Isolation and Identification of ACC Deaminase Producing Bacteria from Rhizosfer and Plant Roots of Maize, Cowpea, and Groundnut Growing under Saline Stress

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Abstract. ACC deaminase producing bacteria reduces the excess ethylene produced by stressed plant because ACC deaminase catalyzes the cleavage of ACC into ammonium and α-ketobutyrate. ACC deaminase is produced by several rhizospheric and endophytic bacteria. This study was aimed to obtain ACC deaminase producing bacteria isolated from rhizosfer and plant roots of maize, cowpea, and groundnut growing under saline stress. Isolation was conducted by surface plating on NA medium. Qualitative selection was based on the growth of isolate on DF salts medium supplemented with AIB. Quantitative selection based on ACC deaminase activity assay. Identification of the isolates was carried out by morphology and sequencing of 16S rRNA gene. Isolation and selection resulted four bacterial isolates (AJG3, RJG6, ATL5, and RTN10). Those bacterial isolates have the ACC deaminase activity between 184.65 to 692.54 nmol α-ketobutyrate. mg⁻¹. h⁻¹. Isolate of AJG3, RJG6, and ATL5 were gram negative bacteria, only one isolate was gram positive bacteria (RTN10). Based on 16S rRNA gene sequence, AJG3, RJG6, ATL5, and RTN10 isolates have a high similarity with Klebsiella variicola, Rhizobium pusense, Agrobacterium tumefaciens, and Bacillus stratosphericus respectively.

1. Introduction
Agricultural land in the world continues to decrease due to an increase in population and environmental damage. Efforts to extend agricultural land to meet food needs continue to be carried out especially by utilizing marginal land or less productive land. Thus various strategies are needed to increase agricultural productivity on marginal land [1]. Marginal land if managed properly can increase agricultural productivity. One of the marginal land that has not been utilized optimally for agricultural land is land along the coastline. This is caused by the high concentration of salt in the form of NaCl which is a limiting factor for plant growth. High concentration of salt causes plants to experience stress. Salinity is a factor that causes low productivity of crops [2], whereas around 23% of agricultural land in the world is saline land [3].

The saline land used as agricultural land causes plant growth to become abnormal. Plant growth can be hampered by the presence of excessive ethylene. Ethylene which is an overgrowth of plant growth hormone is the response of the plant to salinity stress [1]. Therefore, efforts are needed to reduce ethylene production in gripped plants. Excessive ethylene production by gripped plants can be controlled by Plant Growth Promoting Bacteria (PGPB), the ACC producing deaminase bacteria. These bacteria can reduce ethylene production using the ACC deaminase enzyme. The enzyme can deflect the reaction of ethylene formation and produce ammonium as a source of nitrogen (N) which is
useful for plant growth. ACC producing bacteria deaminase can increase plant tolerance to environmental conditions that are less favorable for these plants [4]. Thus, the ACC deaminase enzyme-producing bacteria that interacts with plant roots is very beneficial for plant growth in clogged conditions.

ACC producing bacteria deaminase can be used as biological fertilizers for plants in a gripping condition, so that these plants can grow well. The less favorable saline land can still provide better agricultural results in the presence of these bacteria. This study was carried out to isolate and identify endophytic and rhizospheric bacteria producing ACC deaminase enzymes from maize and legume plant in salinity plantations from the Special Region of Yogyakarta. Maize, peanut, and tolo beans plants are used because these plants have a low tolerance to salinity.

2. Materials and Methods

2.1 Sample collection
Healthy plant samples of a salinity plantation, Zea mays L., Arachis hypogaeae L. and Vigna unguiculata L., collected from Glagah beach and Ngeden beach, the Special Region of Yogyakarta were used as sources for the isolation of plant-associated bacteria. The salinity of crop land which the samples collected was reached 12.75 dS/m and 9.8 dS/m. [5] states that the land with a conductivity value of 12.75 dS / m and 9.8 dS / m is saline soil.

2.2 Isolation of endophytic and rhizospheric bacteria
The root samples were firstly surface sterilized using previously described procedures [6]. Ten grams of sterilized root tissues were aseptically crumbled into smaller fragments using a sterile mortar and pestle and then macerated with sterile distilled water. Then, 100 μl of the tissue extracts and the serial dilutions (10⁻¹ to 10⁻⁴) were plated onto nutrient agar (NA) as isolation media. The plates were incubated at 28°C for 24–48 hours. A representative of each colony as evident from their colony morphology was picked and transferred to fresh nutrient agar medium to establish pure cultures of bacteria and use for further analysis of ACC deaminase enzyme activity.

