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SPECIES AND FUNCTIONAL DIVERSITY OF RHIZOBACTERIA OF RICE PLANT IN THE COASTAL SOILS OF INDONESIA

Keragaman Spesies dan Fungsional Rhizobakteri pada Tanaman Padi di Tanah Sawah Daerah Pesisir di Indonesia

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ABSTRACT

Rhizobacteria are important components of soil and directly or indirectly influence the soils quality and plant growth for maintaining adequate plant nutrition and reducing the negative environmental effects of fertilizers. Applying high dose of chemical fertilizers in most of rice fields in the coastal areas could reduce the quality of the soil in the long time. There are few studies addressed to verify the species and functional diversity of cultivable rhizobacteria associated with rice plant in the coastal soils. The objective of the study was to verify the species and functional diversity of rhizobacteria isolated from the coastal soils of two rice production areas of Subang and Indramayu, West Java. Special focus was given to verify phosphate solubilization, nitrogen fixation, IAA and cellulase production of the selected 78 strains of rice rhizobacteria isolated from the coastal paddy field, as well as taxonomical analyses based on 16S rRNA. The results showed that among 78 bacterial isolates from the coastal paddy field, mostly were belonging to the Firmicutes, most of them affiliated with genera *Bacillus*, 75 strains produced IAA, 32 strains fixed nitrogen, 37 strains solubilized phosphate and 33 strains produced cellulase. Several strains of the rhizobacteria were capable of producing plant growth promoting substances (PGPR), alone or in combination, such as IAA, fixing nitrogen, solubilizing phosphate, and producing cellulase. Taking all of these diverse PGPR characteristics into account, it is clear that the 78 identified isolates have great potential for improving saline soils of the coastal paddy fields in Indonesia.

[Keywords: Rhizobacteria, functional diversity, rice plant, coastal soil**]**

ABSTRAK

Rhizobakteri merupakan komponen penting dalam tanah dan secara langsung atau tidak langsung memengaruhi kualitas tanah dan pertumbuhan tanaman untuk menjaga nutrisi tanaman dan mengurangi dampak negatif penggunaan pupuk kimia yang berlebihan. Aplikasi pupuk kimia yang berlebihan dalam kurun waktu yang lama di sebagian besar lahan sawah di daerah pesisir dapat mengurangi kualitas tanah. Studi mengenai keragaman fungsional mikroba tanah yang berasosiasi dengan tanaman padi di lahan sawah daerah pesisir masih terbatas. Tujuan penelitian ini adalah untuk memverifikasi keragaman spesies dan potensi rhizobakteri yang diisolasi dari rizosfer tanaman padi pada tanah pesisir Subang dan Indramayu, Jawa Barat. Studi ini difokuskan pada verifikasi isolat rhizobakteri yang mampu melarutkan fosfat, menambat nitrogen, menghasilkan IAA dan enzim selulase dari 78 strain rhizosfer tanaman padi sawah di daerah pesisir, serta analisis taksonominya berdasarkan 16S rRNA. Hasil penelitian menunjukkan bahwa di antara 78 isolat bakteri dari tanah sawah di daerah pesisir, sebagian besar tergolong ke dalam filum Firmicutes, genus Bacillus*, dan secara potensi 75 strain mampu menghasilkan IAA, 32 strain mampu menambat nitrogen, 37 strain mampu melarutkan fosfat, dan 33 strain mampu menghasilkan selulase. Beberapa strain bakteri rizosfer mempunyai kemampuan untuk menghasilkan senyawa perangsang pertumbuhan tanaman, secara tunggal atau kombinasi, seperti IAA, memfiksasi nitrogen, melarutkan P, dan memproduksi enzim selulase. Hasil penelitian mengindikasikan potensi isolat rhizobakteri dari tanah sawah pesisir untuk meningkatkan potensi lahan salin di Indonesia.*

[**Kata kunci:** Rhizobakteri, keragaman fungsional, tanaman padi, tanah pesisir]

INTRODUCTION

Indonesia has approximately 8.1 million ha of rice fields; 3.25 million ha or 40.3% of these are spread over the northern coastal areas of Java Island. Some of the rice production centres in the northern coastal area of West Java, such as Subang and Indramayu, contributed significantly to the national rice production (Central Bureau of Statistics 2011). The lowland areas of these regions, however, have different soil salinity as a result of the seawater intrusion.

Rice plants are highly sensitive to salinity, especially during the germination period and at the

beginning of vegetative growth. Therefore, the presence of seawater intrusion continuously would be a serious threat which leads to plant toxicity, poor growth, and reduced yield (Suriyan and Chalermpol 2009). At the long time, this may affect agroecosystem sustainability.

In general, farmers apply high dose of chemical fertilizers in most of rice fields in the coastal areas of Subang and Indramayu, West Java. Such practices could inhibit the interaction of microorganisms and their host plants. In the long term, it would reduce the quality of the soil due to saturation of certain soil elements hence not available for plant growth. There are very few studies addressed to verify the species and functional diversity of cultivable rhizobacteria associated with rice plant in the coastal soils.

Plant Growth Promoting Rhizobacteria (PGPR) are important components of soil and directly or indirectly influence soil quality and plant growth for maintaining adequate plant nutrition and reducing the negative environmental effects of chemical fertilizers. These microbes mediate soil processes, such as nitrogen fixation, nutrient mobilization, mineralization, denitrification, and decomposition. Rhizosphere bacteria also produce phytohormones, such as indole acetic acid (IAA), cytokinin, and gibberellin (Madhaiyan *et al*. 2006; Kang *et al*. 2009). The activity of cellulase acts as a key enzyme for the invasion and colonization of plant roots (Hallmann *et al.* 1997; Reinhold-Hurek and Hurek 1998).

The objective of this study was to verify the species and functional diversity of rhizobacteria isolated from coastal soils of two rice production areas of Subang and Indramayu, West Java. Special focus was given on the verification of phosphate solubilization, nitrogen fixation, IAA and cellulase production of selected 78 strains isolated from the coastal paddy field. The study is expected to understand the diversity of rhizobacteria and their potential role in improving saline soil of the coastal paddy field in Indonesia.

MATERIALS AND METHODS

Location and Soil Sampling

Three replicated samples each of rice rhizosphere soils were collected from paddy field near coastal area in Subang (06°14'59"S, 107°54'31"E) and Indramayu (06°18'48", 108°02'15"E) during December 2012 and January 2013. The physicochemical properties of the soil samples were analysed at the Indonesian Soil Research Institute, Bogor. The microbiological analysis of the soil samples was carried out immediately after sampling to minimize the storage effects.

