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Identification of bacterial isolates of Tumpang and Bumiasri (East Java, Indonesia) using 16S rRNA gene sequencing and screening of their active compounds as a biofertilizer

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Keywords: 16S rRNA, biofertilizer, *Lysinibacillus fusiformis*, *Lysinibacillus macroides*, soil bacteria

INTRODUCTION


The need for food will continue to increase in line with the growth of the human population. It is important to note that this increase in demand is inversely proportional to the availability of land for cultivation. In addition, land that has been planted with plants for a long time will reduce the quality of the soil. This is indicated by a decrease in nutrients such as sodium (Na), phosphorus (P) and potassium (K) resulting in a decrease in plant productivity (Sriwardhani et al. 2006). Therefore, technological innovation is needed to anticipate and increase plant productivity, one of which is biofertilizer technology. Biofertilizer is a fertilizer that contains living microorganism that benefits both the soil and plant. Based on its function, biofertilizer is classified into nitrogen fixer, phosphate solubilizer, organic ingredients solubilizer, growth booster, and disease control (Daniel et al. 2022). Biofertilizer has been used since a hundred years ago and has been proven impacted the environment, plant and consumers in a beneficial way and the first microorganism utilized as a biofertilizer is *Rhizobium* (Sriwardhani et al. 2006). Apart from that, bacteria can also be utilized as a biofertilizer, such as rhizobacteria that was isolated from

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The main ingredient of biofertilizer is microorganism that can grow and have the ability to benefit both soil and plant (Sriwardhani et al. 2006). Some microorganisms that will be used as biofertilizer are isolated from the soil or plant's part that becomes the fertilization target. TPG (Tumpang) and BAS (Bumiasri) isolates are some bacterial isolates isolated from agricultural land in Malang District, East Java Province, Indonesia. TPG isolates was

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The main ingredient of biofertilizer is microorganism that can grow and have the ability to benefit both soil and plant (Simanungkalit et al. 2006). Some microorganisms that will be used as biofertilizer are isolated from the soil or plant's part that becomes the fertilization target. TPG (Tumpang) and BAS (Bumi Asri) isolates are some bacterial isolates isolated from agricultural land in Malang District, East Java Province, Indonesia. TPG isolates was

isolated from the Tumpang area and BAS isolates was isolated from the Bumi Asri area by Prihartini (2007). Those bacteria are able to completely degrade lignin of rice straw within 7 days (Prihartini 2007). This was much higher compare to *Pleurotus flabellatus* that can only degrade 25% of the lignin (Rajarathnam et al. 1987). These bacteria also higher than *P. djamor* that degrade 70.31%, *P. eous* that degrade 64.71% and *P. flabellatus* that degrade 66.69% of the lignin within 20 days (Theradimani et al. 2019). On the other hand, Renganathan et al. (2020) reported that *Pleutorus* spp. is able to degrade lignin from 18.75 to 21.15% within 7 days, which is also lower than the TPG and BAS ability. Prihartini et al. (2021) also reported that TPG isolate is able to improve the digestion of rice straw.

These bacterial isolates have been used as biofertilizer with the rice straw as the carbon source and it can increase rice production through improving soil nutrients and stimulating growth hormone. As a biofertilizer, it is important to clearly understand the characteristics (genus, family, order and class) and active compounds produced of these isolates. However, until now the isolates of these bacteria have not been reported on their characteristics and also their active compounds produced from the degradation process of rice straw. So, this study aimed to identification of isolates of TPG and BAS bacteria and screening of their active compounds as biofertilizer.

MATERIALS AND METHODS

Isolation of bacteria

This research used a single biofertilizer, which consisted of two biofertilizers with two different bacterial isolates. The first is Tumpang (TPG) bacteria was isolated from Tumpang area (-8.002636, 112.756562), referred to as biofertilizer C1 and the second Bumi Asri (BAS) bacteria was isolated from Bumi Asri area (-7.911925, 112.579687), referred to as biofertilizer C2. These isolates were found by Prihartini (2007) in a rice field in Malang Regency, East Java, Indonesia. The identification of isolates of TPG and BAS used 16S rRNA (Church et al. 2020) and the screening of active compounds used liquid chromatography tandem-mass spectrometry (LC-MS/MS QTOF) (Ismail et al. 2021b).

