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Potentiality of Endophytic Actinomycetes Isolated from Sugar Cane

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Abstract

Endophytic actinomycetes residing in plants may be originated from soil around plant root. They are not act as symbiosis but mutually associated as free living microbes. These actinomycetes may be an efficient source promoting growth through secretion of plant growth regulators. The objectives of this study were isolation characterization and determination the beneficial properties; the production of bioactive compound, indole-3-acetic acid (IAA), siderophore and biosolubilizing activities of insoluble phosphate and leonardite, of endophytic actinomycetes. The results showed that 102 endophytic actinomycetes were isolated from root, leaf and stem of sugar cane. The major population was found in root followed by stem and leaf, respectively. Based on its morphological and chemotaxonomical analysis, 86 isolates were belonged to member of genus *Streptomyces* while the other 16 isolates were classified as non-Streptomyces. Among 102 of isolated endophytic actinomycetes, 46 isolates were able to produce indole-3-acetic acid (IAA), a plant growth promoting substance. The maximum production of 100 ± 0.34 µg/mL was produced from isolate SCR5. The siderophore, an iron chelating compound was detected for 93 isolate from those of endophytic actinomycetes when cultured on CAS agar medium with positive zone less than 20 mm. Thirty seven isolates showed abilities to solubilize insoluble phosphate and only one isolate can solubilize leonardite as low quality coal. The isolate SCR5 was the potential strain as a plant growth promoting activity such as phosphate solubilizing activity, IAA and siderophore production.

Keywords: endophytic actinomycetes, sugar cane, indole-3-acetic acid, siderophore, phosphate solubilizing, leonardite

1. Introduction

Thailand is one of the largest sugar cane producers in Southeast Asia region. The sugar cane was used as raw material for sugar and ethanol production. The production has increased drastically every year to approximately more than 100 million tons in 2013 according to demand of consumer. The important factors that effected to plant health is the microorganisms that living in and out-side of the plant [1-2]. To protect sugar cane from diseases, the control strategies are the use of resistant variety of sugar cane and the application of fungicide. However, the application of chemical control leads to environmental impact. The environmental friendly application was consider for controlling the sugarcane disease.

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Actinobacteria are filamentous Gram-positive bacteria. Most members contained the % G+C content higher than 55% [3]. Actinomycetes were accepted source for medical enzymes as uricase, xylanase and bioactive metabolites [4-6]. Many Actinomycetes produce bioactive compounds such as antibiotics, including actinomycin and tetracycline [7-9].

Endophytic bacteria were classified into 2 groups of obligate and facultative endophyte base on its life character [10]. However, the origin of endophyte came from soil. Endophytic actinomycetes residing in plants are not act as symbiosis but mutually associated as free living microbe [11]. These actinomycetes may be an interested source to finding efficient biocontrol agents for plant health and promoting growth through secretion of plant growth regulators. The studies of endophytic actinomycetes were done in various kind of plant such as orchid, mandarin orange, zingiber, cinnamimum leguminous plant, rice etc. [12]. However, endophytic actinomycetes in sugar cane have not been reported. The aim of this study were isolation characterization and determination the beneficial properties; the production of indole-3-acetic acid (IAA), siderophore and biosolubilizing activities of insoluble phosphate and leonardite, of endophytic actinomycetes.

2. Materials and Methods

2.1 Endophytic actinomycetes

From our previous study, endophytic actinomycetes were isolated from each part of sugar cane in Thailand. [13] The distribution was found in root, stem and leave respectively. The major population was found in root followed by stem and leaf, respectively [13]. One-hundred and two endophytic actinomycetes were isolated and classified into genus level based on it morphological character and chemotaxonomic properties according to the methods of Hasegawa *et al.* (1983) [14] and Lechevalier & Lechevalier (1980) [15]. Major population was belonging to the genus *Streptomyces* followed by *Micromonospora* [13]. However, the biological role of these microorganism still unknown. In this study we focus on biological activities of endophytic actinomycetes from sugar cane.