Source of Rhizosphere Bacterial Isolates

The rhizosphere soil was obtained by taking the whole rice plant with soil on it to the laboratory. The rhizosphere soil was shaken manually to remove the soil from the roots, while the fine layer of soil firmly attached to the roots was immersed into 100 mL sterile distilled water to remove them. The resulting mixed solution was referred as rhizosphere soil. Soil microbes were isolated using a standard dilution plating technique on a Plate Count Agar (PCA) on Soil Extract Agar (SEA) consisted of 0.1% glucose, 0.05% dipotassium phosphate, and 1.775% yeast extract. The incubation was done at 28°C for 7 days. All isolates had already been maintained in the Indonesian Culture Collection (InaCC) of the Indonesian Institute of Sciences (LIPI) and the Biogen Culture Collection (BiogenCC) of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD). The isolates were routinely cultured on SEA for the functional characterizations.

Identification of Bacterial Isolates

DNA Extraction

Bacterial DNA was extracted from all isolates of the rhizosphere bacteria. Each isolate was grown in a liquid medium 1/10 strength of Luria Bertani Broth composed of tripton 1%, 0.5%, yeast extract and 1% NaCl. Overnight cultures were centrifuged at 13,000 rpm for 2 minutes to pellet the cells and remove the supernatant. Genomic DNA of each isolate was then extracted from bacterial cell pellet by using the Wizard DNA genomic extraction kits (Promega). Quality and quantity of the isolated DNA were checked by using nanodrops and gel electrophoresis on a 0.8% agarose gel.

16S rDNA Gene Amplification and Sequencing

PCR amplification targeting the 16S rDNA gene was carried out on samples of the bacterial DNA using 16S-27F (5'AGAGTTTGATCCTGGCTCAG3') and 16S-

1492R (5'GGTTACCTTGTTACGACTT3') primers. Phylogenetic affiliations and taxonomical hierarchy based on 16S rRNA were determined with 95% confidence by using CLASSIFIER tool (<http://rdp.cme.msu.edu>) of RDP-II database (Cole *et al.* 2009).

Functional Characterization of Bacterial Isolates

PO_4 Solubilization

Rhizobacterial ability to solubilize inorganic phosphate (tricalcium phosphate) was assessed using phosphate-solubilizing medium (Pikovskaya agar medium) and incubated at 28°C for 7 days. Colonies with clearing zones were scored as positive for phosphate solubilization (Pikovskaya 1948). The experiments were performed with three replicates for each bacterial strain.

N_2 Fixation

Selection of N_2 fixer bacteria was carried out under semi-solid nitrogen-free bromthymol blue (NFb) medium (0.5% DL-mallic acid, 0.4% KOH, 0.05% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $MnSO_4.H_2O$, 0.002% NaCl, 0.001% $CaCl_2$, 0.005% $FeSO_4.7H_2O$, 0.0002% $Na_2MoO_4.2H_2O$ and 0.175% bacto agar, and 2 ml 0.5% bromtimol blue (BTB). Five days after incubation at 28°C, the isolates showed a veil-like pellicle near the surface of the medium were considered positive as N_2 fixer isolates (Dobereiner 1995).

Cellulolytic Activity

Screening of the cellulolytic isolates was performed on carboxy methyl Cellulose (CMC) agar media containing 0.5% caboxymethyl cellulose, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.1% KCl, 0.05% $MgSO_4.7H_2O$, 0.05% yeast extract, 1.5% bacto agar, pH 8.0. Five days after incubation at 28°C, a solution of Congo red was poured on the surface of the agar to detect the cellulolytic enzyme activity as described by Teather and Wood (1982). The cellulolytic activity was indicated by a clear zone around the colony of bacteria.

IAA Producer

IAA was detected calorimetrically in the supernatants of the bacterial cultures using Gordon and Weber's reagent (1 ml 8.12% $FeCl_3.6H_2O$, 50 ml 35% $HClO_4$ in dark bottle). The isolates were grown overnight in a modified nutrient broth M26 (0.5% NaCl, 1% peptone, 1% beef extract). As many as 100 µl of the overnight culture was added to 10 ml of minimal salt medium (0.136% KH_2PO_4, 0.213% Na_2HPO_4, 0.02% $MgSO_4.7H_2O$, and 10 ml trace element). Trace element consisted of 700 mg $CaCl_2.2H_2O$, 300 mg $FeSO_4.7H_2O$, 20 mg $MnSO_4.H_2O$, 40 mg $CuSO_4.5H_2O$, 20 mg $ZnSO_4.7H_2O$, 3 mg H_3BO_3, 7 mg $CoCl_2.6H_2O$, 4 mg $Na_2MoO_4.H_2O$, and 1 ml H_2SO_4 per 1 litre) supplemented by 1 ml L-tryptophan (10% glucose, 1% L-triptofan and 0.1% yeast extract, filtered by using milliphore of 0.2 µm). After further incubation for 48 hours, IAA production was assessed as follows. Bacterial cells were removed from the culture medium by centrifugation (8,000 rpm, 4°C, 10 minutes), and then 2 ml of the above reagent was mixed with 1 ml of culture supernatant, followed by incubation at room temperature for 25 minuters. The optical density of the culture grown in the minimal salt medium supplemented with tryptophan was measured using a spectrophotometer at 530 nm. The concentration of IAA in each culture medium was determined by comparison to a standard curve generated from known concentration of IAA (Gordon and Weber 1951). The readings were performed with three replicates for each bacterial strain.

RESULTS AND DISCUSSION

The physicochemical properties of the two soil types collected from Subang and Indramayu coastal soils varied in terms of their textures, pH, salinity (EC or TDS), total carbon, and total nitrogen (Table 1). Microbiological properties of the two sites showed that population number in Indramayu was greater than that in Subang.

Functional Characterization of Cultivable Rhizosphere Bacteria from Coastal Soil

The functional groups of rhizosphere microbes are difficult or even impossible to assess directly. Therefore, this study was based on dilution plating, which is a quick, inexpensive and reliable technique.

Table 1. Physical and chemical characteristics of paddy soils from the coastal area of Subang and Indramayu, West Java.

