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Ability Test of IAA (Indole-3-Acetic Acid) Hormone-Producing Endophytic Bacteria from Lamongan Mangrove

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ABSTRACT

Most of the plant-associated bacteria can synthesize active biological components of phytohormones such as auxin. This study aimed to examine the potency of 61 endophytic bacteria isolates from the mangroves at Kutang Beach, Lamongan in producing IAA hormone and to identify types of isolates effecting the concentration of IAA, morphological characteristics of isolates, as well as endophytic bacterial species that have the most potential to produce IAA hormone. Screening of endophytic bacteria isolates was performed using the colorimetric method and the production of IAA was carried out using the spectrophotometric method. IAA production by endophytic bacteria was analyzed descriptively and statistically. One-Way ANOVA was employed to determine the effect of the isolate type on the concentration of IAA. The most potential isolates to produce IAA hormone are identified by 16s rRNA gene marker. The screening results showed that 12 isolates of endophytic bacteria have the potential to produce IAA hormones (2.0-9.3 ppm), coded with LMG 7, 15, 31, 32, 43, 53, 54, 55, 56, 57, 62, and 63. The results of the One-Way ANOVA test suggested that the type of isolates did not affect the concentration of IAA produced by endophytic bacteria. The twelve isolates had different morphological characters and those were Gram-positive bacilli with cell sizes ranging from 1.5 µm - 3 µm. The highest concentration of IAA was produced by LMG 15 (9.3 ppm). LMG 15 was identified as *Bacillus cereus* strain LMG 15, having 99.33% similarity *to Bacillus cereus* strain IAM 12605.

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INTRODUCTION

Indonesia has a very large agricultural sector. Fertilizer accounts for 20% of the success of agricultural production. Currently, modern farmers are very dependent on the use of synthetic fertilizers to obtain optimum crop productivity. The continuous use of synthetic fertilizers since the 1970s has started to have negative environmental impacts, such as the decrease in soil organic matter content, soil permeability, soil microbial populations, and soil vulnerability to erosion (Herdiyanto *et al.*, 2015). In addition, farmers face the inadequate provision of subsidized fertilizer. In December 1998, the Indonesian Government removed subsidies for fertilizer, which made farmers unable to buy non-subsidized fertilizers due to the

high prices. To avoid soil damage due to the use of synthetic fertilizers and the current high price of fertilizers, researchers attempt to develop a biofertilizer.

Biofertilizer contains live microorganisms for plant nutrients (Herdiyanto *et al.*, 2015). Microorganisms contained in biofertilizers are generally groups of N-fixing microorganisms, phosphate solubilizers, decomposers of organic matter, and phytohormones-producing bacteria such as gibberellins, cytokinins, and IAA (Indole-3-Acetic Acid) (Kartikawati *et al.*, 2017). IAA hormone is an endogenous auxin that plays a role in cell enlargement and the formation of xylem and phloem tissue, inhibits the growth of side shoots, stimulates abscission, and also affects the development and elongation of roots. In preparing the biofertilizer formula, the researchers tried to find potential microbes that produce IAA hormones by exploring various places. Bacteria that have ability to produce IAA consist of soil bacteria (Apine and Jadhav, 2011), epiphytes, endophytes (Duca et al., 2014; Liu et al., 2017; White et al., 2019), marine bacteria (Duca et al., 2014), meliotropes (Duca et al., 2014) and cyanobacteria (Sergeeva et al., 2002). Endophytic microbes can be isolated from plant parts. Each higher plant can contain endophytic microbes that have the potential to produce secondary metabolites which are assumed to be the result of coevolution or genetic transfer from their host plants into endophytic microbes (Radji, 2005).

