson, 1980). Bacterial isolates were grown in biphasic modified liquid mannitol media and incubated for 3 days. Bacterial culture were then sentrifuged to separate cell pellets from the medium. The cell pellets produced were put into venoject and added with 0,5 mL of acetylene to undergo incubation for 24 h. When the incubation had finished, gas inside the venoject was analysed with gas chromatography.

Qualitative ammonium excretion capaity test

Bacterial isolates were grown in 30 mL Burk's medium. Bacterial culture were then incubated in room temperature for 48 h and shaked using rotary shaker. After incubation time ended, the sample were taken and sentrifuged at 12.000 rpm for 10 min (Shanmugam and Valentine, 1975). 5 mL of supernatan produced was taken and the pH value was set to 11 by adding NaOH. After that, medium supernatant were added with 0,11 mL EDTA, 0,11 mL sodium potassium tartrate and 0,22 mL Nessler reagent and homogenized. Raection tube were incubated for 20-30 minutes at 25 ± 5^{o}C then observed for the color change.

Quantitative ammonium excretion capacity test

Sampling were performed periodically every 8 h, started at the early incubation time to undergo sentrifugation at 12000 rpm for 10 min (Shanmugam and Valentine, 1975). The supernatant produced from sentrifugation were put into new tube and pH value was set to 11 by adding NaOH. Supernatant added with 0,07 mL EDTA, 0,07 mL sodium potasium tartrate, and 0,13 mL Nessler reagent was homogenized and incubated at temperature of 25 ± 5^{o}C for 20-30 min. About 2,5 mL sample was taken and the absorbance was determined using spectrophotometer at 435 nm wave length (Yuen and Pollard, 1952).

Nucleotide bases sequence analysis and phylogenetic relationship

Alignment nucleotide sequence reading result was performed using align two sequence of Basic Local Alignment Search Tool (BLAST) algorithm programme. Unsuitable nucleotide sequences were corrected manually utilizing Genetyx programme based on electophoregram graph. Intact 16S rRNA gene sequence obtained from selected isolate was explored for its homologue with 16S rRNA bacterial gene provided in Genebank database using BLAST programme. Multiple alignment and arrangement of phylogenetic tree of selected bacterial isolates' 16S rRNA gene sequence and bacterial control's 16S rRNA gene sequence from genebank were performed by utilizing CLC Free workbench programme based on *unweighted pair-group method using arithmetic averages* (UPGMA) values. Phylogenetic tree consistency was determined by using bootstrap analysis with 1000 times resampling.

Results and Discussion

Genetic diversity of utilized nitrogen fixing bacteria isolates

Amplification of 16S rRNA gene from all analyzed isolates of nitrogen-fixing bacteria produced one DNA ribbon with molecule weight of about 1500 bp (result is not shown). The results of 16S rRNA gene amplification were then separated by using *Hae*III restriction enzyme. Polymorphism pattern as the result of 16S rRNA gene cleavage is shown in Figure 1.

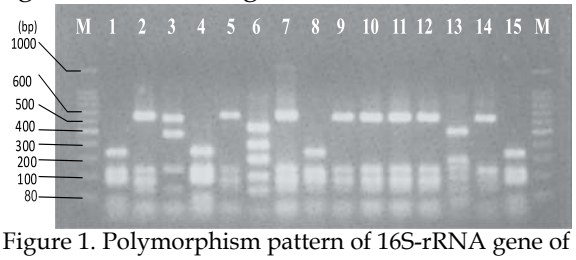


Figure 1. Polymorphism pattern of 16S-rRNA gene of nitrogen fixing bacteria isolates after cleavage using *Hae*III restriction enzymes. From left to right : M: Marker 100 bp DNA Ladder, **1**: GMA1, **2**: GMA2, **3**: GMA3, **4**: GMA41, **5**: GMA42, **6**: GMA5, **7**: GMA6, **8**: GMA7, **9**: GMA8, **10**: GMA9, **11**: GMA92, **12**: GMA10, **13**: GMA11, **14**: GMA12, **15**: GMA13