Soil properties	Coastal paddy field	
	Subang	Indramayu
Sand (%)	16 ± 1.0	13 ± 1.73
Silt (%)	18 ± 1.1	33 ± 3.51
Clay (%)	66 ± 1.0	54 ± 5.13
C total (%)	1.59 ± 0.28	1.61 ± 0.36
N total (%)	16 ± 0.05	0.25 ± 0.13
pH	16 ± 0.11	5.97 ± 0.11
EC (dS m^{-1})	0.261 - 1.594	0.355 - 1.908
TDS (mgl^{-1})	123 - 782	169 - 944
CEC (Na)	2.53 - 8.50	1.14 - 7.42
Total[1)]	4.76 ± 0.39	13 ± 0.71

Values are mean ± SE.

[1)]Total means total bacterial population.

Values (culturable) are expressed as log of bacteria per gram of soil

Seventy eight bacterial taxes collected from rhizosphere of rice plant grown in coastal soil were able to grow on SEA medium. Most of the rhizobacteria were Gram-positive (66.7%) mostly belonging to the Firmicutes, most of them were affiliated with genera *Bacillus*. Different morphotypes of the rhizobacteria from Subang coastal soil were purified, which belonged to 12 genera and 19 species. Rhizobacteria from Indramayu consisted of 18 genera and 34 species (Table 2).

Among the isolates obtained in this study, the most often genera identified was *Bacillus*. Members of *Bacillus* are ubiquitous bacteria that include both free-living rhizobacteria and pathogenic species. *Bacillus* in the rhizosphere of rice plant in this study had the proportion of 32/78. It suggests they are highly competent in the rhizosphere compared with other genera. Rhizobacteria of the genera *Bacillus* have been reported to enhance the growth of several plants such as *Pinus*, wheat, tomato, sugar beet, sorghum, peanut and onion (Caceres *et al.* 1996; Probanza *et al.* 2001; Yan *et al.* 2003). Previous studies reported that numerous PGPR belonging to *Bacillus* were mostly restricted to certain species. However, this study indicated a high diversity of *Bacillus* PGPR species, such as *Bacillus stratosphericus, B. amyloliquefaciens, B. cereus, B. methylotrophicus, B. pumilus*, *B. marisflavi*, *B. megaterium* and *B. flexus*.

Lysinibacillus and *Microbacterium* were the second largest PGPR group found in this study. The four isolates in each of these genera (*Lysinibacillus* and *Microbacterium*) had high plant growth-promoting properties. Two *Lysinibacillus* species were identified; among them were *Lysinibacillus sphaericus* (three isolates), the most dominant, followed by *Lysinibacillus boronitolerans* (one isolate). The other genera was *Microbacterium*, commonly found in the soil samples. *Microbacterium* species identified as PGPR in this study were *M. arborescens*, *M. fluvii*, *M. xylanilyticum*, and *M. awajiense*. Some *Microbacterium* species have function in phytoextraction by assimilating heavy metals, such as Pb and Ni. They have been reported to increase the growth of apple and rape (Karlidag *et al.* 2007; Sheng *et al.* 2008).

The third group of PGPR belonged to the genera *Aeromonas*, *Arthrobacter*, *Enterobacter*, *Pantoea*, *Pseudomonas* and *Streptomyces*. The *Pseudomonas* species identified as PGPR in this study were *P. mosselii* and *P. knackmussii* (one isolate each). The finding of both bacteria as PGPR is the first time, as we known, therefore, lending to the significance of our study. Interestingly, previous studies showed that *Pseudomonas* PGPR are highly resistant to various environmental stresses. For instance, the effects of the PGPR *Pseudomonas fluorescens* MSP-393 are not altered in highly saline soils such as those on coastline (Paul and Nair 2008). Furthermore, production of 1 aminocyclopropane-1-carboxylic acid (ACC) deaminase by *P. fluorescens* increases the resistance of plants to salt stress (Saravanan *et al.* 2007).

PGPR members of the genera *Arthrobacter* are usually found in soil; they role in bioremediation. For instance, *Arthrobacter chlorophenolicus* has the ability to degrade high concentrations of 4-chlorophenol, which is a recalcitrant toxic compound in contaminated soils (Westerberg *et al*. 2000). A PGPR *Pantoea* was shown to promote the growth of pepper plants (Kang *et al.* 2007).

Besides members of these three major genera, the remaining PGPR belonged to 13 genera, consisted of *Exiguobacterium*, *Staphylococcus*, *Citrobacter*, *Rhodobacter*, *Stenotrophomonas*, *Ochrobactrum*, *Salinicola*, *Providencia*, *Brevibacterium*, *Rhodococcus*, *Rhizobium*, *Sinomonas* and *Acinetobacter*. The genera *Acinetobacter* promotes production of wheat, pea, chickpea, maize and barley through nitrogen fixation, siderophore production and mineral solubilization (Gulati *et al.* 2009; Sachdev *et al.* 2010). The genera *Stenotrophomonas* consists one species, i.e. *Stenotrophomonas maltophilia*, known as plant growth promoter and plays roles in bioremediation and phytoremediation (Ryan *et al.* 2009).

The study identified a large variation among isolates of these species with respect to different combinations of PGP traits that they carried. Other

Table 2. Species diversity of rhizosphere bacteria associated with rice plant from Subang and Indramayu coastal soil, West Java.

