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Potential of Acetoin-Producing Rizobacteria from Rhizospheres of Plants of Graminae Grown in Merauke Indonesia to Promote the Growth of Rice

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Abstract
Acetoin (3-hydroxybutan-2-butanone) is a volatile compound produced by bacteria that function as a plant growth promoting agent. Acetoin plays an important role in stimulating the process of plant organogenesis (morphogenesis) for faster formation of plant organs and faster plant growth. Besides being able to increase the growth, acetoin can also induce plant resistance, increase the formation of the number of branches, roots and flowers so that acetoin can increase crop productivity. This study aims to isolate and identify acetoin-producing rhizobacteria which can promote the growth of rice plants. Rhizobacteria isolation was carried out from the rhizospheres of plants of Graminae grown in Merauke, Indonesia. Identification was made molecularly through 16S rRNA gene analysis. This study obtained four rhizobacteria isolates that produce acetoin which act as a promoter for rice plant growth, namely isolates Pd13, Rg21, Pd7, and Bb7. These four rhizobacteria isolates can increase the number of lateral roots of rice seedlings aged 14 days after planting with percentage of increase by 60.68%; 40.24%: 32.61%, and 19.56% compared to controls. The identification results show that the species of isolates of rhizobacteria are *Myroides odoratimimus* Pd13 and Bb7, *Providencia vermicola* Rg21, and *Serratia marcescens* Pd7. They have the potential to be developed as a promoter for plant growth and increase the yield of rice plants, especially in Marauke Papua, Indonesia.
Keywords: Rizobacteria, acetoin, growth promotion, rice yield

1. Introduction

Plant growth promoting rhizobacteria (PGPR) is a group of bacteria that live around plant roots that can be beneficial because they can promote the plant growth. The rizobacteria live in a colony surrounding the roots of plants (Shruti *et al*. 2013). According to Agustian *et al*. (2010), PGPR plays an important role in increasing the plant growth, increase the crop yields, and and improve the soil fertility. PGPR can work directly or indirectly. Directly, PGPR stimulates plant growth by producing growth hormones, vitamins, and organic acids, as well as increasing nutrient uptake for plants (Agrawal, 2013). For plants, the existence of these microorganisms will be very good because these bacteria provide benefits in the process of plant physiology and growth (Gandanegara, 2007).

Some researchers reported that rhizobacteria such as *Bacillus* sp. and *Pseudomonas* sp. are able to dissolve phosphate (Sutariati, 2006), while *Serratia* sp. besides being able to increase the availability of P also can fixate nitrogen (Gholami *et al*., 2008). *Bacillus* sp. isolate was also reported capable of synthesizing IAA growth hormones (Sutariati, 2006), giberelin (Joo *et al*., 2005), and cytokinin (Timmusk *et al.,* 2005). *Pseudomonas fluorescens* isolates are capable of producing IAA (Sutariati, 2006), giberelin and cytokinin (Ahmad *et al*., 2005), as well as isolate *Serratia* sp. Was reported able to synthesize IAA (El Azeem *et al*., 2007). Rhizobacteria can be isolated from rhizosphere of various types of plants, including cabbage, apples and soybeans (Ikhwan, 2010). Rizobacteria can also be isolated from Graminae plants, upland rice, elephant grass, and lemongrass, and able to stimulate the growth of banana plants (Eliza *et al*., 2007).

The use of acetoin-producing rhizobacteria as a promoter for plant growth is obtained in the rhizosphere of plants and root surfaces. Acetoin-producing Rizobacteria which can increase plant growth and yield can be used as PGPR. Rizobacteria that produce acetoin in enhancing plant growth and development are those which can increase seed germination and seed vigor, the number of lateral roots and root hair, water absorption, plant response to chemical fertilizer, the number of root nodules, photosynthesis and content leaf chlorophyll, and the seed protein content, but it can reduce ethylene concentration by producing enzymes (Teng *et al*., 2010).

