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Optimization of indole-3-acetic acid (IAA) production by rhizobacteria isolated from epiphytic orchids

Kajohnpong Dasri[1] *, Jureeporn Kaewharn*[1], *Sungwan Kanso*[2] *and Suphavee Sangchanjiradet*[3]

[1] *Microbiology Program, Faculty of Science, Ubon Ratchathani Rajabhat University*

[2] *Department of Biological Science, Faculty of Science, Ubonrajathanee University*

[3] *Biology Program, Faculty of Science, Ubon Ratchathani Rajabhat University*

* *Correspondent author: john_ubu@hotmail.com*

Abstract

The optimal conditions for indole-3-acetic acid (IAA) production by bacterial isolate DPY-05 (Gram-positive, rods), living in association with aerial orchid roots (*Dendrobium pulchellum*) were studied. The factors that affect the IAA production were investigated including culture media, pH, temperature, agitation and culture period. The results showed that the isolated DPY-05 gave the highest amount of IAA of 67.18 µg/ml when cultured in King-B medium supplemented with tryptophan 2.5 mM (KB+Trp) pH 7.0 at 37°C in static condition for 6 days.

Keywords: Optimization, Indole-3-acetic acid, Orchid rhizosphere, Plant-growth promoting rhizobacteria

1. Introduction

Bacteria living in association with roots (rhizosphere) are important to orchid growth. Known beneficial roles of these bacteria include nitrogen fixation and indole-3-acetic acid (IAA, auxin) production (1). IAA is essential for plant growth, affecting both cell division and cellular expansion. Depending on specific tissue, IAA may promote axial elongation, lateral expansion, or isodiametric expansion (2).

Bacterial diversity on orchid rhizosphere depends on orchid species. Members of genera *Streptomyces*, *Bacillus*, *Pseudomonas*, *Burkholderia*, *Erwinia* and *Nocardia* were found to populate *Paphiopedilum appletonianum* roots while bacterial strains belong to genera *Pseudomonas, Flavobacterium, Erwinia, Burkholderia, Stenotrophomonas, Pantoea, Chryseobacterium, Bacillus, Agrobacterium* and *Paracoccus* were isolated from *Pholidota articulata* (1). Production of IAA by bacterial isolates varies greatly among different species and strains. For example bacteria member of five different genera isolated from *Dendrobium moschatum* roots which were *Sphingomonas*, *Microbacterium*, *Mycobacterium*, *Bacillus*, and *Rhizobium* produced 50.2, 53.1, 92.9, 37.6, and 60.4 µg IAA/ml respectively (3).

Improve productivity of IAA in fermentation was done by varying factors affecting bacterial growth and IAA yield which include pH of the medium, temperature, carbon source, nitrogen source and L-tryptophan supplementation (4, 5). Khamna's teams reported an improvement of IAA production by *Streptomyces* CMU-H009 in which the amount of IAA was raised from 143 µg/ml to 300 µg/ml (6).

Our research team aimed to isolate bacteria from the roots of orchids in UbonRatchathani province and to improve IAA productivity by adjusting various conditions to find optimal IAA production. The results of the experiment will be used as the basis for production of IAA *in vitro* and its subsequent use to promote growth in cultured orchids.

2. Materials and Methods

2.1 Isolation and screening for IAA producing bacteria

Bacteria were isolated from epiphytic orchids, *Dendrobium* "anna", *Dendrobium pulchellum*, *Cattleya* "Queen Sirikhit", and *Aerides falcate*, which were cultivated in natural conditions. Aerial orchid roots were cut aseptically whole, 4-5 roots per plant. Orchid roots of 2.5 g were chopped to 2-5 mm in size and filled in 125-ml flasks with 50 ml distilled water and 2 droplets of tween80. The mixture was shaken at 100 rpm for 30 min. The solutions were serially diluted from 10^{-1} - 10^{-3} and used as inoculums. Bacterial strains were isolated by streaking the solution onto selective medium for Rhizobia (7) yeast-mannitol agar (YMA: formula in gram per liters, yeast extract 0.5, mannitol 10.0, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, Agar 15.0, pH 7.0).YMA plates were then incubated at room temperature (25-30^{o}C) until colonies could be detected. The bacterial isolates were stored on YMA slant at 4-5^{o}C.