Genera	Subang	Indramayu
Bacillus	*Bacillus stratosphericus* (Ptb I B1.4; Ptb I B2.2; Ptb I B3.2; Ptb II B1.7; Ptb II B2.6; Ptb II B3.2), *B. amyloliquefaciens* (Ptb I B2.9), *B. cereus* (Ptb I B3.6; Ptb II B1.3; Ptb II B2.5; Ptb II B3.9); *B. methylotrophicus* (Ptb I B3.7); *B. pumilus* (Ptb II B1.2); *B. marisflavi* (Ptb II B1.5; Ptb II B3.3)	*B. megaterium* (Er I B1.2; Er I B2.12; Er I B3.4); *B. marisflavi* (Er I B1.4; Er II B2.3); *B. methylotrophicus* (Er I B1.5); *B. cereus* (Er I B2.1; Er II B2.14; Er II B3.11); *B.stratosphericus* (Er I B2.4; Er I B3.1; Er II B1.6; Er II B2.2; Er II B3.2); *B. amyloliquefaciens* (Er I B3.8); *B. flexus* (Er I B3.12); *B. pumilus* (Er II B1.2)
Aeromonas	*Aeromonas taiwanensis* (Ptb I B1.5; Ptb I B2.1)	*Aeromonas hydrophila* (Er I B1.7a)
Exiguobacterium	*Exiguobacterium indicum* (Ptb I B2.3)	
Arthrobacter	*Arthrobacter defluvii* (Ptb I B2.10; Ptb I B3.3)	*Arthrobacter alpinus* (Er I B2.8)
Enterobacter	*Enterobacter cloacae* (Ptb I B3.1); *Enterobacter ludwigii* (Ptb II B2.11)	*Enterobacter cloacae* (Er II B3.5)
Pantoea	*Pantoea agglomerans* (Ptb I B3.5); *Pantoea dispersa* (Ptb II B3.11);	*Pantoea agglomerans* (Er II B1.7);
Staphylococcus	*Staphylococcus gallinarum* (Ptb I B3.10)	*Staphylococcus gallinarum* (Er II B3.8)
Citrobacter	*Citrobacter freundii* (Ptb II B1.6; Ptb II B2.3)	
Pseudomonas	*Pseudomonas pseudoalcaligenes* (Ptb II B2.10)	*Pseudomonas mosselii* (ErIB2.5); *Pseudomonas knackmussii* (Er II B3.4)
Rhodobacter	*Rhodobacter aestuarii* (Ptb II B2.12)	
Stenotrophomonas	*Stenotrophomonas maltophilia* (Ptb II B3.1)	*Stenotrophomonas maltophilia* (Er I B3.6)
Ochrobactrum	*Ochrobactrum cytisi* (Ptb II B3.5a)	
Salinicola		*Salinicola salarius* (Er I B1.1; Er II B1.1)
Providencia		*Providencia rettgeri* (Er I B1.9; Er I B2.10)
Lysinibacillus		*Lysinibacillus sphaericus* (Er I B1.6; Er II B1.3; Er II B2.9); *Lysinibacillus boronitolerans* (Er II B3.15)
Brevibacterium		*Brevibacterium iodinum* (Er I B1.3)
Streptomyces		*Streptomyces coelicoflavus* (Er I B1.7b) *Streptomyces humidus* (Er I B2.2) *Streptomyces albidoflavus* (Er I B2.11)
Rhodococcus		*Rhodococcus ruber* (Er I B2.3)
Rhizobium		*Rhizobium radiobacter* (Er I B3.5)
Sinomonas		*Sinomonas flava* (Er I B3.7); *Sinomonas atrocyanea* (Er II B1.4)
Acinetobacter		*Acinetobacter calcoaceticus* (Er I B3.15); *Acinetobacter soli* (Er II B1.5)
Microbacterium		*Microbacterium arborescens* (Er II B2.5); *Microbacterium fluvii* (Er II B3.1); *Microbacterium xylanilyticum* (Er II B3.9); *Microbacterium awajiense* (Er II B3.10)

researchers reported that indigenous rhizobacteria commonly possess a variety of PGP traits, alone or in combination (Kumar *et al.* 2011; Sharma *et al.* 2011; Timmusk *et al*. 2011).

The study confirmed that all of 78 bacterial taxa to four PGP traits; several of them possessed more than one trait. Fifteen isolates from Subang were positive for IAA production and phosphate solubilization, whilst seven were positive for nitrogen fixation in addition to the previous three traits, i.e. *Bacillus stratosphericus* (Ptb I B2.2 and Ptb II B3.2), *B. amyloliquefaciens* (Ptb I B2.9), *Pantoea agglomerans* (Ptb I B3.5), *Pseudomonas pseudoalcaligenes* (Ptb II B2.10), *Enterobacter ludwigii* (Ptb II B2.11), and *Pantoea dispersa* (Ptb II B3.11) (Fig.1; Table 3). Four isolates from Subang coastal soils, i.e. *B.*

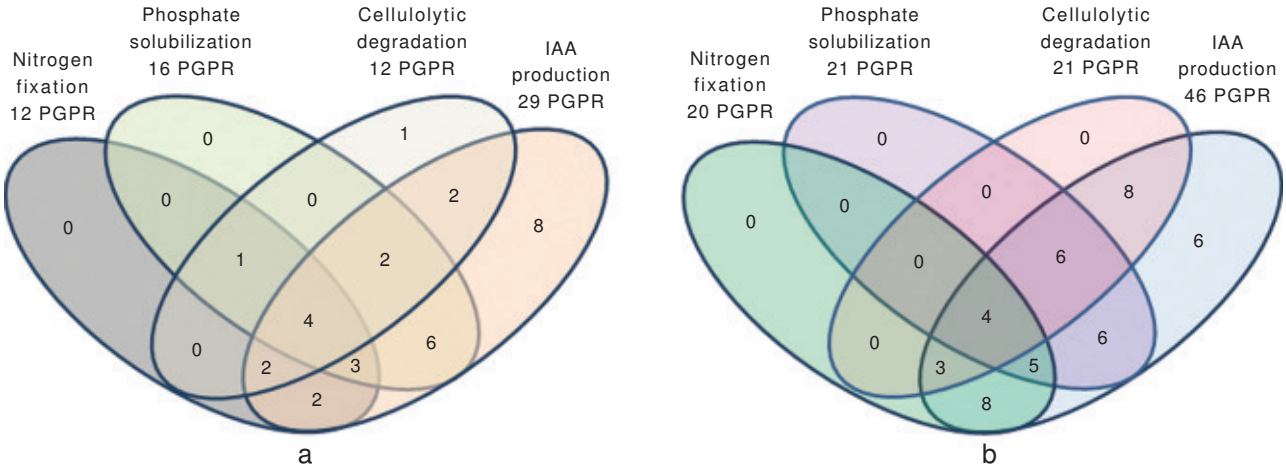

Fig. 1. The Venn diagram illustrates the distribution of the number of rhizobacteria associated with rice plant in the coastal soils of Subang and Indramayu, West Java: Isolates from coastal soil of Subang (a) and Indramayu (b) showing nitrogen fixation, phosphate solubilization, cellulolytic degradation, and IAA production traits.

stratosphericus (Ptb I B2.2), *B. amyloliquefaciens* (Ptb I B2.9), *P. pseudoalcaligenes* (Ptb II B2.10), and *P. dispersa* (Ptb II B3.11) had four beneficial traits. Meanwhile, four isolates from Indramayu coastal soils that also showed four beneficial traits were *Bacillus megaterium* (Er I B3.4), *Sinomonas atrocyanea* (Er II B1.4), *Acinetobacter soli* (Er II B1.5), and *Lysinibacillus boronitolerans* (Er II 3.15) (Fig 1; Table 3). *Sinomonas atrocyanea* Er II B1.4) and *Acinetobacter soli* (Er II B1.5) were obtained from moderate saline soil from the Indramayu coastal soil.

