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ISOLATION AND CHARACTERIZATION OF INDOLE-3-ACETIC ACID PRODUCING BACTERIA FROM COW URINE

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Abstract. *Cow urine contains urea as nitrogen source, therefore it can be expected to isolate the beneficial bacteria for plants, for example indole-3-acetic acid (IAA) or auxin producing bacteria. The objective of research was to obtain IAA producing bacteria from cow urine, to characterize bacterial isolate, and to measure its ability to stimulate the growth of green bean seedlings (Vigna radiata). The methods used in this study were collecting urine from cow cattle, obtaining IAA-producing bacteria from urine, measuring IAA using Salkowski method, and applying selected bacterial supernatants on green bean seedling plants. The number of IAA producing bacteria that was successfully purified was 18 isolates. There are five isolates, namely US 5, BS1, BS 2, BS 4 and BS 5 which have the ability to solubelize phosphate on Pikovskaya agar. The five isolates were also able to fix free nitrogen on N Free media and did not show hypersensitivity on tobacco leaves. The results of the growth of isolates in blood agar showed positive for US 5 and BS 2 as beta hemolysin producers. Furthermore, isolate BS 4 was chosen to produce exogenous IAA quantitatively. Isolate BS 4 produced IAA 6.364 ppm at the 45 h incubation at stationary phase. The use of BS 4 supernatant on green bean seedlings showed an effect on plant height and lateral root length better than control (without treatment) on 6 days after planting. Morphological characteristic of isolate BS 4 was rod shape, Gram positive, endospore producing, aerobic, and had similarity with genus Bacillus.*

Keywords: *bacteria, cow urine, green bean, indole-3-acetic acid*

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INTRODUCTION

Cow urine has been widely used as a liquid organic fertilizer through a fermentation process by microorganisms. Cows weighing ± 250 kg produce urine 7.5-9.0 liters per day (Adijaya et al., 2008). In addition to being used as fertilizer, cow urine contain N, P and K which is larger than manure and contains substances that can be used to promote plant growth, one of which is an Indole-3-Acetic Acid (IAA) or auxin. IAA plays an important role in organogenesis, cell elongation, root growth, lateral root initiation and initiation of plant primordial organs (Benjamins & Scheres, 2008). IAA endogenously produced by plants and is also exogenously produced by bacteria. IAA substances in most plants, only a small percentage are unbound (free). Free IAA is the active form of IAA, while bound

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IAA is involved in transport, storage and protection from enzyme degradation (Spaepen et al., 2007). Some IAA producing bacteria, viz. *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, *Pseudomonas putida* (Torres-Rubio et al., 2000), *Beijerinckia fluminensis*, *Caulobacter segnis*, *Rhizobium grahamii* (Harca et al., 2014), *Serratia marcescens* and *Bacillus thuringiensis* (Astriani et al., 2016).

The exogenous IAA has greater effect in plant growth which can trigger root hair growth by increasing the number and length of primary roots and lateral roots in plants (Duca et al., 2014). In addition, IAA produced by bacteria can directly stimulate the extension of root cells or tissues (Patten & Glick, 2002). Garuda et al. (2014) reported that IAA-producing bacteria, such as Bacillus, Pseudomonas, Azospirillum and Azotobacter also have the ability to dissolve phosphates and to fix nitrogen.

A number of microorganisms could involve in cow urine fermentation, including IAA producing microorganisms. Currently, it has not been reported related to IAA-producing bacteria isolated from cow urine which has been measured in their ability to produce IAA. The objective of research was to obtain IAA producing bacteria from cow urine, to characterize the bacterial isolates and to measure their ability to stimulate the growth of green bean seedlings (*Vigna radiata*).

MATERIALS AND METHODS

Sampling

There were three types of samples used, namely fresh cow urine released in the morning, taken as much as 100 mL and collected in sterile dark-colored glass bottles. The second, urine that has been incubated for one night in a sterile bottle (overnight urine) and then fermented urine that has been through the fermentation process is taken from the reservoir and then put in a bottle. All samples were obtained from PT Swen Inovasi Transfer, Jl. Cikerti No.25 Ciomas, Bogor 16610. All samples are maintained for further analysis in Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University.