In the screening for indole-3-acetic (IAA) producing bacteria, all strains were grown on yeast-mannitol broth medium (YMB: same formula as YMA, but without agar), incubated at 30^{O}C with shaking at 100 rpm for 48 h. Cell suspension, at 10% inoculums, was transferred in duplicate to the 50 ml of media, supplemented with 2.5 mM L-tryptophan and without in 125-ml flasks (8), incubated at 37^{o}C for 3 days. Quantification of IAA was performed using Salkowski's colorimetric assay (9). Duncan's Multiple Rang Test was used for statistical analysis of data.

2.2 Study of condition for IAA production

Selected strains were cultured in three different culture media, Nutrient broth (NB: formula in gram per liters, beef extract 3.0, peptone 5.0, pH 7.0), King-B medium (KB: formula in gram per liters, peptone 20.0, glycerol 15 ml, K_2HPO_4 1.5, $MgSO_4 \cdot 7H_2O$ 1.5, pH 7.0) and YMB, supplemented with 2.5 mM L-tryptophan. Triplicate of 50 ml medium in 125-ml flasks were inoculated with bacterial strain at 10%, incubated at 37^{o}C for 3 days. IAA assay was determined by Salkowski's colorimetric assay. The mean differences of data were analyzed by Duncan's Multiple Range Test.

The optimal conditions for IAA production were investigated using King-B medium by varying medium pH (range 4.0-9.0), temperature (20-45^{o}C), agitation (0 and 100 rpm), and incubation period (1-11days).

2.3 IAA production in optimal conditions

Production of IAA in optimal condition was performed in triplicate, 300 ml of medium in 500-ml flasks. Bacterial growth, pH of the media and IAA production were monitored using a spectrophotometer (measuring OD_{600}), pH meter and Salkowski's colorimetric assay, respectively, every 24 hours for 11 days, until the maximum of IAA concentration in the medium had been reached.

2.4 Quantification of IAA

Culture supernatant was recovered after centrifugation at 5,000 rpm for 5 min. Two milliliters of supernatant were mixed with 3 ml of Salkowski's reagent R2 (4.5 g of $FeCl_3$ per liter in 10.8 M H_2SO_4), incubated at room temperature for 30 min in the dark, measured OD value at 550 nm by spectrophotometer (9).

2.5 Data analysis

The experiments were designed by Completely Randomized Design (CRD) and results were analyzed with Analysis of Variance (ANOVA). The results were determined and compared the differences between test groups by DMRT (Duncan's Multiple Range Test).

3. Results and Discussion

3.1 Isolation and screening for IAA production bacteria

Twenty-five bacterial isolates obtained from 4 samples of aerial orchid roots were screened for an ability to produce IAA. Five isolates, namely AFY-04, AFY-07, DPY-05, QSCY-04 and DAY-04, gave positive results. Sample sources of the five isolates were shown in Table 1. The phytohormone production can be found in both Gram-positive and Gram-negative bacteria corresponding to data presented by other authors (1, 3-6). All five isolates produced higher amount of IAA in culture media supplemented with 2.5 mM L-tryptophan comparing to without L-tryptophan supplementation as shown in Figure 1. Tryptophan has been identified as a main precursor for IAA biosynthesis in bacteria (8). With addition of this compound into the culture medium, IAA production has increased in all experiments. Isolated DPY-05 produced the highest amount of IAA at 11.49 µg/ml in the screening experiment. DPY-05 colony was white and 1 mm. in diameter. It was a Gram positive rod, single or pair, with central endospores. DPY-05 has not been identified. The aerial roots with the presence of the spongy velamen may serve as one of the most favorable habitats for bacteria (1).

