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Production of Indole Acetic Acid (IAA) by *Serratia marcescens* subsp. *marcescens* and *Rhodococcus* aff. *qingshengii*

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Abstract *In vitro* screening and characterization assay on indole acetic acid (IAA) production generally provides a reliable base for selection of useful plant growth promoting bacteria. Characterization at different parameters such as temperature, pH, and other physiological conditions is useful in finding optimum condition for the IAA production by bacteria. In this study, six endophytic bacterial isolates from *Cocos nucifera* L. and two strains of *Rhodococcus* aff. *qingshengii* were examined for their IAA production activity at different pH, temperature, and L-tryptophan concentration. Identification of effective IAA-producing bacteria was conducted by molecular phylogenetic analysis based on nucleotide sequences generated from 16S rRNA gene. The results showed that highest IAA concentration was produced by *S. marcescens* subsp. *marcescens* strain KB01, *S. marcescens* subsp. *marcescens* strain KB05, and *R.* aff. *qingshengii* strain 100A with 64.75 µg/mL, 56.60 µg/mL, and 18.06 µg/mL IAA, respectively. The IAA production by these bacteria was affected by L-tryptophan concentrations and was optimum at basic condition (pH 8-9).

Keywords: bacteria; Indole-3-acetic acid; L-tryptophan; plant growth promoting; 16S rRNA

Introduction

The growth of plants is directly influenced by their interaction with microbial endophytes such as *Azospirillum*, *Rhizobium*, *Pseudomonas*, *Bacillus*, *Micrococcus*, and *Enterobacter* (Sudha *et al*., 2012). Members of these bacteria reside in the proximity of plant roots, and they are commonly called plant growth promoting bacteria (PGP) (Couillerot *et al*., 2012). The PGP bacteria have been known for their ability to produce beneficial hormones or other biostimulants for growth and health of the plants such as Indole-3-acetic acid (IAA). The indole-3-acetic acid is the most abundantly produced auxin by majority strains of PGP bacteria (Spaepen *et al*., 2007). The auxins have direct effect on controlling several stages of plant growth and development, such as

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initiates cells division and elongation, tissue differentiation, apical dominance, lateral and adventitious root formation; mediates responses to light, affects photosynthesis and pigment formation; and induces resistance to stressful conditions (Tsavkelova *et al*., 2006; Duca *et al*., 2014).

Among PGP bacteria, members of *Serratia* and *Rhodococcus* are distributed in various regions as soil-borne bacteria and endophytes. Several species of endophytic *Serratia* such as *S. marcescens*, *S. nematodiphila*, and *S. plymuthica* have been known for their ability in promoting plant growth of various crops (Dastager *et al*., 2011, Kang *et al*., 2015; Liu *et al*., 2016). While in *Rhodococcus*, the most widely known species for their plant growth promoting ability is *R. fascians* (Stes *et al*., 2011). The ability of *R. fascians* in producing IAA has further been utilized for germplasm storage and for plant propagation (Vereecke *et al*., 2000).

Considerable microorganism strains with PGP ability isolated from different plants and various regions in Indonesia are deposited and preserved at the Indonesian Culture Collection (InaCC), a national culture collection of microorganisms of Indonesia. In this study, we report molecular identification and quantification of IAA production of selected IAA-producing bacteria preserved at InaCC, in order to find powerful bacterial isolates for development of microbial biofertilizer and biostimulant formula for various crops application.

Materials and methods

Microorganisms

Six endophytic bacterial isolates from coconut tree (*Cocos nucifera* L.) and two *Rhodococcus* aff. *qingshengii* isolates (strain 100A and 100D) (Hastuty *et al*., 2014) were obtained from the Indonesian culture collection (InaCC). Isolates were maintained on Nutrient Agar (NA) medium with the following composition: peptone 5 g, yeast extract 3 g, NaCl 5 g, beef extract 1 g, and distilled water 1,000 mL. All isolates incubated at room temperature.

