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The use of Sprout as Precursor for the Production of Indole Acetic Acid by Selected Plant Growth Promoting Rhizobacteria Grown in the Fermentor

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Indole-3-acetic acid (IAA) is the main member of the auxin family that controls many important physiological processes in plant. Such beneficial IAA that produced by plant growth promoting rhizobacteria (PGPR), enhances plant growth and was believed to increase the access to more nutrients in the soil. The precursor for syntetizing IAA is tryptophan, and it was also found in the sprout or other sources of protein. The aim of this study was to investigate the best bacteria and growth medium supplemented with extract of bean sprout or fish meal as the sources of precursor for the IAA production. Several bacterial isolates were screened for IAA production. IAA production was measured with High Performance Liquid Chromatography. All of isolates were able to produce IAA and isolates PS1 was selected for the further assay by cultivating under fermentor system. Sequencing of 16S rDNA of PS1 isolate indicated as *Acinetobacter sp*. The result showed that the highest IAA production during fermetation was 62,428 ppm found in under medium supplemented with mung bean sprout extracts grown in fermentor, after 24 hours incubation.

Key words: Plant growth promoting rhizobacteria, indole acetic acid, mung bean sprout extract, *Acinetobacter* sp.

Asam indole-3-asetat (AIA) adalah bagian utama dari kelompok auksin yang mengontrol banyak proses fisiologis penting dalam tanaman. Peran penting AIA yang dihasilkan oleh Rizobakteri Pemacu Pertumbuhan Tanaman (RPPT) meliputi meningkatkan pertumbuhan tanaman dan dipercaya semakin menngkatkan penyerapan nutrisi dalam tanah. Prekursor untuk memproduksi IAA adalah triptofan, dan itu juga ditemukan dalam kecambah atau sumber protein lainnya. Tujuan dari penelitian ini adalah untuk mendapatkan bakteri terbaik dan media pertumbuhan yang dilengkapi dengan ekstrak kecambah atau tepung ikan sebagai sumber prekursor untuk produksi IAA. Beberapa isolat bakteri diseleksi untuk produksi IAA tertinggi. Produksi IAA diukur dengan *High Performance Liquid Chromatography*. Semua isolat yangdigunakan mampu menghasilkan IAA dan isolat PS1 dipilih untuk pengujian lebih lanjut dengan menumbuhkannya dalam fermentor. Analisa sekuen 16S rDNA dari isolat PS1 diketahui tergolong sebagai *Acinetobacter sp*. Hasil penelitian menunjukkan bahwa produksi IAA tertinggi PS1 selama fermentasi adalah 62.428 ppm, dengan ditumbuhkan pada media yang mengandung ekstrak kecambah kacang hijau pada Ferementor setelah inkubasi 24 jam.

Kata kunci: Rizobakteri Pemacu Pertumbuhan Tanaman (RPPT), Asam indole-3-asetat, ekstrak kecambah kacang hijau, *Acinetobacter* sp.

The phytohormone auxins play a key role in plant growth and development as a regulator of numerous biological processes, such as cell division, elongation and differentiation to tropic responses. Auxins are employed to induce rooting, callus formation, flowering and so on (Teale *et al.* 2006). The action and interaction of some growth regulators like auxins regulate most of the physiological activities and growth in plants. Aplication of liquid or solid organic fertilizer containing IAA producing bacteria could improve not only the growth of various vegetables and crops, but also increased the yield under green house and field trial (Antonius & Agustiyani 2011a; Antonius and Agustiyani 2011b; Antonius *et al.* 2012; Antonius *et al.* 2015). Naturally occurring substances with indole nucleus possessing growth-promoting activity are referred to as auxins, chemically it is indole acetic acid (IAA). The auxins and cytokinins could be synthesized by microorganisms as well plants. The ability to synthesize phytohormone is widely found among plant-associated bacteria (Spaepen *et al.* 2007). About 80% of the bacteria isolated from plant rhizosphere able to produce IAA. Tryptophan is used by plants and bacteria as physiological precursors to produce IAA. The high content of tryptophan in an organism or bacterial growth media, normally following with high content of IAA.