Isolation of IAA Producing Bacteria

One milliliter of each sample was taken and diluted from 10^{-1} to 10^{-4} using physiological saline (NaCl 0.85%). A total of 0.1 mL of the mixture was spread on Nutrient Agar (NA) containing 1 mM L-tryptophan and incubated for 24 h at an incubator at 38°C. The growing bacterial colonies were counted using the total plate count (TPC) method and then the colonies with different morphologies were purified using the quadrant method on NA media containing 1 mM L-tryptophan. A single colony obtained is then maintained on slant agar media (Harca et al., 2014).

Screening and Selection of Potential Bacterial Isolates

Screening and selection of potential IAA bacterial isolates were carried out based on the colorimetric method using the Salkowski-containing reagent 150 mL H_2SO_4, 7.5 mL $FeCl_3.6H_2O$ 0.5 M and 250 mL sterile water. The bacterial isolates obtained were measured for their ability to produce IAA. Each bacterial isolate was inoculated into Nutrient Broth (NB) containing 1 mM L-tryptophan and incubated in 100 rpm rotary incubator for 8 h at room temperature (± 27°C), then 2 mL of culture inoculated into 18 mL NB media at Erlenmeyer. The culture was incubated at 100 rpm for 24 h at room temperature and then centrifuged at 10.000 rpm (Galaxy 7D centrifuge) for 10 minutes. A total of

1 mL the supernatant was homogenated with 4 mL of Salkowski reagent and incubated for 15 minutes in the dark. The absorbance was measured by using the Genesys spectrophotometer at 520 nm. IAA concentrations of the bacterial isolate were determined based on the IAA standard curve with a range of 0-100 ppm (Astriani et al., 2016).

Characterization and Identification of IAA Producing Bacteria

Identification of bacteria was carried out based on morphological of bacterial colonies and cell form, gram staining and endospores staining. Characterization of bacterial isolates based on the characteristic of pathogenicity, hypersensitifity, the ability of phosphate solubilization and nitrogen fixing.

The Pathogenicity Test

Pathogenicity tests were carried out using blood agar of 24 h isolates. Bacterial isolates are streaked on blood agar media and then observations were carried out after 24 h. Hemolysin producing isolates and suspected pathogens are indicated by the presence of clear or greenish zones around colony of isolates.

Hypersensitivity Test Using Tobacco Leaves

One loop of 24 h isolate was inoculated into 25 mL Nutrient broth (NB) containing 1 mM L-tryptophan and incubated with an agitation of 100 rpm to a concentration of ± 10^8 CFU/mL. Distilled water and media without culture were used as a negative control and *Pseudomonas syringae* as a positive control. One mL of culture and each control was injected into mesophyll between the leaf veins of the tobacco leaves. Hypersensitivity reactions or necrotic symptoms were observed at 48 h after injection. Distilled water was injected as negative control while the positive control was *Pseudomonas syringae*. Bacterial isolates which showed no necrotic symptoms (negative reaction) in the tobacco leaves were then used to further analysis (Wahyudi et al., 2011).

Estimation of Phosphate Solubilization and Nitrogen Fixation

One loop of isolate was spotted on Pikovskaya agar media (10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.0025 g $MnSO_4 \cdot H_2O$, 0.0025 g $FeSO_4 \cdot 7H_2O$, and 20 g bacto agar, pH 7 for 1 L medium) and incubated for 7 days at room temperature. The clear zone formed around the colony was measured and then calculated for phosphate solubilization index that is the diameter of clear zone reduced by the diameter of the colony. The resulted value was divided by diameter of colony. Estimation of nitrogen fixation was carried out by growing selected isolates on N-free agar media and incubated for 7 days. Nitrogen fixing isolates could grow well on N-free media (Fitriyanti et al., 2017).

Growth Curve and IAA Synthesis

The measurement of bacterial growth and IAA production was carried out only a chosen isolate which produced the highest IAA on screening stage. One loop of 24 h selected isolate was inoculated into 25 mL NB containing 1 mM L- tryptophan and incubated in shaking incubator until the cells reached 10^8 CFU/mL. 2 mL culture was inoculated into 100 mL NB containing 1 mM L- tryptophan and incubated in shaking incubator at 100 rpm in room temperature. The measurement of cell density and IAA production was performed every 3 h by using a spectrophotometer with 600 nm wavelength for 48 h (Astriani et al., 2016).

