

Isolation, Screening and Identification of Plant Growth-Promoting Endophytic Bacteria from *Theobroma cacao*

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Abstract. Cacao (*Theobroma cacao*) is one of the main plantation commodities in Indonesia which has an important role for the national economy. The low productivity of cacao plants in Indonesia is due to the condition of old plants, pests and diseases attacks which affect the quality of the fruit and decrease the plant's productivity. The objective of this study was to isolate and identify of endophytic bacteria from stem, flower, leaf and fruit of *T. cacao* by using 16S rRNA gene as genetic marker. Twenty seven endophytic bacterial isolates were collected from local plantation in Yogyakarta area. From this study, 8 endophytic bacterial strains exhibited the higher PGP traits. The isolates produced Indole Acetic Acid level by 0.3 to 5.21 ppm/hour. All of isolates had nitrogen fixation activity but have not phosphate solubilization activity. Among them, isolates CSDT 4 and CGKBH 4 showed promising potential as PGP bacteria. Based on the 16S rRNA gene sequences, those bacterial strains were identified as *Brevibacillus brevis* (CSDT 4) and *Pantoea* sp. (CGKBH 4). We propose that the *B. brevis* and *Pantoea* sp. which is reported for the first time for their PGP potential in cacao, exerts its beneficial effects on cacao crop through combined of activities. The potential PGP bacteria from the Cacao plant was used to make a specific bio-fertilizer formula for the Cacao plant, because of the different needs and condition that every plant requires.

Key words: Endophytic Bacteria; IAA; Nitrogen Fixation; Phosphate Solubilisation; *Theobroma cacao*

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INTRODUCTION

Cacao (*Theobroma cacao* L.) is an annual plant with the humid tropical habitat. This plant is exported commodities that generate high economic income for Indonesia. The cacao are source of bioactive compounds that gives a wide variety of valuable food products and beneficial impact for human health. It can also be used for livestock feeds. The end product from this fruit that most known and universally relished product is chocolate and cocoa powder (Araujo et al., 2016).

Drought is environment stress for cacao plants besides pests and diseases. In a study conducted by Prihastanti (2010), drought stress affects the chlorophyll content of cacao seedling leaves. Plants grown on 50% groundwater content have lower chlorophyll a and b content than those grown on 75% groundwater content and the productivity consequently decreases.

Plant Growth-Promoting Bacteria (PGPB) are beneficial bacteria that isolated from plant crops worldwide. Many of them are used as agricultural inoculants, which can improve the growth and development of plant both directly and indirectly. Direct stimulation includes providing nitrogen, phytohormone, ACC deaminase activity, siderophore, indolic

compounds and phosphate solubilization. Meanwhile, indirect stimulation of plant growth is preventing pathogenic bacteria (biocontrol), thereby increasing the growth and development of plant (Ambrosini & Passaglia, 2017). PGPB can be isolated from intercellular spaces of different plant tissues (endophytic), as well as from the rhizoplane, rhizosphere, and plant-surfaces (epiphytic) (Kandel et al., 2017).

Endophytic bacteria has an important role for plant growth promotion and usually they are harbored inside of healthy plant but do not lead any pathogenic reaction (Ding & Melcher, 2016). It is means that colonization of endophytic bacteria are important part of the plant systems. Endophytic bacteria can induce plants defence reactions and tolerance to stress environmental such as cold, salinity, drought, nutrition limitation, pathogenic attacking, etc. (Kozyrovska, 2013). Although the clear mechanism on how the endophytic bacteria support the plant growth promotion remains elusive, production of metabolic compounds from endophytic bacteria is believed to be one mechanism of the plant growth promotion. Several bacteria either endophytic or non-endophytic that promoting the plant growth have been isolated and well characterized (Glick, 2014). Successful studies on the field application of endophytic bacteria to ame-

liorate the plant growth have also been reported previously (Jasim et al., 2013; Simarmata et al., 2018; Putriani et al., 2019). Therefore, it is important to discover more endophytic bacteria and select the effective bacteria strains for promoting the cacao plant growth because every specific plant requires the different needs and condition. The objectives of this study were to isolate and identify the endophytic bacteria from the cacao plants of local plantation in Yogyakarta area and screen their plant growth promotion (PGP) activity such as indole acetic acid (IAA) production, nitrogen fixation, phosphate-solubilization and their enzymatic activity. The study could be used to promote cacao plant growth, protect the plants from environmental and give the information to cacao farmer about the potential PGPB for their plant growth and The potential bacteria will be used as an inoculum of bio-fertilizer for cacao plant.