In order to produce IAA by using rhizobacteria, it is necessary to find correct natural resources as substrate that may contain amino acid as precursor for IAA

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production. According to Mubarak (2005), germination process also lowers antinutrition component and increase the content of amino acids. Applying of mung bean sprouts extract and fish meal might be possible to get high containing tryptophan. According Kay (1979) sprout contained high amino acid, tryptophan and serin. This paper discusses the results of screening of IAA producing rhizobacteria by applying mung bean sprout extract and fish meal as sources of precursor and its IAA production ability by selected best isolate with the suitable precursor under fermentor conditions.

MATERIAL AND METHODS

Bacterial Screening of IAA Producing Activity by Using Sprout Extract or Fish Meal as Precursor. Seven bacterial isolates that were screened of IAA producing activity qualitatively in the laboratory of agricultural microbiology, Research Center for Biology-LIPI were used for this study. The medium used for growing the bacteria and IAA production was Tryptic Soy Broth (TSB) 50% (half strength), with the following composition: 10 g of Peptone 10 g, 2.5 g of NaCl, 20 g of agar, and 1000 mL of distilled water. After the medium was sterilized, filter sterilized precursor L-Tryptophan 200 ppm was added to the medium (Glickmann and Deseaux 1995). One loop of the pure culture of IAA producing bacteria was inoculated into 25 mL TSB medium in 50 mL flask and incubated at 25 °C, 120 rpm for 72 h. Each of bacteria culture at 0, 24, 48, and 72 h with 0.6 optical density (10^7 cells mL^{-1}) was picked up 3 mL and centrifuged at 12 000 rpm for 15 min for measured the IAA concentration. To study the potency of mung bean sprout as precursor, instead of tryptophan, 50 % volume of media was suplemented the sprout extract or fish meal.

DNA Extraction and Amplification of 16S rDNA. PS1 isolates was growth on the Nutrient Agar (NA) medium and incubated for 48 h. The DNA of colonies of bacteria was extracted by using GES method (Pitcher *et al* 1989). The DNA amplification was performed using Polymerase Chain Reaction (PCR). A total PCR volume used was 25 µL, containing 12.5 µL Go*Taq* Green® Master Mix (Promega), 1 µL of 10 µM 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1 µL of 10 µM 1492R (5'-TACGGYTACCTTGTT ACGACTT-3') ; and 10.5 µL Nucleus Free Water and 1 µL DNA template. The PCR protocol was as follows pre-denaturation at 95 °C for 2 min, followed by denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 1,5 min, final-extension at 72 °C for 10 min. The PCR cycle was used 30 cycles. The PCR product was verified by electrophoresis for 30 min and 100 V, on 1 % agarose in 1X TAE buffer. Formed band profiles were observed under the UV transilluminator.

DNA Sequencing and Phylogenetic Analysis. The PCR product was further analyzed by DNA sequencing (1st BASE, Singapore). The sequenced data were processed using Bioedit programme. The homology of 16S rDNA sequence were searched using BLAST software (blastn) on the National Center Biotechnology Information site (NCBI) (http://www.ncbi.nlm.nih.gov/). Constructions of phylogenetic tree was done using neighbor-joining tree method (NJT) implemented in MEGA 5.05 software (Tamura *et al* 2011). Model of K2+G+I (Kimura2-parameter and Gamma distributed) was selected as the best-fit substitution model for the current analysis. Strength of internal branches of the phylogenetic tree was tested with bootstrap analysis using 1000 replications.

Sprout Extract Preparation. A total of 1 kg of mung bean (*Vigna radiate*) seed was prepared and germinated for ± 3 d to get out the young plants with a length of 4 cm sprouts. Sprouts were then boiled in 1 L of distilled water for 30 min or until the half water volume left. The rest of sprout boiled water collected by filtered. The extract obtained was collected into the bottle and stored in the refrigerator.