Ten grams soil sample was serially diluted, spread plated on full strength nutrient agar and incubated at 28°C for 48 h. The different colonies were isolated on nutrient agar (NA) and were purified. Strains were assessed for morphology and Gram reaction and other characterization.

2.3 ACC deaminase activity assay
To determine the presence of ACC deaminase, the ability of the isolates to use ACC as nitrogen source was checked by growing them onto DF salts minimal agar medium supplemented with amino isobutyric acid (AIB) instead of (NH₄)₂SO₄ as nitrogen source [7]. The ACC deaminase activity of cell-free extracts was determined by estimating the amount of α-ketobutyrate (α-KB) generated by the enzymatic hydrolysis of ACC [8] and the amount of α-ketobutyrate produced was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 nmol. The total protein in supernatant of cell free extract were determined by the BioRad Assay method with used the bovine serum albumin (BSA) as standard. A total of 2 μL crude enzymes, 200 μL BioRad solution and 798 aquades were homogenized and measured at absorbance 595 nm. The standard protein is used by measuring the absorbance of various concentrations of BSA (Bovine Serum Albumin) [9]. After determining the amount of protein and α-KB, the enzyme activity was expressed as nanomoles of α-KB per milligram of protein per hour of the active isolates.

2.4 Antibiotic resistance assay
To test antibiotic resistance, the bacterial strains were tested against ampicillin, kanamycin, and ciprofloxacin. Bacterial cells were cultivated by streaked plate method on nutrient agar (NA) media supplemented with serial concentration of each antibiotic. The minimum inhibitory concentration (MIC) of bacterial strains against the three antibiotics determined using the micro dilution method as described by [10]. Each antibiotic were serial diluted into a series of concentration from 0 ppm to 200 ppm. The culture media were incubated at room temperature for 168 hours and each culture media was
made in duplicate. The lowest concentration of antibiotics that inhibited the visible growth of the bacterial strain was recorded as the MIC value.

2.5 Identification of ACC deaminase producing bacteria
The selected bacterial isolate identification was carried out by observation phenotypic appearance and also based on the sequence of 16SrRNA gene. Phenotypic appearances were determined according to the Bergey’s Manual Determinative Bacteriology. In order to amplify the 16SrRNA gene, the genomic DNA from selected bacteria was 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 Go Taq 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 MEGA 7 software [11]. The stability of the clades in the trees was appraised using a bootstrap value with 1,000 repeats.

3. Results and Discussion
3.1 Isolation plant-associated bacteria
The sample used for bacterial isolation is a plant that gripped by salinity and the soil around the roots of the plant (rhizosphere). ACC producing bacteria deaminase is one of Plant Growth Promoting Rhizobacteria (PGPR) that it can be found in the roots and in the soil around the roots (rhizosphere)[12]. In general, plant growth in a population is relatively same, but some plants can show better growth. The plant with better growth among the stressed plant populations may be associated with ACC producing deaminase bacteria that it have better abilities. The sample plants were chosen because they have better growth than similar plants in the vicinity. In addition, sample plants were chosen because they have leaves greener and larger than similar plants in the vicinity. Part of the plant sample used as a source of isolates is the root.

According to [13], bacterial isolates isolated from soil samples that stressed by salinity or saline environment have higher resistance to salinity stress than isolates isolated from non-saline areas. Under salinity stress, soil microorganisms, including bacteria, have several defense mechanisms. They are capable of lowering ethylene production in their host plants, they should also render the plants more tolerant to salt-induced stress. Bacterial strains were isolated from stressed plant contain ACC deaminase, should also act as plant growth promoters. Hence, it was expected that maize, cowpea, and groundnut plants planted in saline environment could harbor salinity–resistant bacteria. It has been known that the population of endophytic bacteria are affected by biological and non–biological agents such as salinity stress [14]. In other words, the bacteria surviving in these environments are resistant to environmental stresses, including salinity stress. Plants under various environmental stresses, including salinity, absorb bacteria that are resistant to stress and can help plant establishment under such conditions [15].