Qualitative assay of Indole Acetic Acid (IAA) production

IAA screening was conducted on Luria-Bertani (LB) broth medium amended with L-tryptophan as precursor for the IAA production by bacteria. The medium composition contains: tryptone 10 g, yeast extract 5 g, NaCl 5 g, and distilled water 1,000 mL. Bacterial colony was inoculated using inoculation loop to the LB broth medium and incubated at 30 ℃ on rotary shaker (120 rpm) for 24 hrs. Each bacterial inoculum was further transferred into 10 mL LB broth

medium amended with 1% of 5 mM L-tryptophan, and then incubated at 30 ℃ on rotary shaker (120 rpm) for 96 hrs.

IAA production assay was conducted using Salkowski's reagent (Ehmann, 1977). One mL of sample was centrifuged at 13,000 rpm for 5 min. Supernatant was further transferred into tubes amended with 1 mL Salkowski's reagent. The tube was incubated at 30 ℃ for 30 min. Positive reaction is indicated by change in the color of medium to pink or violaceous color.

Optimization of Indole Acetic Acid (IAA) production

Optimization of IAA production was conducted using LB broth medium at various concentrations of L-tryptophan (5, 10, 15, 20, and 25 mM), pH (6, 7, 8, and 9), and temperatures (35, 37, 40, and 45^{o}C). The IAA was measured at 530 nm using spectrophotometer (UV-PharmaSpec 1700, Shimadzu) every 24 hrs for 96 hrs incubation.

Molecular identification of the most effective IAA producing bacteria

All PSB isolates were qualitatively examined for their ability to produce phytase by using phytase screening medium (PSM) agar (L^{-1}): 0.5% calcium phytate as substrate, 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.01% NaCl, 0.05% KCl, 0.001% $FeSO_4$, 0.01% $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ 0.01%, 0.001% $MnSO_4$, 1.5% agar, pH 6.5 (Kerovuo *et al.*, 1998). 10 µL of bacterial culture was inoculated at the center of PSM agar medium and then incubated for 7 days at room temperature. Bacterial colonies capable of hydrolyzing calcium phytate (extracellular phytase) are characterized by halo zone surrounding the colony. The colony diameter and halo zone were measured after 7 days incubation. PSB isolates with halo zone was further examined for their phytase activities in PSM liquid medium. One enzyme unit (U) is defined as amount of enzyme that liberates 1 µmol inorganic phosphate in one minute.

Results

Qualitative assay of Indole Acetic Acid (IAA) production

From a total eight isolates of bacteria capable in producing IAA in the medium amended with 5 mM L-tryptophan, three isolates showed highest IAA production, namely, strain KB01, strain KB05, and *Rhodococcus* aff. *qingshengii* strain 100A (Table 1, Fig. 1). These bacterial isolates were further selected for optimization of the IAA production assay.

Table 1. Qualitative assay of IAA production by selected bacteria.

No.	Strain code	Screening result	Sources of isolate
1	K5-B-2-05B	+	Coconut tree
2	K5-B-2-06B	+	Coconut tree
3	K7-B-1-05B	+	Coconut tree
4	K7-B-1-06B	+	Coconut tree
5	KB05	++++	Coconut tree
6	KB01	++++	Coconut tree
7	*Rhodococcus* aff. *qingshengii* strain 100A	+++	Polluted river
8	*Rhodococcus* aff. *qingshengii* strain 100D	+	Polluted river

