Indonesian Journal of Biotechnology, December, 2013 Vol. 18, No. 2, pp.161-167

Legume Nodulating Bacterium, *Achromobacter xylosoxidans* Found in Tropical Shrub Agroecosystem, Sumatera, Indonesia

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Abstract

Legume nodulating bacteria (LNB), known also as rhizobia, are soil bacteria, which are able to form root nodules and fix nitrogen in the leguminous plants. The LNB availability in the soil depends on the type of agroecosystem, where plant grows. In this study, we isolated LNB from the shrub agroecosystem in Sumatera, Indonesia, and obtained four selected bacterial strains. Among them, the isolate UGM48a formed root nodule in *Macroptilium atropurpureum* and showed highest number of nitrogenase activity. UGM48a also contains *nifH* and *nodA* genes. An analysis of the PCR-amplified 16S rDNA and BLASTn analysis showed that UGM48a displayed 96% similarity with *Achromobacter xylosoxidans*. In addition, UGM48a were successfully nodulated *Glycine max* (L.) merr *var*. wilis. This is the first report detecting *A. xylosoxidans* as nodule-forming species for *Glycine max* possesing the positive copy of *nodA* gene.

Keywords : Legume Nodulating Bacteria, shrub agroecosystem, *Achromobacter xylosoxidans, nodA, Glycine max*

Introduction

Legume nodulating bacteria (LNB), known also as rhizobia, are soil bacteria able to form root nodules and fix nitrogen in the leguminous plants. Leguminous plants usually establish a symbiosis with rhizobia and benefit from its ability to fix atmospheric nitrogen, which allows them to grow more efficiently on nutrient-limited soils.

LNB availability in the soil may change due to history of land use. Various agroecosystems in Sumatera, Indonesia have different land use, with 87% used for coffee plantation. Shrub agroecosystem means the soil was in fallow for about 5 years after coffee plantation (Evizal *et al*., 2013). This condition may influence the availability of LNB in the soil, because there are shrubbery that dominated this area.

LNB or Rhizobia are divided into several groups, which include alphaproteobacteria comprised of 13 genera of Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium, Allorhizobium, Sinorhizobium/Ensifer, Methylobacterium, Blastobacter, Devosia, Ochrobactrum, Phyllobacterium, Shinella (Lin *et al*., 2008) and Microvirga (Ardley *et al*., 2012). Nowadays, there are many studies showing that LNB does not always belong to Alphaproteobacteria. Betaproteobacteria are comprised of three genera of Burkholderia (Sheu *et al*., 2012), Cupriavidus and Herbaspirillum (Euzeby's, 2006). In addition to alphaproteobacteria and betaproteobacteria, there are bacteria belonging to the class gammaproteobacteria (Benhizia *et al*., 2004; Shiraishi *et al*., 2010), but their symbiont properties have not been yet demonstrated.

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This study reported four LNB based on cell and colony's morphology, as well as the nodulation assay in *Macroptilium atropurpureum*. Based on sequence analysis of 16S rDNA gene and its nitrogenase activity in *M. atropurpureum,* it was identified as strain of *Achromobacterium xylosoxidans*. This bacterium is one of the genera belonging to betaproteobacteria, which have been reported as legume nodulating bacteria for the first time by Benata *et al*. (2008). *A. xylosoxidans* formed root nodule in *Prosopis juliflora* (Benata *et al*., 2008); *Mucuna bracteata* (Salwani *et al*., 2012); and cowpea (Guimarães *et al*., 2012). In this study, we also confirmed the existence of *nifH* and *nodA* copy genes. Our finding also observed the nodulation ability of *A. xylosoxidans* in soybean

Materials and Methods

Bacterial Isolates

Four isolates used in this study were screened from shrub agroecosystem in Sumatera, Indonesia. The pure cultures were maintained on yeast-extract mannitol agar (YEMA) slants at 4° C using standard procedures (Somasegaran, 1994).

Nodulation and N_2-fixation Assay

Four isolates were examined through nodulation and N_2-fixation assay using *Macroptilium atropurpureum*. Surface-sterilized seeds of *M. atropurpureum* were germinated in petridishes. The seedling were transferred to the test chamber called minirhizotron which consists zeolite based medium (Joachim *et al*., 2006). The seedlings were watered daily with N-free nutrient solution as described by Somasegaran (1994). At the 40th day, the plants were analyzed for the number of fresh nodules, and their nitrogenase activity was determined using Acetylene Reduction Assay (ARA).