According to the given results of ARDRA in Figure 1, 16S rRNA gene cleavage from analyzed bacterial isolates shows seven different patterns of DNA polymorphism. Each DNA polymorphism pattern

consists of 4-6 DNA band with molecule weight varies from 80 bp to 600 bp. Cruz *et al.* (2001) reported that 16S rRNA gene cleavage using *Hae*III enzymes on 38 nitrogen fixing bacteria isolated from *Musa* spp dan *Ananas comosus* (L) Merril resulted in 10 different patterns of DNA polymorphism.

DNA polymorphism pattern derived from ARDRA method was not only used to differentiate bacterial in species level and higher taxonomy (Reinhardt *et al.*, 2008) but also to define the familial relationship between analyzed bacterial isolates (Cruz *et al.*, 2001). Familial relationship of analyzed bacterial isolates in this research was performed by constructing phylogenetic tree in dendogram form (Figure 2).

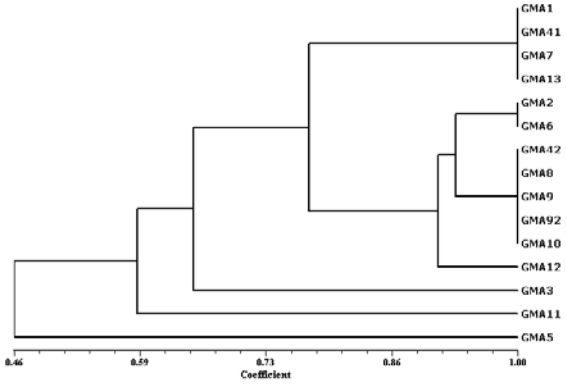


Figure 2. Phylogenetic tree of 16S rRNA gene cleavage result using *Hae*III enzymes in nitrogen-fixing bacteria isolates

Phylogenetic tree in Figure 2 above shows that some of bacterial isolates had 16S rRNA gene polymorphism pattern with high similarity level, indicated by similarity coefficient 1. The coefficient 1 were obtained from GMA1, GMA41, GMA13 and GMA7 isolates. Bacterial isolates also showing high similarity level of 16S rRNA gene polymorphism pattern were GMA42, GMA8, GMA9, GMA92 GMA10, GMA2 and GMA6 isolates. Those bacterial isolates showed that they have the same taxonomy or very close familial relationship.

In contrast, bacterial isolates of GMA3, GMA5, GMA11, and GMA12 showed a low 16S rRNA gene polymorphism pattern with similarity coefficient less than 1 compared to other isolates. These bacterial isolates were supposed as genetically different isolates compared to others and were selected for the next test.

Bacterial isolates group that consisted of those with the same ARDRA pattern and high similarity coefficient were supposed as genetically similar isolates and one of them was selected for the next test. In this study we chose GMA1, GMA6, GMA 9 and GMA13 isolates because they had highest nitrogen- fixing capacity compared to other isolates based on previous research.

Growth Pattern of Selected Nitrogen-Fixing Bacterial Isolates in Free Nitrogen Medium

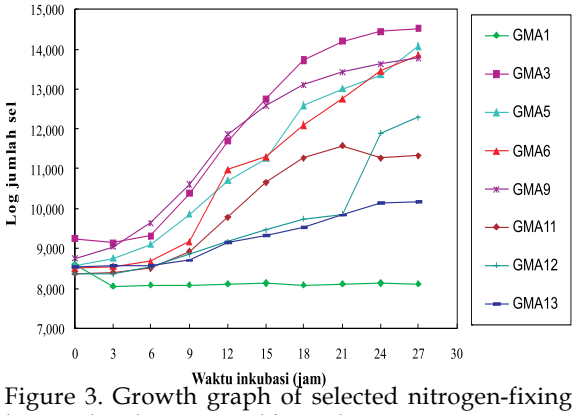


Figure 3. Growth graph of selected nitrogen-fixing bacterial isolates in Burk's media