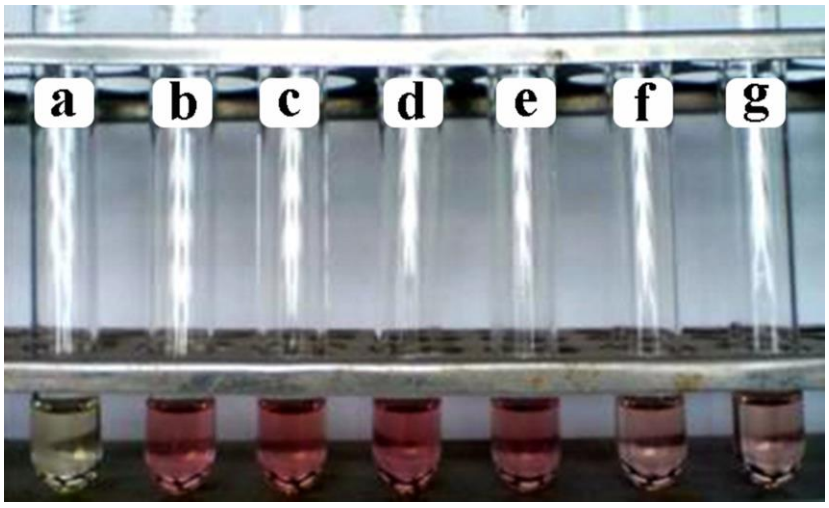

Figure 1. Qualitative screening of IAA production. (a) Negative control, (b-c) strain KB01, (d-e) strain KB05, and (e-f). *R.* aff. *qingshengii* strain 100A.

Optimization of Indole Acetic Acid (IAA) production

Effects of L-tryptophan concentrations

This assay showed that concentrations of IAA produced by selected bacteria were in line with increasing of L-tryptophan concentrations (Fig. 2). Highest IAA was found at the medium amended with 25 mM L-tryptophan in all bacterial strains. At this L-tryptophan concentration, bacteria strain KB05, strain KB01, and *R.* aff. *qingshengii* strain 100A produced 10.36 µg/mL, 11.18 µg/mL, and 11.19 µg/mL IAA, respectively.

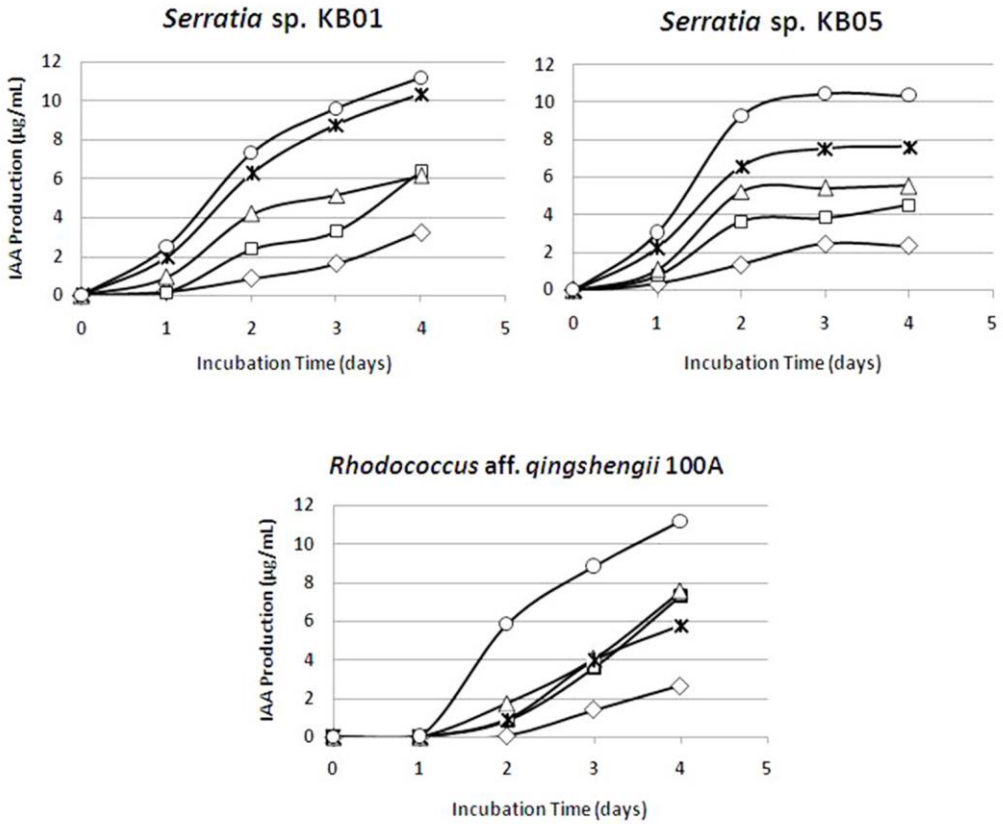

Figure 2. Effects of various L-tryptophan concentrations on IAA production by bacteria strain KB05, strain KB01, and *R.* aff. *qingshengii* strain 100A isolates. (◊) 5 mM, (□) 10 mM, (Δ) 15 mM, (ж) 20 mM, (O) 25 mM.