Production of IAA Pilot Scale. The 20 L volume of fermentor (Shanghai Bailun-Bio technology co., Ltd) was filed with TSB media and sprout extract or fish meal (1:1) and on site sterilized. The pH of media was adjusted at 7 and under room temperature was inoculated with 1 L bacterial inoculant. The fermentor was suplied oxygen and adjusted the oxygen desolve near about 80 % with the rotor speed 100-150 rpm. The growth of bacteria and the IAA production were monitored by taking sample for measuring optical density and IAA concentration until late stationare phase.

Sample Preparation and Determination of IAA Concentration. A 3 mL of clarified supernatant of mung bean sprout extract were adjusted to pH 2.8 with HCl and extracted three times using 3 mL ethyl acetate in each extraction. The extract was drying by rotary evaporator to eliminate the solvent. In parallel, the recovery took place with percentage of 98%. The indol compounds were dissolved in 1.0 mL methanol solution and analyzed by HPLC. The resulting solution was injected into a Hitachi HPLC system consisting UV-Vis detector, autosampler. Chromatographic

separation was performed on analytical column Microsorb C18 (150 mm × 4.6 mm), eluted with methanol:acetic acid:water (30:1:70) with isocratic elution at a flow rate of 1.0 mL min-1 at room temperature. The IAA presence was detected at 280 nm wavelength for 15 min (Mehnaz dan Laravoits 2006). The IAA concentration was calculated using the peak area and the calibration curve between 0-10 ppm. Each standard of calibration curve was injected by triplicates. Each extract was run with duplicates.

RESULTS

Screening of the PGPR that Has the Highest IAA Producing Activity Quantitatively. First step screening of bacterial isolates for IAA producing activity is usually based on quality analysis. In this case, the information was only positive or negative regarding the bacterial isolate ability to produce IAA, it needed therefore to screened based on quantitative IAA producing activity in relation with the growth.

Seven different bacterial isolates (PS, ES, RS, RL, MS, PS1, and AE) collection of Agriculture microbiology laboratory-LIPI that originated from various ecosystem and showed IAA producing activity qualitatively before, were chosen to use in the study. All isolates were selected based on their ability to produce IAA by measuring the activity quantitively. In order to know the correlation of growth phase of each isolate with the optimum IAA production, the growth curve of all isolate were monitored (Fig 1). Measurement of IAA by using HPLC showed that growth medium of all the isolates contained substantial amount of IAA. Spectrophotometrically monitoring of bacterial growth indicated that PS isolate showed the best growth by showing the highest optical density data.

However the production of IAA by PS isolate was relatively low, with the maximum IAA production of about 18 ppm (Fig 2). The other isolates with code ES, RS, RL, MS, and AE produced various conctration of IAA in the growth media and PS1 isolate showed the highest production of IAA. The highest production of IAA by PS1 was about 140 ppm, acheived after 72 h incubation (Fig 2). Based on sequence analisis of 16S rDNA of PS1 isolate indicated as *Acinetobacter sp.*

Phylogenetic Characterization of Indole-3-Acetic Acid Producing Bacteria. The homology search by the BLAST program showed that PS1 isolate closely related to *Acinetobacter* sp. The phylogenetic tree was inferred using the Neighbor-Joining method and the result showed that PS1 and *Acinetobacter baylyi* strain B2 (Fig 3) were clustered together with 16S rDNA sequence-similarity values of 99%.

Production of IAA by *Acinetobacter* sp. with Sprout Extract and Fish Meal as Precursor. It is well known that tryptophan is a good precursor for production of IAA by PGPR. It has been reported that production of IAA and ILA (indole-3-lactic acid) increased with increasing concentrations of tryptophan (1-100 μg mL^{-1}) by *Azospirillum brasilense* Sp13t and SR2, respectively (Tien *et al*. 1979).