The use of acetoin-producing bacteria is one of the technological innovations to improve the ability of plants to absorb nutrients so that the quality and quantity of plants can be increased (Nicolas *et al*., 2001). The use of

acetoin and urease-producing bacteria have been reported to increase uptake of chemical fertilizers and soybean and rice crops. Khalimi *et al*. (2011) reported that *Pantoea agglomerans* BS2a was able to increase soybean root dry weight by 190.59% when compared to control. Khalimi *et al.* (2012) reported that *Enterobacter cloacae* EA was able to increase the number of soybean pods by 73.85% when compared to control while *P.agglomerans* PAJ was reported to be able to increase nitrogen uptake by 59.09%, phosphate uptake by 57.14%, and potassium uptake by 33.33% when compared to control on rice plants. Suprapta *et al*. (2014) reported that *Enterobacter cloacae* Al6G was able to increase the pithy grain weight per rice clump by 26.12% when compared to control. The use of acetoin and urease-producing bacteria is also reported to increase co-inoculation of *Rhizobium* sp. so that it can increase the number of root nodules.

Various types of bacteria have been identified as PGPR. Most come from the gram-negative group with the most number of strains from the genus Pseudomonas and some from the genus *Serratia* (Kloepper, 1993). In addition to the two genera, it is reported among others from the genera *Azotobacter, Azospirillum, Acetobacter, Burkholderia*, and *Bacillus* (Glick, 1995). This study was done in order to find and identify the acetoin-producing rhizobacteria from rhizospheres of plants of Graminae that are grown in Merauke, Papua, Indonesia that potentially can be used as plant growth promoter to increase the yield of rice.

2. Materials and Methods

Sampling and isolation of rhizobacteria

Sampling was carried out from the rhizospheres of five types of plants belonging to the Graminae family, namely rice (*Oryza sativa* L.), reeds (*Imperata cylindrical* L.Brauv), elephant grass (*Pennisetum purpureum*), and bamboo (*Schizostachum mosum*). Isolation of rhizobacteria from plant roots was carried out by following the modified procedure developed by Geetha *et al*. (2014). A total of 10 g of rhizosphere from each sample was macerated on mortal then diluted with 100 ml of phosphate saline buffer (PBS). Furthermore, a series of dilution with PBS was made until the 10^{-7} dilution. The media used to isolate rhizobacteria are Nutrient Agar (NA) media containing 0.3% beef extract, 0.5% peptone, 1.5% agar and distilled water. This medium is added with benomyl (120 mg / ml) or Nystatin (50 mg / liter) to inhibit fungal growth. A total of 0.2 ml of suspension from each dilution was put into a Petri dish and then mixed with 10 ml of NA media with a temperature of about 45^{o} – 50^{o}C. This culture is incubated at room temperature for 24 hours. Growing colonies are then transferred to the new NA media, for the isolation process. The rhizobacterial isolates obtained were coded and moved on the NA media obliquely and ready to be used for further testing.

Testing of Rizobacterial Isolates that produce Acetoin

Biochemical tests of rhizobacterial isolates that produce acetoin were carried out using the method Methyl Red-Voges Proskauer (MR-VP) (Djide and Sartini, 2006). This test is useful in identifying groups of bacteria that are capable of producing acetoin. If bacteria utilize carbohydrates to 2,3 butanadiol as the main product, there will be a buildup of the material in the growth medium. Then it was added with 40% KOH and 5% alpha naphthol. If the rhizobacterial suspension changes color in the medium to red, then the rhizobacterial isolate shows the presence of 2,3 butanadiol products as a result of fermentation and the rhizobacteria are tested positive for producing acetoin (acetyl methyl carbonyl).

Selection of Acetoin-producing Rhizobacteria isolates that act as a promoter for lateral root formation

A total of 17 rhizobacterial isolates that were positive for producing acetoin were then tested for their ability to increase the number of lateral roots of rice seedlings. The results of selection of acetoin-producing rhizobacteria isolates that act as plant growth promoters found 4 rhizobacteria isolates which showed the best results in increasing the number of lateral roots of 17 tested rhizobacteria isolates. The four rhizobacteria isolates were Rg21 isolates, Pd13 isolates, Bb7 isolates and Pd7 isolates (Table 2).