The best isolate showing highest number of nitrogenase activity were subsequently observed in various plants. In this report, soybean (*Glicyne max* [L.] *merr* var. *wilis*) was used as tested plant, to observe the nodulation ability of the selected isolate. The purpose of using this plant was investigate nodulating ability in soybean, since soybean can only be infected by few bacteria. Furthermore, *Glicyne max* (L.) *merr* var. *wilis* is one of the superior types of soybean released in Indonesia since 1983. Surface-sterilized seeds of wilis were germinated in petridishes after soaking the seeds overnight in a sterile water. Observation was performed 40 days after cultivation.

Morphological Observation

Bacterial colonies and colours were observed by using standard microbiological method as described by Yang *et al*. (2008), Vincent (1972; 1982), and Somasegaran and Hoben (1985). The colonies of pure cultures were maintained on YEMA slants at 4° C and also stored at 4°C.

Genomic DNA Isolation

Total DNAs were extracted from 5 ml bacterial cultures grown in Luria Bertani. The cultures were centrifuged at 11000 rpm for 8 min and washed in TE buffer (10mM Tris, 1 mM EDTA; pH 8). The pellets were suspended in 30 µl SDS 10%, incubated at 37°C for 30 min, then 100 µl NaCl 5 M and 200 µl CTAB were added, and incubated at 65°C for 15 min. The mixtures were centrifuged at 11000 rpm for 8 min. The top layer was transferred to a new tube. This aqueous phase containing DNA was precipitated with 500 µl isopropanol and then centrifuged at 11000 rpm for 8 min. The precipitated DNA was washed with 100 µl ethanol, vacuum dried, and subsequently dissolved in TE buffer. This method was modification as described method by Ausubel (1995).

PCR amplification of nodA and nifH genes

To amplify *nifH* gene fragments, degenerate primers were used, forward primer 5'-GCI WTI TAY GGN AAR GGN GG-3' and reverse primer 5'-GCR TAI ABN GCC ATC ATY TC-3' as described by Burgmann and coworkers (2004). In this study, *nodA-1* and *nod A-2* were used

to amplify *nodA* gene fragments. Forward primer (*NodA-1*) : 5'-TGC RGT GGA ARN RNN CTG GG<3' and reverse primer (*nodA-2*) : 5'-GGN CCG TCR TCR AAW GTC ARG-3' were used for PCR amplification of the *nodA* gene (Kaisa *et al*., 1998). These were also degenerate primer with notation W=A or T; B=C, G or T; R=A or G; N=A, C, G, or T; and I=inosine. To amplify both gene fragments, each reaction was performed on a 25 µl sample of the PCR reaction mixture contained 21,5 µl Vivantis Kit PCR Mix, the template genomic DNA (50 ng. μl^{-1}), 0,5 µl Taq DNA Polymerase, and 25 pmoles each DNA primers. PCR was performed using PCR thermal cycler (T100™ Thermal Cycler, Bio-Rad). PCR condition for amplification of *nifH* gene was set as follows : pre denaturation at 95° C for 5 min, denaturation at 94° for 30 second, annealing at 56° C for 30 second, elongation at 72° C for 1 min (35 cycles), and final elongation at 72° for 10 min. In addition PCR cycling program for *NodA* amplification was set as follows : 5 min pre denaturation at 94° C, followed by 30 cycles of 30 seconds denaturation at 94° C, 1 min annealing at 55° C, and 1 min polymerization at 68° C. Final elongation was 10 min at 68° C. Amplified bands were resolved by electrophoresis in a 1,5% (w/v) agarose gel in TBE buffer and visualized by ethidium bromide staining in UV-transiluminator. Size was compared by using 100 kb Vivantis DNA Ladder.

PCR amplification and Analysis of 16S rDNA Sequences

The 16S rDNA sequences were amplified from the genomic DNA of the isolates using the universal primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R primer (5'-GGT TAC CTT GTT ACG ACTT-3') as described by Lane (1991). PCR was performed using PCR thermal cycler (T100™ Thermal Cycler, Bio-Rad) with total volume 25µl PuRe Taq Ready-To-Go™ PCR Beads consist of : 22µl dH_2O (nuclease free water), 1µl DNA template (50 ng. μl^{-1}), 1 µl each DNA primers (25 pmoles). The PCR condition was set as follows : pre denaturation at 94° C for 4 min, denaturation at 94° for 30 second, annealing at 55° C for 1 minute 30 second, elongation at 72° C for 30 seconds (30 cycles), and final elongation at 72° for 10 min. Amplified bands were resolved by electrophoresis in a 1,5% (w/v) agarose gel in TBE buffer and visualized by ethidium bromide staining in UV-transiluminator. Size was being compared by using 1 kb Vivantis DNA Ladder.