Identification of isolate of TPG and BAS

The genomic DNA extraction of isolates of TPG and BAS was conducted by sending samples to 1st base with the following method used Quick-DNA Fungal/Bacterial mini kit 9Zymo Research, D6005. The amplification PCR used MyTaq Red Mix (Bioline). The PCR master mix used was 25 μ L with dd H₂O (9.5 μ L), MyTaq Red (12.5 μ L), 20 μ M 27F Primer (5'-AGAGTTTGATCMTGGCTCAG-3') (1 x25 μ L), 20 μ M 1492R 5'-TACGGYTACCTTGTTACG ACTT-3') (1 μ L), and DNA template (1 μ L). The PCR condition were initial denaturation 95°C for 1 min, denaturation 96°C for 15 sec, annealing 52°C for 30 sec, Extension 72°C for 45 sec, and hold 4°C for unlimited times.

Screening of active compounds as a biofertilizer

The materials used consisted of TPG and BAS bacterial isolates as microorganisms and mineral mix (P, K, Fe, Mg and S), rice straw extract, and water as a medium. Biotin and chloramphenicol (Sigma Aldrich) were used as standards to check the function of LC-MS/MS QTOF. Methanol was used as the LC-MS/MS mobile phase. Biofertilizer was dried by freeze dryer before screening active compounds used LC-MS/MS QTOF (de Torres et al. 2010; Ismail et al. 2021a). Then the active compounds screening conducted using LC-MS/MS QTOF (Waters) (Qiao et al. 2013). The steps are as follows: 0.5 g of the sample was dissolved in 10 mL methanol, then extracted using ultrasonic for 30 min. The extract was filtered using a 'membrane filter GHP/PTFE 0.22 μ m' and then injected into the UPLC system.

Liquid chromatography setting

The Liquid Chromatography (LC) was set using C18 column with temperature 40°C, auto sampler temperature 15°C, injection volume 10 μ L and MS setting: mode of operation was ToF MSE, ionization was ESI (-)/ ESI (+), acquisition range was 50-1200 Da. Active compound identification criteria: Mass error reading of the analyte <5 ppm error, Isotope match MZ RMS <6, analyte intensity >300 and there was one fraction with brake value <4 in the fragment match elucidation system (Qiao et al. 2013).

Data analysis

All of data obtained were done with Duplo. The results of the sequence then compared with the sequences in GenBank public database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Identification of the species and genus of isolates based on the similarity value to the bacterial 16S rRNA gene sequences that have been submitted to GenBank. The 11 bacterial species that have the same nucleotide base arrangement were taken and added to the contig and controlling samples (TPG and BAS sequencing), then a phylogenetic tree was constructed using the 'MEGA' application based on the comparison of the nucleotide base arrangement obtained from the NCBI GenBank. The results of sequencing of isolates and screening of active compounds were displayed in tables and figures with descriptive analysis according to Ismail et al. (2021b).

RESULTS AND DISCUSSION

Identification of isolate of TPG and BAS using 16S rRNA gene sequencing

The results of genomic DNA quantification (nanodrop) of isolates of TPG and BAS perform on Table 1 and the gel photo-PCR products perform on (Figure 1).

The results of DNA isolation using nanodrops showed that TPG and BAS isolates at absorbance (A_{260/280}) were 1.80 and 1.69 respectively (Table 1). These results indicate that the TPG isolate was pure because it had a value of 1.8, while BAS isolate was suspected to be slightly contaminated with protein because it had a value of (A_{260/280}) was 1.69 (<1.8) (Desjardins and Conklin 2011).

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In the BAS isolate, although it was suspected to have protein contamination, the amplification process did not become a problem because the read sequence length was around 1.500 bp (Kozieł and Gałązka 2019).