Effectiveness Rizobacteria in Increasing Lateral Root Growth of Rice Seedling

The effectiveness test of four isolates in increasing the growth of lateral roots of rice seedling was done in plastic cups. Plastic cup used was 10 cm x 7 cm (diameter x height). Rice seeds that were planted are rice seeds that have germinated. The seeds are first sterilized through soaking with 1% sodium hypochlorite for 5 minutes. Furthermore, as much as 10 g of rice seeds were soaked for 2 hours in Erlenmeyer containing a rhizobacteria suspension with a density of 10^{6} CFU / ml. Rice seeds soaked in sterile water were used as control. The seed was sown on a plastic cup that had been filled with soil as much as 50 g and 0.1% of NPK solution. Each cup was planted with 10 rice seeds. This test uses a randomized block design (RBD) with 5 treatments consisting of 4 risobacteria isolates namely Rg21, Pd13 isolates, Bb7 isolates, Pd7 isolates and control. Each treatment was

repeated 3 times so that there were 15 cups of plastic prepared in this study. The study was carried out in a green house on the farm of the Faculty of Agriculture, Udayana University.

Molecular identification of acetoin-producing Rizobacteria

DNA Extraction

Rhizobacteria isolates were grown in Erlenmeyer containing Tryptic Soy Broth media (17g Tryptone, 3 g Phytone, 5 g NaCl, 2.5 g K2HPO4, 2.5 g glucose, and fill up to 1,000 ml) incubated for 16 hours and shaken at speeds 5xg at room temperature. Furthermore the rhizobacteria cells were put into 2ml-Eppendorf tube and centrifuged at 5,000xg for 10 minutes. Furthermore, the supernatant was removed. The rhizobacteria cell was re-suspended with 180 µl digestion solution, 20 µl protease solution was added and mixed until it was homogeneous by ascending the dropper or in vortex and incubated at 56^{o}C until the tissue was completely perfected (clear liquid). Added with 20 µl of RNase A solution, vortexed then incubated for 10 minutes at room temperature. This mixture was added 200 µl of lysis solution, vortexed for 15 seconds until the mixture became homogeneous. This mixture was added with 400 µl of 50% ethanol and vortexed. The mixture was transferred to the Genomic DNA Purification Column which was placed in the collection tube. Column centrifugation for 1 minute at 6,000x g. Remove the collection tube containing the solution, place the column in a new collection tube and add 500 µl of wash buffer I (which has been added to ethanol. If the residual solution is left in the column, empty the collection tube and centrifuge the column for 1 minute at 12,000xg, remove the collection tube containing the solution and move the column to a new and sterile 1.5 ml micro-tube. Add 200 µl of elution buffer right in the middle of the column to dissolve DNA. Incubate for 2 minutes at room temperature and centrifuge for 1 minute at 8,000 x g. Then the column was removed, the pure DNA can be used immediately or stored at -20^{o}C until it is used for further analysis.

DNA amplification with PCR

The 16S rRNA gene was amplified by PCR using a 16S primer pair (63F 5'-CAG GCC TAA CAC GCA-3 'ATA CAA and 1387R (5'-GGG CGG WGT GTA CAA GGC-3'). The reaction took place using SENSOQUEST Labcycler using 2x Kappa PCR Ready Mix (Kappa Biosystem), at 94^{o}C conditions for 5 minutes, followed by 30 cycles in a row at 94^{o}C for 30 seconds, 55^{o}C for 45 seconds, and 72^{o}C for 2 minutes, last added 72^{o}C for 10 minutes.

Sequencing of 16S rRNA genes and DNA sequence computer analysis

The nucleotide sequence was determined using ABI-Prism 3100-Avant Genetic Analyzer. The sequential DNA sequencing results were then trimmed and assembled using the ChromasPro version 1.5 program. The Data that had been assembled subsequently in BLAST with data that had been registered at the NCBI (National Center for Biotechnology Information) through the website http://www.ncbi.nlm.nih.gov/BLAST. Some data on homologous results from blast which are the closest species were taken from Genbank data at NCBI. The data were analyzed again by aligning the sequence using the MEGA version 6.0 program. Furthermore, the data were analyzed using the PAUP 4.0b program with the Maximum Parsimony method with 1,000 repeat bootstraps (Calvo, 2005). Then the phylogeny tree was designed using Tree Graph 2.0 (Stover and Muller, 2010).