Results and Discussiom

Morphological observation

Based on morphological observations of both cells and colonies, the four isolates (Table 1) had similar colonies morphology on YEMA plates (picture not shown). They were transparent to creamy (white) colonies with 0,5-2 mm in diameter after 3 days incubation at room temperature. All bacterial isolates were Gram negative and rod-shaped with different shape of edge, elevation, and inner cell's structure (Table 1).

Nodulation and N-fixation assay of four isolates on M. atropurpureum

The nodulation assay of four isolates on legume *M. atropurpureum* from shrub

Table 1. The observation of colony and cell morphology

No	Colony's morphology						Cell's morphology			
	Isolates	Elevation	Shape of colony	The edge of the colony	Colony's inner structure	Colony growth on YMA	Shape of cell	Gram	Size of the cell (µm)	Motility
1	UGM48a	Convex	Circulair	Entire	Coarsely granular	White	Rod	Negative	2,5x2,5	Motile
2	UGM50b	Low convex	Circulair	Undulate	Coarsely granular	White	Rod	Negative	0,5x0,5	No motile
3	UGM50c	Low convex	Irregulair	Entire	Coarsely granular	White	Rod	Negative	1x1,2	No motile
4	UGM50d	Low convex	Amoeboid	Entire	Coarsely granular	White	Rod	Negative	0,5x1	No motile
5	USDA122	Convex	Curled	Lobate	Wavy entelaced	White	Rod	Negative	2,5x2,5	Motile

agroecosystem in Sumatera, Indonesia were obtained by using minirhizotron method. All bacterial isolates successfully formed nodule with different number of nodules. The fresh nodules were used for measuring the nitrogenase activity. Among them, UGM48a isolates showed the highest number of nitrogenase activity in 0,806 mmol C_2H_4.g nodule^{-1}.h^{-1} with the highest nodule dry weight (Table 2).

Minirhizotron is zeolite based medium in the chamber (Joachim *et al*., 2006). The seedlings in the chamber were watered daily with N-free nutrient solution. Based on findings by Busch *et al*. (2006), *Rhizotron* method is the easiest reliable method to plant and to harvest the belowground biomass in distinct soil layers because of the simple access to the intact soil and root system at harvest. The tip of lateral root spreads out to all part of the chamber of rhizotron, include the surface. It is easier for isolates to initiate flavanoid (compound that triggers the secretion of Nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses, such as ion fluxes and the formation of a root nodule). At harvest, which was the 40th day after cultivation, the plants were analyzed for the number of fresh nodules and their nitrogenase activity was determined using Acetylene Reduction Assay (ARA). The result showed that UGM48a isolates have the highest number of nitrogenase activity in 0,806 mmol C_2H_4.g nodule^{-1}.h^{-1}. This isolate was used as the selected isolate, which we observed thoroughly.

Table 2. Number, Dry Weight, and Nitrogenase Activity of Nodules Formed by Bacterial Isolates in M. atropurpureum

Isolates	Number of nodules (per plant)	Nodule dry weight (g/plant)	Nitrogenase activity (mmol C_2H_4.g nodule^{-1}. h^{-1})
UGM48a	20	0,016	0,806
UGM50b	21	0,012	0,645
UGM50c	12	0,011	0,587
UGM50d	17	0,008	0,584

Analysis of the 16S rDNA sequences

Pure DNA genome of selected isolate, UGM48a, was used in 16s rDNA sequence analysis. This is one of the most effective tools for identifying bacteria. The 16S rDNA fragments of UGM48a was successfully amplified. Based on BLASTn result at NCBI (http://blast.ncbi), UGM48a has 97% in similarity with *Achromobacter xylosoxidans* with identity 1272 bp query length. *A. xylosoxidans* is one of genera belong to betaproteobacteria that has been reported as legume nodulating bacteria for the first time by Benata *et al*. (2008).

NifH and NodA amplification

The existence of *NifH* and *NodA* gene copy were also amplified. The molecular weight of the amplified *NifH* and *NodA* gene were about 300 bp and 200 bp (Figure 1), whereas the molecular weight of the amplified *NifH* genes was between 371-464 bp (Burgmann *et al*., 2004). The amplification proved that the bacterium has the potential to fix up free nitrogen. The other publications also mentioned that extensively *NifH* gene amplification in nitrogen-fixing bacteria, non-symbiotic, or associative. Achouak *et al*. (1999) successfully amplify *NifH* genes in bacteria *Paenibacillus polimyxa, P. macerans*, and *P. azotofixans* with a molecular weight of about 370 bp. In the other hand, Mirza *et al*. (2006) successfully amplify *NifH* genes in Pseudomonas sp strain K1 with a molecular weight of 360 bp.