Exploration of endophytic microbes from mangroves in Indonesia has not been widely studied. Oktafiyanto *et al.* (2017) have succeeded in isolating endophytic bacteria from mangrove forests originating from the coast of Indramayu, Jakarta, Yogyakarta, and Banyuwangi. The result suggests that endophytic bacteria isolates can be used as biocontrols for wilt disease caused by *Ralstonia solanacearum* as well as *Meloidogyne incognita*, the main pathogen that causes stunted plants, easy wilting, leaves yellowing, and roots ringing in tomato.

There were not many studies that revealed the potential of endophytic bacteria in mangroves to produce IAA hormones. Irawan *et al.* (2019) have succeeded in isolating 61 endophytic bacteria from the stems, leaves, and roots of the mangroves at Kutang Beach, Lamongan. This study aimed to determine the potential of those 61 isolates to produce IAA hormones, to know the types of isolates affecting the concentration produced, to characterize the macroscopic and microscopic morphology of the isolates, and to identify the most potential endophytic bacteria species in producing IAA hormone. The most potential IAA producing bacteria can be used as one of the constituents for biofertilizer formula as an alternative way in overcoming problems caused by the use of chemical fertilizers.

MATERIALS AND METHODS

Rejuvenation of endophyte bacteria isolates

Sixty-one isolates of pure endophytic bacteria were rejuvenated in slanted NA media, then incubated at 37°C for 24 hours.

Screening of IAA hormone-producing endophytic bacteria isolates

Screening of 61 isolates of endophytic bacteria that have been isolated from the mangrove's plants at Kutang Beach was based on their ability to produce IAA hormone. Each isolate was cultured into bottles containing 24 mL of NB media (700 ppm L-Tryptophan) and incubated at room temperature for 24 hours. Fifteen mL of isolate culture was centrifuged for 20 minutes at 5,000 rpm to obtain the supernatant. A total of 1 mL of the supernatant was reacted with 4 mL of Salkowski's reagent and incubated in a dark room for 15 minutes. IAA hormone-producing bacteria was seen by observing the color change in the supernatant culture of bacterial isolates from yellow to pink or transparent pink.

Measurement of IAA concentration

The turbidity of the 61 bacterial isolates culture was equalized (OD = 0.5) at a wavelength of 580 nm. Each bacterial isolate was cultivated in the same way as the screening stage. The concentration of IAA produced by the isolates was measured using a spectrophotometer at a wavelength of 530 nm. The concentration of IAA was calculated after being compared with the standard IAA curve (Pattern and Glick, 2002 with modification). The IAA standard curve was made by creating a synthetic IAA stock solution with a concentration of 200 ppm, or 0.01 g synthetic IAA in 50 mL methanol. The IAA standard curve from the spectrophotometric results shows the relationship between the IAA standard solution (x) and its absorbance (y). The equation obtained was as follows: $y = a + bx$ (y = absorbance; a = intercept; b = slope/regression coefficient; x = concentration).

Macroscopic and microscopic characterization of endophytic bacteria isolates

Bacterial isolates that have the potential to produce IAA hormone were characterized macroscopically by observing colony morphology which included color, shape, elevation, and colony margins. Meanwhile, microscopic characterization was carried out by Gram staining and measurement of bacterial cells.

Identification of bacteria species using 16S rRNA gene

Isolation of genomic DNA from bacterial isolates that have the most potential to produce IAA hormone was carried out according to the procedures listed in the Promega Genomic DNA Purification Kit Wizard. The results of genomic DNA observations can be seen on agarose gel electrophoresis with 1% agarose through the appearance of bands indicating that the DNA sample has been isolated.

The isolated DNA was then quantitatively tested using a spectrophotometer at a wavelength of 260 nm and 280 nm to determine its purity level. Measurement of isolated DNA was carried out at a wavelength of 260 nm, while measurement of protein was carried out at a wavelength of 280 nm. Then, PCR was performed on 16S rRNA gene using universal primers 27F and 1492 R.