3. Results and Discussion

A total of 105 rhizobacteria isolates were obtained in this study consisting of 30 isolates from rhizospheres reeds, 24 isolates from rhizospheres of rice plants, 22 isolates from bamboo rhizospheres and 29 isolates from rhizospheres of elephant grass. According to Eliza *et al*. (2007) rhizobacteria can be isolated from Graminae plants such as upland rice, elephant grass, and lemongrass while Ikhwan (2010) stated that rhizobacteria can also be isolated from the rhizospheres of other types of plants such as cabbage, apples and soybeans.

The results of biochemical testing with the method of Methyl Red – Voges Proskauer (Mac Faddin, 1976) showed that 18 isolates of rhizobacteria were proven to produce acetoin. This can be seen in the rhizobacteria suspension mixture after the addition of 40% KOH and 5% alpha naphthol underwent a color change to red cherry. Acetoin ($C_4H_8O_2$) which was contained in the oxidized medium in the presence of air and KOH becamediacetyl. Diacetyl reacted with guanidine from peptone, and the presence of apha-naphthol produced a red color that functions as a catalyst and color enhancer (Figure 2). The appearance of red cherry showed positive (+) results and yellow brown color showed negative results (-) (Table 1) (Sridhar, 2006).

A total of 18 rhizobacteria isolates capable of producing acetoin (+) were tested for their ability to increase lateral root growth of rice seedlings (Table 2) and obtained 4 rhizobacteria which showed a percentage increase in lateral roots, i.e. isolate Rg21 increasing lateral roots by 50.00%, isolate Pd13 increased root lateral was 64.28%, isolate Bb7 increased lateral roots by 57.14% and isolate Pd7 increased lateral roots by 42.85%

compared to control. The results of the effectiveness test of rhizobacteria in increasing the number of lateral roots, root base weight and plant height at 2 weeks after planting showed that the treatment of rhizobacteria significantly increased the number of lateral roots compared to control (Table 3). Table 3 shows that the rhizobacteria treatment increases the number of lateral roots between 19.56% and 60.86% compared to control. Pd13 treatment increases the number of lateral roots by 60.86%, Rg21 treatment increases the number of lateral roots by 40.24%, Pd7 treatment increases the number of lateral roots 30.61% and Bb7 increases the number of lateral roots by 19.56%. The effect of rhizobacteria on the immersion of rice seeds that have germinated for 2 hours with a rhizobacteria suspension can accelerate the symbiosis between the roots and the rhizobacteria so as to produce more lateral roots as a place for root hair growth (Glick *et al*., 2016)

The effect of rhizobacteria increases root weight between 83.33% and 188.88% compared to control. Each treatment increased root weight, i.e. Bb7 of 188.88%, Rg21 of 177.77%. Pd13 is 177.77% and Pd7 is 83.33% when compared to control. The effect of rhizobacteria treatment on increasing plant height ranged from 4.92% to 12.71%. The treatment of Bb7 did not experience an increase in plant height compared to control. Rg21 treatment increases plant height by 12.71%, Pd13 increases plant height by 11.66% and Pd7 increases plant height by 4.92% when compared to control.