METHODS

Isolation of endophytic bacteria

Samples of young leaves, elderly leaves, fruit, and stem from cacao (*Theobroma cacao*) were obtained and collected from the area of Gunung Kidul, Yogyakarta. The plant samples were cut to a size of 1 x 1 cm and subjected for surface sterilization and then were placed onto nutrient agar (NA) media. Surface sterilization of the samples were as follows, the samples were washed in running water and followed by the immersion sequence of 75% ethanol for 1 min, 20% NaClO for 3 min and 75% ethanol for 0.5 min (Ambrosini & Passaglia., 2017). The inoculated agar media were incubated at room temperature for 24 hours. The growth colonies were further purified to obtained pure single colonies. The selected colonies were further tested for the plant growth promotion activity.

Plant growth promotion study

All isolates were assessed for PGP (Plant Growth Promoting) traits such as the ability to produce indole-3-acetic acid (IAA), solubilization of inorganic phosphate, nitrogen fixation and enzymatic (cellulolytic, amylolytic, proteolytic, catalytic) activity.

IAA production assay

Production of IAA by bacteria was evaluated with colorimetric technique proposed by Gordon and Weber method (1951). The isolates were grown in tubes containing 3 ml of Luria Bertani (LB) broth with 100 µg/ml tryptophan (10^7 - 10^8 cfu.ml⁻¹) individually and incubated under shaking conditions (120 rpm) at 28 °C for 24 h. The supernatant culture was recovered after centrifuged at 5000 rpm for 15 min at room temperature. Then, 2 ml of Salkowski reagent was added to 2 ml of culture supernatant and the resulting mix-

ture was incubated at 30°C for 30 mins in dark room. Development of pink color indicated IAA was produced by the test bacteria. The absorbance of the samples was recorded at 530 nm using UV/VIS spectrophotometer and the blank medium was used as negative control (Egamberdieva, 2008). Determination of IAA production in the sample was carried out by calculating the IAA value of sample in the IAA standard curve. The pellet was dried in an oven until the weight was constant to determine the dry weight of the cell.

Phosphate (P) Solubilizing Activity

Phosphate solubilizing activity was carried out with subculturing the bacterial strains by spot inoculation on Pikovskaya medium that containing Ca₃(PO₄)₂ (5g/L of distilled water), then incubated for 3-7 days at room temperature. The bacteria strains that were able to dissolve P from Ca²⁺ were characterized by the development of clear zones around the growing colonies, then an analysis of phosphate solubilizing ability was carried out by Latif Khan et al., (2016) method.

The assay of amylase, cellulose, protease and catalase enzymes activity

The assay of amylase enzymes activity was carried out by means of bacterial isolates being scratched on Starch Agar medium. Bacterial isolates were incubated for 24-48 hours at 37 ° C. Then, a few drops of Gram's iodine were dropped on the bacterial culture, and the formation of clear zone was observed. The qualitative testing of cellulolytic activity of bacteria was carried out by testing the ability of bacterial isolates to degrade cellulose. Bacterial isolates were inoculated in a medium containing pure cellulose substrate, namely carboxy methyl cellulose (CMC) (Patil et al, 2015), and incubated for 48 hours at 37°C. Growing colonies were washed with 0.1% Congo red solution for ten minutes and rinsed using 1% NaCl. Positive results were indicated by the formation of clear zones in the area around the colony. Testing the proteolytic activity was carried out by growing the bacterial isolates on Skim Milk Agar medium. After incubating 48 hours at 37°C, the positive results were indicated by a clear zone around bacterial isolates. Amylolytic, cellulolytic and proteolytic activity index were measured in the following way (Baehaki & Budiman., 2011):

$$IE = \frac{X1 - X2}{X2}$$

IA/IS/IP = Amylolytic, cellulolytic and proteolytic activity index

X1 = diameter of clear zone

X2 = diameter of colony

Catalytic enzyme activity test qualitatively was carried out by inoculating the bacteria into DSMZ 261 (pH 4) media. The media was incubated at 55° C until turbid. The turbid media was dripped on glass preparations and then added with H₂O₂. Catalase activity of microbes was determined by the formation of O₂ gas bubbles.