The growth pattern of selected nitrogen-fixing bacterial isolates in figure 3 shows most of bacterial isolates had undergone adaptation phase for 6 hours as shown in GMA3, GMA5, GMA6, GMA9, GMA11 and GMA12 isolates. Generally, all selected bacterial isolates enter the exponential growth phase 9 hours after incubation, except in GMA1 and GMA13 isolates. Some of bacterial isolates in this study had different growth time in exponential phase. GMA5, GMA6 and GMA12 grew exponentially until the end of observation time. GMA3,

GMA9 and GMA13 grew for 18 hours in exponential phase whereas UGM13 grew for 15 hours. GMA11 underwent stationary phase 21 hours after incubation, whereas GMA3, GMA9 and GMA13 passed through that phase after 24 h and continued until the observation period ended.

Detection of Fe protein encoding nifH gene (Dinitrogenase Reductase)

Detection of *nifH* gene in nitrogen-fixing bacterial isolates was conducted by using PCR technique with primary specific *nifH-univ* (for A-site and for rev-site). This primer was designed to amplify *nifH* gene widely in nitrogen-fixing bacteria (Burgmann, 2003). The result of *nifH* gene amplification in selected nitrogen-fixing bacteria isolates are shown in Figure 4.

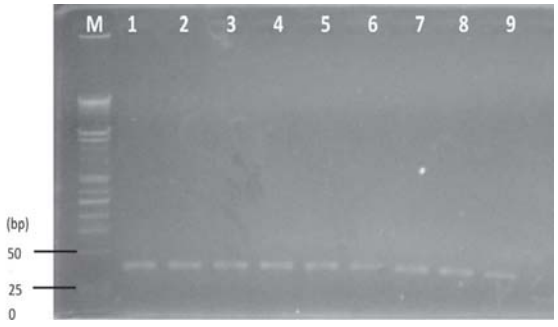
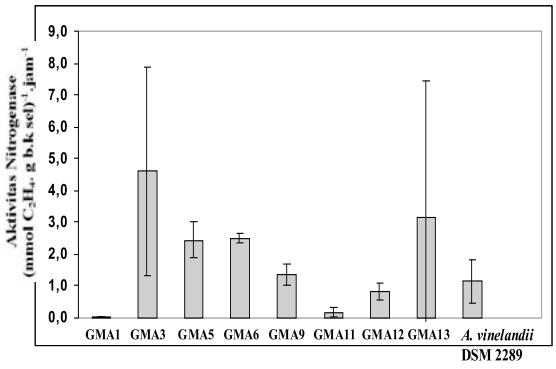


Figure 4.Amplification result of *nifH* gene in selected nitrogen-fixing bacteria isolates. M: Marker 1 kb DNA Ladder, **1**: GMA1, **2**: GMA3, **3**: GMA5, **4**: GMA6, **5**: GMA9, **6**: GMA11, **7**: GMA12, **8**: GMA13, **9**: Positive control (*Azotobacter vinelandii* DSM 2289)

According to Figure 4, all selected bacterial isolates had *nifH* gene with molecular weight about 400 bp. This result was relevant with Burgmann (2003) statement that amplification of *nifH* gene with specific *nifH-univ* primer (for A-site dan for rev-site) would produce PCR products with molecular wight about 469 bp. This result also approved that genetically all selected bacterial isolates were potential to fixate free nitrogen.

Nitrogenase activity analysis using acetylene reduction assay (ARA) method

Nitrogen-fixing activity by nitrogenase complex in selected bacterial isolates is shown in Figure 5.