Table 1
Rhizobacterial isolates tested for their ability to produce acetoin

NO	Isolate	Acetoin	No	Isolate	Acetoin	No	Isolate	Acetoin
01	Al 1	+	36	Pd 6	-	71	Bb 17	-
02	Al 2	-	37	Pd 7	+	72	Bb 18	-
03	Al 3	-	38	Pd 8	-	73	Bb 19	-
04	Al 4	-	39	Pd 9	+	74	Bb 20	-
05	Al 5	+	40	Pd 10	-	75	Bb 21	
06	Al 6	+	41	Pd 11	-	76	Bb 22	+
07	Al 7	-	42	Pd 12	-	77	Rg 1	-
08	Al 8	+	43	Pd 13	+	78	Rg 2	-
09	Al 9	-	44	Pd 14	+	79	Rg 3	-
10	Al 10	+	45	Pd15	+	80	Rg 4	
11	Al 11	-	46	Pd 16	-	81	Rg 5	+
12	Al 12	-	47	Pd 17	-	82	Rg 6	-
13	Al 13	-	48	Pd 18	-	83	Rg 7	-
14	Al 14	-	59	Pd 19	-	84	Rg 8	-
15	Al 15	-	50	Pd 20	-	85	Rg 9	-
16	Al 16	+	51	Pd 21	-	86	Rg 10	-
17	Al 17	-	52	Pd 22	-	87	Rg 11	-
18	Al 18	-	53	Pd 23	-	88	Rg 12	-
19	Al 19	-	54	Pd 24	-	89	Rg 13	-
20	Al 20	-	55	Bb 1	-	90	Rg 14	-
21	Al 21	-	56	Bb 2	-	91	Rg 15	-
22	Al 22	-	57	Bb 3	-	92	Rg 16	-
23	Al 23	-	58	Bb 4	-	93	Rg 17	-
24	Al 24	-	59	Bb 5	-	94	Rg 18	-
25	Al 25	-	60	Bb 6	-	95	Rg 19	-
26	Al 26	-	61	Bb 7	+	96	Rg 20	-
27	Al 27	-	62	Bb 8	-	97	Rg 21	+
28	Al 28	-	63	Bb 9	-	98	Rg 22	-
29	Al 29	-	64	Bb 10	-	99	Rg 23	-
30	Al 30	-	65	Bb 11	-	100	Rg 24	-
31	Pd 1	-	66	Bb 12	-	101	Rg 25	-
32	Pd 2	-	67	Bb 13	-	102	Rg 26	+
33	Pd 3	+	68	Bb 14	-	103	Rg 27	-
34	Pd 4	-	69	Bb 15	+	104	Rg 28	-
35	Pd 5	-	70	Bb 16	-	105	Rg 29	-

Description: +: produce acetoin; -: do not produce acetoin

Table 2.
Effectiveness of 18 isolates of acetoin-producing Rhizobacteria to increase lateral roots of rice seedlings

No	Isolate	Number of Lateral root	Percentage of compared to control (%)
1	Control	14	-
2	Al1	5	-
3	Al5	12	-
4	Al6	9	-
5	Al8	10	-
6	Al10	9	-
7	Al16	11	-
8	Al 17	7	-
9	Pd 2	6	-
10	Pd 3	12	-
11	Pd 7	20	42,85
12	Pd 9	11	-
13	Pd 13	23	64,28
14	Pd 14	13	-
15	Pd15	14	-
16	Bb 7	22	57,14
17	Bb	12	-
18	Rg 21	21	50,00
19	Rg 26	9	-

Table. 3
Effect of rhizobacteria on the increase and number of lateral roots, fresh root weight and plant height

No	Treatment	Number of Lateral root	Weight of fresh root (g)	Plant Height (cm)
1	Control	15.33 c	0.36 a	25.80 a
2	Rg21	21.50 ab (40.24%)	1.00 a (177.77%)	29.08 a (12.71%)
3	Pd13	24.66 a (60.86%)	1.00 a (177.77%)	28,81 a (11.66%)
4	Bb7	18.33 bc (19.56%)	1.04 a (188.88%)	25.79 a (-)
5	Pd7	20,33 ab (30.61%)	0.66 a (83.33%)	27.07 a (4.92%)

* The values followed by the same letter in the same column shows non-significant differences (P> 0.05) according to Duncan's Multiple Range Test.
** The value in the sign ( ) in the same column shows the percentage increase when compared with control.