The results of the DNA sequence of the 16S rRNA gene were trimmed using the Bioedit program to remove the low-quality DNA (<15%) and to remove the gap. Then, the forward and reverse sequences were aligned, the consensus was made, and the gaps in consensus are eliminated. The consensus is translated by changing the translation frame until it does not find a stop codon. Then, multiple sequence alignment was performed to determine the difference in nucleotide base sequences between samples (Kearse *et al.*, 2012). The DNA sequences of the bacterial samples were matched with the data on GeneBank using blast N via the NCBI (National Center for Biotechnology Information) website to determine the degree of similarity of the sampled nucleotide base sequences to the closest bacterial species.

Data Analysis

Data were analyzed using one-way ANOVA with a significant degree of 5%. The DNA samples of endophytic bacteria that have the most potential to produce IAA hormone were identified molecularly with the 16S rRNA gene marker. Data from 16S rRNA gene sequencing were analyzed using Bioedit 7.04 software to obtain nitrogen base consensus data. Then, DNA sequences of endophytic bacteria that have the most potential to produce IAA hormone were matched on GeneBank using blast N through the *NCBI (National Center for Biotechnology Information)* website.

RESULTS AND DISCUSSION

Screening of IAA hormone-producing endophytic bacteria isolates

Screening of endophytic bacterial isolates was tested qualitatively using the colorimetric method by observing the color change in the bacterial culture supernatant from yellow to pink after being reacted with Salkowski's reagent. Based on the results of the qualitative IAA screening test (Figure 1), there were 12 isolates of endophytic bacteria from the mangroves at Kutang Beach, Lamongan that produced the IAA hormone, coded with LMG (7, 15, 31, 32, 43, 53, 54, 55, 56, 57, 62, 63) with IAA concentrations ranging from 2 - 9.3 ppm. The color change in the bacterial culture supernatant from yellow to pink after being reacted with Salkowski's reagent occurred due to a reaction between IAA produced by bacteria and Fe contained in Salkowski's reagent, forming a complex compound $[Fe_2(OH)_2(IA)_4]$, indicated by IA, which is indole-3-acetate.

Figure 1. Results of qualitative test on potential IAA hormone-producing endophytic bacteria isolates

Table 1. IAA hormone concentration produced by endophytic bacteria

Codes	Average of IAA Concentration (ppm)
LMG 7	3.9
LMG 15	9.3
LMG 31	4.1
LMG 32	3.3
LMG 43	7.4
LMG 53	3.2
LMG 54	4.0
LMG 55	2.8
LMG 56	2.6
LMG 57	2.0
LMG 62	3.3
LMG 63	2.8

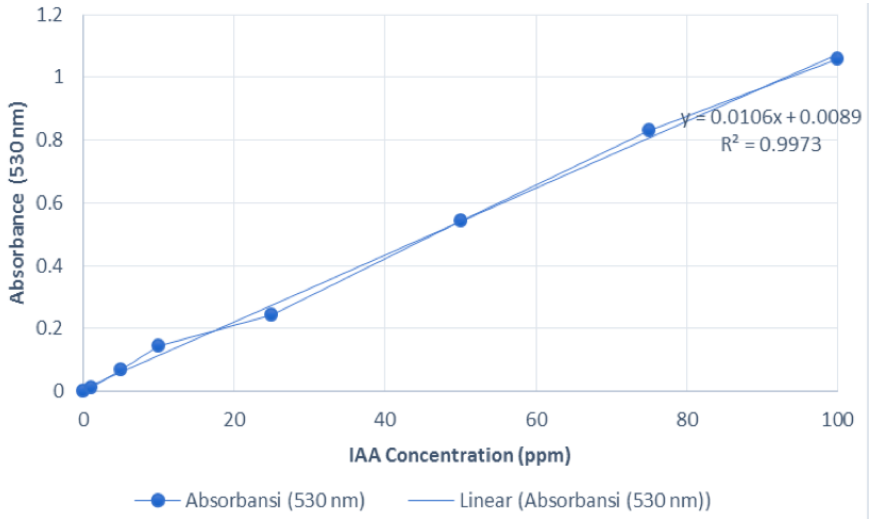

Figure 2. IAA Standard Curve

The production of IAA by isolates of potential endophytic bacteria

The production of IAA by 12 isolates of potential endophytic bacteria was quantitatively carried out using the spectrophotometric method. Table 1 show the concentration of IAA hormone produced by isolates of endophytic bacteria.