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Test of Bacterial IAA on Green Bean Seedlings

Green beans were washed with water to remove the impurities and soaked in bacterial supernatant for 1 hour. The sinking seeds are collected and planted on sterilized planting media. The treatments used were control (distilled water) and the supernatant containing IAA from the selected isolate. Each treatment was carried out with ten replications. The parameters measurement i.e. plant height, main root length and number of lateral roots on 6 days after planting. The results were analyzed statistically using one-way ANOVA.

RESULTS AND DISCUSSION

IAA Producing Bacteria from Cow Urine

Isolation of IAA producing bacteria from fresh urine samples, overnight urine and fermented urine was carried out using nutrient agar (NA) containing 1 mM L-tryptophan. A total of 18 isolates produced exogenous IAA obtained from the three urine samples. There were 10 isolates were obtained from fresh urine, 2 isolates from overnight urine and 6 isolates from fermented urine (Table 1). All isolates were quantitatively measured in IAA producing at NB medium containing 1 mM L-tryptophan for 24 h of incubation. Then 6 Isolates were selected which IAA production more than 1 ppm, viz. US 5, US 10, BS 1, BS2, BS 4 and BS 5.

Table 1. IAA production of 18 bacteria isolated from cow urine

No	Isolate	Source	Concentration of IAA (ppm)
1	US 1	Fresh urine	0.321
2	US 2	Fresh urine	0.689
3	US 3	Fresh urine	0.389
4	US 4	Fresh urine	0.605
5	US 5	Fresh urine	2.155
6	US 6	Fresh urine	0.456
7	US 7	Fresh urine	0.609
8	US 8	Fresh urine	0.629
9	US 9	Fresh urine	0.590
10	US 10	Fresh urine	1.787
11	UOS 1	Urine stored overnight	0.412
12	UOS 2	Urine stored overnight	0.316
13	BS 1	Fermented urine	4.082
14	BS 2	Fermented urine	3.096
15	BS 3	Fermented urine	0.084
16	BS 4	Fermented urine	6.808
17	BS 5	Fermented urine	1.853
18	BS 6	Fermented urine	0.045

Characterization of IAA Producing Bacteria

Six isolates were tested growing on Pikovskaya media containing $Ca_3(PO_4)_2$ and N free media, respectively. Solubilizing phosphate bacteria was surrounded by a clear zone around the colonies. N-fixing bacterial isolates could grow well on N-free media, whereas non N-fixing isolate could not grow on the media (Figure 1). Isolate BS 4 showed the highest phosphate solubilization index, which is equal to 1.00 (Table 2). There was only one isolate could not dissolve the phosphate and fix the nitrogen, viz. US 10.

All isolates showed negative hypersensitivity symptoms on tobacco leaf after 48 h of incubation. These assumed that all isolates were not pathogenic in plants (Figure 2). Further characterization showed that two bacterial isolates, US 5 and BS2, produced hemolysin. Clear zone or greenish zone around bacterial colony indicated lysis of red blood cell in blood agar media (Table 2).

(a) (b) (c)

Figure 1. The growth of six bacterial isolates on media (a) free of N, (b) Pikovskaya and (c) blood agar.

(a) (b) (c)

Figure 2. Hypersensitivity testing on tobacco leaves (a) bacterial tested, (b) negative control (distilled water) (c) positive control (*Pseudomonas syringae*). White patches showed necrosis (positive hypersensitivity)

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Table 2. Characterization of the six tests bacterial isolates

Isolate	Concentration of IAA (ppm)	Phosphate dissolution index (IP)	The ability to fix N qualitatively	Hypersensitivity reaction	Haemolysin producer
US 5	2.155	0.13	+	-	+
US 10	1.787	0.00	-	-	-
BS 1	4.082	0.16	+	-	-
BS 2	3.096	0.18	+	-	+
BS 4	6.808	1.00	+	-	-
BS 5	1.853	0.20	+	-	-

+ positive reaction, - negative reaction

The Growth and Synthesis of IAA from Selected Bacteria

The growth of isolate BS 4 increased from the initial inoculation to 33 h incubation at stationary phase. After 6 h incubations, the IAA production increased 35 times compared to the initial incubation of 3.5 ppm and then there was a slight decrease until the 15th hour. The highest IAA production at 45 h was 6.364 ppm (Figure 3).