Our study confirmed 16 taxa of rhizosphere bacteria being positive for nitrogen fixation, IAA production and P solubilization. It found that only 20.5% (16 out of 78) of strains tested had the ability to produce IAA, fix nitrogen and solubilize P. These included seven isolates from Subang and nine isolates from Indramayu coastal soils. Among them, *Providencia rettgeri* (Er I B1.9 and Er I B2.10) had the ability to fix nitrogen, solubilize phosphate and produce high amount of IAA (50 ppm) (Fig 1; Table 3).

Diversity in IAA Production Traits of Rhizobacteria

The rhizobacteria were functionally diversed and some possessed more than one PGP trait. The bacteria from Subang coastal soil were relatively poor in IAA producer trait. Among the rhizobacteria, a total of 97.9% isolates from Indramayu coastal soil showed IAA producer as compared to only 93.5% of the isolates from Subang coastal soil (Table 3). It is reported that 80% of microbial isolates from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Pattern and Glick 1996).

Most of these isolates exhibited as producers of IAA with low to moderate concentrations. Some of the rhizobacteria produced the highest levels of IAA (>50 ppm). *Providencia rettgery* (Er I B1.9), *B. megaterium* (Er I B1.2), *Brevibacterium iodinum* (Er I B1.3), *P. rettgery* (Er I B2.10), *Arthrobacter alpinus* (Er I B2.8), *Rhizobium radiobacter* (Er I B3.5) and *Lysinibacillus sphaericus* (Er II B1.3) isolated from Indramayu coastal soil had the ability to produce IAA >50 ppm (Table 3). Moreover, only one isolate from Subang coastal soil, *Ochrobactrum cytisi* (Ptb II B3.5a) produced IAA of >50 ppm.

High levels of bacterial indolic compounds stimulate the formation of lateral and adventitious roots (Patten and Glick 1996), which could increase the absorption of nutrients including phosphate. Generally, IAA secreted by rhizobacteria interferes with the many plant developmental processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA secreted by bacteria (Spaepen *et al.* 2007; Glick 2012).

Several species of the genera *Bacillus* could produce IAA, but with variable efficiency. *Bacillus amyloliquefaciens* Ptb I B2.9 produced IAA of more than 50 ppm, but *B.amyloliquefaciens* (Er I B3.8) produced IAA of less than 50 ppm. Other species such as *B. cereus* (Er I B2.1) also produced much higher IAA concentration than *B. cereus* (Er II B2.14, Er II B3.11), and *B. megaterium* (Er I B1.2) produced

Table 3. Functional diversity of rhizosphere bacteria associated with rice plant from the coastal soils of Subang and Indramayu, West Java.

Species (strain)	N_2	P-	Cellulose	IAA
Subang				
Bacillus stratosphericus (Ptb I B1.4)	+	-	+	18.20
Aeromonas taiwanensis (Ptb I B1.5)	-	-	-	34.50
Aeromonas taiwanensis (Ptb I B2.1)	+	-	-	36.80
Bacillus stratosphericus (Ptb I B2.2)	+	+	+	4.60
Exiguobacterium indicum (Ptb I B2.3)	-	-	-	4.90
Bacillus amyloliquefaciens (Ptb I B2.9)	+	+	+	51.30
Arthrobacter defluvii (Ptb I B2.10)	-	+	-	4.60
Enterobacter cloacae (Ptb I B3.1)	+	-	-	9.90
Bacillus stratosphericus (Ptb I B3.2)	-	+	-	8.00
Arthrobacter defluvii (Ptb I B3.3)	-	-	-	14.10
Pantoea agglomerans (Ptb I B3.5)	+	+	-	41.20
Bacillus cereus (Ptb I B3.6)	+	+	+	-
Bacillus methylotrophicus (Ptb I B3.7)	-	-	-	23.40
Staphylococcus gallinarum (Ptb I B3.10)	+	-	+	26.95
Bacillus pumilus (Ptb II B1.2)	-	+	+	13.00
Bacillus cereus (Ptb II B1.3)	-	-	-	6.60
Bacillus marisflavi (Ptb II B1.5)	-	-	-	10.40
Citrobacter freundii (Ptb II B1.6)	-	+	-	25.70
Bacillus stratosphericus (Ptb II B1.7)	-	-	-	7.00
Citrobacter freundii (Ptb II B2.3)	-	+	+	5.10
Bacillus cereus (Ptb II B2.5)	-	-	-	1.00
Bacillus stratosphericus (Ptb II B2.6)	-	+	-	12.90
Pseudomonas pseudoalcaligenes (Ptb II B2.10)	+	+	+	21.40
Enterobacter ludwigii (Ptb II B2.11)	+	+	-	28.60
Rhodobacter aestuarii (AM748926)	-	-	+	-
Stenotrophomonas maltophilia (Ptb II B3.1)	-	-	+	12.30
Bacillus stratosphericus (Ptb II B3.2)	+	+	-	14.50
Bacillus marisflavi (Ptb II B3.3)	-	-	+	6.00
Ochrobactrum cytisi (Ptb II B3.5a)	-	+	-	59.80
Bacillus cereus (Ptb II B3.9)	-	+	-	24.00
Pantoea dispersa (Ptb II B3.11)	+	+	+	26.50
Indramayu				
Salinicola salarius (Er I B1.1)	-	-	-	2.90
Aeromonas hydrophila (Er I B1.7a)	-	+	-	36.96
Providencia rettgeri (Er I B1.9)	+	+	-	55.30
Bacillus megaterium (Er I B1.2)	-	+	-	75.95
Bacillus marisflavi (Er I B1.4)	+	-	-	6.90
Bacillus methylotrophicus (Er I B1.5)	-	-	-	3.15
Lysinibacillus sphaericus (Er I B1.6)	-	-	-	7.40
Brevibacterium iodinum (Er I B1.3)	+	-	-	54.10
Streptomyces coelicoflavus (Er I B1.7b)	+	-	+	25.00
Pseudomonas mosselii (Er I B2.5)	-	+	+	8.40
Providencia rettgery (Er I B2.10)	+	+	-	93.45
Bacillus cereus (Er I B2.1)	-	-	+	52.60
Bacillus stratosphericus (Er I B2.4)	-	+	+	2.35
Bacillus megaterium (Er I B2.12)	-	+	-	21.20
Streptomyces humidus (Er I B2.2)	+	-	-	5.10
Rhodococcus ruber (Er I B2.3)	-	-	+	27.15
Arthrobacter alpinus (Er I B2.8)	-	-	+	435.20
Streptomyces albidoflavus (Er I B2.11)	-	-	+	2.65
Bacillus stratosphericus (Er I B3.1)	-	+	+	2.95
Bacillus megaterium (Er I B3.4)	+	+	+	15.55
Rhizobium radiobacter (Er I B3.5)	+	-	-	109.80
Stenotrophomonas maltophilia (Er I B3.6)	-	-	-	4.10
Sinomonas flava (Er I B3.7)	-	-	-	11.39
Bacillus amyloliquefaciens (Er I B3.8)	+	-	+	2.00
Bacillus flexus (Er I B3.12)	-	+	-	40.80