Effects of temperature variation

Optimum temperature for IAA production varies among bacteria strain KB05, strain KB01, and *R.* aff. *qingshengii* strain 100A (Fig. 3). Highest IAA production by strain KB05 (11.23 µg/mL) was achieved at 40 ℃, while highest IAA production by strain KB01 (8.54 µg/mL) and *R.* aff. *qingshengii* strain 100A (4.90 µg/mL) was achieved at 37 ℃ and 35 ℃, respectively.

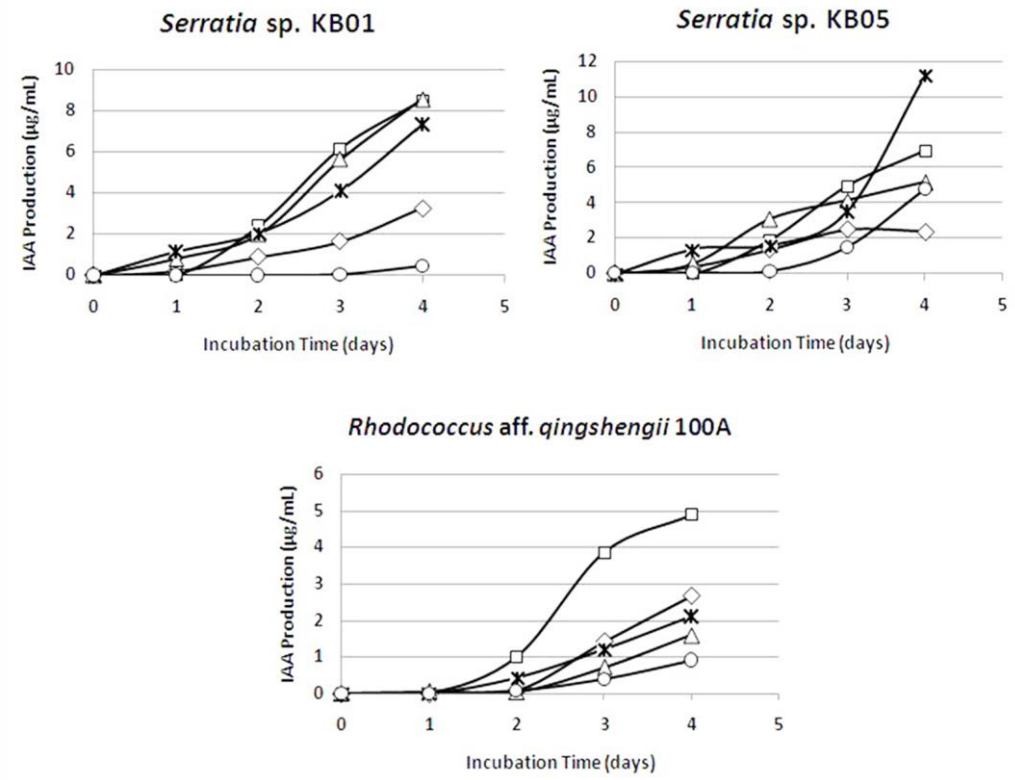

Figure 3. Effects of temperature variation on IAA production by bacteria strain KB05, strain KB01, and *R.* aff. *qingshengii* strain 100A isolates. (◊) 30ºC, (□) 35º, (Δ) 37ºC, (ж) 40ºC, (O) 45ºC.

Effects of pH medium variation

Effects of pH variation on the IAA production by bacteria strain KB05, strain KB01, and *R.* aff. *qingshengii* strain 100A is showed in figure 4. All isolates exhibited optimum activity at basic condition. Highest IAA production by bacteria strain KB05 (3.39 µg/mL) and strain KB01 (3.56 µg/mL) was found at pH 9, while highest IAA production by *R.* aff. *qingshengii* strain 100A (3.39 µg/mL) was found at pH 8.

