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Production of Indole-3-Acetic Acid by *Enterobacter* sp. DMKU-RP206 Using Sweet Whey as a Low-Cost Feed Stock[S]

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In this study, we investigated Indole-3-acetic acid (IAA) production by a rice phylloplane bacteria, *Enterobacter* sp. DMKU-RP206, using sweet whey as a feed stock instead of lactose. We succeeded in using sweet whey for *Enterobacter* sp. DMKU-RP206 to produce 3,963.0 mg IAA/l with the optimal medium containing 1.48% sweet whey, 1.42% yeast extract and 0.88% L-tryptophan. The medium pH was adjusted to 6 and the culture conditions were shaking at 200 rpm on an orbital shaker at 30°C for 3 days. We also evaluated the effect of IAA in culture filtrates of *Enterobacter* sp. DMKU-RP206 on the promotion of jasmine rice growth in a pot experiment. Compared with the negative control (without IAA), the result showed that biosynthetic IAA produced by *Enterobacter* sp. DMKU-RP206 significantly increased the growth of jasmine rice (*Oryza sativa* L. cv. KDML105) in terms of length and dry weight of shoot. This work thus reveals the impact of IAA produced by *Enterobacter* sp. on the promotion of jasmine rice growth.

Keywords: Indole-3-acetic acid, *Enterobacter*, sweet whey, plant growth promotion, jasmine rice

Introduction

Rice (*Oryza sativa* L.) is an important food crop worldwide, particularly in Asia. Thai rice, especially the variety of Khao Dawk Mali 105 (KDML105), is famous worldwide. Khao Dawk Mali Thai rice is commonly known in food markets as ''Jasmine Rice,'' due to its shiny white color like a jasmine flower and its soft and tender texture with an aromatic fragrance when cooked. The demand for jasmine rice (*O. sativa* L. cv. KDML105) in the markets is increasing year by year. Hence, it is worth focusing on the promotion of jasmine rice growth.

Several studies have shown that *Enterobacter* can promote plant growth both in dicot and monocot [1–5]. It has therefore been claimed that *Enterobacter* is one of the bacteria that promote plant growth through either direct or indirect mechanisms. This was supported by the study of the draft genome of *Enterobacter* spp., which showed interactions between the bacteria and plant [6–9]. The genome of *Enterobacter* consists of genes coding for putative proteins involved with oxidative stress, nutrient uptake, root adhesion and colonization. The bacteria produced siderophore, 2-phenylethanol and 4-hydroxybenzoate to protect plants against bacterial and fungal infections. Moreover, the bacteria also produced acetoin and 2,3-butanediol to promote plant growth [6].

Indole-3-acetic acid (IAA) is recognized as the key plant-growth-promoting substance that regulates plant physiological and developmental functions. It has been shown that *Enterobacter* spp. produced a wide range of 5.6 mg IAA/l [10] – 382.2 mg IAA/l [11]. However, the cost of the carbon source for bacterial growth is a key factor that influences the price of any IAA produced. The use of cheap carbon sources, in particular those generated from industrial or agricultural by-products, will save production costs. Low-cost substrates, *e.g.* cassava fibrous residue and chickpea have been used for this purpose [12, 13].

Whey, a by-product from the dairy industry, is classified into two types according to the method of casein coagulation: sweet whey (rennet-induced coagulation) with pH of about 6.0–6.5, and acid whey (isoelectric precipitation) with pH of about 4.5 [14]. Sweet whey contains 70% lactose, 12%

proteins, 1.5% fat, 8.5% other solids and 4% moisture [15]. As the lactose content in whey causes pollution, one possible way of reducing such lactose content is microbial whey fermentation. Sweet whey was used as a substrate for fermentation of various products such as ethanol [16], lactobionic acid [17] and galactooligosaccharides [18]. These reviews suggested that sweet whey is an interesting alternative substrate for microbial biotechnology. However, there has not yet been any report on IAA production from sweet whey. This study thus investigated the usage of sweet whey as an alternative substrate for IAA production by *Enterobacter* sp. DMKU-RP206. In addition, the impact of bacterial IAA on the promotion of rice growth was also evaluated.

Materials and Methods

Microorganism

The potential IAA-producing bacteria *Enterobacter* sp. DMKU-RP206 used in this study was isolated from the rice phylloplane and identified by analysis of the 16S rRNA gene sequence and multilocus sequence (*gyrB*, *rpoB*, *atpD*, and *infB* genes) similarities, as described in Nutaratat *et al.* [19]. This bacterium was previously reported as a high IAA producer using lactose as a carbon source [19].