Based on the morphological colonies and cells appearance, we have successfully isolated 40 bacterial strains from root tissue and rhizospheric area of plants. The selected bacterial strains were further tested for their ability to grow on minimal media DF salt agar containing either (NH₄)₂SO₄, AIB, or ACC as nitrogen sources. The bacterial growth was observed qualitatively based on the colonies appearances, which then scored by the following criteria good (5+), average (4+), fair (3+) and poor (≤2+). Among the 40 bacterial strains only 4 bacterial strains, which showed the positive response when grown on DF salt media (Fig.1). Almost 90% of them exhibited good to fair growth appearance when the (NH₄)₂SO₄ was used as nitrogen source. Interestingly, the growth ability on (NH₄)₂SO₄ was comparable to the growth on negative control media. However when the (NH₄)₂SO₄ was replaced by ACC as nitrogen source only 75% of the bacterial strains exhibited good growth. The good growth was observed when the structural analog of ACC, AIB, was used as nitrogen source (Fig. 1).

Although most of the selected bacterial strains exhibited poor growth on DF salt agar media, it is worth to check the ACC deaminase production those bacterial strains.
3.2 ACC deaminase activity of isolated plant-associated bacteria

The results of qualitative selection were not enough to prove that the selected isolates were able to produce the ACC deaminase enzyme. Therefore, quantitative testing is needed. In the present study, a total of 40 plant-associated bacterial strains were isolated. Eleven of them isolated from the inner tissues of plant and 29 isolates obtained from rhizosfer. Out of these, only 4 isolates were able to grow on DF agar medium supplemented with AIB, indicating that they have the ACC deaminase activities.

Based on the quantitative assays, 4 isolates showed different level of ACC deaminase activity (Fig 1). Highest ACC deaminase activity was exhibited by the isolate AJG3 (692.54 ± 14.91nmol α-KB/mg Pr·h), followed by strains RJG6 (616.95 ± 7.34 nmol α-KB/mg Pr·h), ATL5 (503.82 ± 10.30 μmol α-KB/mg Pr·h), and RTN10 (184.65 ± 5.36 nmol α-KB/mg Pr·h). The ACC deaminase production was induced by ACC substrate and the ACCdeaminase activity was determined by measuring the amount of ammonium released from the breakdown of ACC molecule. The enzymatic activity assay result could be categorized into low, fair and high which showed the ACC deaminase activity ≤ 100; 110-200 and > 200 nmol/mg/h, respectively. The results showed that 1 of bacterial strains exhibited fair, meanwhile 3isolates exhibited high ACC deaminase activity, respectively (Fig. 2). Four bacterial strains that exhibited fair and high ACC deaminase activity might be a good candidate for further exploration on ACC deaminase producing bacterium.

**Figure 1.** The qualitative growth of bacterial strains in minimal medium DF salt supplied with various nitrogen sources ammonium (NH4+), amino isobutyric acid (AIB) and amino cyclopropane carboxylic acid (ACC). The number of positive (+) mark indicated quality of the bacterial growth.
Figure 2. The activity of ACC deaminase extracted from bacterial strains. ACC deaminase activity was measured by calculating the amount of an $\alpha$-ketobutyrate released in the reaction mixture.

Isolate of RTN10 had the lowest ACC deaminase activity (184.65 ± 5.36 nmol $\alpha$-KB/mg/hour). The ACC deaminase activity of RTN10 isolates was still higher than the ACC deaminase activity of Microbacterium sp bacteria isolated from the rhizosphere that is 122 nmol $\alpha$-KB/mg/hour [16], [17] also showed that ACC deaminase enzyme activity from various strains of Rhizobium between 76 to 274 nmol $\alpha$- KB/mg/hour. According to [8], bacteria that have ACC deaminase activity above 400 nmol $\alpha$- KB/mg/hour classified as high activity and potential to be used as PGPB (Plant Growth Promoting Bacteria). Thus, isolates of AJG3, RJG6, and ATL5 have high potential to be used as ACC producing deaminase bacteria.

3.3 Antibiotic resistance assay
In order to further discriminate among the bacterial strains and as well as with other common bacteria, we have tested the selective resistance of each bacterial strains against certain antibiotics. Ampicillin, kanamycin, and ciprofloxacin were used for the antibiotic resistance test.