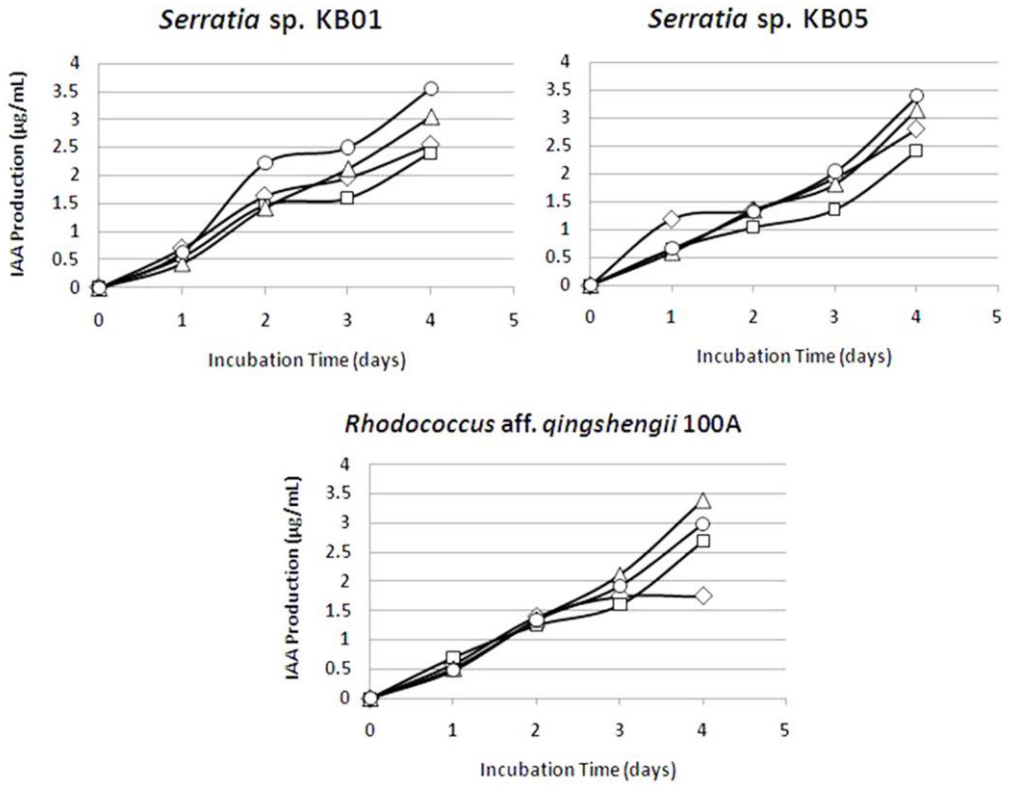

Figure 4. Effects of pH medium variation on the IAA production by bacteria strain KB05, strain KB01, and *R.* aff. *qingshengii* strain 100A. (◊) pH 6, (□) pH 7, (Δ) pH 8, (O) pH 9.

IAA production assay at optimum condition

Optimum condition of pH, temperature, and L-tryptophan concentration in the previous assays (Table 2) was used in further assay. Based on this condition, highest IAA yield was produced by bacterium strain KB01, followed by bacterium strain KB05 and *R.* aff. *qingshengii* strain 100A with 64.75 µg/mL, 56.60 µg/mL, and 18.06 µg/mL IAA, respectively (Fig. 5).

Table 2. Optimum condition for IAA production by bacteria strain KB05, strain KB01, and *R.* aff. *qingshengii* strain 100A.