Optimization of Indole-3-Acetic Acid Production by *Enterobacter* sp. DMKU-RP206 Using Sweet Whey as a Substrate

Lactose, the carbon and energy source used in the ordinary culture media, was replaced by sweet whey powder (Lactoproducion, France) in order to minimize the cost of IAA production by *Enterobacter* sp. DMKU-RP206. Sweet whey composition is displayed in Table 1. To further reduce the production cost, laboratory-grade yeast extract was replaced by technical-grade yeast extract (Becton, Dickinson and Company, USA), which possesses acceptable clarity.

IAA production was optimized using the Box-Behnken design with four factors of interest which were sweet whey (0.5–1.5%), yeast extract (0.5–1.5%), L-tryptophan (0.1–1%) (Reagent grade, Acros Organics, USA) and pH (6–8). Twenty-seven treatments were performed in 50 ml production medium (in 250 ml Erlenmeyer flask) and cultivated at 30°C on an orbital shaker at 200 rpm (Table S1). All experiments were done in triplicate. Samples were taken during 3 days' incubation and culture supernatants were collected by centrifugation at $5,000 \times g$ for 5 min. IAA in the culture supernatants were analyzed as described by Nutaratat *et al.* [20]. The regression model was determined for its adequacy using the coefficient of determination (R^2) and the analysis of variance (ANOVA) with the lack-of-fit test. The Design Expert software version 10.0 (State-Ease, US) was used to analyze the data and draw the contour plots. The model and values obtained were validated in triplicate.

Table 1. The composition of sweet whey powder.

Component	Value
Crude protein (%, min.)	11.00
Crude fat (%, max.)	1.50
Crude ash (%, min.)	8.00
Lactose (%, max.)	70.00
Minerals	
Chloride (%)	3.50
Sodium (%)	0.90
Potassium (%)	2.80
Calcium (%)	0.90
Phosphorus (%)	0.90
Iron (ppm)	7
Copper (ppm)	2
Magnesium (%)	0.10
Amino acids	
Lysine (%)	0.87
Methionine + Cystine (%)	0.40
Threonine (%)	0.67
Tryptophan (%)	0.17

Effect of IAA in Culture Filtrates of *Enterobacter* sp. DMKU-RP206 on the Promotion of Jasmine Rice Growth

The bacterial IAA biological activity was evaluated through the effect of IAA in culture filtrates of *Enterobacter* sp. DMKU-RP206 on jasmine rice (*Oryza sativa* L. cv. KDML105) growth. In this study, we decided to use crude biosynthetic IAA to reduce the production cost on extraction and purification of IAA product. Concerning the crude biosynthetic IAA, the effect of other components in crude biosynthetic IAA was considerably negligible because the crude IAA was serial diluted to more than 1300-fold. Therefore, the influence of other components in culture filtrates may not interfere with plant growth promoting activity. The five different treatments were set up as follows: Yoshida's solution (YS) [21] as negative control; YS + synthetic IAA (10 and 50 µg/l) (Reagent grade, Sigma-Aldrich, USA) as positive control; and YS + biosynthetic IAA (10 and 50 µg/l) as test. The rice seeds were surface-sterilized for 30 sec with 70% ethanol, then by 1% NaClO for 2.5 min and 70% ethanol for 30 sec again [22]. After sterilization, the sterile seeds were rinsed in sterile water three times. The last rinse water was checked for complete external disinfection. The sterile rice seeds were soaked with sterile water for 1 day and transferred onto sterilized moistened tissue paper for 1 day in order to study the seed germination. The germinated rice seeds were then planted in pots containing sand and vermiculite (2:1) in a greenhouse with natural photoperiod in Bangkok during June 2017 (atmospheric temperature 24–34°C; https://www.accuweather.com). The sand and vermiculite were autoclaved three times at 121°C for 30 min. Fifteen milliliters of

Table 2. Analysis of variance (ANOVA) of Box–Behnken design for quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	18403280.24	14	1314520.02	5.869	0.002
X_1-Sweet whey	1216982.60	1	1216982.60	5.433	0.038
X_2-Yeast extract	1268767.82	1	1268767.82	5.664	0.035
X_3-L-tryptophan	8989361.67	1	8989361.67	40.132	0.000
X_4-pH	1552076.97	1	1552076.97	6.929	0.022
X_1X_2	664.35	1	664.35	0.003	0.957
X_1X_3	1671125.91	1	1671125.91	7.461	0.018
X_1X_4	98658.81	1	98658.81	0.440	0.519
X_2X_3	28825.25	1	28825.25	0.129	0.726
X_2X_4	95505.72	1	95505.72	0.426	0.526
X_3X_4	2560928.53	1	2560928.53	11.433	0.005
X_1^2	14326.25	1	14326.25	0.064	0.805
X_2^2	203624.25	1	203624.25	0.909	0.359
X_3^2	243698.94	1	243698.94	1.088	0.317
X_4^2	170747.72	1	170747.72	0.762	0.400
Residual	2687937.38	12	223994.78		
Lack of fit	2641282.41	10	264128.24	11.323	0.084
Pure error	46654.97	2	23327.49		
Cor total	21091217.62	26			