The IAA concentration of the endophytic bacterial samples was calculated based on the IAA standard curve. IAA standard curve was made to obtain an equation (value x) for calculating the IAA concentration from the bacterial isolate supernatant. The IAA concentration value obtained is expressed in ppm units. Figure 2 is the standard IAA curve that has been made, and the linear regression value is 0.9973.

Based on Table 1, it can be seen that the levels of IAA hormone produced by the endophytic bacteria from the mangroves at Kutang Beach varied greatly at 24 hours incubation time. The highest levels of IAA were produced by isolates coded LMG 15 at 9.3 ppm. And based on the results of the ANOVA test, the types of isolates have no significant effect on the concentration of IAA produced. However, the isolate with the code LMG 15 was identified as the best isolate because it was able to produce IAA with the highest concentration at 24 hours of incubation. It is possible that the bacteria were in a stationary phase, and thus the IAA produced was high. Ramadhani *et al.* (2020) report that bacteria with the highest concentration of IAA at a certain incubation period indicated that bacteria were in the stationary phase.

The difference in the concentration of IAA produced by bacteria is also influenced by the metabolic ability of each bacterium in utilizing media containing tryptophan. Tryptophan is an amino acid that functions as a precursor in the biosynthesis of IAA in plants and microorganisms (Patil, 2011). In general, IAA biosynthetic pathways are divided into two groups, trp-independent and trp-dependent. In the trp-independent pathway, bacteria do not use tryptophan as a precursor but instead use indole-3-glycerol phosphate (IGP). However, the intermediate pathway and the genes involved in trp-independent are still not defined. Meanwhile, in trp-dependent pathway, there are five biosynthetic pathways in bacteria. They are indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), tryptamine (TRA), tryptophan side-chain oxidase, and indole-3-acetonitrile (IAN), with IPA, IAM, and IAN become the main biosynthetic pathways of IAA in bacteria. In general, the bacteria capable of synthesizing IAA are Plant Growth Promoting Bacteria (PGPB) such as *Rhizobium*, *Bradyrhizobium*, *Bukholderia*, *Azotobacter*, *Acinetobacter*, *Bacillus*, and *Paenibacillus polymixy* (Ahemad et al., 2014).

Acuna *et al.* (2011) report that *Paenibacillus sp.* and *Bacillus sp.* are able to produce IAA with low concentrations (1.4-1.9 ppm) in a medium that does not contain tryptophan. The range of IAA concentrations produced by endophytic bacteria to increase plant growth varies greatly, from low to high concentrations. Bacteria that produce high concentrations of IAA can increase the growth and yield of wheat (Khalid *et al.*, 2004). Bacteria that produce constant low concentrations of IAA can also increase plant growth (Tsavkelova *et al.*, 2007). This is in accordance with Astriani *et al.* (2016) who reports that the rhizobacteria *Bacillus thuringiensis* producing an IAA of 3.99 ppm is capable of promoting root elongation in oil palm seeds. Lwin *et al.* (2012) report that *Bacillus sp.* with an IAA range from 53.1 ppm to an optimal 71.1 ppm is capable of spurring soybean growth. Wahyudi *et al.* (2011) add that *Bacillus sp.* with an IAA concentration of 15.2 ppm was able to increase the growth of shoots, primary roots, and lateral roots.