Table 1. Antibiotic resistance of bacterial strains against ampicillin, ciprofloxacin, and kanamycin. Antibiotic resistance was calculated based on the minimum inhibitory concentration (MIC). Antibiotic concentrations were varied from 0-80 µg/ml.

| No. | Bacterial strains | Minimum Inhibitory Concentration (MIC) (µg/ml) |
|-----|------------------|-----------------------------------------------|
|     |                  | Ciprofloxacin | Kanamycin | Ampicillin |
| 1   | AJG3             | 1             | 4         | 80         |
| 2   | RJG              | 1             | 12        | 10         |
| 3   | ATL5             | 1             | 12        | 15         |
| 4   | RTN10            | 6             | 2         | 10         |

Based on table 1, each selected isolate has different resistance to several antibiotics. Ciprofloxacin is the antibiotic that most strongly suppresses the growth of isolates AJG3, AJG6, and ATL5. The three isolates showed no growth at the lowest concentration of ciprofloxacin, but RTN10 isolates had resistance to ciprofloxacin. Kanamycin is able to suppress the growth of AJG3, AJG6, and ATL5 isolates, although it is not as strong as ciprofloxacin, but kanamycin is the most powerful antibiotic that suppresses the growth of RTN10 isolates. Ampicillin is less able to suppress the growth of selected bacterial isolates. All selected isolates had resistance to ampicillin, especially in AJG3.
isolates which showed MIC values of around 80 µg/ml. The antibiotic resistance test, which was expressed as minimum inhibitory concentration (MIC) indicated that the ciprofloxacin and kanamycin were the best candidate as selective marker, because of lower MIC value compared to the ampicillin (Table 1).

3.4 Identification of ACC deaminase producing bacteria
Thirteen selected bacterial strains showing the ACC deaminase activity isolated from rhizospheric of plants or crops of local agricultural plantations were further characterized by morphological and molecular level. Generally it can be said that there was strong correlation between the morphological (Table 2) and molecular characterization (Fig. 3).

Table 2. Phenotypic characterization of bacterial strains based on their cell morphology and gram staining. The cell morphology was examined microscopically with 1000x magnification. The Gram character was determined by gram staining method.

| No. | Bacterial strains | Colony colour | Colony shape | Colony elevation | Colony Edge | Colony Surface | Gram staining |
|-----|-------------------|---------------|--------------|-----------------|-------------|----------------|---------------|
| 1   | AJG3              | Shiny white   | Circulair    | Convex          | Entire      | Translucent    | Negative      |
| 2   | RJG6              | Cream         | Circulair    | Low convex      | Entire      | Transparent    | Negative      |
| 3   | ATL5              | Cream         | Circulair    | Convex          | Entire      | Transparent    | Negative      |
| 4   | RTN10             | White         | Circulair    | Umbonate        | Undulate    | Opaque         | Positive      |

From the molecular characterization, we could identify bacterial strain individually up to species level. The constructed phylogenetic tree (Fig. 3) showed that among the 4 bacterial strains could be classified into three main different phylum, i.e. Proteobacteria and Firmicutes. Among the 4 bacterial strains, 3 strains belonged to Proteobacteria, and 1 strains belonged to Firmicutes.

After DNA extraction and PCR amplification, phylogenetic analysis was carried out. The 4 ACC deaminase producing bacteria were identified by partial and full length sequencing of the 16S rRNA gene sequences. The percentage of 16S rRNA gene sequence similarities (95.7–100 %) of these isolates to the closest type strains was presented in Fig3. Based on the sequence of the 16S rRNA gene, these strains distributed under four different genera: Bacillus (one isolate), Klebsiella (one isolate), Rhizobium (one isolate), and Agrobacterium (one isolate).
Figure 3. The evolutionary history of bacterial strains was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is 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 Kimura 2-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Evolutionary analyses were conducted in MEGA7.

*Klebsiella variicola* was found previously by [18] as ACC producing deaminase bacteria with lower activity than AJG3 isolates. Klebsiella sp. ECI-10A strains are also known to have ACC deaminase activity (539.1 nmol α-KB/mg/hour)[16]. [19] also stated that *Agrobacterium tumefaciens* D3 had *acdS* and *acdR* genes, and had ACC deaminase activity around 470 nmol α-KB/mg/hour. From the member of Firmicutes which consist of *Bacillus* sp.RTN10 was produce lower ACC deaminase activity compared to the other isolates. Therefore among the 4 bacterial strains the *Klebsiella* sp. AJG3 was the most promising candidate for ACC deaminase producing bacterium. It is necessary then to check whether *Klebsiella* sp. AJG3 is able to support the plant growth in the stress condition by examining the ACC reduction.