3.2 Study of condition for IAA production

Three different culture media (NB, KB and YMB) supplement with 2.5 mM of tryptophan (NB+Trp, KB+Trp and YMB+Trp) were used to grow isolated DPY-05 and amounts of IAA production in those media were compared. Isolated DPY-05 produced highest IAA from King-B medium that was supplemented with 2.5mM L-tryptophan (Figure 2). This complex medium, supplemented with tryptophan had often been used to assess auxin production by plant growth-promoting rhizobacteria (10), especially the members of the family Pseudomonaceae such as *Pseudomonas syringae* (9). The effect of temperature on IAA production was studied. The highest amount of IAA was found at 30 and 37°C as shown in Figure 3. As the amounts of IAA produced at 30 and 37°C were not significantly different, the effect of pH on IAA production was studied at 37°C because it showed less variation at this temperature.

Effect of initial pH of the medium was not significant when analyzed by statistical analysis (α=0.05). If the culture is slightly acid to strongly alkaline medium (pH 6.0-9.0) as shown in Figure 4, which was similar to other reports (6). In contrast Spaepen *et al*., 2007 reported that diverse group of bacteria produced more IAA under acidic condition (8). However, we chose to use the culture

Table 1 Distributions and characteristics of bacteria isolated from the roots of epiphytic orchids

Plant	Root's part	Bacterial Isolates	Characteristics	
			Colony[a]	Cells
Aerides falcata	Rhizoplane	AFY-04	diameter 1 mm, white, entire, smooth, convex	Gram negative, Cocci
		AFY-07	cream, entire, rough, riased	Gram negative, Cocci
Dendrobium "anna"	Rhizoplane	DAY-04	diameter 1 mm, cream, entire, rough, convex	Gram negative, Coccobacilli
Dendrobium pulchellum	Rhizoplane	DPY-05	cream, erose, rough, flat	Gram positive, Bacilli, Endospore
Cattleya "Queen Sirikit"	Rhizoplane	QSCY-04	diameter 1 mm, white, entire, smooth, flat	Gram negative, Coccobacilli

[a] Characteristics of bacterial colony which were colony diameter, color, margin, surface texture and elevation

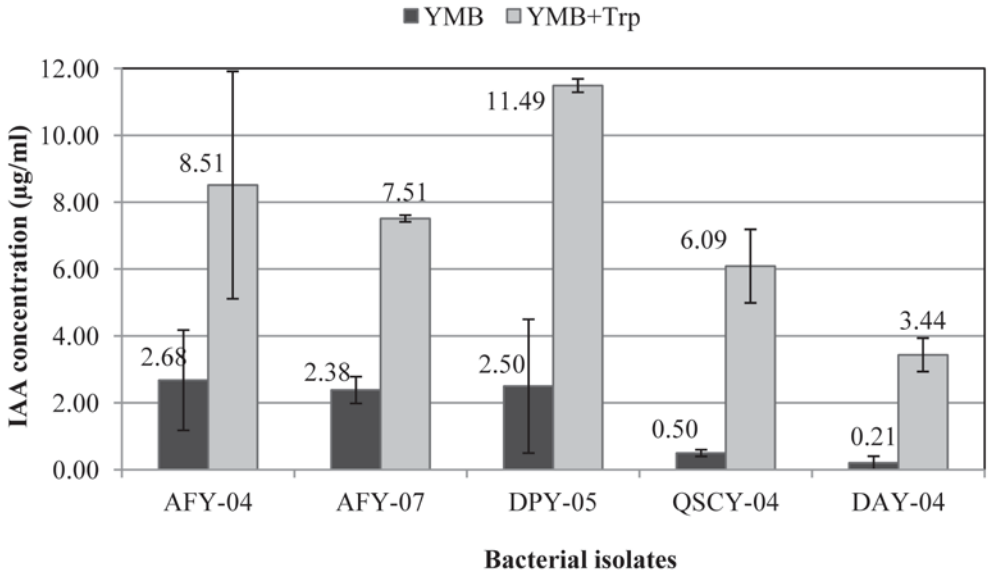

Figure 1 IAA production by bacterial isolated from orchid roots in YMB medium supplemented with or without 2.5 mM L-tryptophan, growing at 37 °C for 3 days. The experiments were performed in duplicates and the bars indicate standard deviations.