Macroscopic and microscopic characterization of endophytic bacteria isolates

Twelve isolates that have the potential to produce IAA hormone were characterized macroscopically and microscopically. The macroscopic characterizations observed include color, shape, elevation, edge, and consistency of endophytic bacterial colonies on Nutrient Agar (NA) media. While the microscopic characterization observed include cell shape and Gram staining using a 1000x magnification microscope. Figure 3 and Figure 4 show the colony and cell morphology of 12 potential IAA hormone-producing bacteria isolates. Meanwhile, macroscopic and microscopic characteristics of endophytic bacteria from the mangroves at Kutang Beach are presented in Table 2.

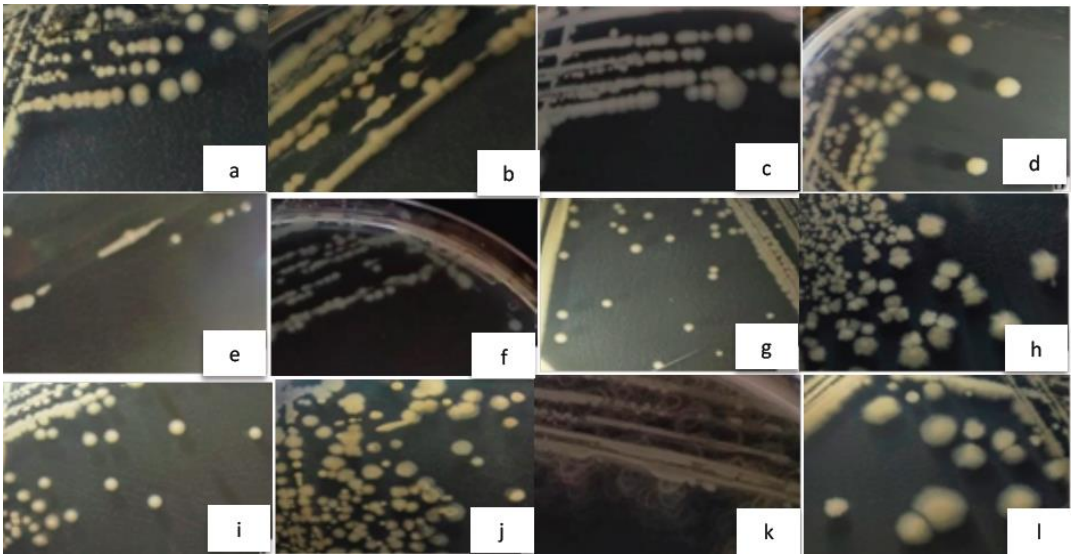

Figure 3. Colony morphology of endophytic bacteria isolates; a: LMG-7; b: LMG-15, c: LMG-31; d: LMG-32; e: LMG-43; f: LMG-53; g: LMG-54; h: LMG-55; i: LMG-56; j: LMG-57; k: LMG-62; l: LMG-63

