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Contributed Paper

Low-Cost Production of Indole-3-Acetic Acid Fermentation by *Enterobacter* sp.

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ABSTRACT

Enterobacter sp. DMKU-RP206 is a plant growth-promoting bacterium that can promote plant growth through direct and indirect mechanisms. At the time of writing, the bacterium *Enterobacter* sp. DMKU-RP206 has been claimed as the best IAA producer among the members of the genus *Enterobacter*. However, the drawback with regard to IAA production from this bacterium is the high cost of the production medium. A major cost component is reagent grade precursor, L-tryptophan, which is an essential and very expensive part of the IAA production medium. Therefore, the present study investigated the scaled-up IAA production by *Enterobacter* sp. DMKU-RP206 using a low-cost precursor. This study showed that it is possible to successfully reduce production costs by 52-fold as a result of the substitution of reagent grade L-tryptophan with a low-cost L-tryptophan (feed grade). The use of the low-cost medium gave the highest IAA concentration of 3,340.8 mg/L (equivalent to an IAA productivity of 27.8 mg/L/h and IAA yield of 1.97 mg/mg L-tryptophan) when 10% bacterial inoculum was used.

Keywords: *Enterobacter* sp., feed grade L-tryptophan, indole-3-acetic acid, low cost production, sweet whey

1. INTRODUCTION

Indole-3-acetic acid (IAA) is a plant growth hormone that is important with regard to stimulating plant physiology. The proper concentration of IAA supports cell division, cell elongation and cell differentiation [1] that increase plant growth and biomass. In addition, IAA may also enhance phytoremediation efficiency by promoting growth and the heavy metals uptake ability of hyperaccumulator plants [2]. Moreover, IAA has been used in human health therapy. Although IAA itself is not toxic to humans, it produces free radicals as a result of exposure to visible and ultraviolet light, which means that it then becomes cytotoxic. Na *et al.* [3] and Huh *et al.* [4] demonstrated the potential use of IAA as a photosensitizer for the treatment of acne. IAA can be activated not only by the use of visible and ultraviolet light, but also by the use of horseradish peroxidase (HRP) which Kim *et al.* [5] proposed as a cancer therapy in combination with IAA. These applications indicate that IAA has extensive benefits and it becomes of increasing interest in the health sciences.

IAA can be synthesized by both plants and microorganisms (bacteria, actinomycete, yeast and fungi). A high IAA concentration has been extensively reported in bacteria [6,

7] and in some yeasts [8]. Production cost - which is a key proportion of the overall cost of the product - can be reduced as a result of a reduction in fermentation time and raw material costs. Nutrient cost is an important aspect of IAA fermentation. Thus, employing low-cost substrates for IAA production is crucial for cost reduction. Several low-cost substrates such as agro-industrial residues (e.g. cassava fibrous residue) [9], agro waste substrate (e.g. chickpea, mungo bean and mung bean) [6], Jatropha seedcake [10] and sweet whey [11] have been used for IAA production. We successfully optimized the production medium using response surface methodology [7]. We then continued our research in an economic manner and formulated a low-cost medium by replacing lactose and yeast extract (laboratory grade) with sweet whey and yeast extract (technical grade), respectively [11]. However, the level of production costs reduction is still not satisfactory.

In our previous study, optimal L-tryptophan concentration increased the IAA yield more than 3-fold compared with a non-optimal L-tryptophan concentration [8]. This indicates that L-tryptophan is an important factor for IAA production. However, L-tryptophan is very expensive which resulted in the high production costs. The cost of L-tryptophan depends on the commercial brand used, such as $586.16 US/kg (ACROS Organic; http://www.fishersci.com) and $1,517.25 US/kg (Sigma-Aldrich; http://www.sigmaaldrich.com). In this study, we investigated scaled-up IAA production with a reduction in IAA production costs using low cost L-tryptophan.

2. MATERIALS AND METHODS

2.1 Microorganism and Culture Conditions

Enterobacter sp. DMKU-RP206 was isolated from the rice phyllosphere and maintained on Tryptone Soy Agar (TSA) [7]. The IAA production medium - Sweet whey Yeast extract L-Tryptophan (SYT) medium - was optimized in a previous study [11]. It consisted of 1.48% sweet whey (Lactoproducion, France), 1.42% yeast extract (technical grade, Becton, Dickinson and Company, USA), and 0.88% L-tryptophan (Reagent grade, Acros Organics, USA) with the pH adjustment to 6.