$R^2 = 0.87$

individual treatments were poured only on the first day of plantation followed by pouring 15 ml daily of YS for 2 weeks in all treatments. After 2 weeks of cultivation, the length and dry weight of root and shoot were measured. The experiment was done in duplicate.

Results

Optimization of Indole-3-Acetic Acid Production by *Enterobacter* sp. DMKU-RP206 Using Sweet Whey as a Substrate

During three days' cultivation of *Enterobacter* sp. DMKU-RP206, the production of IAA was 210.6–4,054.0 mg/l. The analysis of variance (ANOVA) is presented in Table 2. Based on the ANOVA, the p-values for the model ($p = 0.002$) and for lack-of-fit ($p = 0.084$) indicated that the experimental data fitted well with the model. The final response equation for IAA production is shown below:

$$Y = -80.04 + 1618.70X_1 + 4117.71X_2 + 9958.90X_3 - 1263.53X_4 + 51.55X_1X_2 + 2872.71X_1X_3 - 314.10X_1X_4 + 377.29X_2X_3 - 309.04X_2X_4 - 1778.10X_3X_4 - 207.31X_1^2 - 781.58X_2^2 + 1055.61X_3^2 + 178.93X_4^2$$

where Y is indole-3-acetic acid (mg/l), X_1 is sweet whey, X_2 is yeast extract, X_3 is L-tryptophan and X_4 is pH.

This model was supported by the high value (0.87) of the correlation coefficient (R^2) obtained. The R^2 value that was close to 1 implies that the strong statistical model and good response prediction were obtained [23]. The regression coefficients showed that IAA production by *Enterobacter* sp. DMKU-RP206 was influenced by interactions between sweet whey and L-tryptophan, and pH and L-tryptophan. The interactions between these variables on IAA yield were illustrated as a surface response and a contour plot (Fig. S1), which showed a significant ($p < 0.05$) effect on IAA production by this bacterium (Table 2). The results showed that high levels of sweet whey, yeast extract and L-tryptophan increased IAA yield. However, low pH was also found to enhance the IAA yield.

The RSM model revealed the conditions for maximum IAA production (4,084.8 mg/l) by *Enterobacter* sp. DMKU-RP206 as 1.48% sweet whey, 1.42% yeast extract, 0.88% L-tryptophan, pH 6 and 3 days' shaking on an orbital shaker at 200 rpm and 30°C. The experimental model was validated using the optimum conditions described above and in the production of IAA at 3,963.0 mg/l was found. A good correlation was obtained when comparing the predicted with the observed values. This implies that the RSM model

Table 3. Effect of IAA in culture filtrates of *Enterobacter* sp. DMKU-RP206 on jasmine rice growth promotion.

Treatments	No. of plants	Root length (cm)	Shoot length (cm)	Root dry weight (g)	Shoot dry weight (g)
Yoshida's solution (Negative control)	248	11.6 ± 0.9[a]	18.1 ± 1.2[a]	0.002 ± 0.0[a]	0.003 ± 0.0[a]
YS + 10 µg/l synthetic IAA (Positive control)	248	11.8 ± 0.8[a]	22.7 ± 1.3[c]	0.002 ± 0.0[a]	0.009 ± 0.0[b]
YS + 10 µg/l biosynthetic IAA	246	11.8 ± 0.9[a]	22.7 ± 1.5[c]	0.002 ± 0.0[a]	0.009 ± 0.0[b]
YS + 50 µg/l synthetic IAA (Positive control)	242	11.6 ± 0.9[a]	18.8 ± 1.1[b]	0.002 ± 0.0[a]	0.003 ± 0.0[a]
YS + 50 µg/l biosynthetic IAA	244	11.6 ± 0.9[a]	18.7 ± 1.0[b]	0.002 ± 0.0[a]	0.003 ± 0.0[a]

Different superscript letters (a, b, and c) within the same column indicate significant difference between treatments using Duncan's multiple range test ($p < 0.05$).

can be used to describe the relationship between the IAA production and the influenced factors.