Strain code	Optimum condition		
	pH	Temperature (°C)	L-tryptophan concentration (mM)
KB01	9	37	25
KB05	9	40	25
Rhodococcus aff. *qingshengii* strain 100A	8	35	25

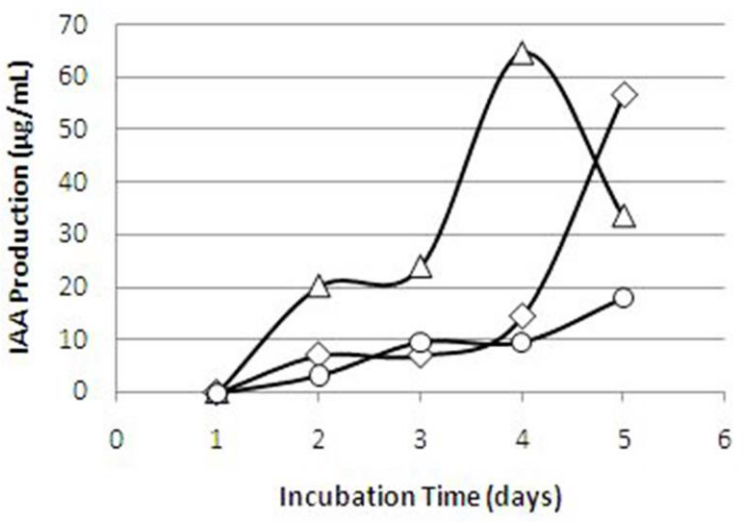

Figure 5. IAA production based on optimum parameters. (Δ) strain KB01, (◊) strain KB05, (O) *R.* aff. *qingshengii* strain 100A.

Molecular phylogenetic analysis

The dataset contains 23 sequences of *Serratia* type species including *E. coli* strain ATCC 11775T as outgroup (Fig. 6). GenBank accession number, species name, and strain code are presented in the Fig. 6. The phylogenetic tree generated from ML analysis showed that bacterial strain KB01 and KB05 belong to *Serratia marcescens* subsp. *marcescens* with 62% bootstrap support (Fig. 6). Therefore, both strains are determined as *S. marcescens* subsp. *marcescens* strain KB01 and *S. marcescens* subsp. *marcescens* strain KB05. This is the first report of *S. marcescens* subsp. *marcescens* as an endophyte of *C. nucifera* from Indonesia.