UGM48a isolates were amplified using primers degenarated NodA : NodA-1 (5' –TGC RGT GGA ARN RNN CTG GG -3') and primer NodA-2 (5'- GGN CCG TCR TCR AAW GTC ARG - 3') with a molecular weight of 200 bp. According to Haukka *et al*. (1998) the expected amplification product is a band with a molecular weight of 666 bp. But the results showed other bands besides the expected band. Generally, the additional band does not preclude the measurement between the bright and the smear band. However, degenerate primer performed

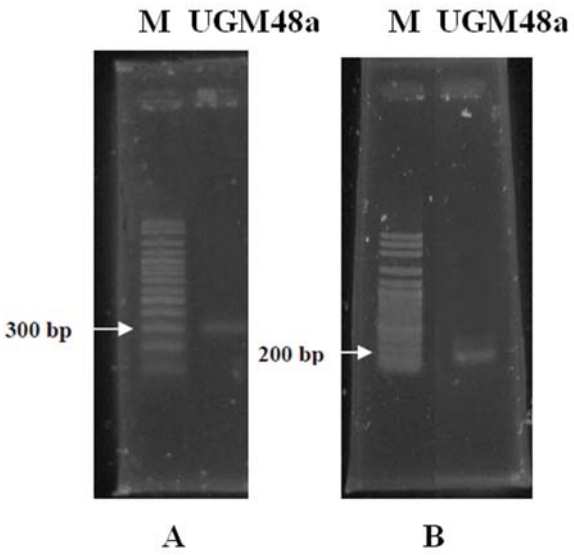

Figure 1. The Agarose Gel Electrophoresis of UGM48a Amplified nifH (A) and nodA (B) genes.

in the attachment of the primary side is not suitable because of the low annealing temperature. In many experiments with degenerate primer, the expected molecular weight from amplified products of PCR can not be predicted with certainty (McPherson and Møller, 2006).

Orthologs of the *nodA* gene, one of the key nod genes encoding an acyl transferase (Kamst *et al*., 1998), have been discovered in symbiotic *Methylobacterium* sp. (Sy *et al*., 2001) and *Burkholderia* sp (Moulin *et al*., 2001). Major nod factor-triggered responses include the formation and deformation of root hairs, intra- and extracellular alkalinization, membrane potential depolarization, changes in ion fluxes, induction of early nodulin gene expression, and formation of nodule primordia (Broughton *et al*., 2000; Perret *et al*., 2000). So that, the *NodA* gene has been shown to be a good nodulation marker indeed (Boivin and Giraud, 1999).

Table 3. Nodulation assay in Glicyne max (L.) merr var. wilis

Isolates	Nodulation assay
UGM48a	+
USDA 122	+
Uninnoculated	-

Note : Positive (+) sign showed that UGM48a able to form root nodule in soybean as well as USDA 122. Negative (-) sign showed that uninnoculated soybean did not form root nodule.

Nodulation assay in Glycine max (L.) merr var. wilis

The last observation was the ability of UGM48a to form root nodule in soybean to ascertain the extent of bacterium ability to form root nodules on different host plants. The soybean is an important food commodity in Indonesia. 40 days after cultivation of soybean, observation showed that UGM48a formed nodule (Table 3). *A. xylosoxidans* has previously been reported having ability to nodulate *Prosopis julifora* (Benata *et al*., 2008); cowpea (Guimarães *et al*., 2012); and *Mucuna bracteata* (Salwani *et al*., 2012). This is the first findings that *A. xylosoxidans* were able to nodulate soybean. There are only seven species capable of symbiosis with soybean (Khan *et al*., 2010) : *Bradyrizhobium japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall *et al*., 1992), *B. liaoningenese* (Xu *et al*., 1995), *B. canariense, B. yuanmingense* (Vinuesa *et al*., 2008), and *E. fredii, Ensifer xinjiangensis* (Young, 2003).

Acknowledgement

This work was funded in part by the Project of Below-Ground Biodiversity (BGBD) Indonesia and by the Directorate General of Higher Education, Republic of Indonesia. We are grateful to Dr. Denisa Kera for her critical reading on the manuscript.

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