The growth indicator of each *Acinetobacter* sp. culture by plating method in TSB media suplemented with tryptophan, mung bean sprout extract or fish extract were comparable (data not shown), however

Table 1 Source of isolates and sample locations

Number	Isolates code	Source of samples	Sampling locations
1	PS	Rhizosphere of healthy banana plant	Lampung, Sumatra
2	ES	Endophytes of diseased banana plant	Lampung, Sumatra
3	RS	Rhizosphere of healthy banana plant	Lampung, Sumatra
4	RL	Rhizosphere of healthy banana plant	Lampung, Sumatra
5	MS	Rhizosphere from mix soil of banana plant	Lampung, Sumatra
6	PS 1	Rhizosphere of healthy banana plant	Lampung, Sumatra
7	AE	Rhizosphere of edamame plant	Cibinong, West Java

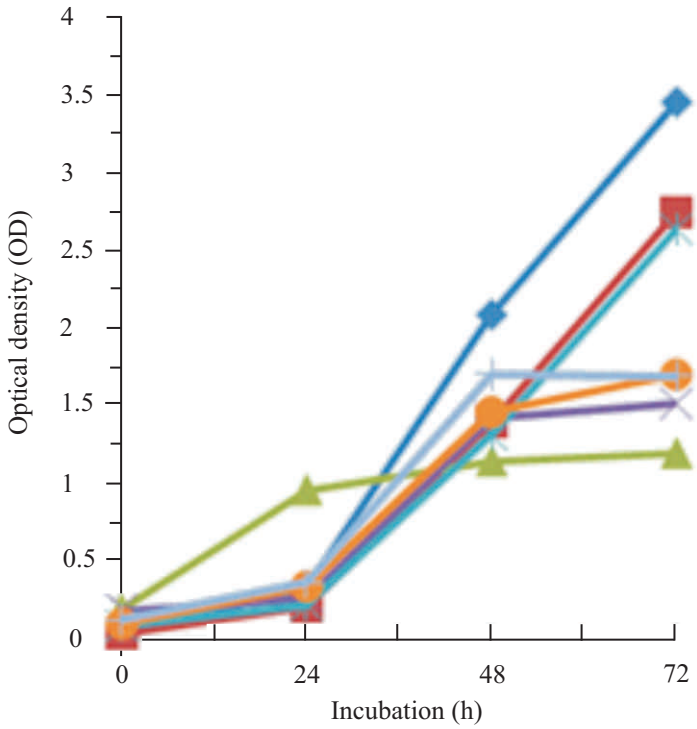

Fig 1 Growth curve of several isolates during screening for the highest production of IAA with tryptophan as precursor. The experiments were performed in triplicates. PS: , ES: , RS: , RL: , MS: , PS1: , and AE: .

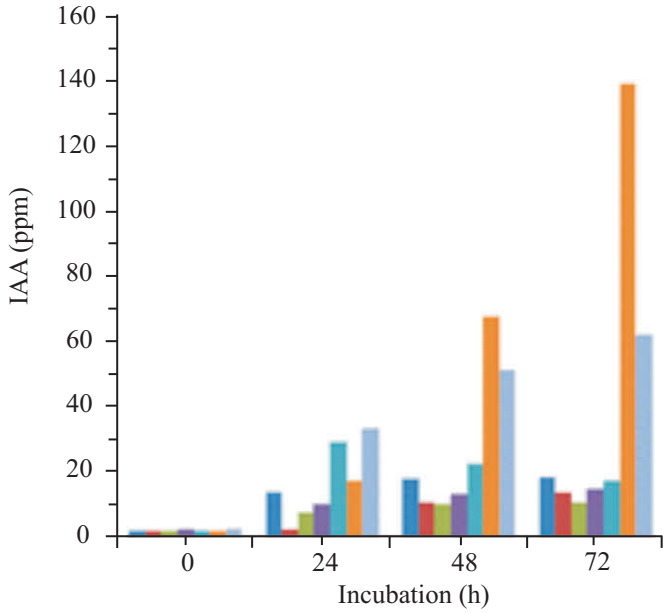

Fig 2 IAA production by several isolates during screening for the highest production of IAA with tryptophan as precursor. The experiments were performed in triplicates. PS: , ES: , RS: , RL: , MS: , PS1: , and AE: .

the production of IAA were much different especially in the media suplemented with 200 ppm of tryptophan, the IAA containing in the media was much higher about 150 ppm (Fig 4).