2.2 2-L Scale of Indole-3-Acetic Acid Production

Batch fermentation of IAA production was carried out in a 2-L stirred tank fermenter (BIOSTAT B, B. Braun Biotech International, Melsungen, Germany) with a 1.5-L working volume of SYT medium at 30°C.

In terms of inoculum preparation, the bacterium was grown on TSA for 18-20 h. One loop of the inoculum was then transferred to a 250 mL Erlenmeyer flask containing 50 mL SY broth, and incubated on an orbital shaker (Jeio Tech, Seoul, South Korea) at 170 rpm at 30 °C for 18-20 h. Finally, all of the culture was transferred to a 1,000 mL Erlenmeyer flask containing 200 mL SY broth and incubated under the same conditions and used as an inoculum for IAA fermentation.

2.2.1 Effect of agitation and aeration on indole-3-acetic acid production

5% of the bacterial inoculum was transferred to a fermenter. The agitation speed was varied (200 rpm and 300 rpm) while the aeration rate was varied at 1.5 and 3 L/min (equivalent to 1 and 2 vvm, respectively). The medium pH was uncontrolled throughout the fermentation process. Samples were taken daily for 5 days. The culture supernatant was collected by centrifugation and analysed for IAA and L-tryptophan using high-performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies, CA, USA), as described previously [12]. The culture pH was measured using a pH electrode (405-DPAS-SC-K8S/225, Mettler Toledo, Greifensee, Switzerland). The

cell growth was determined by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Genesys 20, Thermo spectronic, NY, USA). The experiments were done in triplicate.

2.2.2 Replacement of the reagent grade L-tryptophan with the feed grade L-tryptophan

5% bacterial inoculum was transferred to a fermenter. The fermenter was operated under optimal conditions in terms of agitation speed and aeration rate. Regarding the IAA production medium, feed grade L-tryptophan (Ajinomoto Thailand, Co. Ltd.) was used instead of reagent grade L-tryptophan (Acros Organics, USA). The culture supernatant was analysed for IAA and L-tryptophan using HPLC. The experiments were done in triplicate.

2.3 15-L Scale of Indole-3-Acetic Acid Production

IAA production was carried out in a 15-L stirred tank fermenter (BIOSTAT C, B. Braun Biotech International, Melsungen, Germany) with a 10-L working volume of SY medium supplemented with feed grade L-tryptophan at 30°C. Following this, 5% and 10% bacterial inoculum was transferred to the fermenter. The batch fermentation was carried out at an optimal agitation speed and aeration rate that were obtained from 2-L stirred tank fermentation. The pH of the medium was uncontrolled throughout the course of the fermentation. Samples were taken daily for 5 days. All the parameters were measured. The experiments were performed in duplicate.

3. Results and Discussion

3.1 2-L Scale of Indole-3-Acetic Acid Production

3.1.1 Effect of agitation and aeration on indole-3-acetic acid production

Enterobacter sp. DMKU-RP206 was initially shown to produce 3,804.2 mg IAA/L in a shaking flask with the optimal medium containing 0.85% lactose, 1.3% yeast extract, 1.1% L-tryptophan and 0.4% NaCl [7]. Subsequently, the production cost was shown to be reduced by replacing lactose and laboratory-grade yeast extract with sweet whey and technical grade yeast extract, respectively. After re-optimization, this bacterium yielded 3,963.0 mg IAA/L when cultivated in 1.48% sweet whey, 1.42% technical grade yeast extract and 0.88% L-tryptophan [11]. In the present study, a stirred tank fermenter was used to scale-up the IAA production with an optimized medium amended with sweet whey and technical grade yeast extract.