The result of identification of TPG isolate based on 16S rRNA sequencing was identified as a *Lysinibacillus fusiformis* with a 99% similarity in the GenBank database (Figure 2). Although the nucleotide composition of the TPG isolate also had 99% similarity to the *Bacillus* sp. group (Figure 2), however, when viewed in detail the results of the BLAST at NCBI, the *L. fusiformis* bacteria group had a higher similarity with a total score of 28954 compared to the *Bacillus* sp. group which only has a total score of 2641 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Therefore, the TPG isolate was identified as a group of bacteria *L. fusiformis*. Then this genes sequence has been submitted to GenBank database with the accession number of MW802176 as a *L. fusiformis* BIP-211. Furthermore, the BAS isolate was identified as a *L. macroides* with a 99% similarity in the GenBank database (Figure 3). This is because, based on BLAST results at NCBI, the composition of the nucleotide isolates of BAS has 100% similarity to those of the *L. macroides* group of bacteria. Therefore, this isolate was identified as a group of *L. macroides* bacteria (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Then, this genes sequence has been submitted to GenBank database with the accession number of MW802177 as a *L. macroides* BIP-212.

Screening of active compounds

The active compounds contained in biofertilizer based on TPG (C1) and BAS (C2) bacterial isolates were identified using LC-MS/MS with positive (ESI⁺) and negative (ESI⁻) ionization modes. The results of screening for active compounds in biofertilizers based on bacterial isolates of TPG (C1) and BAS (C2) using the ESI⁻ mode indicated that there were no active compounds in the biofertilizer C1 and C2. This was indicated by the absence of the peaks in the screening process. While, in the positive ionization (ESI⁺) there were two active compounds in the both C1 and C2 biofertilizer. Furthermore, the results of the screening ESI⁺ mode (Figures 4-7).

The results of the active compound screening on C1 biofertilizer using LC-MS/MS QTOF with positive ionization (ESI⁺) mode showed that the C1 biofertilizer there are two active compounds which appear at 0.44 and 17.34 minutes which are indicated by the appearance of peaks at both times (Figure 4). Furthermore, the identification of active compounds based on molecular weight (Figure 5).

Based on the mass spectrum in biofertilizer C1, there are benzoic acid compounds, 4-(butylamino) which appear at 0.44 minutes with a molecular weight of 193.0372 g/mol (pubchem.ncbi.nlm.nih.gov) and 4-methoxycinnamyl P-coumarate compounds that appear at 17.34 minutes with a molecular weight of 310.0470 g/mol (pubchem.ncbi.nlm.nih.gov) (Figure 5). All active compounds identified in biofertilizer C1 (Table 2).

The results of screening for active compounds in biofertilizer isolate BAS (C2) using LC-MS/MS QTOF with positive ionization mode (ESI⁺) indicate that this biofertilizer contains two active compounds which are marked by the

appearance of peaks at 0.43 minutes and 0.44 (Figure 6). Furthermore, the identification of active compounds based on the mass spectrum of the compound is carried out (Figure 7).

The result of screening the molecular weight of the active compound contained in biofertilizer C2 is presented in (Figure 7). Furthermore, the active compound contained in biofertilizer C2 is a betaine compound which appears at 0.43 minutes with a molecular weight of 117.0064 g/mol (pubchem.ncbi.nlm.nih.gov) and a benzoic acid compound, 4-(butylamino) which is identified at 0.44 minutes with a molecular weight of 193.0375 g/mol (pubchem.ncbi.nlm.nih.gov). Furthermore, the active compounds identified in the biological fertilizer C2 are shown in (Table 3).

Table 1. The nucleic acid quantification of TPG and BAS

Isolate	Conc. (ng/μL)	A _{260/280}	A _{260/230}	Volume (μL)
TPG	105.4	1.80	0.88	30
BAS	141.2	1.69	0.20	30

Table 2. Active compounds contained in biofertilizer isolate bacteria TPG (C1)

Compound	Formula	RT (Min)	Response (Se)	Area width (%)	Molecule weight (g/mol)
Benzoic Acid, 4-(Butylamino)	C ₁₁ H ₁₅ NO ₂	0.44	1076	54.07	193.0372
4-Methoxycinnamyl P-coumarate	C ₁₉ H ₁₈ O ₄	17.34	914	45.93	310.0470

Table 3. Active compounds contained in biofertilizer isolate BAS (C2)

Compound	Formula	RT (min)	Response (Se)	Area width (%)	Molecule weight (g/mol)
Betaine	C ₅ H ₁₁ NO ₂	0.43	202	17.24	117.0064
Benzoic Acid, 4-(Butylamino)	C ₁₁ H ₁₅ NO ₂	0.44	970	82.76	193.0375