The growth of *Acinetobacter* sp. culture in the fermentor was very good and after 12 h incubation started to aproaching in the stationare phase. In paralel with the growth of *Acinetobacter* sp., the IAA production was started at the beginning growth and reached maximum at early stationary phase (Fig 6). The maximum production of IAA by *Acinetobacter sp* under fermentor cultivation was obtained in the 24 h incubation of time (Fig 6).

DISCUSSION

The results of quantitative IAA producing activity screening obviously showed that the diverse

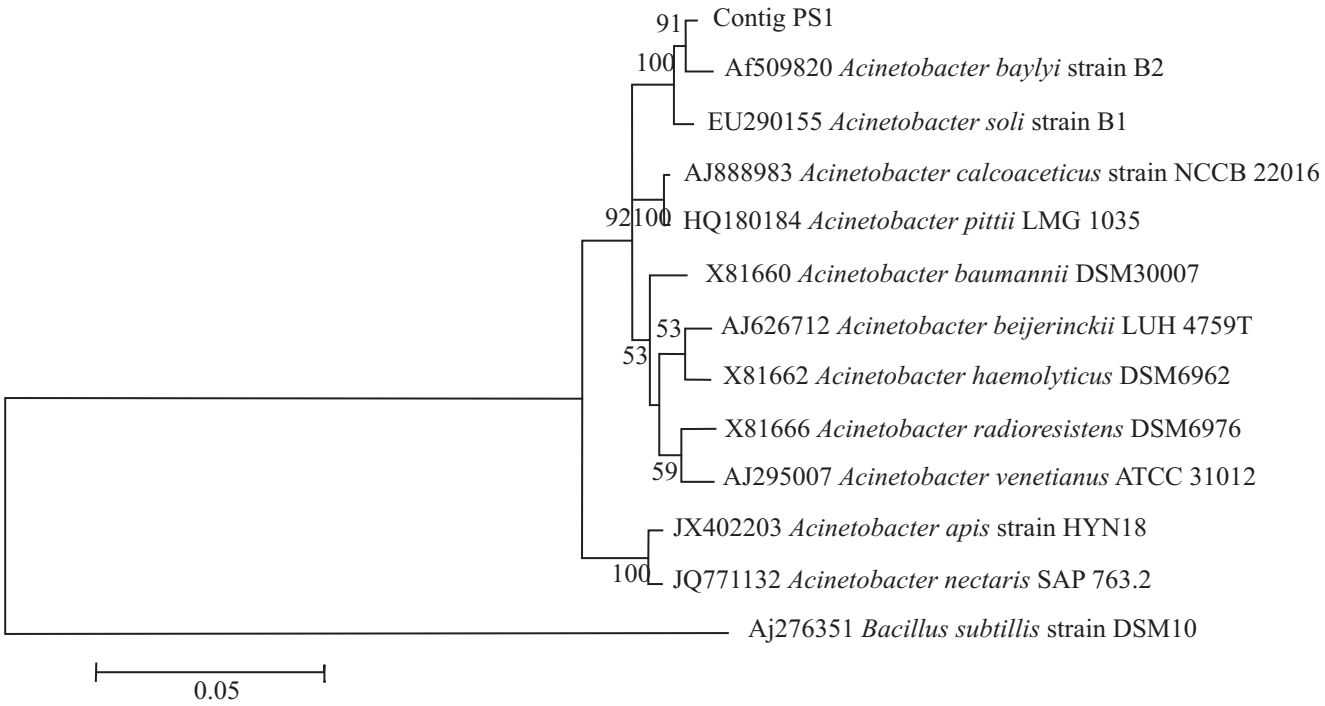

Fig 3 Phylogenetic tree of PS1 isolate based on 16S rDNA sequences of the using neighbor-joining tree method, model Kimura2-parameter and Gamma distributed with 1000 replications.