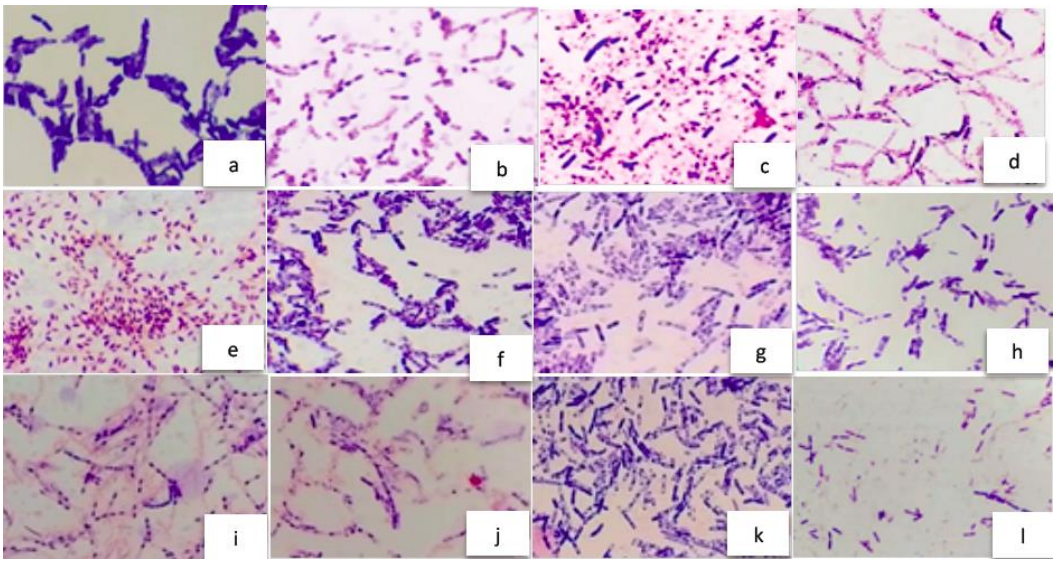

Figure 4 . Cell morphology of endophytic bacteria isolates; a: LMG-7; b: LMG-15, c: LMG-31; d: LMG-32; e: LMG-43; f: LMG-53; g: LMG-54; h: LMG-55; i: LMG-56; j: LMG-57; k: LMG-62; l: LMG-63

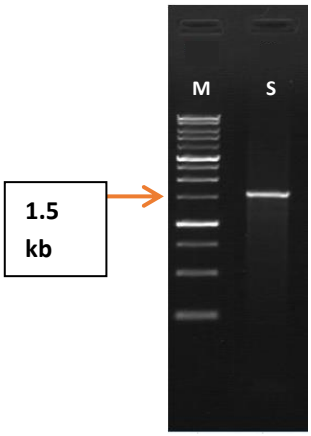

Figure 5. Results of electrophoresis of the DNA PCR product of LMG 15 isolate with universal primers (M=DNA marker, 1 Kb; PCR product of 16S rRNA gene of LMG-15

Table 2. Macroscopic and microscopic characteristics of IAA hormone-producing endophytic bacteria

Codes	Colour	Shape	Edge	Elevation	Cell lenght	Cell shape, Gram Stain
LMG 7	White	Irreguler	Entire	Convex	2.5	Bacile, positive
LMG 15	White	Irreguler	Entire	Raised	1.5	Bacile, positive
LMG 31	White	Irreguler	Undulate	Raised	2.5	Bacile, positive
LMG 32	White	Irreguler	Curled	Raised	2.0	Bacile, positive
LMG 43	White	Circular	Entire	Raised	3.0	Bacile, positive
LMG 53	White	Circular	Entire	Raised	2.0	Bacile, positive
LMG54	White	Circular	Entire	Flat	2.0	Bacile, positive
LMG 55	White	Irreguler	Undulate	Raised	2.0	Bacile, positive
LMG 56	White	Circular	Curled	Flat	2.0	Bacile, positive
LMG 57	White	Irreguler	Rhizoid	Flat	2.0	Bacile, positive
LMG 62	White	Filamentous	Filiform	Flat	2.5	Bacile, positive
LMG 63	White	Irreguler	Curled	Flat	3.0	Bacile, positive

Table 3. Bacterial strains based on the NCBI database of LMG 15 isolate sequences

Strain species	Access code	Max values	Query Cover (%)	E-value	Percent Identification (%)	Notes
Bacillus cereus IAM 12605	NR115526.1	1079	46	0.0	99.33	*Partial sequence*

Based on the macroscopic characterization, 12 isolates of endophytic bacteria that produced IAA had varied shape, elevation, and colony edges. Meanwhile, based on microscopic characterization, it was found that the 12 isolates of endophytic bacteria that produced IAA were Gram-positive bacteria in the form of bacilli with bacterial cell lengths ranging from 1.5 µm to 3 µm. Several studies that have been conducted regarding the isolation and characterization of endophytic bacteria have shown that endophytic bacteria can be either Gram-positive or Gram-negative in various forms (Suhandono *et al.*, 2016; Ying *et al.*, 2016). According to Idris *et al.* (2007), IAA synthesization of Gram-negative bacteria are highly dependent on tryptophan as important intermediates through indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM), and indole-3-acetonitrile (IAN). Meanwhile, Gram-positive bacteria do not depend on the presence of external tryptophan to produce IAA and can synthesize IAA in various ways, but the main route is still the IPA pathway (Vandeputte *et al.*, 2005; Idris *et al.*, 2007).