Gambar 5. Nitrogenase activity of chosen nitrogen-binding bacterial isolates analyzed using ARA method

Selected bacterial isolates showed different nitrogenase activities (Figure 5). GMA3 isolate had highest nitrogenase activity of 4,60 mmol C_2H_4.(g/dry weight cell/h). GMA13 isolates had the second highest nitrogenase activity of 3,15 mmol C_2H_4.(g/dry weight cell/h). Nitrogenase activities of both isolates were higher than nitrogenase activities of positive control *Azotobacter vinelandii* DSM 2289 that was 1,16 mmol C_2H_4.(g/dry weight cell/h). Nitrogenase activities in GMA5, GMA6, GMA9 and GMA12 isolates were in the range of 0,82 mmol C_2H_4.(g/dry weight cell/h) to 2,50 mmol C_2H_4.(g/dry weight cell/h). GMA11 and GMA1 isolates had the lowest activities, 0,04 mmol C_2H_4.(g/dry weight cell/h) and 0,17 mmol C_2H_4.(g/dry weight cell/h).

Some of previous research publications reported their success in isolation of three nitrogen-fixing bacteria with highest nitrogenase activities as high as 187 to 387 nmol C_2H_4.(mg protein)$^{-1}$.h^{-1} (Kim *et al.*, 2005). Molecular identification showed that the three isolates were *Azospirillum brasilense, Azospirillum lipoferum, and Enterobacter sp.*

Qualitative test of ammonium excretion capacity

According to the result shown in Table 1, we notice that strong positive reaction indicated with a change of medium supernatant color into reddish brown after undergoing reaction with Nessler reagent occur in GMA1, GMA3, GMA6, and GMA9 isolates. This result showed the existence of ammonium in high concentration in the growth medium of those bacterial isolates. No color change noticed in supernatant from growth medium of GMA11, GMA12 and GMA13 isolates showed that there was no ammonium detected in those isolates culture medium.

Table 1. Qualitative ammonium excretion capacity of selected nitrogen-fixing bacterial isolates.

No	Isolates	Color Reaction
1	GMA1	+++
2	GMA3	+++
3	GMA5	++
4	GMA6	+++
5	GMA9	+++
6	GMA11	-
7	GMA12	-
8	GMA13	-
9	*Azotobacter vinelandii* DSM 2289	+
10	*Azospirillum brasilense* DSM 1224	-
11	Negative control	-

Note : (+++) indicates positive reaction, (++) intermediate positive reaction, (+) weak positive reaction, and (-) negative reaction

Quantitative ammonium excretion ability

Figure 6 shows that ammonium excretion occured in all bacterial isolates at different concentration. The highest ammonium concentration was detected in 4 culture medium; GMA1, GMA3, GMA6 and GMA9. The highest ammonium concentration was detected in culture medium of GMA6 isolate as much as 1107,692 µMol. The second highest ammonium concentration was found in culture medium of GMA3 as much as 688,718 µMol, followed by GMA9 with 454,103 µMol, and GMA1 with 391,538 µMol. Ammonium in culture medium of GMA5, GMA11, GMA12, and GMA13 isolates were low, only in a range concentration from 0,001 µMol to 26,154 µMol. The ammonium concentration in culture medium of positive control *Azotobacter vinelandii* DSM 2289 was 242,821 µMol and *Azospirillum brasilense* DSM 1224 was 129,744 µMol

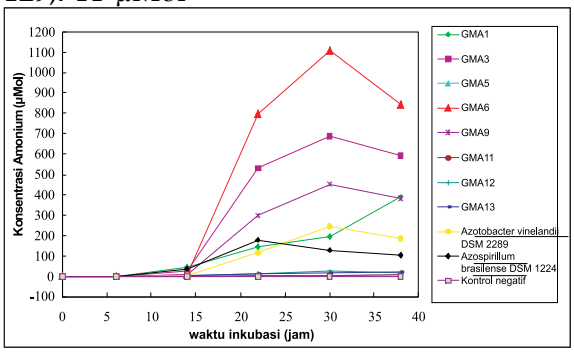


Fig ure 6. Quantitative ammonium excretion capacity in selected nitrogen-fixing bacterial isolates

Some previous studies reported that they could perform ammonium excretion capacity test in some strain of wild type nitrogen-fixing bacteria such as *Azotobacter vinelandii* in concentration about 260,251 µM (Gordon *et al.*, 1983).