Table 3. (continued).

Species (strain)	N_2	P-	Cellulose	IAA
Acinetobacter calcoaceticus (Er I B3.15)	-	-	-	-
Salinicola salarius (Er II B1.1)	-	+	-	26.80
Bacillus pumilus (Er II B1.2)	+	+	-	6.05
Lysinibacillus sphaericus (Er II B1.3)	+	-	+	283.10
Sinomonas atrocyanea (Er II B1.4)	+	+	+	4.80
Acinetobacter soli (Er II B1.5)	+	+	+	15.45
Bacillus stratosphericus (Er II B1.6)	-	+	+	21.50
Pantoea agglomerans (Er II B1.7)	+	+	-	9.00
Bacillus stratosphericus (Er II B2.2)	-	-	+	4.95
Bacillus marisflavi (Er II B2.3)	-	+	+	4.10
Microbacterium arborescens (Er II B2.5)	-	-	+	3.60
Lysinibacillus sphaericus (Er II B2.9)	-	-	+	20.85
Bacillus cereus (Er II B2.14)	+	-	-	28.00
Microbacterium fluvii (Er II B3.1)	-	-	-	1.30
Bacillus stratosphericus (Er II B3.2)	+	+	-	11.80
Pseudomonas knackmussii (Er II B3.4)	+	-	-	5.60
Enterobacter cloacae (Er II B3.5)	+	-	-	15.10
Staphylococcus gallinarum (Er II B3.8)	+	-	-	7.60
Microbacterium xylanilyticum (Er II B3.9)	-	+	-	28.70
Microbacterium awajiense (Er II B3.10)	-	+	+	7.10
Bacillus cereus (Er II B3.11)	-	-	+	7.35
Lysinibacillus boronitolerans (Er II B3.15)	+	+	+	8.45

+ = positive for N_2 fixer or phosphate solubilizer or cellulolytic solubilizer
- = negative for N_2 fixer or phosphate solubilizer or cellulolytic solubilizer

higher level of IAA than *B. megaterium* (Er I B2.12, Er I B3.4). Thus, these strains varied in their potential to produce IAA, and even strains belonging to the same genera or same species. Mirza *et al.* (2001) showed that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability.

Diversity in Phosphate Solubilizing Traits of the Rhizobacteria

Phosphorus is the second major nutrient for plants and exists in nature in a variety of organic and inorganic forms, however, it is the least soluble in soil either by adsorption, chemical precipitation or both (Paul and Clark 1996). Several soil micro-organisms known as PSB have the ability to solubilize insoluble phosphate mineral by producing various organic acids, siderophores, mineral acids, protons, humic substances, CO_2 and H_2S (Ivanova *et al.* 2006). This results in acidification of the surrounding soil, releasing soluble orthophosphate ions ($H_2PO_4^{-1}$, HPO_3^{-2} and PO_4^{-3}) which can be readily taken up by plants.

Thirty seven of the 78 isolates were able to solubilize phosphate (calcium triple phosphate) Table 3). It consisted of 16 isolates from Subang coastal soil and 21 isolates from Indramayu. Isolates that belong to *Bacillus*, *Aeromonas*, *Providencia*, *Pseudomonas*, *Arthrobacter*, *Pantoea*, *Halomonas*, *Acinetobacter*, *Microbacterium*, *Enterobacter*, *Citrobacter*, *Streptomyces*, *Sinomonas* and *Ochrobactrum* genera were among those identified as good phosphate-solubilizing strains (Fig 2). The important genera of PSB include *Achromobacter*, *Aerobacter*, *Alkaligenes*, *Bacillus*, *Pseudomonas*, *Serratia, Xanthomonas, Enterobacter*, *Pantoea*, *Rhizobium* and *Flavobacterium* (Chen *et al.* 2006).

Several species of the genera *Bacillus* could solubilize high amounts of tri-calcium phosphate under *in vitro* conditions, such as *B. stratosphericus* (Ptb I B2.2, Ptb II B3.2, Ptb II b2.6, Ptb II B3.2, Er I B2.4, Er I B3.1, Er II B1.6, Er II B2.2, Er II B3.2); *B. amyloliquefaciens* (Ptb I B2.9), *B. cereus* (Ptb I B3.6, Ptb II B3.9), *B. pumilus* (Ptb II B1.2), *B. megaterium* (Er I B1.2, Er I B2.12, Er I B3.4), *B. flexus* (Er I B3.12), *B. pumilus* (Er II B1.2), and *B. marisflavi* (Er II B2.3). *Bacillus subtilis*, *B. megaterium*, *B. amyloliquefaciens*, *B. atrophaeus* and *B. licheniformis* have already isolated from mangrove plant soil and have the ability