Nitrogen fixation assay

Efficiency of N₂ fixation was carried out by Dobreiner method in Beneduzi (2013). All isolates were grown on LB medium then transferred on 5 ml tube containing semi solid Nitrogen free bromothymol blue (Nfb) media which consisted of 0.05% of malate as carbon source, then incubated at room temperature for 2-4 days. Positive results were indicated with the formation of white veil-like pellicles below the surface of semi-solid Nfb media that indicated with isolate had ability for fixation of nitrogen.

Identification of Endophytic ACCD Producing Bacteria

Bacterial isolate identification was carried out by observation of phenotypic appearance and also based on the sequence of 16SrRNA gene. Phenotypic appearances were determined according to the Bergeys Manual Determinative Bacteriology. Amplification of the 16SrRNA gene was achieved by amplify the genomic DNA from selected bacteria that used as template for PCR amplification with the following universal primers, 27F (5’ AGA GTT TGA TCC TGG CTC AG - 3’) and 1492 R (5’- GTT TAC CTT GTT

ACG ACT T- 3’). The PCR method was carried out using GoTaq Green PCR kit and the PCR products were then directly sequenced. The complete sequencing results were built by using DNA Baser suite and used for further nucleotide BLAST analysis (<https://blast.ncbi.nlm.nih.gov/>). Phylogenetic tree was constructed by using the neighbor joining method. A phylogenetic and molecular evolutionary analysis was performed using MEGA 6 software (Tamura et al., 2013).

RESULTS AND DISCUSSION

Isolation and purification of endophytic bacteria

In this study, we found that cacao plant also harbored an abundance of culturable endophytic bacteria, and varied between stems, leaves, and fruits. A total of 27 morphologically different endophytic bacteria was obtained and isolated from the sterilized surface of roots, stems and leaves of cultivated cacao plants. The efficacy of surface sterilization process was proved by the absence of growth on control plates, even after 7 days of incubation. The endophytic bacterial isolates were distributed as shown in Table 1.

Preliminary characterization of these isolates indicated that studied parts of cacao plant contained both Gram negative and Gram-positive bacteria. In order to obtain the plant growth promoting endophytic-bacteria (PGPE), all isolates were qualitative tested for a number of important properties regarding PGP activity (Figure 1, 2 and 3).

Table 1. Distribution of bacterial endophytes obtained from cacao plants.

Sampling location	Part of plant	Isolate code	Number of isolates
Gunung Kidul	Stem	CGHBt1, CGHBt2, CGHBt3	3
	Old leaves	CGKDT1, CGKDT2, CGKDT3, CGKDT4	4
	Young leaves	CGKDM1, CGKDM2, CGKDM3, CGKDM4, CGKDM5, CGKDM6	6
	Fruit	CGKBH1, CGKBH2, CGKBH3, CGKBH4	4
Sleman	Stem	CSBt1, CSBt2, CSBt3, CSBt4	4
	Old leaves	CSDT1.1, CSDT1.2, CSDT2, CSDT3, CSDT4, CSDT5	6
Total			27

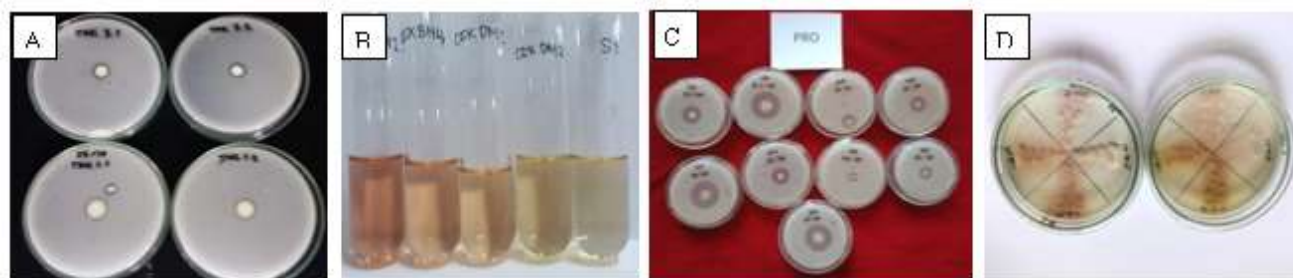


Figure 1. Qualitative test of plant growth promoting activities: (A) P-solubilization, (B) IAA, (C) enzymatic, (D) N-fixation