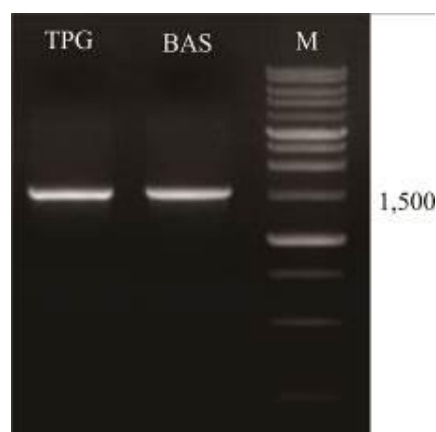


Figure 1. The gel photo-PCR products of TPG and BAS

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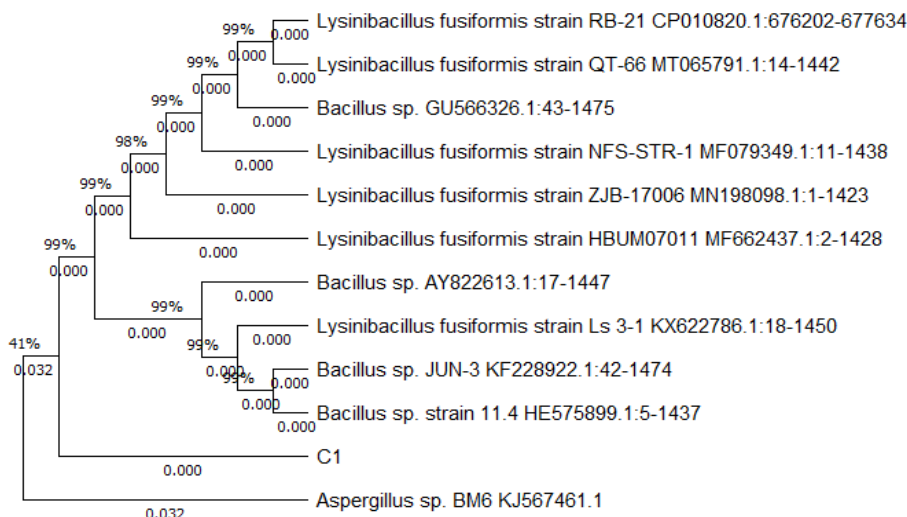