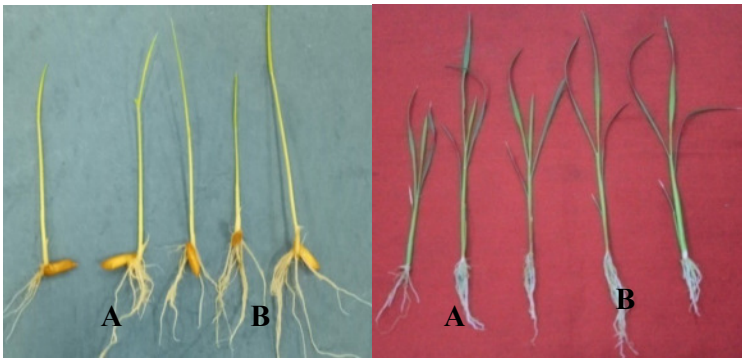

Figure 1
Effect of rhizobacteria treatment on rice seedlings growth.
Control (A) Treated with rizobacteria (B)

Rizobacteria that produce acetoin in increasing plant growth and development are rhizobacteria which can increase seed germination and seed vigor, the number of lateral roots and root hair, water absorption, the number of root nodules, and photosynthesis and leaf chlorophyll content (Teng*et al*., 2010). Maximum root growth will affect nutrient absorption. Activation of hormones can stimulate growth and can stimulate the growth of lateral roots and root hairs (Vasundevan *et al*., 2002).

Results of Identification of rhizobacteria isolates that produce acetoin

The results of 16S rRNA gene amplification showed that DNA fragments were 1500 bp as seen on electrophoregram (Figure 5). The size of the amplicon DNA obtained was in accordance with the primary design of the study conducted by Ahmed *et al.* (2014) that the amplification of rhizobacterial 16S rRNA genes produces a DNA fragment measuring 1500 bp.

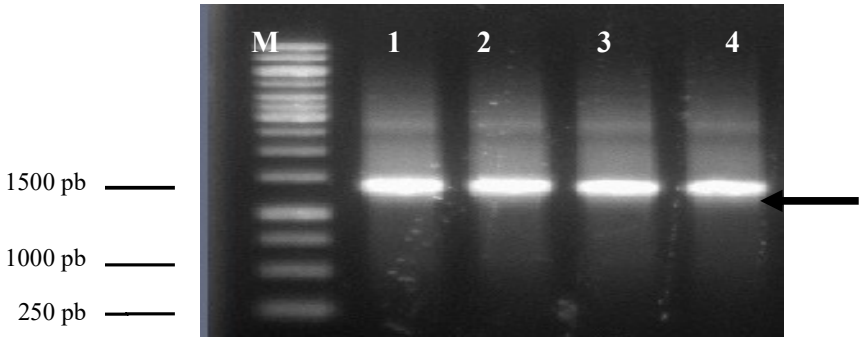

Figure 2
Amplification of 16S rRNA genes of four isolates of rhizobacteria (arrow). M. 1 Kb DNA marker
1. Rg21 2. Pd13, 3 Pd7, 4. Bb7

Based on the alignment of the 16S rRNA gene sequence with the GenBank database using the BlastN program, isolate Rg21 belonged to the *Providencia vermicola* because homologous of Rg21 isolate with *P. vermicola* strains BAB-812 (KC250202.1) and *P. vermicola* strain BA 1 (MF276891.1) with maximum identity level of 89%. Rhizobacteria isolate Pd13 was included in the *Myroides odoratimimus* due to PD13 homology with *M. odoratimimus* strain YCT106 (JF775418 1), *M. odoratimimus* YCG1 strain (JF775411 1) with a maximum identity level of 81%. Rhizobacteria isolate Bb7 was included in the *Myroides odoratimimus* because the B7 isolate was homologous with *M. odoratimimus* Unisa3 strain (KP823030 1), and *M. odoratimimus* strain Unisa5 165 (KP823029 1) with a maximum identity level of 97%. Rhizobacteria isolate Pd7 belonged to the *Serratia marcescens* because Pd7 isolates were homologous with *S. marcescens* strain B8 (KY454856.1), *S. marcescens* strain CS2101 (KY379045.1), *S. marcescens* strain CS2101 (KY379045 1), *S. marcescens* strain MSCP10 (MG066582 1) with an identity level of 99% (Table 4).