Ammonium excretion in all selected bacterial isolates started at 6 h after incubation. These could be compared to bacterial growth graph in Figure 3 that shows ammonium started being excreted at exponential growth phase and the excretion continued until stationary phase. This condition was consistent with previous study showing that ammonium excretion in *A. Chrococcum*, *A. vinelandii*, and *Klebsiella pneumoniae* occurred when the bacteria were in late exponential growth phase before entering the stationary phase when the growth started to end (Narula *et al.*, 1981; Bali *et al.*, 1992). These could be due to high nitrogen fixation activity in order to fulfill cell's needs of nitrogen for fast growing. Another factor probably caused this condition was the low oxygen concentration at the end of stationary phase due to highly oxygen consuming bacterial growth activity in aerobic condition.

After 32 h of incubation, ammonium concentration in culture medium of bacterial isolates relatively remain stable and also decline, showing that ammonium excretion started to decrease. Ammonium excretion probably ended due to some factors; 1) Decreasing of nutrition such as carbon resources that the isolates need for their growth, 2) Accumulation of nitrogenase inhibitors such as ammonium, and 3) Increasing of medium base (pH) value, (Bali *et al*, 1992).

Identification of Selected Nitrogen-fixing Bacterial Isolates

Based on dendogram affiliation of phylogenetic tree (Figure 8), we could notice that GMA1 and GMA13 isolates had the highest homology (99%) with *Stenotrophomonas* sp. strain MFC-C.

Stenotrophomonas sp is one of commonly found in soil bacteria, especially in rhizosper plants area. Reinhardt *et al*. (2008) reported that *Stenotrophomonas* had the ability to fixate nitrogen that was approved by using ARA test and detection of *nifH* gene. Liba *et al*. (2006) stated that the ability to fixate free nitrogen from atmosphere was one of newly found characteristic of *Stenotrophomonas* sp that had not been reported yet in previous studies. The ammonium excretion capacity of this bacteria has not been reported yet.

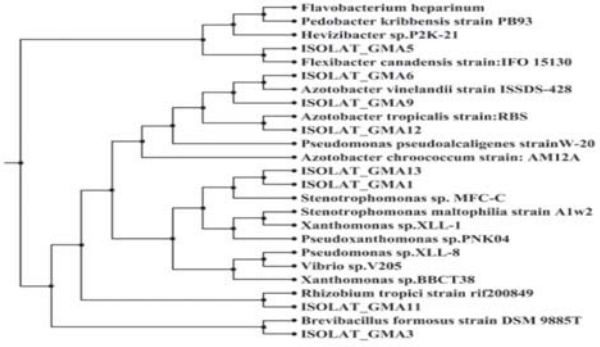


Figure 7. Phylogenetic tree of selected bacterial isolates arranged using *CLC Free Workbench* programme based on *unweighted pair-group method using arithmetic averages* (UPGMA) values. The consistency of the tree was determined using *bootsrap* analysis with 1000 times *resampling.*

GMA3 isolates has the highest homology (99%) with *Brevibacillus formosus* strain DSM 9885T bacteria. This strain was published for the first time by Shida *et al.* (1995) as *Bacillus formosus.* It then reclassificatin as *Brevibacillus formosus* with the same characteristic as reported before, with some additional molecular data (Shida *et al*., 1996). Some species of genus *Bacillus* such as *Bacillus pumilus* (Lie *et al*., 2008) and many more species from genus *Paenibacillus* such as *Paenibacillus forsythiae* (Ma and Chen, 2008) were known to have nitrogen-fixing capacity but none of them were reported to have the ability to excrete ammonium.