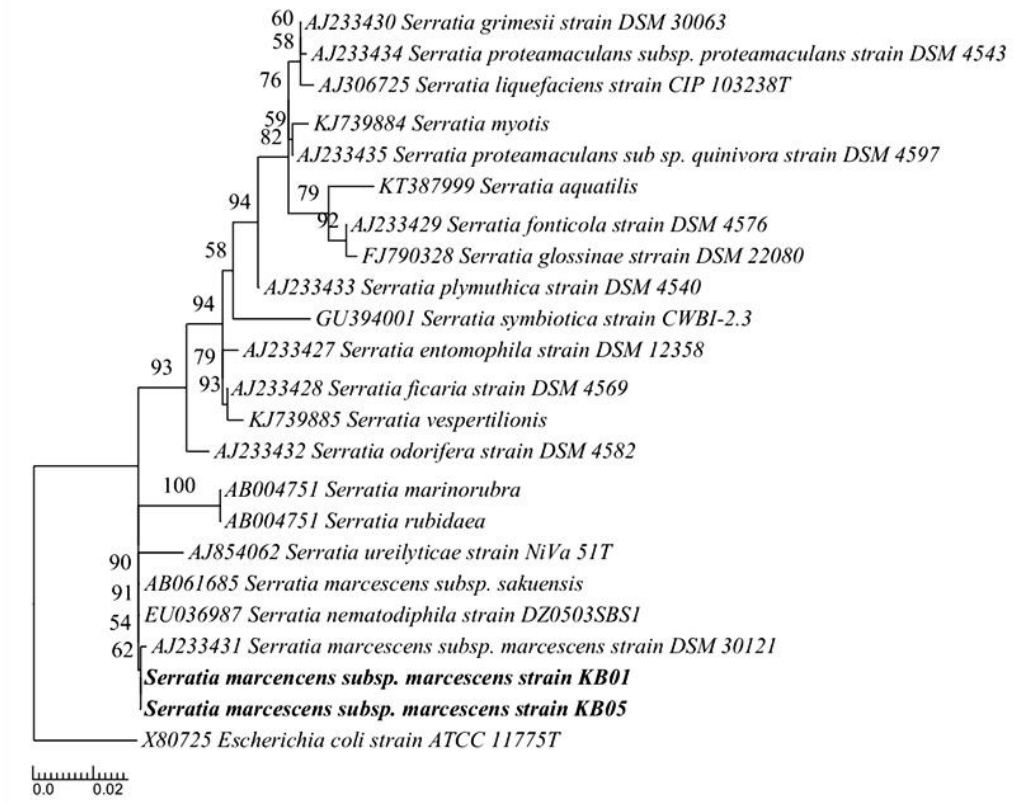

Figure 6. Maximum Likelihood (ML) tree showing phylogenetic affinities of bacteria strain KB01 and strain KB05 within *Serratia* type species based on the 16S rDNA sequences. Bootstrap value > 50% is shown at the branches node.

Discussion

Qualitative assay of Indole Acetic Acid (IAA) production

Indole acetic acid (IAA) is one of important phytohormones that play a central role in plant growth and development. In the qualitative IAA production screening by microorganisms, capability of the IAA-producing bacteria in metabolizing L-tryptophan (TRP) into IAA or some analogous compounds of IAA is indicated by pinkish color formation in the medium (Fig. 1). The color formation is reported due to Salkowski's reagent when mixed with IAA, forming tris-(indole-3-acetato)-iron(III) complex that exhibit pink coloration (Gordon and Weber, 1950). In the qualitative assay, intensity of pink color

formation determined the robustness of IAA formation produced by bacterial isolates. Based on this parameter, it was obvious that three isolates, viz, *S. marcescens* subsp. *marcescens* strain KB01, *S. marcescens* subsp. *marcescens* strain KB05, and *R.* aff. *qingshengii* strain 100A qualitatively exhibited highest IAA production (Fig. 1). Indeed, the IAA produced by bacteria varies between different species and strains. This variation is possibly affected by several factors, such as physiological condition of bacterial isolates, growth stage, and substrate availability (Mohite, 2013).

Optimization of Indole Acetic Acid (IAA) production

Tryptophan is one of the compounds present in exudates produced by several plant species. Figure 2 showed that L-tryptophan concentrations quantitatively affected IAA production by *S. marcescens* subsp. *marcescens* strain KB01, *S. marcescens* subsp. *marcescens* strain KB05, and *R.* aff. *qingshengii* strain 100A. Concentration of IAA produced by these bacteria was found in line with L-tryptophan concentrations amended in the medium. Study on L-tryptophan relationship with IAA production reported that L-tryptophan is a key precursor in IAA production by various microorganisms (Costacurta and Venderleyden, 1995). The mechanism of IAA production by microorganisms is different, depends on the species or strain of the microorganisms. For example, IAA production via indole-3 acitamide was found in *Pseudomonas syringae* (Kosuge and Sanger, 1987), via indole 3-actaldehyde in *P. fluorescens* (Oberhansli *et al*., 1991), via indole-3 pyruvic acid in *Enterobacter cloacae* (Koga *et al*., 1991), via tryptamine in *Agrobacterium tumefaciens* and via indole 3-acetonitreile in *Alcaligenes faecalis* and *A. tumefaciens* (Costacurta and Vanderleyden, 1995). Studies on inactivation of genes involved in tryptophan biosynthesis and in a putative tryptophan-dependent IAA biosynthesis pathway showed a reduction in IAA concentration and plant-growth-promoting activity (Idris *et al*., 2007).

In temperature effects on IAA production assay, highest IAA production by *S. marcescens* subsp. *marcescens* strain KB05 (11.23 µg/mL) was achieved at high temperature (40 ℃), while highest IAA production by *S. marcescens* subsp. *marcescens* strain KB01 and *R.* aff. *qingshengii* strain 100A was achieved at 37 ℃ and 35 ℃, respectively (Fig. 3). This result is in agreement with Hussein *et al.* (2016) who found that highest IAA production (133.27 mg/mL) by *B. subtilis* was optimum at 40 ℃. However, optimum temperature of IAA-producing bacteria such as *Azotobacter*, *Rhizobium*, *Bacillus*, and *Pseudomonas*, is usually around 36 ± 2 ℃ (Joseph *et al*., 2007). In practical condition of IAA-producing bacteria application, expression of IAA production

at high temperature, such as 40℃ or higher temperature, by bacteria endophytes is important due to temperature stress to the plant in the field.