Table. 4
Comparison of percentage of similarity of 16S rRNA genes of rhizobacteria isolates Rg21, Pd13. Bb7 and Pd7 with several DNA sequences in GenBank using the BLAST program

Rizobacteria isolates (Rg21, Pd13, Bb7, Pd7	Similarity Percentage (%)	Accession Number
Providencia vermicola strain BAB-812	89	KC250202 1
Providencia vermicola strain BA	89	MF276891 1
Myroides odoratimimus strain YCT106	81	JF775418 1
*Myroides odoratimimus*strain YCG1	81	JF775411 1
Myroides odoratimimus strain Unisa3	97	KP823030 1
*Myroides odoratimimus*strain Unisa5 165	97	(KP823029 1
Serratia marcescens strain CS2101	99	KY379045 1
Serratia marcescens strain MSCP10	99	MG066582 1

While the results of the phylogeny tree analysis from the four bacterial isolates above using the Maximum Parsimony (MP) method with 1,000 Bootstrap replications showed that the Rhizobacteria isolate Rg21 was *Providencia vermicola* because it is one clade with *P. vermicola* bacterial sequences. Rizobacteria isolates Pd13 and Bb7 were *Myroides odoratimimus* because they are one clade with *M. odoratimimus* sequences and Pd7 rhizobacteria is *Serratia marcescens* because it is one clade with *S. marcescens* found in the GenBank database with 100% Bootstrap Support (BS) support (Figure 5) (Samson *et al*., 2014).

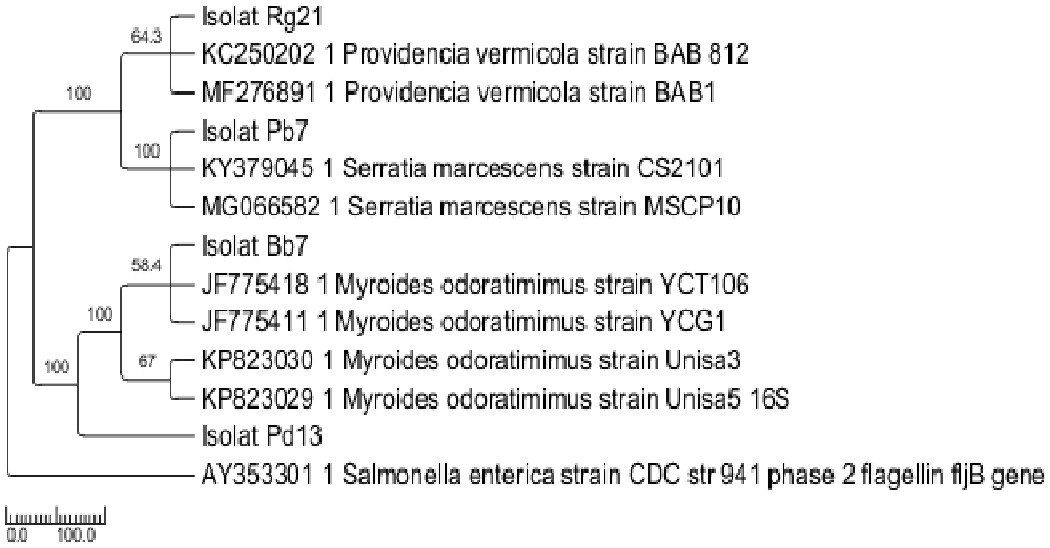

Figure 3
Phylogeny tree based on MP method for the 16S rRNA gene sequence which shows the kinship between the 4 rhizobacterial isolates and bacteria in GenBank Database. The bootstrap value (%) is based on 1,000 replications

4. Conclusion

The results showed that there were four acetoin-producing rhizobacteria isolates that acted as plant growth promoting rhizobacteria (PGPR) which could increase the number of lateral roots, root weight, and plant height compared to controls. Sequencing results of Rg21 isolates belong to the *Providencia vermicola*. Isolates Pd13 and Bb7 belong to the *Myroides odoratimimus* while Pd7 isolate belong to the *Serratia marcescens*.

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