The effect of the agitation speed and aeration rate on IAA production was carried out in a 2-L stirred tank fermenter. *Enterobacter* sp. DMKU-RP206 was able to produce IAA under all the evaluated fermentation conditions. However, the IAA concentration varied according to the agitation speeds and aeration rates employed (Figure 1). Figure 1 showed the IAA production, pH and bacterial growth obtained in each experimental condition. Our results demonstrated that the maximum agitation speed (300 rpm) and aeration rate (2 vvm) supported the maximum IAA production (3,051.8 mg/L), followed by agitation speeds and aeration rates of 200 rpm and 2 vvm (2,817.4 mg/L), and 200 rpm and 1 vvm (2,781.3 mg/L) (Figure 1A). Regarding the pH and growth profile, a similar pattern of changes in pH and growth were shown in Figures 1B and 1C. There was a slight difference in the culture pH in that a pH range of 7.5-8.5 was observed in all tested treatments after 2 days and throughout the course of fermentation. In terms of growth, the maximum growth was reached after 2 days' cultivation, and decreased gradually over 3 to 5 days' cultivation under each condition. The effect of oxygen on the growth of bacteria, including IAA production, has been reported. In 1997, Brandl and Lindow [13] did not see

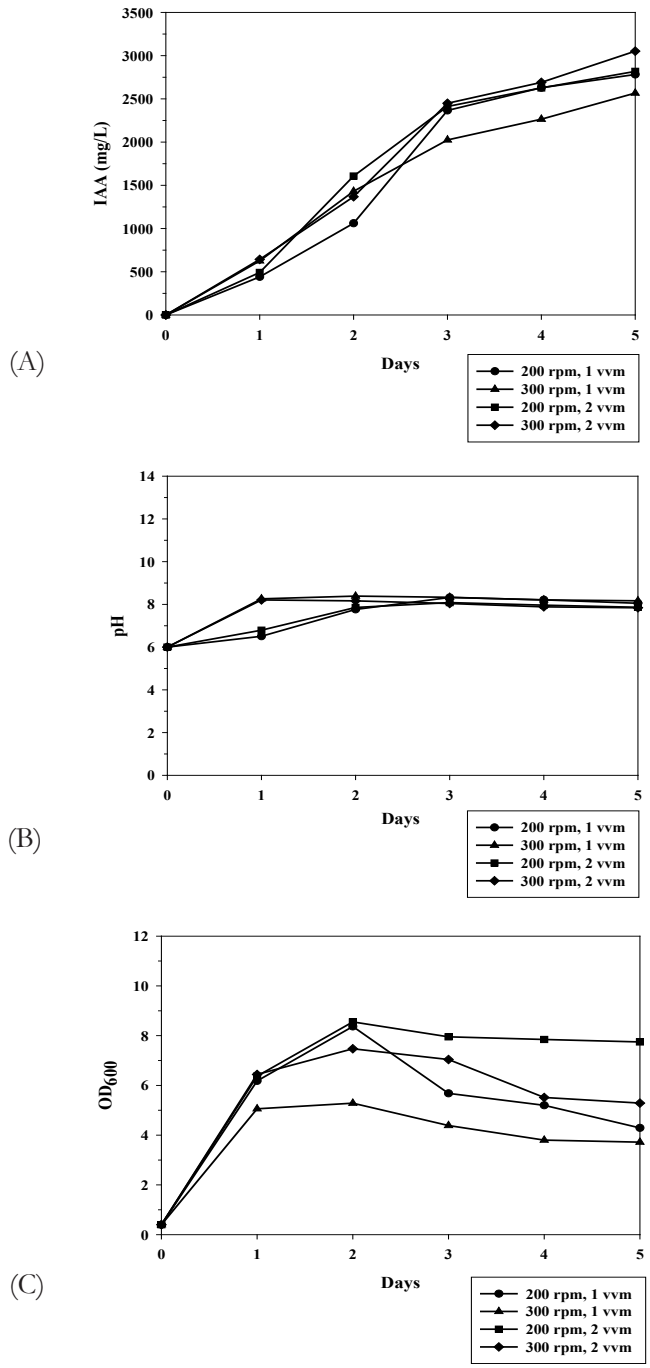

Figure 1. Profile of IAA concentration (A), pH (B) and growth (C) in all the evaluated fermentation conditions by *Enterobacter* sp. DMKU-RP206.

any effect in terms of oxygen availability on IAA biosynthesis and the expression of the *ipdC* (indole-3-pyruvate decarboxylase) promoter in *Erwinia herbicola*. However, Ona *et al.* [14] showed that low levels of oxygen (micro-aerobic condition) can be the determining factor in regulating bacterial growth rate and the production of IAA in *Azospirillum brasilense*. While, aerobic

condition (30% DO), the production of IAA and the expression of the *ipdC* were drastically reduced. These studies suggested that the effect of oxygen on IAA production was bacterial strain-dependent. In addition, the demand for oxygen also depend on the concentration and form of nutrients in the medium. Our results indicated that the maximum IAA production was observed under high aeration rate condition (2 vvm) in conjunction with a high agitation rate (300 rpm).