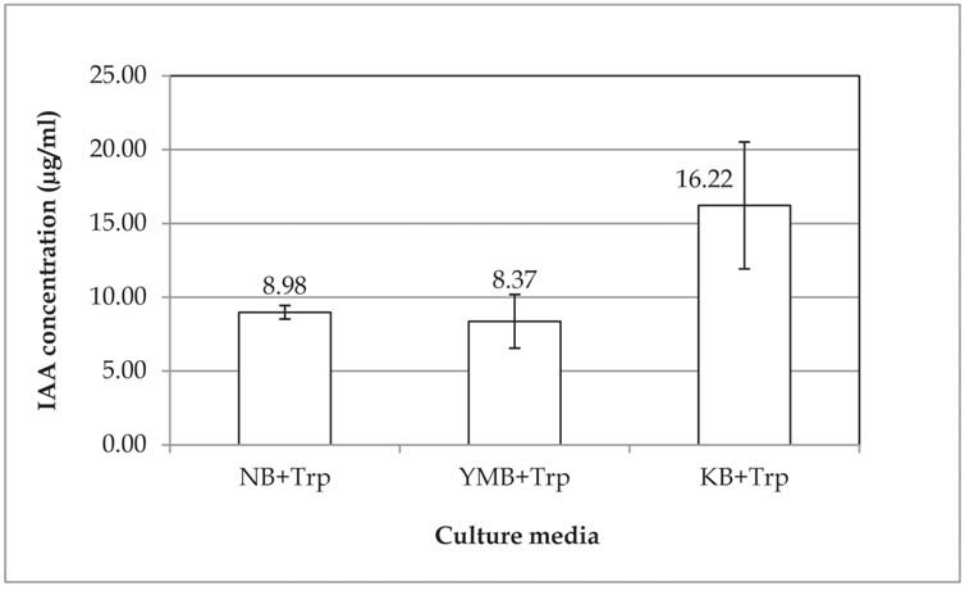

Figure 2 IAA productions by isolated DPY-05 growing in 3 different culture media supplemented with 2.5 mM L-tryptophan, pH 7.0, incubated at 37°C for 3 days. The experiments were performed in duplicates and the bars indicate standard deviations.

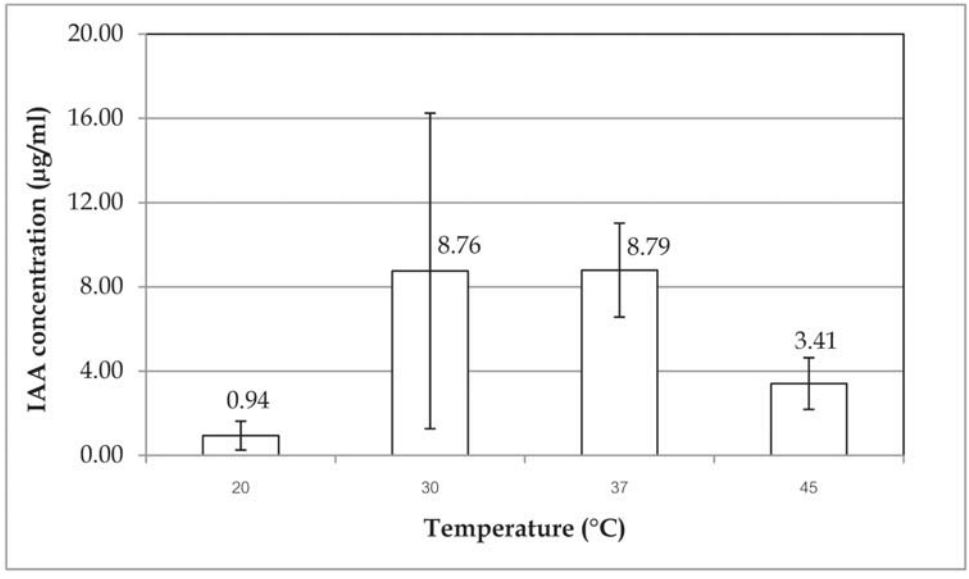

Figure 3 Effect of temperature on IAA production by isolated DPY-05 growing in King B media supplemented with 2.5 mM L-tryptophan, pH 7.0, incubated at 37°C for 3 days. The experiments were performed in triplicates and the bars indicate standard deviations.