Plant growth-promoting activities of endophytic bacteria

Seven endophytic bacterial isolates showed a positive reaction for IAA production by producing pink to red color. The quantitative estimation of IAA at 530 nm was also done spectrophotometrically. Highest IAA production was observed from endophytic isolate CSDT4 (5.21 ppm) followed by CGKBH4 (4.58

ppm). Least IAA production was recorded from CSDT5 strain (0.3 ppm) (Figure 2).

Among 18 isolates, some of them were found to be nitrogen fixing since when they were transferred on to Doberiner's nitrogen free semi solid BTB agar, they showed a color change of the medium from green to blue and formation of surface pellicles after 4 days of incubation.

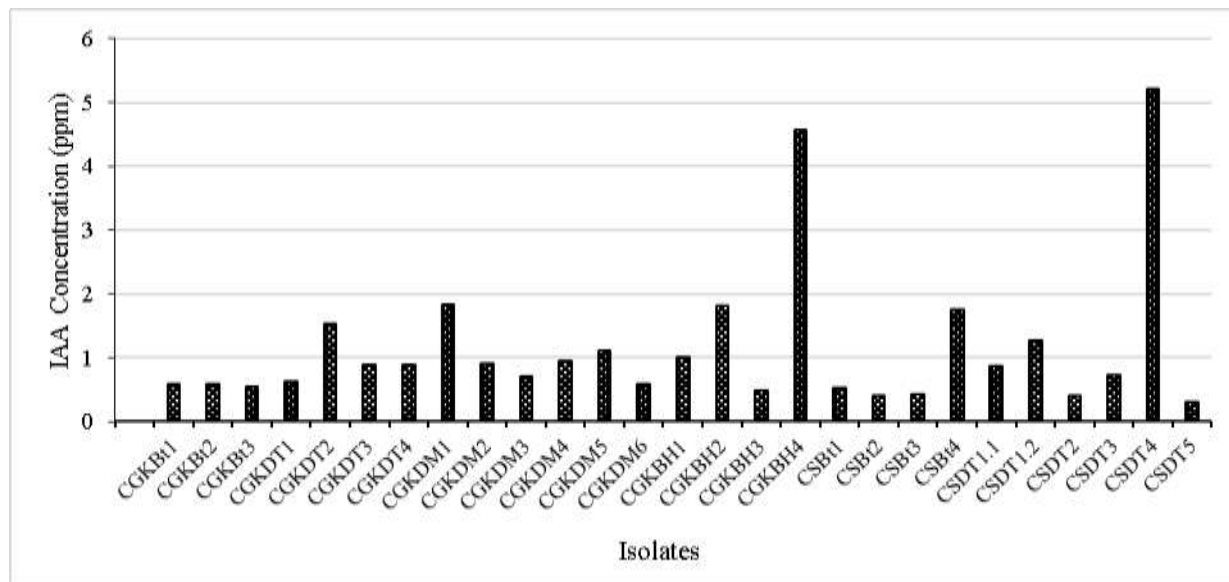


Figure 2. IAA production of endophytic bacteria from cacao

Among twenty-seven isolates of endophytic bacteria that were screened for enzymatic activity, 19 isolates were showed enzymatic activity. Each bacterium produce different enzyme complexes, depending on gene and carbon source used. In this study, the maximum protease activity was observed in CGKBH1 isolate indicated by formation of clear zone around the colony because of degradation of gelatin, while the other ten isolates indicated negative results. Proteases are used in clinical applications especially in the treatments like diabetes. Out of 27 endophytic bacteria, only 6 isolates showed positive results for amylase activity. The isolate of CSDT3 was the maximum producer of amylase activity. All isolates grew on liquid media CMC containing 1% (w/v) CMC as the inducer component. Ten bacteria showed the cellulose activity indicated by formation of clear zone around the colony. The formation of clear zone showed that isolates can utilize the carbon cellulose source by synthesizing the cellulose enzyme. Among the tested organisms, maximum cellulose activity was observed in CGKDM4 and CGKDM6 isolate while other iso-

lates exhibited moderate to low activity. Based on Patil et al. (2015), the production of extra-cellular enzymes was greater in the liquid medium as compared to the plate based assays. Enzymes that were not detected in plate based assays for some particular isolates were found positive in liquid culture conditions.