The Use of Bacterial IAA in Green Bean Plant Seedling

Parameter observations were carried out when the green bean plant was 6 days after planting. The use of supernatant from BS 4 isolates significantly affected plant height and the number of lateral roots compared to control (distilled water) (Table 3).

A total of 18 isolates of IAA-producing bacteria were isolated from fresh cow urine, urine stored overnight and the final product of fermented urine. The number of IAA-producing bacteria isolated from fresh cow urine was higher than urine stored overnight and fermented urine. Shah et al. (2011) reported that fresh cow urine contained anti-bacterial substances which could inhibit the pathogenic bacteria such as *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus*.

Isolation of IAA producing bacteria was carried out using NA media containing 1 mM L-tryptophan. L-tryptophan was known as IAA precursor (Kafrawi et al., 2014). The selection of isolates was carried out based on the highest of IAA production, followed by the pathogenicity test on blood agar. Isolates US 5 and BS 2 revealed beta-hemolytic positive which produced clear zone around the colony (Ariyanti et al., 2011). Both isolates showed tendency to be pathogenic in human and animal.

Isolates US 5, US 10, BS 1, BS 2, BS 4 and BS 5 showed negative hypersensitivity test on tobacco leaves. Hypersensitivity reactions are fast and localized cell death programs. Induction of hypersensitive reactions and pathogenicity is influenced by hrp gene (hypersensitive and pathogenicity) which is commonly found in Gram negative plant pathogens such as *Pseudomonas syringae* (Wahyudi et al., 2011). Furthermore, each of isolates US 5, BS 1, BS 2, BS 4 and BS 5 could dissolve phosphate in Pikovskaya agar media and were able to grow on nitrogen-free media. Phosphate is an element found in nucleic acids and ATP as energy sources. N fixing isolates provided N to synthesize amino acids and as constituents of cell proteins.

Isolate BS 4 produced the highest IAA at 45 h incubation at stationary phase with a concentration of 6,364 ppm (Figure 3). Based

on the characterization and its potency, isolate BS 4 was selected for further analysis. Morphological characteristic of isolate BS 4 was rod shape, Gram positive and endospore producing (Figure 4). Based on morphological characteristic, this isolate is assumed belong to the genus Bacillus. Analysis of physiological and molecular characteristics needs to be confirmed the species of the isolate. Anandham et al. (2015) reported that using the 16S rRNA gene for molecular identification of bacterial isolate, cow urine contained 100% of the genus Bacillus (Firmibacteria). *Bacillus* sp. known as a IAA producer, solubilizing phosphate and biocontrol agents (Compant et al., 2005).

The use of exogenous IAA produced from isolate BS on green bean seedlings showed a better effect on plant height, primary root length and number of lateral roots than control (distilled water). Concentration of exogenous IAA above 0.5-26.5 ppm can directly stimulate the extension of primary roots and lateral root growth when bacteria are associated with plants (Patten & Glick, 2002).

Eighteen isolates were obtained from cow urine, BS 4 isolate could produce exogenous Indole-3-acetic acid (IAA). BS 4 isolate had characteristic of morphological cell like Bacillus, but further analysis of physiological and molecular characteristics needs to be confirmed the species of the isolate. The isolate BS 4 could dissolve phosphate, fixed nitrogen and did not pathogenic on plant and animals or humans. The isolate suggested could be used as biological fertilizer for horticulture plants. It is recommended to perform further study on testing IAA of *Bacillus* sp. BS4 on green bean plant until reproductive stage.