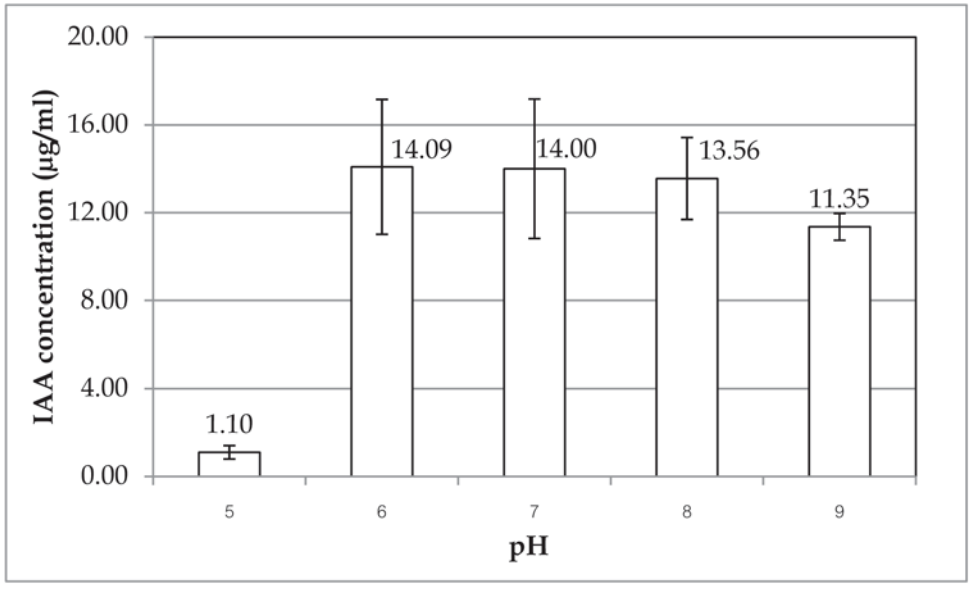

Figure 4 Effect of pH on IAA production by isolated DPY-05 growing in King B media supplemented with 2.5 mM L-tryptophan incubated at 37°C for 3 days. The experiments were performed in triplicates and the bars indicate standard deviations.

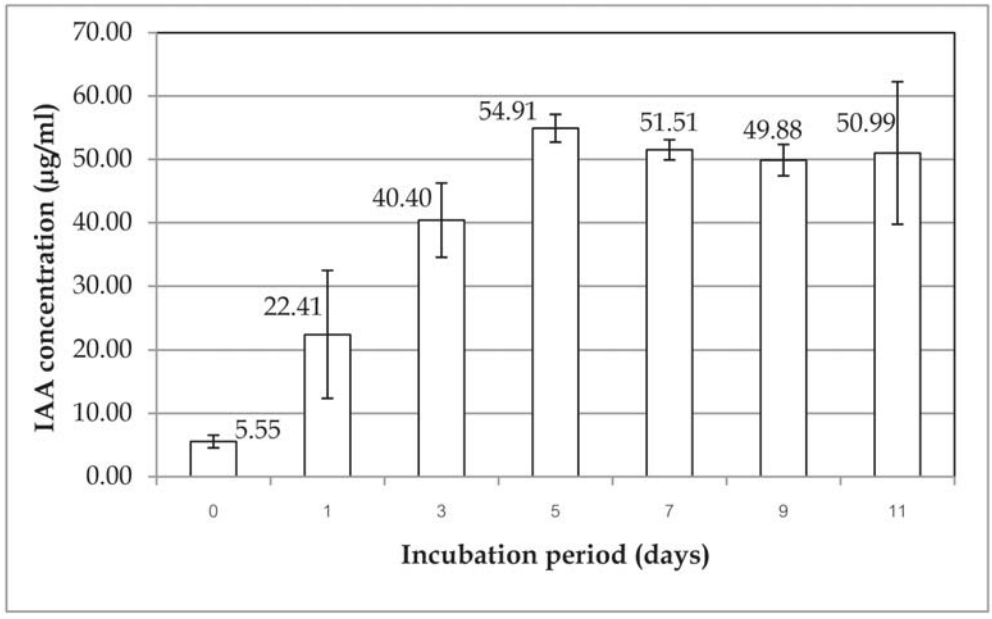

Figure 5 Time course of IAA production by isolated DPY-05 in King-B medium supplemented with 2.5 mM L- tryptophan (0.5 g/l), pH 7, at 37°C. The experiments were performed in triplicates and the bars indicate standard deviations.