Effect of IAA in Culture Filtrates of *Enterobacter* sp. DMKU-RP206 on the Promotion of Jasmine Rice Growth in a Pot Experiment

Growth parameters (the length and dry weight of root and shoot) after 2 weeks' plantation were measured to assess the growth promoting ability of IAA produced by *Enterobacter* sp. DMKU-RP206. Table 3 shows that jasmine rice supplemented with IAA has a significantly increased length and dry weight of shoot compared with the negative control (without IAA); however, no difference was observed on the length and dry weight of root. Regarding the two different concentrations of IAA, the low concentration of IAA (10 µg/l) supported better jasmine rice growth both of length and dry weight of shoot than the high concentration of IAA (50 µg/l). With regard to synthetic and biosynthetic IAA, a similar effect on jasmine rice growth was shown when the same concentrations of synthetic and biosynthetic IAA were used. Thus, this result indicates that IAA in culture filtrates of *Enterobacter* sp. DMKU-RP206 can be used instead of the synthetic IAA.

Discussion

Indole-3-acetic acid (IAA) possesses both positive and negative effects on plants depending on the dose used and kind of plant. Regarding the positive effect, IAA stimulates division, elongation and differentiation of cells including growth of fruit [24]. Regarding the negative effect, IAA can inhibit plant growth by stimulation of ethylene production, which causes stress conditions [25]. These indicate that IAA, at its optimal level, is essential to promote plant growth. IAA was produced by various microorganisms – *i.e.* bacteria [26], actinomycetes [27], cyanobacteria [28], yeasts [20] and fungi [29]. Regarding IAA production, most IAA production by microorganisms has used a synthetic medium. Only a few reports have shown the use of alternative

low-cost materials for IAA production. For example, Peng *et al.* [30] used corn flour as the carbon source and soybean meal as the nitrogen source to study IAA production by *Pseudomonas putida* Rs-198. In addition, agro-industrial residue such as cassava fibrous residue and agro-waste substrate such as chickpea have been used to reduce costs of IAA production [12, 13]. Sweet whey has been used as a carbon source for production of various microbial products [16–18]. However, IAA production from sweet whey has not yet been reported.

In this study, the highest amount of IAA production was 3,963.0 mg/l when cultivated *Enterobacter* sp. DMKU-RP206 in 1.48% sweet whey, 1.42% technical grade yeast extract, 0.88% L-tryptophan, pH was adjusted to 6 and incubated at 30°C for 3 days on an orbital shaker at 200 rpm. In our previous study [19], 3,804.2 mg/l of IAA was obtained as the maximum IAA production in the culture medium containing 0.85% lactose, 1.3% yeast extract, 1.1% L-tryptophan, 0.4% NaCl, pH was adjusted to 5.8 and incubated for 5 days at 30°C and 200 rpm. From the result, it has been clearly shown that sweet whey can be used to replace lactose for IAA production. In addition, using sweet whey also reduced the addition of NaCl into the fermentation medium due to NaCl being contained in sweet whey. The present study is therefore revealed as a success in its reduction of both time and cost of IAA production when compared with the previous study [19]. Moreover, this study is the first report on using sweet whey as a carbon source for IAA production. Finally, we found a higher amount of IAA than that previously reported for the genus *Enterobacter* [10, 11].

In the pot experiment, we found that both synthetic and biosynthetic IAA promoted rice growth in terms of shoot, in particular shoot length, but had no effect on the length and dry weight of root. At low concentration of IAA (10 µg/l), it significantly enhanced the length and dry weight of shoot, while at high concentration of IAA (50 µg/l) only shoot length was significantly enhanced when compared with the negative control. The result indicated

that more than 50 μg/l of IAA may inhibit jasmine rice growth. This result was consistent with that of Leveau and Lindow [31], which indicated that the lowest concentration of IAA (1 nmol/l) significantly stimulated radish root growth compared with higher concentrations (1 μmol/l and 1 mmol/l) and control (without IAA). Park *et al.* [32] recently demonstrated that 10^{-6} mol/l IAA significantly promoted the length and width of leaf and root length of lettuce, whereas 10^{-4} mol/l IAA strongly inhibited plant growth. In addition, the present study clearly indicated that biosynthetic IAA from *Enterobacter* sp. DMKU-RP206 had the potential to promote jasmine rice growth similarly to commercially synthetic IAA. Thus, the biosynthetic IAA produced by *Enterobacter* sp. DMKU-RP206 can be used to replace a commercially synthetic IAA.

In conclusion, this study succeeded in using sweet whey as a carbon source to produce IAA by *Enterobacter* sp. DMKU-RP206 and the IAA produced by this bacterium showed the potentiality to promote jasmine rice growth.

Acknowledgment

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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