Production of indole acetic acid by *Enterobacter cloacea* H3 isolated from Mungbean (*Vigna radiata*) and its potential supporting the growth of soybean seedling

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Abstract. Many plant-associated bacteria synthesize the phytohormone indole acetic acid (IAA). Three bacterial isolates (H1, H2 and H3) were isolated from root nodules of Mungbean (*Vigna radiata*) and tested for their productivity of indole acetic acid by spectrophotometric method. Isolate H3 showed the highest IAA production (12.28 µg/ml) in culture medium supplemented with L-Tryptophan. Based on 16S rRNA gene analysis, isolate H3 was identified as *Enterobacter cloacae*. The cultures condition optimized for maximum IAA production by using different pH and tryptophan concentrations. IAA production of isolate H3 was maximum at pH 6 and 5 mg/ml of tryptophan concentration. The highest IAA producing isolate selected for determining its capability and compatibility to support the germination of soybean. The results showed that germination percentage and rate of soybean were not significantly different between control and isolate H3 treatment.

1. Introduction

Plant Growth Promoting Rhizobacteria (PGPR) is bacteria that are able colonize the roots or rhizosphere of plants following inoculation into seedlings or rhizosphere and enhance plant growth [1]. PGPR may promote plant growth by direct and indirect mechanism. One of the PGPR mechanisms in promoting plant growth is synthesizing phytohormones such as indole-3-acetic acid (IAA). IAA influence of plant function such as plant cell division, extension and differentiation, stimulates seed and tuber germination, increases the rate of xylem and root development, controls processes of vegetative growth, initiates lateral and adventitious root formation mediates responses to light, gravity and florescence, affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions [2].

Many plant-associated bacteria synthesize the phytohormone indole acetic acid (IAA). The microorganisms isolated from rhizosphere region and plant roots of various crops have an ability to produce indole acetic acid as secondary metabolites due to rich supply of substrates. Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals that are involved in nutrient uptake [3].

Mungbean (*Vigna radiata*) is one of the important legumes and well-known economic crops. It often used in crop rotation and mixed cropping system to provide nitrogen, improve soil fertility and control pest and diseases. As leguminous plant, mungbean could be nodulated by Rhizobia, causing the formation of nodules and establishing a nitrogen fixing system. In addition to symbiotic nitrogen
fixation, rhizobia also plant growth regulator including auxin. Auxin biosynthesis by rhizobia is increased many folds in supplementation with suitable precursor L-tryptophan [4].

The objectives of this research are to study the IAA hormone production by bacterium isolated from Mungbean, to find out the cultural requirement for maximum IAA production and to determine effect of IAA hormone on seed germination of soybean.

2. Materials and Methods

2.1. Isolation and screening for IAA production
The fresh nodules collected from roots of Mungbean. Roots nodules of Mungbean obtained from Wonosari, Jogjakarta (DIY). Root nodules washed with running water, soaked in ethanol 95% for 5-10 seconds and washed thoroughly with sterile water 10 times. Nodules crushed with sterile glass rod after adding 1 ml sterile water. A loopful of inoculums was streak on to Yeast Mannitol Agar (YMA) medium with Congo Red and petridish were incubated at 28°C for 3 days.

Bacterial isolates grown on 10 mL of Nutrient Broth (NB) medium containing 0.5 mM L-tryptophan. These cultures incubated at 28°C with shaking at 150 rpm for 24 hours and then harvested by centrifugation at 10,000 rpm for 10 minutes. Two mL of the supernatant mixed with 2 mL of reagent Salkowsky. Optical Density (OD) read at λ 530 nm after 60 min. The appearance of pink colours indicated IAA production. The level of IAA produced estimated and compared with the IAA standard.

2.2. Culture optimization for the production of IAA
The isolate that produce the highest IAA, studied to identify the optimal condition for IAA production. The effect of pH on IAA production, studied by cultivating the isolate in NB medium containing L-tryptophan at different levels ranging from 3.0 – 9.0. The effect of L-tryptophan on IAA production studied using NB medium supplemented with L-tryptophan concentration of 1, 2, 3, 4 and 5 mg/mL.