All of the endophytic isolates did not showed any zone of clearance around the bacterial colonies in the Pikovskaya's agar plate indicating an absence of phosphate solubilization ability.

Enzymatic activity of endophytic bacteria was tested in all the isolates from cacao plants (Figure 3) The results showed that the activity of protease enzyme was detected in 59% of the isolates, amylase in 22% of the isolates, celluloses in 37% of the isolates and catalases activity was detected in 15% of the isolates. The study showed that proteases and cellulose activity were dominated isolates from leaves (Figure 3). It is suggesting a potential role of endophytic bacteria in colonizing the leaf tissues.

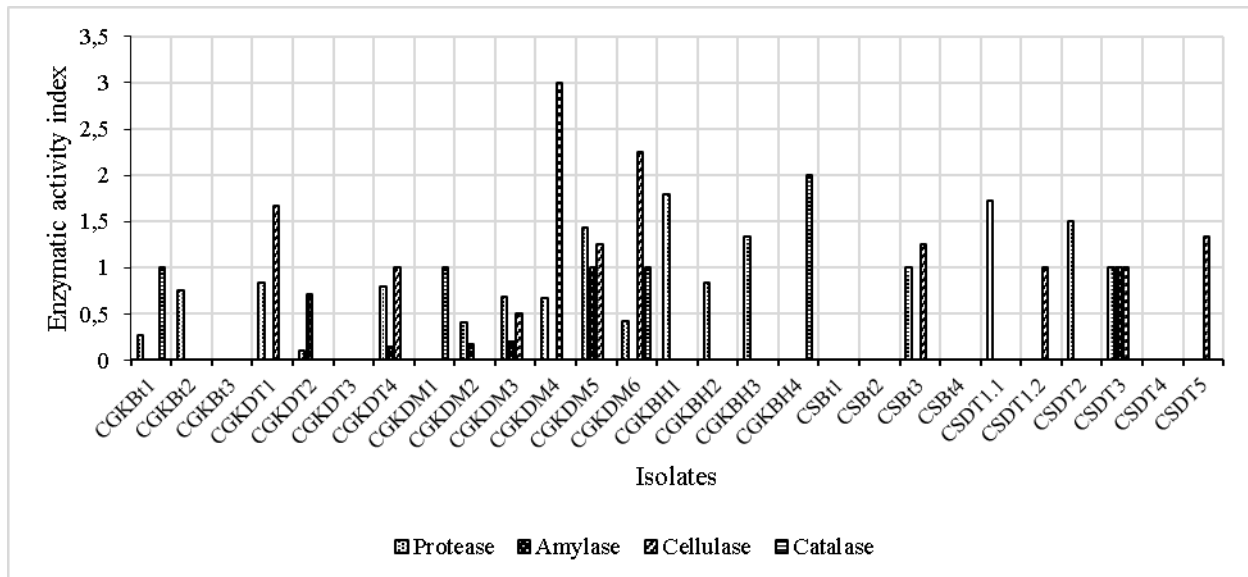


Figure 3. Enzymatic activity of endophytic bacteria from cacao

Table 2. Nitrogen Fixing ability of endophytic bacteria from cacao

Isolates	Ring Formation	Color
CGKBt1	+	Blue
CGKBt2	+	Blue
CGKBt3	+	Blue
CGKDt1	+	Blue
CGKDt2	+	Blue
CGKDt3	-	Green
CGKDt4	-	Blue
CGKDM1	++	Blue
CGKDM2	-	Blue
CGKDM3	-	Blue
CGKDM4	-	Blue
CGKDM5	-	Blue
CGKDM6	-	Blue
CGKBH1	+	Blue
CGKBH2	-	Blue
CGKBH3	-	Green
CGKBH4	-	Blue
CSBt1	-	Blue
CSBt2	+	Blue
CSBt3	-	Blue
CSBt4	-	Green
CSDT1.1	-	Blue
CSDT1.2	-	Blue
CSDT2	-	Blue
CSDT3	-	Blue
CSDT4	-	Blue
CSDT5	-	Blue