relationship between the growth rate and the ability to produce IAA. It has been reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mutluru and Konada 2007). The ideal selected bacterial isolate should be best on growth parameter and its IAA production activity. However, there was no isolate such best criteria and PS1 isolate was chosen to further study. This PS1 isolate showed the highest IAA production activity (140 ppm) compare to other isolates although maximum growth was not so high (about OD λ 436: 1.5). The highest growth was PS isolate (about OD λ 436:1.5) even though only showed relatively low IAA production (18 ppm). Interestingly, both isolates PS1 and PS were originally from Rhizosphere of healthy banana plant. The other isolate were endophytes or soil bacteria showed no special IAA producing activity nor maximum growth. Sarwar and Kremer (1992) reported that isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil. Based on phylogenetic analysis (Fig 3) PS 1 isolate has closely relatedwith *Acinetobacter* sp., It was probably due to their activity to producing IAA (Kwon and Song 2014). Rokhbakhsh-Zamin *et al.* (2011) found that *Acinetobacter* sp. was isolated from rhizosphere has ability to produce IAA.

The production of IAA with the supplement of 200 ppm of tryptophan was much higher (about 150 ppm) than the growth media supplemented with mung bean sprout extract or fish meal as natural source of tryptophan. Sprout extract and fish meal contain high concentration of protein, it might contain high concentration of amino acid including tryptophan. Therefore both source of amino acid were used in the experiments as precursor of IAA production by *Acinetobacter sp*.

Basically, the hypothesis reaction was protein of raw material converted first to amino acid (tryptophan), then use as precursor for IAA production. It was possible that conversion of the mung bean sprout extract and fish extract to tryptophan was much lower than 200 ppm and consequently the production IAA were about 25 ppm. According to Lee *et al.* (2004), in presence of higher concentration of tryptophan, the microbes release greater quantities of IAA and related compounds. The using of tryptophan in this work was as positive control. Although comparing to tryptophan, applying mung bean sprout extract as precursor for the production of IAA was much lower, it was still higher than applying fish meal as precursor. It was great interest to produce IAA by *Acinetobacter* sp. with applying mung bean sprout extract as precursor under pilot plant in the fermentor.

Antonius *et al.* (2012) reported that production of liquid organic biofertilizer with row material containing mung bean sprout could promote the higher concentration of IAA in the final product after 3 weeks

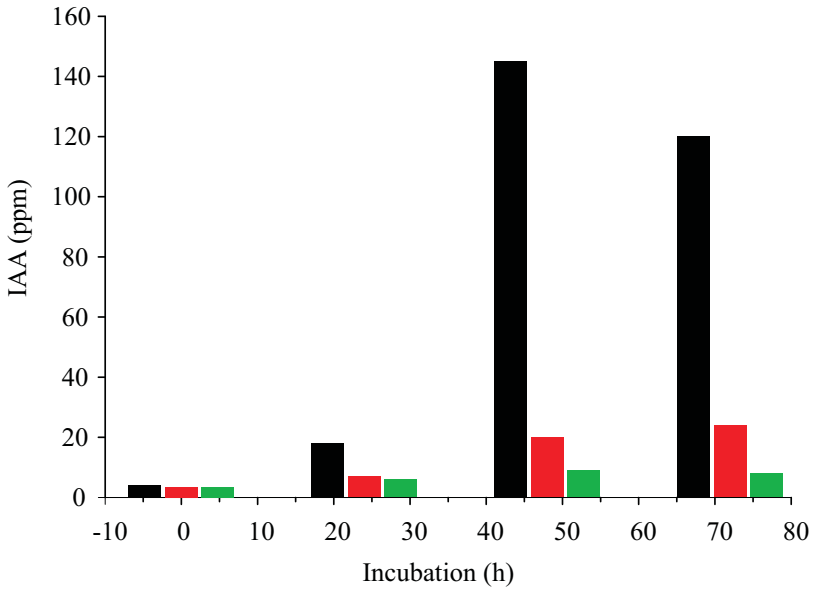

Fig 4 IAA production by *Acinetobacter* sp. with tryptophan (black bar), mung bean sprout extract red bar) and Fisch meal (green bar) as precursor. The experiments were performed in triplicates and the bars indicate standard deviation. ■ : tryptophan, ■ : mung bea sprout, and ■ :fish meal.