2.3. Germination test
The highest IAA producing strain selected for germination test. Seeds of soybean var. Anjasmoro were soaked in 10 ml of bacterial suspension for 30 minutes. For control, seed were soaked in aquadest. The straw papers were soaked in water until all the pieces were wet and then drained. One sheet of plastic spread and then 3 pieces of straw papers put on it. Twenty-five seeds of soybean placed on straw papers, covered with two sheets of papers and then rolled. The rolls of paper placed in germinator with standing position. Observation done 4 days after planting, by counting the seeds of normal. Each treatment was four replicated.

Germination Percentage (G%) was recorded according to [5] by equation 1:

\[ G\% = \frac{\sum X_n \times 100\%}{N} \]  

\( X_n \): number of normal seedlings
\( N \): number of seedlings

Germination rate (GR) of seedlings determined by equation 2:

\[ GR = \frac{X_1 + (X_2 - X_1) + \ldots + (X_n - 1)}{Y_1 \quad Y_2 \quad Y_n} \]  

\( X_n \): The number of germinated seeds to the n-th day
\( Y_n \): The number of day from cultivation time until final time
2.4. Identification of IAA producing bacteria

Isolate H3 that produced the highest of IAA was identified by 16S rRNA gene sequence. One colony of isolate were taken with sterile toothpick then inserted into eppendorf tube containing 100 mL dH₂O and it was vortex. One mL suspension was used for the amplification with polymerase chain reaction (PCR) technique. PCR (TC 5000 TECHNE) of 16S RNA gene by using universal primer 520 F (5’-GTGCCAGCAGCCGCGG-3’) and 920 R (5’GTCAATTCCTTTGAGTTT-3’). A total volume of 50 µL of PCR volume were contain 1µL DNA template, 2 µL of primer for each forward and reverse, 2 µL of 2X KAPA taq ready mix (KAPA Bioystem) and ddH₂O 20 µL. Amplification was performed for 30 cycles that include initial denaturation stage at a temperature 96°C for 5 min, denaturation at a temperature 96°C for 30 sec, annealing at a temperature 55°C for 30 sec, extension at a temperature 72°C for 1 min, final extension at a temperature 72°C for 7 min. The PCR were checked by electrophoresis for 30 min with a voltage of 100V 500mA (BIO-RAD Mupid exU-75577 Advance Japan). Sequence analysis was conducted by comparing the sequences with GenBank database using Blastn in the National Center for Biotechnology Information (NCBI) to determine similarity of the sequence homology.

3. Results and Discussion

Bacteria isolates varied in their auxins producing with L-tryptophan supplementation to the medium. Three isolates selected in its ability to produce the IAA hormone. The results on the production of growth promoting hormone indicated that only one isolate was able to produce IAA. Isolate H3 produced the highest amount of IAA (12.28 µg/mL), followed by H2 and H1 (Table 1). This could be due to better utilization of medium components for IAA production by isolate H3 compared to other isolates.

| Code of isolates | Production of IAA (µg/ml) |
|------------------|--------------------------|
| H1               | 1.45                     |
| H2               | 1.83                     |
| H3               | 12.28                    |

Various studies have conducted on Plant-Growth Promoting Bacteria (PGPR), since it can used as biofertilizer to promote sustainable agricultural practices. PGPR colonize the plant roots, they are able to promote plant growth based on the ability to excrete plant growth regulator such as IAA [6]. All three isolates are positive for IAA production but only one isolate H3 selected as potential IAA producers.