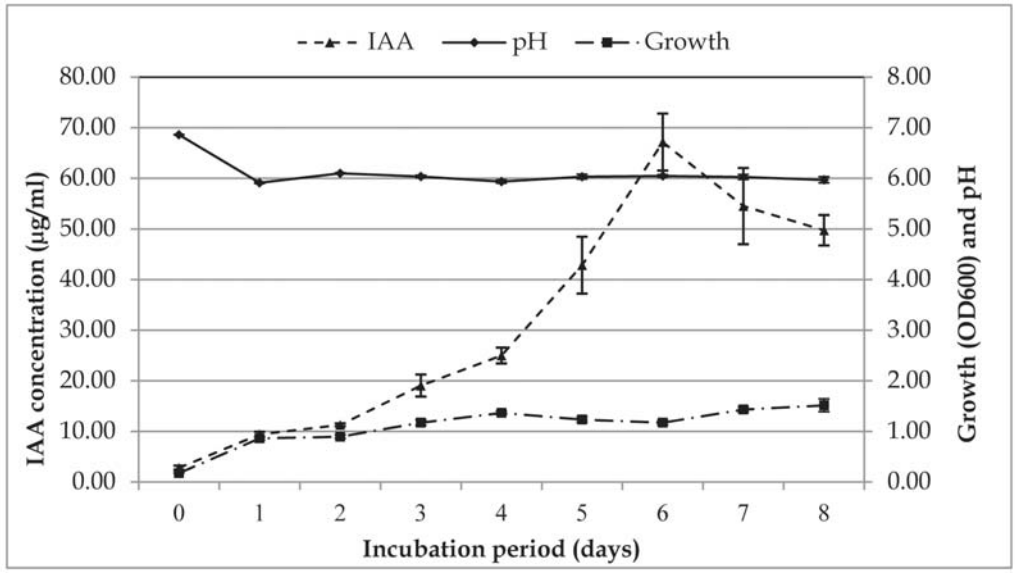

Figure 6 IAA production and growth of isolated DPY-05 under optimal condition (in King-B medium supplemented with 2.5 mM L-tryptophan (0.5 g/l), pH 7, at 37°C in static condition) in working volume of 300 ml. The experiments were performed in triplicates and the bars indicate standard deviations.

medium in neutral condition (pH 7.0) for the next experiments.

The comparison of agitation speed at 100 rpm and without agitation by DPY-05 in King-B medium supplemented with 2.5 mM of tryptophan (KB+Trp) showed that no significant difference (α=0.05) was observed in IAA production of 49.73 µg/ml and 51.51 µg/ml, respectively (data not shown). Therefore, we chose static incubation for the following experiment to find an optimal incubation period for IAA production. Amounts of IAA were measured maximum at the fifth day and then slightly decreased at the end of fermentation as shown in Figure 5.

3.3 IAA production in optimal condition

The isolated DPY-05 began to produce IAA at the beginning of its growth and reached the maximum at the stationary phase as shown by Figure 6 similar to results reported by others (1, 8, 11). Thus, the optimal condition for maximum production of IAA (of 67.18 µg/ml) was culturing DPY-05 in King-B medium supplemented with 2.5 mM tryptophan (0.5 g/l), pH 7, at 37°C in static condition for 6 days.

4. Conclusion

IAA producing bacteria can be found in rhizosphere of epiphytic orchids. The ability of IAA production depends on the supplementation of culture media with L-tryptophan. When the isolated DPY-05 was cultured in the optimal condition (King-B medium supplemented with 2.5 mM tryptophan (0.5 g/l), pH 7, at 37°C in static condition for 6 days), the maximum IAA production measured was 67.18 µg/ml, which was up to 5.85 folds greater than the control experiment. The productions of IAA were found in stationary phase of bacterial growth.

5. Acknowledgement

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