The results of qualitative extracellular catalase enzyme tests that had been carried out showed positive results on isolates CGKBt1, CGKDM1, CGKDM6, and CGKBH4 (figure 3). This was indicated by the presence of gas bubbles in each endophytic bacterial isolate that positively produced the catalase enzyme when dripped with H₂O₂. The four bacterial isolates were aerobic bacteria. Most facultative and aerobic bacteria contain a high concentration of catalase enzyme (Reiner, 2010).

Two isolates namely CSDT4 and CGKBH4 possessed multiple of PGP properties and enzymatic activity were selected for subsequent studies. On the basis of morphological and biochemical characteristics as well as comparative analysis of the partial sequence of 16S rRNA gene, the CSDT4 and CGKBH4 showed 100% homology with *Brevibacillus* sp., and 99% with *Pantoea* sp., respectively. The potential of endophytic diazotrophic bacteria with multiple plant growth promotion traits have been well documented in the present study. The use of such beneficial bacteria as bioinoculants for cacao plants can support the indigenous bacteria to fix nitrogen. In addition, the endophytic bioinoculants are able compensate in PGP traits compared to the chemical fertilizer (Data in another publication)(Widowati et al, 2019). the application of this study is expected provide a significance and promising thing for cacao farmers.

Many species of endophytic bacteria can be isolated from a single plant and many factors, such as soil condition, phytopathogen population, plant age (Islam et al., 2010), host genotype, and plant variety can contribute to significant differences in endophytic bacterial diversity (Shore & Sathisha, 2010).

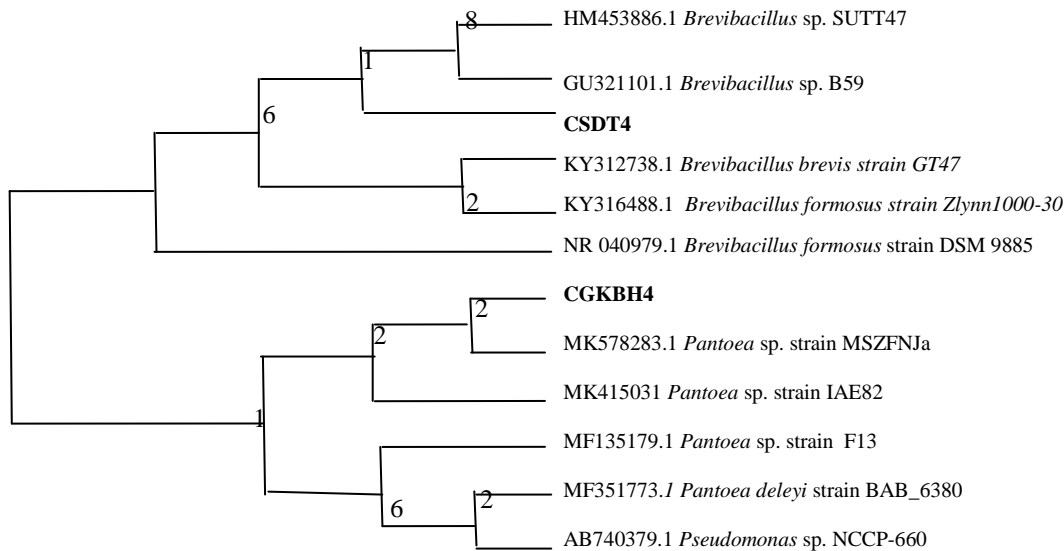


Figure 4. Phylogenetic tree showing relatedness of endophytic bacteria isolated from *Theobroma cacao* and their relatives in the Proteobacteria by neighbor-joining grouping of the aligned sequences of the 16 S rRNA gene. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA 6.

Santoyo et al. (2016) stated that bacterial endophytes can stimulate the growth of the host plant and protect from pathogens. Plant and endophytic colonization requires the capacity of bacteria to compete in the rhizosphere soil to find a place to communicate and interact with the plant roots. The community of endophytic bacteria associated to the roots is richer than rhizospheric.