In IAA production assay at different pH condition, highest production of IAA by *S. marcescens* subsp. *marcescens* strain KB01, *S. marcescens* subsp. *marcescens* strain KB05, and *R.* aff. *qingshengii* strain 100A was found at basic pH (8-9) (Fig. 4). This result is in agreement with several studies that reported acidic pH being unfavorable for IAA production by several bacteria (Mohite, 2013). It is probably related to the nature of the IAA-producing bacteria (Sachdev *et al*., 2009).

IAA production assay at optimum condition

Serratia species have been reported potential in producing IAA and other plant growth regulating hormone (Kalbe *et al*., 1996; Dastager *et al*., 2011; Zaheer *et al*., 2016). This includes *S. marcescens, S. rubidaea, S. liquefaciens, S. plymuthica, S. nematodiphila,* and *S. fonticola* (Kalbe *et al*., 1996; Dastager *et al*., 2011; Kang *et al*., 2015; Liu *et al*., 2016; Zaheer *et al*., 2016; Jung *et al*., 2017). In the current quantitative analysis of the IAA production by *Serratia* strains isolated from *C. nucifera, S. marcescens* subsp. *marcescens* strain KB01 and *S. marcescens* subsp. *marcescens* strain KB05 produced 64.75 µg/mL and 56.60 µg/mL IAA at their optimum condition, respectively (Table 3, Fig. 5). The current IAA production is higher than Lwin *et al*. (2012) who found that IAA concentration produced by four *Serratia* strains (*Serratia* sp. strain S1, S2, S3, S4) range between 9.71-20.05 µg/mL at 2.43 mM L-tryptophan. However, in another studies, Serepa *et al*. (2015) reported that *S. marcescens* strain MCB capable in producing IAA 94 µg/mL at 0.15 mM L-tryptophan, and Devi *et al*. (2017) found that *S. marcescens* strain AL2-16 was capable in producing IAA 133.2 µg/mL at 48.9 mM L-tryptophan. Based on the ratio of IAA concentrations produced by bacterial isolates with quantity of L-tryptophan amended in the medium, production of IAA by *S. marcescens* subsp. *marcescens* strain KB01, *S. marcescens* subsp. *marcescens* strain KB05, and *R.* aff. *qingshengii* strain 100A is still need more optimization to increase yields.

Study on IAA production by *Rhodococcus* strains is relatively less frequent than *Serratia* strains, and this is the first report of IAA-producing *Rhodococcus* strains from Indonesia. In this study, the IAA concentration produced by *Rhodococcus* aff. *qingshengii* strain 100A (18.06 µg/mL) is lower than the IAA produced by *Serratia* spp. In another study, higher IAA production by *R. fascians* strain D188 (49.93 µg/mL at 0.1 mM L-tryptophan) was reported by Vandepute *et al*. (2005). This showed that members of *Rhodococcus* are also potential in the IAA production using fermentation

medium. *Rhodococcus qingshengii* was first reported as carbendazim-degrading bacterium (Xu *et al*., 2007), and α-nitrile hydratase- and amidase-producing bacterium (Hastuty *et al*., 2014). Another study also reported *R. qingshengii* as Salmon fish (*Salmo salar*) pathogen (Avendaño-Herrera *et al*., 2011). In addition, *R. fascians* is usually found as pathogen that infects dicotyledonous and monocotyledonous plants, and affects plant hormone balances, in particular cytokinine (Stes *et al*., 2011). Because of this ability, *R. fascians* has been used in plant cell differentiation and organogenesis (Vereecke *et al*., 2000), and the improvement of a wide range of plant tissue culture propagation (Goethals *et al*., 1998).

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