GMA5 isolates has the highest homology (97%) with *Flexibacter canadensis* strain IFO 1513 bacteria. This strain is a soil living bacteria. There has not been any report about the ability to fixate nitrogen and excrete ammonium of this strain.

GMA6 and GMA9 isolates have the highest homology (99%) with *Azotobacter vinelandii* strain ISSDS-428 bacteria, and GMA12 isolates has the highest homology (99%) with *Azotobacter tropicalis* strain RBS bacteria. The nitrogen fixing capacity in aerobic condition is one of main characteristic of genus *Azotobacter* (Brenner *et al*., 2005). Previous study reported that *Azotobacter vinelandii* strain *wild type* isolates could excrete ammonium in concentration about 200 µM (Bali *et al*., 1992) and 260,251 µM (Gordon *et al*., 1983).

GMA11 isolates has the highest homology (99%) with *Rhizobium tropici* strain rif 200849 bacteria. *Rhizobium tropici* is one of nitrogen-fixing bacteria that has symbiotic relationship with legume plant through formation of nodule in plant roots. *Rhizobium* was known to have the ammonium excretion ability for its host plants through peribacteroid membrane (Day *et al*., 2001).

Ammonium excretion analysis revealed 4 isolates which has remarkable ability of producing high level of ammonium namely

GMA1, GMA3, GMA6 and GMA9. The 16S rRNA sequence analysis shown that isolate GMA3, GMA5, GMA11 and GMA12 had a closed relation with *Brevibacillus formosus* strain DSM 9885T, *Flexibacter canadensis* strain ISSDS-428, *Rhizobium tropici* strain rif 200849 and *Azotobacter tropicalis* strain RBS respectively, isolate GMA1 and GMA13 had a close relation with *Sthenotropphomonas* sp. Strain MFC-C, while isolate GMA6 and GMA9 had a close relation with *Azotobacter vinelandii* strain ISSDS-428.

References

Bali, A., G. Blanco, S. Hill, and C. Kennedy. 1992. Excretion of ammonium by a nifL mutant of Azotobacter vinelandii ûxing nitrogen. *Appl. Environ. Microbiol*. **58**,1711–1718.

Boussiba, S. 1991. Nitrogen fixing Cyanobaeteria potential uses. *Plant and Soil*. **137**, 177-180.

Brenner, D, J., Noel, R., K., James, T. S., 2005. Bergeys manual Systematic Bacteriology. 2nd edition. Springer Science Business Media, Inc. New York. USA.

Bürgmann, H. 2003. Activity and diversity of nitrogen-fixing microorganism: novel tools to characterize population in soil. Dissertation. Swiss Federal Institute of Technology Zurich. Switzerland.

Cruz, L. M., E. M. De Souza, O. B. Weber, J. I. Baldani, J. Döbereiner, and F. D. O. Pedrosa. 2001. 16S Ribosomal DNA Characterization of Nitrogen Fixing Bacteria Isolated from Banana (*Musa spp*.) and Pineapple (*Ananas comosus* (L.) Merril). *Appl. Environ. Microbiol.* **67**, 2375–2379.

Colnaghi, R., A. Green, H. Luhong, P. Rudnick, and C. Kennedy. 1997. Strategies for increased ammonium production in free-living or plant associated nitrogen fixing bacteria. *Plant and Soil*. **194**,145–154.

Day, D. A., P. S. Poole, S. D. Tyermanc, and L. Rosendahl. 2001. Ammonia and amino acid transport across symbiotic membranes in nitrogen fixing legume nodules. *Cell. Mol. Life Sci*. **58**,61–71.

Dobbelaere, S., J. Vanderleyden, and Y. Okon. 2003. Plant Growth-Promoting Effects of Diazotrophs in the Rhizosphere. *Crit.Revi. Plant Sci*. **22**(2), 107–149.