In this research, bacterial isolates produced ACC deaminase. Earlier studies have reported that beneficial ACC deaminase producing bacteria can infected the seeds, and can absorb some of the ACC secreted by the plant and degrade it by the ACC deaminase enzyme. It is believed that the bacteria can reduces the stress caused by salinity and increasing the elongation of the root because produce ACC deaminase enzyme at the root of the plant and act as a reservoir for ACC, so reduces the level of exogenous ethylene [20].

The ACC deaminase–producing isolates founded in this study led to the conclusion that host plants are a selective agent for specific bacterial species that select partners from soil or seeds. The ability of such bacteria to colonize both external and internal plant tissues is a desirable feature for inoculation of seeds because such bacteria have a greater chance of having an effect on host growth [21].

These bacteria may be used to enhance maize, cowpea and groundnut plant growth and resistance under salinity stress conditions. The PGP characteristics of these bacteria should to analysis to know the activity of these bacteria somehow reduce the negative effect of salinity to the plant.
4. Conclusion
From this work we have successfully isolate and identified 4 ACC deaminase producing bacterial strains. Among them, *Klebsiella* sp. AJG3, *Rhizobium* sp. RJG6, and *Agrobacterium* sp. ATL 5, showed promising ACC deaminase activity and therefore it could be as a good candidate for further application in plant growth promoting in stress conditions especially in saline stress condition.

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6. References
[1] Glick B R 2014 *Microbiological Research* 169: 30–39.
[2] Ladeiro B 2012 *Journal of Botany* 12: 1-7.
[3] Sparks D L 2003 Academic Press, San Diego.
[4] Ali S, Zulfikar V, Sandhya, Rao L V 2014 *Annals of Microbiology* 2: 493–502.
[5] Waitling K 2007 *Natural Resource and Water*, Queens land
[6] Chen W, Tang Y, Mori K, Wu X 2012 *Journal Aquatic Biology* 15: 99-110.
[7] Simarmata R, Ngadiman, Rohman M S, Simanjuntak P 2019 *Biotropic* 3(1): 13 – 23
[8] Penrose D M, Glick B R 2003 *The Journal of Physiologia Plantarum* 118: 10 – 15.
[9] Bradford M M 1976 *Analytical Biochemistry*, 72(1-2): 248-254.
[10] Jorgensen JH, Turnidge JD, Washington JA 1999 *Manual of clinical microbiology*. Washington DC: ASM Press. 1:1526-43
[11] Kumar S, Stecher G, Tamura K 2016 *Molecular Biology and Evolution* 33(7): 1870-1874.
[12] Bal H B, Das S, Dangar T K, Adhya 2013 *Journal of Basic Microbiology* 53: 972-984.
[13] Mayak S, Tiros T, Glick B R 2004 *Plant physiology and Biochemistry* 42(6), 565-572.
[14] Yaish M W, Al-Lawati A, Jana G A, Patankar H V, Glick B R 2016 *PLoS One* 11(7), e0159007.
[15] Hardoim P R, van Overbeek L S, van Elsas J D 2008 *Trends in microbiology* 16(10), 463-471.
[16] Shrivastava P, Kumar R 2013 *Saudi Journal of Biological Sciences* 2: 123-131.
[17] Duan J, Muller K M, Charles T C, Vesely S, Glick B R 2009 *Microbial Ecology* 57: 423–436.
[18] Zheng P L, Zhang L, Tian L, Zhang F, Chen, Li B Z, Cui Z 2014 *Pakistan Journal of Botany* 5: 1905-1910.
[19] Hao Y T C, Charles, Glick B R 2011 *Canadian Journal of Microbiology* 286: 278–286.
[20] Simarmata R, Ngadiman, Rohman S, Simanjuntak P 2018 *Annales Bogorienses* 22(2): 81-93.
[21] Etesami H, Alikhani H A, Hosseini H M 2015 *Bacterial metabolites in sustainable agroecosystem*, Springer, Cham :183-258.