A phylogenetic analysis was performed using the 16S rRNA gene sequences obtained in this study and those available in GenBank. Our results revealed that two potential endophytic bacteria in this research are associated with the phylum Proteobacteria and Firmicutes (Figure 4). Based on phylogenetic analysis, isolate CGKBH4 had similarity with *Pantoea* sp. *Pantoea* sp. was belonged to Proteobacteria phylum and related to the γ -Proteobacteria class. *Pantoea* sp. is also PGPB, which is known to contribute to the plant growth, can control plant disease (Etmiani & Harighi, 2018) and have ability to solubilize phosphates (Hernandez et al., 2018). It also cold tolerance (Miliute et al., 2015) and resistant to drought (Trivedi et al., 2017) and salinity (Simarmata et al., 2018).

Endophytic bacteria affiliated with the phylum Firmicutes and included to the genera *Brevibacillus* was isolate CSDT4. This isolate matched those of *Paenibacillus*. *Paenibacillus* and *Bacillus* that are known to protect plants against phytopathogenic fungi using different mechanisms, including chitinolytic activity (Singh et al., 2016), via the production of fungistatic antibiotics (Hernandez et al., 2018) and 1-

Aminocyclopropane-1-carboxylic acid (ACC) deaminase (Simarmata et al., 2019). Bacteria of the genus *Bacillus* are widely dispersed in nature, easy to multiply, have a long shelf life when sporulated and are nonpathogenic.

All isolates were screened for their plant growth promoting attributes, which included direct-plant growth promoting (solubilization of phosphorus; production of IAA and nitrogen fixation), and indirect-plant growth promotion (antagonistic, production of lytic enzymes). This study is the first report for the presence of *Brevibacillus* and *Pantoea* endophytic bacteria in cacao tissue that exhibit multifunctional PGP attributes. The diverse and specific host of PGP endophytic bacteria may contribute to plant growth promotion in several environment condition. According to the result assays, *Pantoea* sp. and *Brevibacillus* sp. are inoculant candidates that can be use as plant growth promoter. The application of multi strain inoculum or consortium could be effective for reducing the impact of stress on plant (Aghai et al., 2019).

Endophytic bacteria are non-pathogenic inhabitants of healthy plant tissue that can give benefit for plants. Many endophytic bacteria contribute to plant growth and health (Putriani et al., 2019), reduction of plant stress and degradation of contaminants in plant. This study examines the community of bacteria from healthy cacao plants that have potency as plant growth promoter. The *Pantoea* spp. and *Brevibacillus* sp. isolates showed beneficial characteristics, such as phosphate solubilization, indole-3-acetic acid pro-

duction, nitrogen fixation and enzyme activity. Our results revealed that the functional ability of the analyzed bacterial isolates was influenced by the location of plant tissue from which the endophytic bacteria were isolated. The endophytic bacteria isolated from old leaf and fruit tissue of cacao resulted more of promoting plant growth.

This study offer information about the diversity of endophytic bacterial from cacao plants and their potency for plant growth. The potentially bacterial can be developed as biocontrol agent to inhibit plant pathogens and biofertilizer agent to facilitate in nutrient uptake and minerals which contribute to the growth of cacao plants. The use of PGPB in cacao plantation practices may be a solution to decrease utilization of chemical fertilizer and pesticide which influence on the quality of soil, water, ecological balance and biological diversity.

CONCLUSION

In the end of our study, a total of 27 endophytic bacteria isolated from different tissues of cacao (*Theobroma cacao*) were screened for their putative beneficial characteristics as PGPB. According to phylogeny tree, of isolates CSdT4 and CGKBH4 have the closest relationship to *Brevibacillus* sp. SUT47 and *Pantoea* sp. MSZFNJa strains. Both of isolates are reported for the first time as potential PGP from endophytic bacteria of cacao plant. The two isolates of CSdT4 (*Brevibacillus* sp.) and CGKBH4 (*Pantoea* sp.) produce high IAA, nitrogen fixing, and have thermostable enzymes including extracellular amylase, protease, cellulose, and catalase.

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