Figure 2. Phylogenetic tree of TPG (*Lysinibacillus fusiformis*) isolate

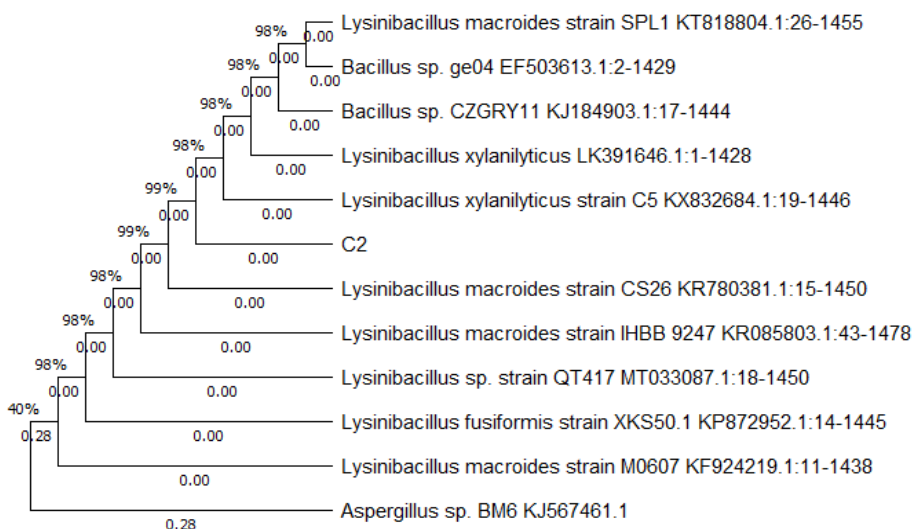


Figure 3. Phylogenetic tree of BAS (*Lysinibacillus macroides*) isolate

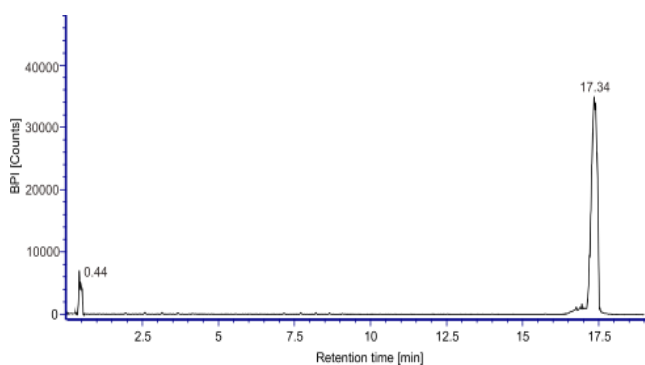


Figure 4. LC-MS/MS total ion chromatogram of active compounds on positive ionization (ESI+) mode of C1

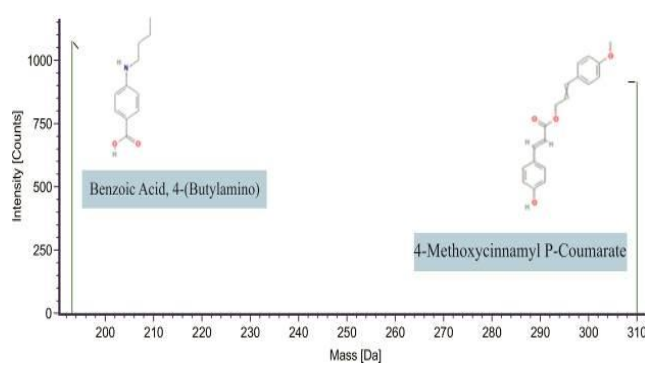


Figure 5. LC-MS/MS mass spectrum of active compounds on positive ionization (ESI-) mode of C1

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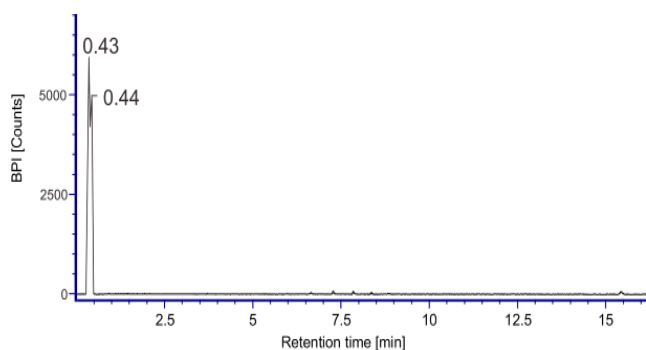


Figure 6. LC-MS/MS total ion chromatogram of active compounds on positive ionization (ESI+) mode of C2

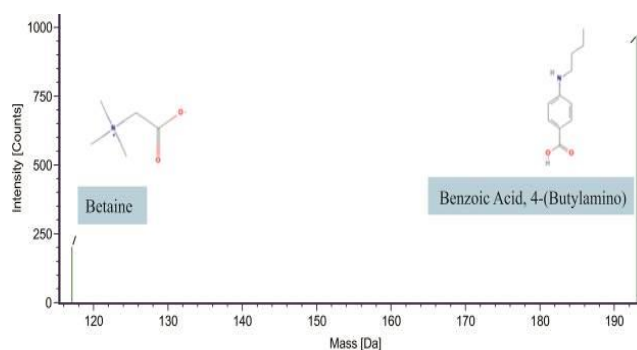


Figure 7. LC-MS/MS Mass spectrum of active compound on positive ionization (ESI+) mode of C2

The result of screening active compounds in the biofertilizer isolate bacteria TPG (C1) and BAS (C2) based on ESI+ and ESI- mode was different, which in the ESI+ mode identified two active compounds in each biofertilizer (C1 and C2), while on the ESI- unidentified active compound. This difference is due to the different ions used to split the sample in LC-MS/MS. In the negative ion mode, there is a reduction in the hydrogen ion $[M-H^+]$ while in the positive ion mode there is an addition of the hydrogen ion $[M+H^+]$. This difference is also in accordance with the results reported by Ramakrishnan et al. (2018) and Ismail et al. (2021b) that the difference in ions used to split the sample in LC-MS/MS can produce different active compounds. The same result had been reported by Zeng et al. (2018) that the type of ion used to screen for active compounds in LC-MS/MS will affect the active compound detected.