Gordon, J. K., and M. R. Jacobson. 1983. Isolation and characterization of Azotobacter vinelandii mutant strains with potential as bacterial fertilizer. *Can. J. Microbiol*. **29**, 973–978.

Gordon, J. K., and R. A. Moore. 1981. Ammonium and methylammonium transport by the nitrogen-fixing bacterium Azotobacter vinelandii. *J. Bacteriol*. **148**, 435–442.

Kleiner, D. 1982. Ammonium (methylammonium) transport by *Klebsiella pneumoniae*. *Biochim. Biophys. Acta*. **688**, 702- 708.

Kim, C., M. L. Kecskés, R. J. Deaker, K. Gilchrist, P. B. New, I. R. Kennedy, S. Kim, and T. Sa. 2005. Wheat root colonization and nitrogenase activity by *Azospirillum* isolates from crop plants in Korea. *Can. J. Microbiol.* **5**, 948–956.

Liba, C. M., F. I. S. Ferrara, G. P. Manfio, F. Fantinatti-Garboggini, R. C. Albuquerque, C. Pavan, P. L. Ramos, C. A. Moreira-Filho, H. R. Barbosa. 2006. Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *J. Appl. Microbiol.* **101**, 1076-86.

Lie, J. H., E. T. Wang, W. F. Chen, W. X. Chen. 2008. Genetic diversity and

potential for promotion of plant growth detected in nodule endopytic bacteria of soybean grown in Heilongjiang province of China. *Soil Biol. Biochem*. **40**, 238-246.

Ma, Y. C., and S. F. Chen. 2008. Paenibacillus forsythiae sp. nov., a nitrogen-fixing species isolated from rhizosphere soil of Forsythia mira. Int *J Syst Evol Microbiol*. **58**, 319-323.

Narula N., K. Lakshminarayana, and P. Tauro. 1981. Ammonia excretion by *Azotobacter chroococcum*. *Biotech. Bioeng*. **23**, 467-470.

Roberts, G. P., and P. W. Ludden. 1992. Control of nitrogen fixation in photosynthetic bacteria. In Biological Nitrogen Fixation. Eds. G Stacey, R H Burris and H J Evans. pp 135–165. Chapman and Hall, New York.

Reinhardt, É. L., P. L. Ramos., G. P. Manfio., H. R. Barbosa., C. Pavan., C. A. M. Filho.2008.Molecular characterization of nitrogen-fixing bacteria isolated from Brazilian agricultural plants at São Paulo state. *Braz. Jour. of Microbiol*. **39**, 414-422.

Shida, O., H. Takagi, K. Kadowaki, S. Udaka, L. K. Nakamura, and K. Komagata. 1995. Proposal of *Bacillus reusten sp. nov*., *Bacillus formosus sp. nov., nom. rev.*, and *Bacillus horstelensis sp. nov., nom. Rev. Int. J. Syst. Bacteriol.* **45**, 93-100.

Shida, O., H. Takagi, K. Kadowaki, and K. Komagata. 1996. Proposal for Two New Genera, *Brevibacillus gen. nov*. and *Aneurinibacillus gen. nov. Rev. Int. J. Syst. Bacteriol.* **46**, 939-946.

Shanmugam, K. T., and R. C. Valentine. 1975. Microbial production of ammonium ion from nitrogen. *Proc. Natl. Acad. Sci. USA*. **72**, 136–139.

Turner, G. I. And Gibson. 1980. Measurement of nitrogen fixation by indirect means. In: Method for evaluating Biological nitrogen fixation. F. J. Bergersen (Ed). John Wiley and Sons, Inc. New York.

Yuen, S. H., and A. G. Pollard. 1952. The Determination of nitrogen in agricultural materials by the Nessler reagent. I. Preparation of the reagent. *J. Sci. Food Agric*. **3**, 441-422.

Zahran, H. H. 1999. Rhizobium legume symbiosis and nitrogen fixation under severe conditions and in arid climate. *Microbiol and Mol. Biol. Rev*. **63**, 968-989.