3.1.2 Replacement of the reagent grade L-tryptophan with the feed grade L-tryptophan

To continue our previous study [11], we decided to lower the cost of IAA production. Firstly, L-tryptophan was eliminated from the production medium to test for tryptophan-independent IAA production by *Enterobacter* sp. DMKU-RP206. The result showed that *Enterobacter* sp. DMKU-RP206 did not produce IAA in the medium without tryptophan supplement (data not shown). This therefore indicated that the bacterium produced IAA through tryptophan-dependent pathway similarly to other high IAA-producing bacteria those possess a tryptophan-dependent pathway for IAA biosynthesis. However, a tryptophan-independent pathway for IAA biosynthesis was also reported in some bacteria (such as *Azospirillum brasilense*) and showed the low IAA level. Thus, substitution of the reagent grade L-tryptophan with a low-cost L-tryptophan (feed grade L-tryptophan) would reduce the IAA production costs as compared to that of Srisuk *et al.* [11]. Figure 2 demonstrated that 2,890.1 IAA mg/L was obtained when the optimal medium contained the feed grade L-tryptophan. In addition, a pattern of changes in culture pH and growth was similar to using the medium supplemented with reagent grade L-tryptophan. From these results, we succeeded in using feed grade L-tryptophan as an IAA precursor instead of the reagent grade L-tryptophan, although the IAA amount was decreased by 5.3%. Regarding cost per product, the use of feed grade L-tryptophan (370 baht/kg equivalent to $11 US/kg) reduced IAA production costs by 52-fold compared to the use of reagent grade L-tryptophan (20,400 baht/kg equivalent to $615 US/kg). This study indicated that feed grade L-tryptophan has a key role in reducing the cost of IAA production.

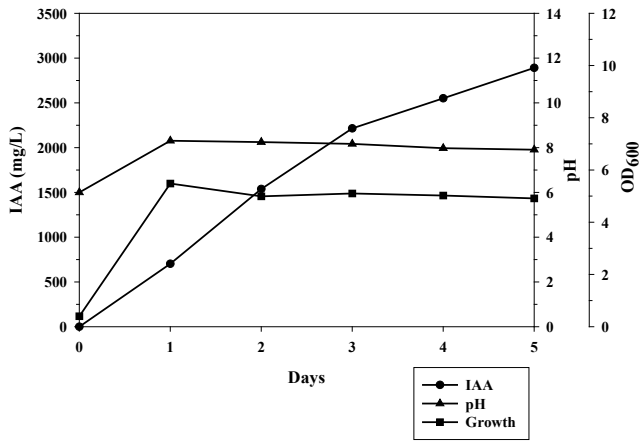

Figure 2. The IAA production by *Enterobacter* sp. DMKU-RP206 when cultivated in optimal medium with feed grade L-tryptophan.

3.2 15-L Scale of Indole-3-Acetic Acid Production

To confirm the supplementation of feed grade L-tryptophan as an IAA precursor for IAA production on a larger scale, we performed a 15-L stirred tank fermentation of IAA. The time course of IAA production, the pH change and the bacterial growth including L-tryptophan consumption were shown in Figure 3. The highest IAA concentration was 3,340.8 mg/L (equivalent to an IAA productivity of 27.8 mg/L/h and IAA yield of 1.97 mg/mg L-tryptophan) which was obtained with 10% bacterial inoculum. While under the same condition with an initial bacterial inoculum of 5%, the IAA concentration was 3,108.4 mg/L (equivalent to IAA productivity of 25.9 mg/L/h and IAA yield of 1.15 mg/mg L-tryptophan). Regarding the culture pH, a similar pattern of change in terms of culture pH was shown in both conditions. This study therefore indicated that a 10% initial bacterial inoculum did not