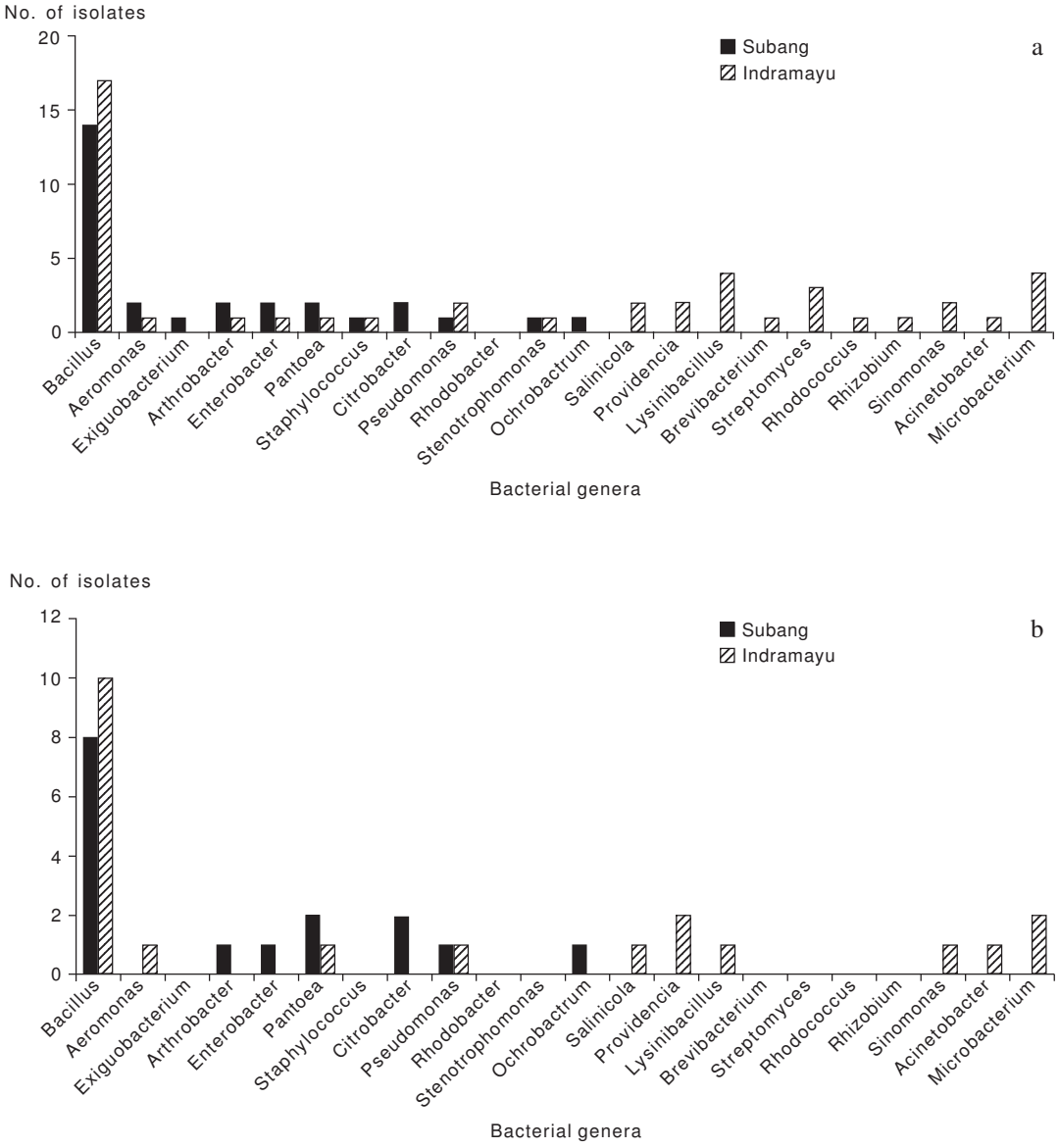

Fig. 2. Plant growth promoting traits, especially on IAA producing (a) and phosphate solubilizing (b) of rhizobacteria associated with rice plant in the coastal soils of Subang and Indramayu, West Java.

to sulubilize phosphate (Vazquez *et al.* 2000; Ravikumar *et al.* 2007, 2009; Nadeem *et al.* 2012). The sole mechanism of solubilization of mineral phosphate is the production of organic acid such as succinic acid, keto-glucinic acid, gluconic acid, oxalic acid and citric acid (Chen *et al.* 2006).

Diversity in CMC-ase (Cellulolytic Degradation) Traits of the Rhizobacteria

A key role in the decomposition and transformation of organic matter such as plant residues in the soil ecosystem is attributed to aerobic microorganisms, especially cellulolytic bacteria that are ubiquitous in soils (Mullings and Parish 1984; Szegi 1988). Cellulolytic bacterial isolates obtained from this study belong to the genera of *Streptomyces*, *Bacillus*, *Rhodococcus*, *Pseudomonas*, *Arthrobacter*, *Stenotrophomonas*, *Staphylococcus*, *Acinetobacter*, *Rhodobacter*, *Pantoea*, *Sinomonas*, *Microbacterium* and *Citrobacter* (Fig 3). These results correspond to the findings of Eriksson *et al*. (1992), who discussed the striking role of *Bacillus*, *Cellulomonas*, *Cytophaga*, *Pseudomonas*, *Sporocytophaga* and *Streptomyces* in cellulose decomposition in soil.

Among all the bacteria isolated from the rice rhizosphere, isolates belonging to the *Bacillus*

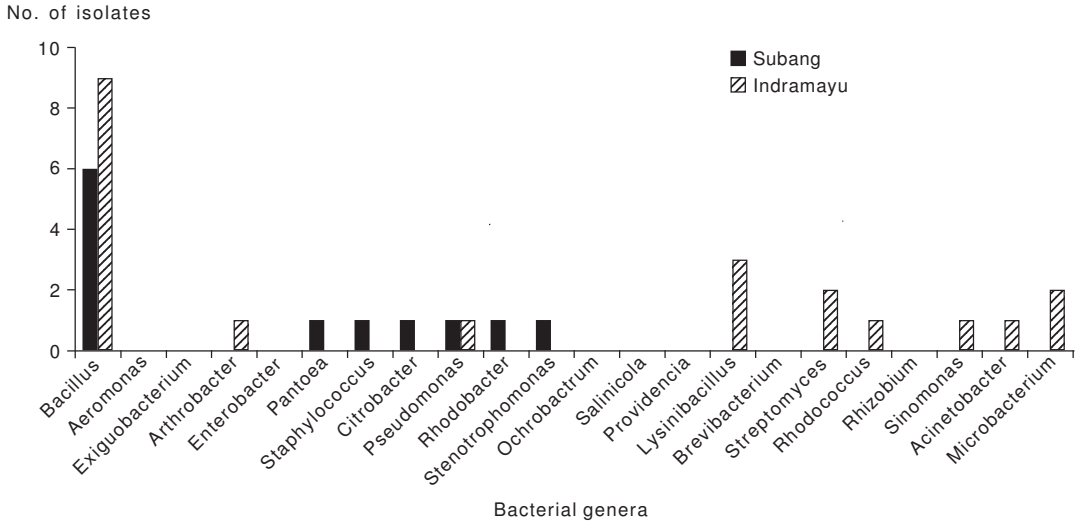

Fig. 3. Plant growth promoting traits, especially on CMC-ase producing of rhizobacteria in the coastal soils of Subang and Indramayu, West Java.