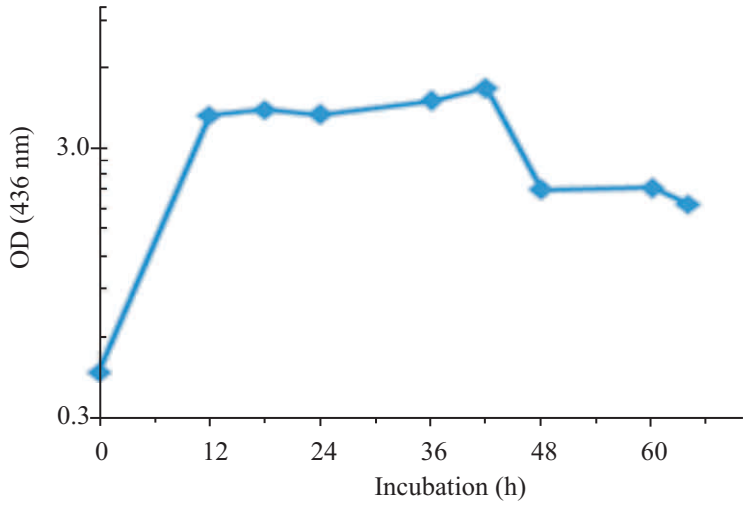

Fig 5 Growth curve of *Achinetobacter* sp. during scale up production of IAA with mung bean sprout extract as precursor in the 20 L fermentor.

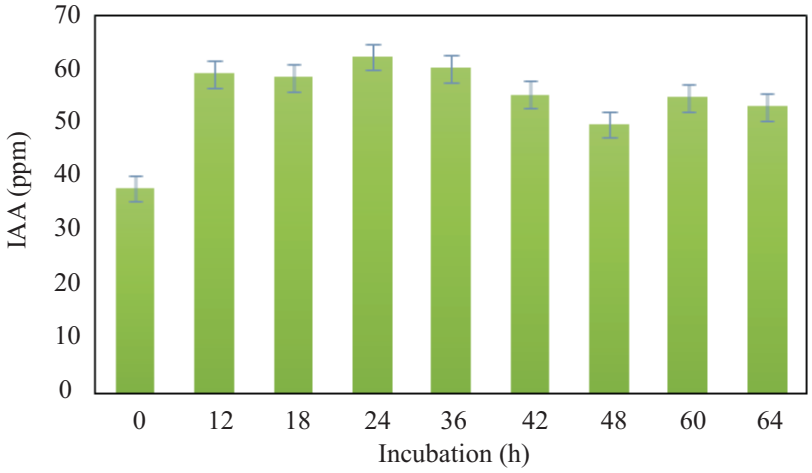

Fig 6 IAA production by by *Acinetobacter* sp. during scale up production of IAA with mung bean sprout extract as precursor in the 20 L fermentor. The experiments were performed in triplicates and the bars indicate standard deviations.

fermentation. Although the production of IAA was not so high, natural row material mung bean extract as precursor for the production of IAA was chosen for pilot IAA production under fermentor system. Such natural raw material is not only cheap but also easily to be found in every market.

The pilot scale of IAA production under fermentor system was obviously much higher compare to the erlenmeyer culturing system incubated in the rotary shaker. Under this fermentor system, the solubility and homogeneity of the substrate and precursor were much better and also very good in the diffusion of oxygen, consequently the bacterial could growth and produce IAA much better. It was likely that optimization of medium composition and the fermentation condition needed.

From this study, it is clear that seven qualitatively selected isolates of IAA producing bacteria has the ability to produce various amount of IAA in a tryptophan-supplemented medium. PS1 isolate show best IAA producing activity (about 150 ppm) in tryptophan-supplemented medium. Sequencing of 16S rDNA of PS1 isolate indicated as *Acinetobacter* sp. Natural sources mung bean sprout extract was able to be used as precursor for IAA production by *Acinetobacter* sp. with concentration of the product about 30 ppm and 60 ppm under erlenmeyer shaking culture and fermentor pilot scale production, respectively. It is concluded that mung bean as precursor could be effective and potent for the precursor of IAA production quantitatively. This research information was the first evidence that mung bean sprouts extract could be used as precursor for the production of IAA by *Acinetobacter* sp.

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