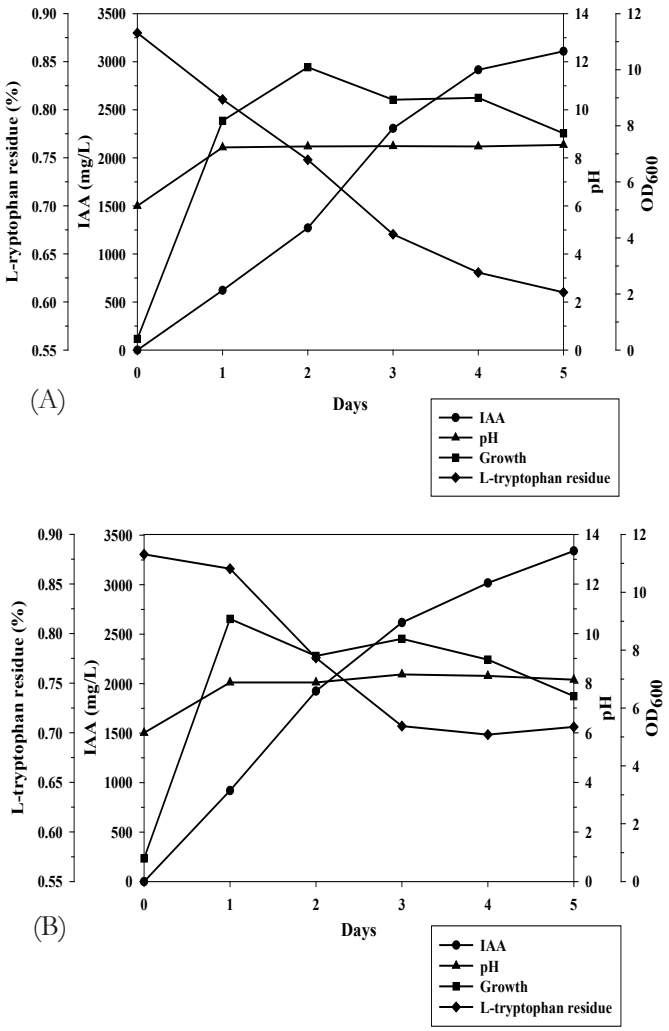

Figure 3. Profiles of IAA concentration (mg/L), pH, L-tryptophan residue and cell growth (OD_{600}) in batch fermentation by *Enterobacter* sp. DMKU-RP206 in a 15-L stirred tank fermenter under varied inoculum size. (A) 5% bacterial inoculum. (B) 10% bacterial inoculum.

promote high growth of bacteria, but supported IAA production when compared with the growth and IAA production under another fermentation condition (Figure 3). This result was consistent with that of Tallapragada et al. [15] which indicated that production of IAA increased with increasing bacterial inoculum size. However, Hanh and Mongkolthanaruk [16] reported positive relationship between growth and IAA production when varying the concentration of inoculum was added in culture medium. In addition, the results confirmed that feed grade L-tryptophan can be used as an IAA precursor instead of the reagent grade L-tryptophan which is a lot more expensive. Moreover, this result suggested that it was a challenge for *Enterobacter* sp. DMKU-RP206 to produce IAA on pilot and industrial scales by using feed grade L-tryptophan. However, L-tryptophan still remained in culture medium after 5 days' fermentation (Figure 3). In further study, repeated batch fermentation will be of interest in terms of solving this problem. Currently, Ozdal *et al.* [17] have demonstrated successful IAA production using immobilized *Arthrobacter agilis* cells. The immobilization of microbial cells has been shown to have many advantages such as higher cell density and stability, reduced reaction time, long term reuse, easy separation from the production medium, and higher product yield [18-20]. For these reasons, the immobilization of *Enterobacter* sp. DMKU-RP206 cells may be used as an alternative way to reduce production costs.

4. Conclusions

In this paper we have presented the use of feed grade L-tryptophan as having a key role in terms of reducing the cost of IAA production. We successfully reduced the production cost to 52-fold of the ordinary cost of production. In addition, we demonstrated that the adequate condition of aeration is of great relevance when it comes to increasing the production of IAA using *Enterobacter* sp. DMKU-RP206. Moreover, the inoculum size also had an effect on IAA production using this bacterium.

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Conflicts of interests

The authors declare that they have no conflict of interest in the publication.

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