genera presented more higher number as cellulase producer. Strains *B. stratosphericus* (Ptb I B1.4, Ptb I B2.2, Er I B2.4, Er I B3.1, Er II B1.6, Er II B2.2), *B. amyloliquefaciens* (Ptb I B2.9, Er I B3.8), *B. cereus* (Ptb I B3.6, Er I B2.1, Er II B3.11), *B. pumilus* (Ptb II B1.2), *B. marisflavi* (Ptb II B3.3, Er II B2.3), *B. megaterium* Er I B3.4 had the ability to produce cellulase. The second highest in number of isolates producing cellulase was *Lysinibacillus*, consisted of *L. sphaericus* (Er II B1.3, Er II B2.9) and *L. boronitolerans* (Er II B3.15) (Fig. 3).

Diversity in N_2 Fixing Traits of the Rhizobacteria

Nitrogen is one of the most limiting essential nutrients into the plant. Prevalence of nitrogen-fixing ability among bacteria colonizing rhizosphere might ensure their survival and multiplication. A high proportion of nitrogen-fixing bacteria in rhizosphere may also be attributed to the host-associated factors. However, the contribution of rhizobacteria is yet to be estimated (Beneduzi *et al*. 2008).

A total of 78 rhizosphere bacteria obtained were selectively isolated from two rice field soil samples with three different levels of salinity. The putative nitrogen fixing bacteria were selected based on their growth on selective semi-solid media without nitrogen (NFb). Twelve bacterial isolates capable of fixing N_2 were obtained from the coastal soil of Subang and 20 isolates were found from Indramayu. The partial sequencing of 16S rRNA gene of nitrogen fixation classified 12 different genera. Strains belong to *Bacillus*, *Brevibacterium*, *Streptomyces*, *Rhizobium*, *Aeromonas*, *Enterobacter*, *Pantoea*, *Staphylococcus*, *Acinetobacter*, *Pseudomonas*, *Providencia* and *Sinomonas* were the N_2 fixers obtained from this study (Fig 4). This is consistent with the result obtained by Farina *et al.* (2012) that *Agrobacterium*, *Enterobacter*, *Pseudomonas*, *Acinetobacter* and *Streptomyces* were the most abundant PGPR found as nitrogen fixers associated with canola plants.

Isolates that scored positive for nitrogen fixation were classified into eight genera; of which were identified as 11 species of *Bacillus*, such as *B. stratosphericus* (Ptb I B1.4, Ptb I B2.2, Ptb II B3.2, Er II B3.2), *B. amyloliquefaciens* (Ptb I B2.9, Er I B3.8), *B. cereus* (Ptb I B3.6, Er II B2.14), *B. marisflavi* (Er I B1.4), *B. megaterium* (Er I B3.4), and *B. pumilus* (Er II B1.2). Three isolates of *Enterobacter* and *Pantoea* were also capable of fixing nitrogen (Table 3).

Our results showed that *Bacillus* is the most dominant as nitrogen fixer, phosphate and cellulose solubilizer, and IAA producer. This might be due to its ability to efficiently use nutrients provided by the plant through exudates, and to inhibit the growth of other strains. Many strains of *Bacillus* have been reported to produce substances that act as growth inhibitors for other microorganisms (Lilinares *et al.* 1994).

It might be interesting to test in the future, the resistance of the selected PGPR of this recent study to various environmental stresses and also the functional characterization of PGPR for practical applications in the rice field in coastal areas.

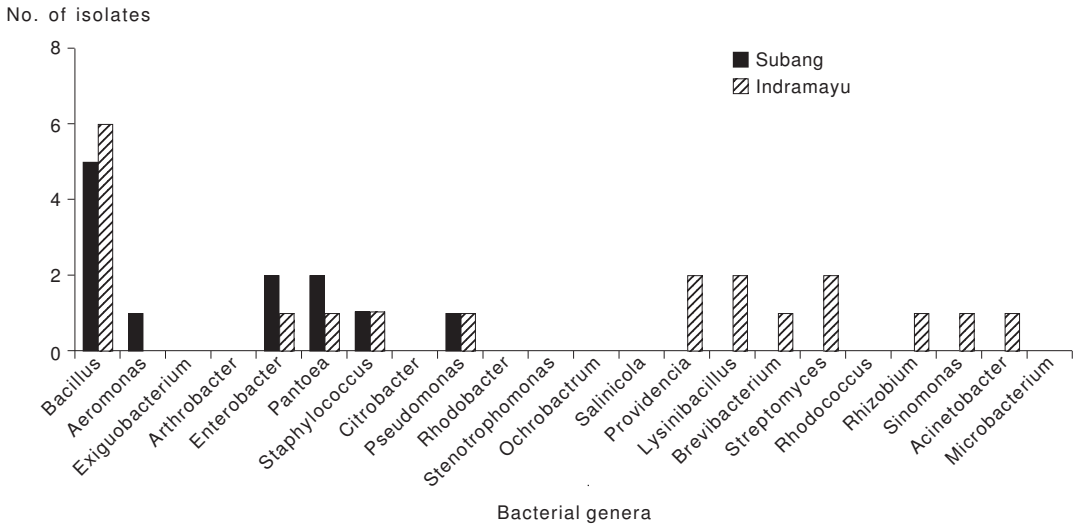

Fig. 4. Plant growth promoting traits, especially on N_2 fixing of rhizobacteria in the coastal soils of Subang and Indramayu, West Java.

CONCLUSION

Taking all of these diverse PGPR characteristics into account, it is clear that the 78 isolates of rhizobacteria identified have great potential for future research. Several strains of the rhizobacteria produced plant growth promoting activities, alone or in combination, including IAA production, nitrogen fixation, phosphate solubilization and cellulase production. Our study on the species and functional diversity of rhizobacteria associated with rice plant from coastal soils may be useful for selecting proper PGPR strains for improving saline soils.

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