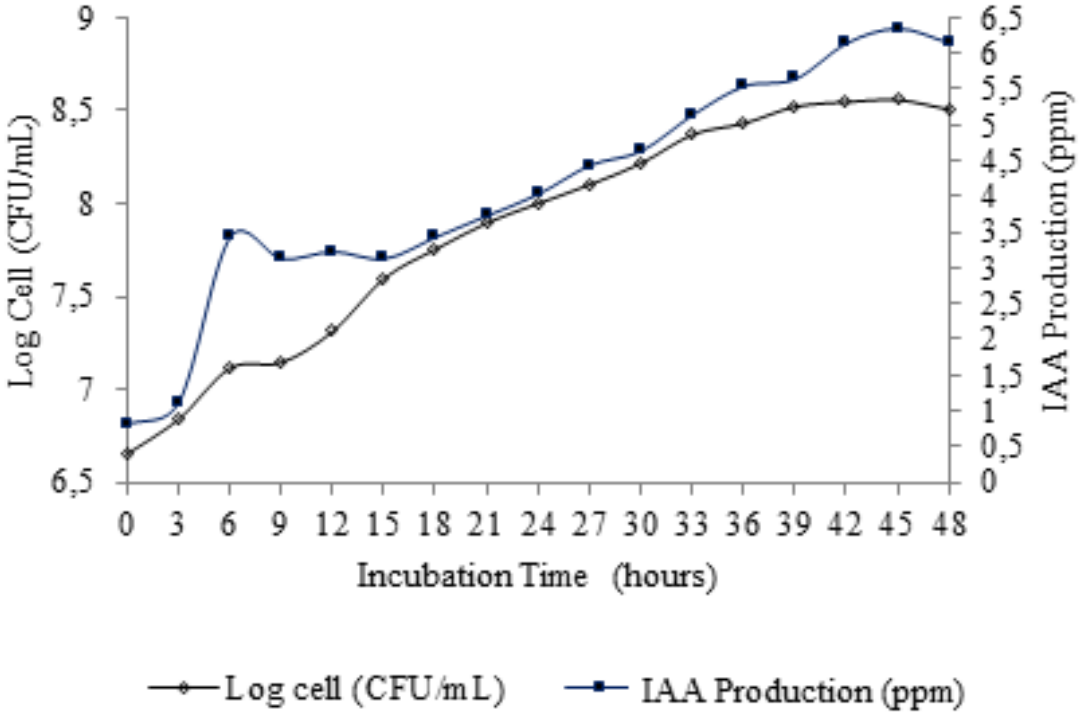

Figure 3. Growth curve and IAA production of isolate BS 4 on NB medium containing 1 mM L-tryptophan for 48 h incubation at room temperature.

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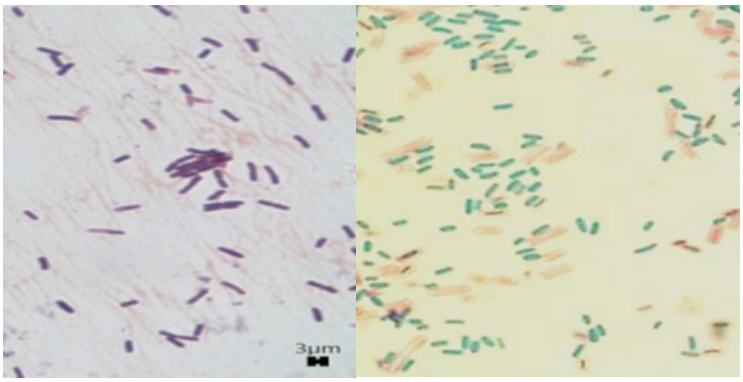
Table 3. Effect of IAA-producing isolates on green bean seedling plants

Treatments	Length of plant (cm)	Length of primary root (cm)	Number of lateral roots
Distilled water (control)	14.81 ± 1.19^{b}	5.82 ± 1.92^{ab}	28.40 ± 6.26^{b}
BS 4	15.86 ± 1.08^{a}	8.69 ± 1.64^{a}	33.30 ± 6.87^{a}

*Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test (α = 0.05). Data were the mean value of six replication ± deviation standard.



(a) (b)

Figure 4. (a) Gram and (b) endospore staining of isolate BS4 at 1000x magnification. Green colour structure is endospore.

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