Benzoic acid compound, 4-(Butylamino) was identified in biofertilizer C1 and C2. It is assumed that from the compound content of rice straw originates as raw material. According to Menzel et al. (2020), rice straw contains the active compound benzoic acid. Furthermore, compound 4-(Butylamino) is thought to be the result of the degradation of rice straw by TPG isolate which then bind to Benzoic acid compounds. This is in accordance with the presence of benzoic compound in biofertilizers.

The identification of 4-methoxycinnamyl P-coumarate in biofertilizer C1 is assumed that from the compound content of straw originates as raw material. The presence of P-coumaric acid in rice straw has been reported by Cui et al. (2019), and Menzel et al. (2020). Furthermore, Goodman (2020) explained that P-coumaryl alcohol is one of the main constituents of lignin in rice straw, apart from coniferyl alcohol and synapyl alcohol.

Betaine or glycine betaine which identified in the biofertilizer C2 is presumed formed from glycine which a non-essential amino acid and it is also presumed one of the constituents of protein in the rice straw. He et al. (2019) reported that the crude protein content in rice straw was 8.16%. As for benzoic acid, 4-(butylamino) as explained above, this compound is thought to have come from rice straw which is used as raw material.

TPG (C1) and BAS (C2) isolates which were used as biofertilizers based on rice straw have different characteristics in degrading rice straw (Prihartini 2007).

The difference in these characteristics is marked by differences in the content of active compounds resulting from the degradation process of rice straw, in which the 4-Methoxycinnamyl P-coumarate compound was detected in the biofertilizer C2 while the C2 biofertilizer was not detected. On the other hand, in C2, betaine was identified, but in C1, it was not detected. Apart from different active compounds, TPG and BAS have the same ability to degrade rice straw into benzoic acid, 4-(Butylamino). This is indicated by the detection of this compound in both biofertilizers (C1 and C2).

Benzoic acid detected in both of the biofertilizers (C1 and C2), and it has been known the capability to stimulate plants to minimize stress due to heat environment, drought and cold pressure on plants, so that these plants can survive and produce optimally (Senaratna et al. 2003). Omar et al. (2018) also reported that benzoic acid can inhibit fungi that are pathogenic to plants, such as *Fusarium moniliforme*, *F. oxysporium*, *F. solani*, *F. graminearum*, *F. sambucinum*, *F. equisetum*, *F. semitectum*, *Rhizoctenia solani*, and *Verticillium dahliae*. Furthermore, P-coumarate detected in a biofertilizer C1 has been reported by Li et al. (2018) that methyl P-coumarate have function as an anti-fungal by damaging cell membranes and also oxidizing stress in these fungi, where the fungus used is *Alternaria* which causes black spots on the roots and often becomes a problem for fruit that has been harvested. Betaine or be called glycine betaine that detected in biofertilizer C2, has also been shown to have a very important role to mitigate the plant stress because of heat environment, drought and cold pressure on plants. According to Civelek and Yildirim (2019), the glycine betaine can reduce the effect of stress because of heat, drought, and cold pressure in the tomato. Our results presented that biofertilizers TPG (C1) and BAS (C2) isolates contain active compounds with various very beneficial functions. This is why the use of TPG and BAS isolate biofertilizers can function as bioremediation, bioactivation, bio decomposer, biofertilizer and bio protection agent in the rice plants as mentioned above in the introduction.

In conclusion, TPG isolate was identified as *L. fusiformis* BIP-211 and BAS isolate as *L. macroides* BIP-212. Biofertilizer isolate bacteria TPG (C1) have two active compounds consisting of benzoic acid, 4-(butylamino) and

4-methoxycinnamyl P-coumarate. Meanwhile, BAS (C2) isolates consisted of betaine and benzoic acid, 4-(Butylamino). So TPG and BAS bacterial isolates have the potential to be used as biofertilizers to increase plant growth. But, further testing on the effectiveness of TPG and BAS bacterial isolates as in vivo biofertilizers is also needed as a basis for field applications for farmers. Testing of the mechanism of degradation of rice straw by TPG and BAS bacterial isolates also needs further investigation to complement the ability of these bacterial isolates.

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