Based on molecular identification, isolate H3 showed 99% similarity with Enterobacter cloacae sequences from GenBank (Table 2). The presence of 99-100% similarity indicated H3 may have chromosome number, genom size and gene function same as with E. cloacae.

| Description                  | Total Score | Query coverage | E.value | Max ident |
|------------------------------|-------------|----------------|---------|-----------|
| Enterobacter cloacae strain S20504 | 2737        | 99%            | 0.0     | 99%       |
| Enterobacter sp. MACL08B     | 2719        | 99%            | 0.0     | 99%       |
| Enterobacter cloacae strain LSRC11 | 2715        | 99%            | 0.0     | 99%       |
| Bacterium MJ09               | 2713        | 99%            | 0.0     | 99%       |
| Enterobacter sp. d8(2011)    | 2713        | 99%            | 0.0     | 99%       |
IAA production of *E. cloacae* H3 is lower than *E. cloacae* NII-0931 (104.8 µg/mL) that isolated from non-rhizosphere soil [7] and *E. cloacae* MSR1 (112 µg/mL) that isolated from roots of non-nodulating of *Medicago sativa* [8]. It has been reported that *E. cloacae* UW 5 produce IAA when exogenous tryptophan was available and IAA accumulates in culture medium during stationary phase [9]. Some rhizobacteria known as plant growth promoting rhizobacteria (PGPR), plays an important role in enhancing plant growth through nitrogen fixation, phosphate solubilisation, phytohormones production, siderophore production and production of pathogen cell wall-degrading enzymes to prevent plant pathogens. Others study showed that *E. asburiae*, *E. cancerogenus* and *E. cloacae* support plant growth and is considered PGPR [10].

![Figure 1. Effect of different concentration of L-tryptophan on IAA production](image1.png)

The concentration of IAA secreted by isolates was different each other. It has been reported that IAA production by bacteria can vary among different species and strains and it is influenced by culture condition, growth stage and substrate availability [11]. The effect of different concentration of L-tryptophan revealed that the maximum growth and IAA production observed in all the three isolates at 5 mg/ml concentration (Figure 1). The production of IAA increased with concentration addition of tryptophan.

![Figure 2. Effect of different levels of pH on IAA production](image2.png)
The variations in the IAA production at different concentrations of L-tryptophan by the all isolates indicate the intrinsic ability of the isolates towards the IAA production. Though the bacteria were able to produce IAA in the absence of L-tryptophan, they produced higher amount of IAA in culture media supplemented with tryptophan. The function of L-tryptophan is as precursor for biosynthesis in plants and microorganisms [12]. The production of IAA was much less as compared to the amount of L-tryptophan applied. This was probably due to the utilization of this essential amino acid partly in protein synthesis and partly for the formation of other indole compounds in addition to IAA [13]. The impact of different levels of pH (3 – 9) was determined. All the three isolates produced maximum IAA at pH 6 (Figure 2). Acidic or alkaline pH found to be unfavourable to IAA production.

A significant correlation also observed between bacterial growth and IAA production. The pH affects the function of enzyme systems and the solubility of many substances that are important for bacteria growth [14]. The effect of inoculation on germination seed shown in Table 3. After 4 days of inoculation showed that germination, percentage and rate of treatment were not significantly different from control.

**Table 3. Effect of IAA producing bacteria on germination of soybean**

| Treatments | Germination Percentage (%) | Germination Rate (%/et mal) |
|------------|----------------------------|-----------------------------|
| Control    | 95                         | 30                          |
| H3         | 93                         | 30                          |

In our study, it found that was no inoculants effect on germination seeds. It might be due to the isolate disability to synthesize seed germination hormone. According to [15], percent germination not influenced but other parameters affected by inoculation. The PGPR effect inducted by rhizobacteria on the seeds or plants depends on species and strain of microorganism, culture conditions and varieties of seeds or plants.

**4. Conclusion**

Three bacterial isolates from root nodules of Mungbean were tested for their productivity of Indole Acetic Acid. Isolate H3 that produced the highest of IAA was identified as *Enterobacter cloacae*. IAA production of isolate H3 was maximum at pH 6 and 5 mg/mL of tryptophan concentration. The germination percent and rate of soybean that inoculated by isolate H3 were not significantly different from control.

**5. References**

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