Identification of bacteria species using 16S rRNA gene

Based on the ability to produce IAA hormone, it was found that isolate with the most potential to produce IAA hormone was LMG 15. LMG 15 was identified using a molecular marker of the 16S rRNA gene. The DNA sample isolation process was carried out using the Wizard Genomic DNA Purification Kit (Promega). The results of DNA isolation through confirmation with a microdrop spectrophotometer showed a purity level of 1.9, which indicated good DNA purity of LMG 15.

Confirmation of DNA isolation is significant to determine whether the sample will be used in the next stage. The samples were then sent to 1st Base PT. Genetics Science in the form of DNA amplicons for sequencing. The results of DNA amplification of endophytic bacteria with the most potential for producing IAA hormones using universal primers 27F (5' – AGA GTT TGA TCM TGG CTC AG – 3') and 1492R (5' –CGG TTA CCT TGT TAC GAC TT–3') were observed through electrophoresis indicating that the DNA bands are parallel to the marker. The results of electrophoresis of the DNA PCR product of LMG 15 isolate with universal primers can be seen in Figure 5.

Furthermore, the obtained sequences were compared with the nucleotide sequences contained in the NCBI database through the BLAST program to determine the level of similarity between the sequences of the LMG 15 isolate and the sequences contained in the database. One bacterial strain with the highest value, query cover, percent identification, and the lowest E-value with isolate LMG 15 is presented in Table 3.

From the data obtained, it is known that the LMG 15 value has the highest similarity with *Bacillus cereus* strain IAM 12605 (percent identification value 99.33% and query cover 46%). The query cover value indicates the percentage of 16S rRNA gene sequences in the sample isolate that were successfully aligned with the gene sequences

contained in the NCBI database. Meanwhile, the equations of the two sequences are expressed in percent identification (NCBI News, 2006/7). In this study, the contig results obtained are around 1272 bp. According to Janda and Abbott (2007), the ideal sequence for microbial identification is around 1300-1500 bp.

LMG 15 was the isolate that produced the highest concentration of IAA (9.3 ppm). The isolate was then identified based on the 16S rRNA gene sequence. The 16S rRNA gene sequence analyzed in this study was the result of PCR amplicon sequencing from LMG 15 DNA using primers 27F (5' – AGAGTTTGATCMTGGCTCAG – 3') and 1492R (5' – CGGTTACCTTGTTACGACTT – 3'). The obtained sequence is compared to the nucleotide sequences contained in the NCBI database through the BLAST program to determine the level of similarity of the LMG 15 sequences to the sequences contained in the database.

It is expected that the LMG 15 isolate coded as *Bacillus cereus* strain LMG 15 can be used as one of the constituents for biofertilizer formulas as an alternative way in overcoming problems caused by the use of chemical fertilizers.

CONCLUSION

There were 12 isolates of endophytic bacteria from the Lamongan mangrove plant that produced the hormone IAA with concentrations ranging from 2-9,3 ppm. Based on macroscopic characterization, potential IAA-producing endophytic bacteria isolates had varied shapes, elevations, and colony edges. Meanwhile, based on microscopic characterization, potential IAA-producing endophytic bacteria isolates were Gram-positive bacteria in the form of bacilli with bacterial cell lengths ranging from 1.5 µm-3 µm. Based on16S rRNA gene-analysis, LMG 15 isolate was identified as *Bacillus cereus* strain LMG 15, having a similarity of 99.33% to Bacillus cereus strain IAM 12605.

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