2.2 Indo acetic acid (IAA) production

All endophytic actinomycetes were screened for their IAA production using colorimetric assay according to the methods of Goudjal et al.; Gangwar *et al.* [16-17]. The actinomycetes were grown on yeast malt extract broth containing 0.2% L-tryptophan and incubated at room temperature (30-35^{o}C) with shaking at 120 rpm for 5 days. Cultures were centrifuged 9000 rpm for 5 min. One milliliter of supernatant mixed with 2 mL of the Salkowski reagent. Absorbent was read at 530 nm using spectrophotometer. The level of IAA production was estimated by comparison with IAA standard.

2.3 Siderophore production

The endophytic actinomycetes were inoculated on Chrome azurol S (CAS) agar medium and incubated at room temperature for 5 days. The colonies with orange zone were determined as positive.

2.4 Phosphate solubilization

The endophytic actinomycetes were inoculated on Pikovskaya medium containing $Ca_3 (PO_4)_2$ according to Nautiyal [18]. The presence of clear zone around the colony after incubated at room temperature for 5 days was determined as positive.

2.5 Leonardite solubilization

The endophytic actinomycetes were inoculated on Pikovskaya agar medium containing $Ca_3(PO_4)_2$ and incubated at room temperature for 5 days. Then small pieces of sterile leonardite were placed on the agar cultures plate. The solubilization was evidenced by the appearance of black liquid surrounding the coals within 3 days. This method modified from Dahlberg *et al.* [19].

3. Results and Discussion

3.1 The production of indole acetic acid (IAA)

Most of the isolates in this study were classified as genus *Streptomyces*. The isolates exhibit wall chemotype I which contained LL-diaminopimelic acid in the peptidoglycan of the cell wall and no characteristic sugars. However, 10 isolates were belonging to member of genus *Micromonospora*. The strain contained *meso*-diaminopimelic acid in the cell wall and ribose, mannose, galactose, xylose, glucose and arabinose were found in whole-cell sugars. The indole-3-acetic acid (IAA) production was found in 46 isolates (45%) of endophytic actinomycetes in a range of 1.96-100 µg/mL (Table 1). The highest production of 100 µg/mL was produced from isolate SCR 5 and the lowest production only 1.96 µg/mL was produced from isolate 1-SCR 12. From our result, it seem to be that endophytic actinomycetes from sugar cane can produce IAA in a higher range than that report of Nimnoi *et al.* [20].

IAA was a microbial product that affected to stimulate plant growth. Its structure was similar to auxin, plant growth hormone. The role of IAA was effected to plant growth by stimulating and controlling the mechanism of cell division, cell elongation and cell differentiation resulted to decrease of root length while increase number of root hair for more nutrient uptake. [21-23].

Actinomycetes were found to produce a variety of metabolite including plant growth regulating agent such as auxin and gibberellins [24].

At present, the interesting in plant growth regulating agents from actinomycetes was increased. Various genera of actinomycetes had ability to produce IAA such as *Nocardia jiangxiensis* and *Actinomadura glauciflara* with 15.14 µg/mL and 9.85 µg/mL, respectively. Generally, most of IAA producing microorganisms was resident in rhizosphere and soil resulted to stimulate plant growth such as weight of maize, cucumber, tomato, sorghum, and carrot [25-27]. However, IAA obtained from microorganism could induce by the culture media containing tryptophan [28].

Table 1. Abilities of some endophytic actinomycetes from sugar cane on production of IAA, siderophore and solubilizing of insoluble-phosphate and leonardite

Genus	Isolated	IAA production (μg/mL)	Siderophore	Phosphate solubility	Leonardite solubility
Streptomyces	5-SCR 23	22.32 ± 0.35	-	-	-
eptomyces	5-SCR 24	37.26 ± 0.42	+	+	-
Streptomyces	5-SCS 26	0	+++	+	-
Streptomyces	2-SCR 7	26.70 ± 0.40	+	-	-
Streptomyces	5-SCR 5	16.14 ± 0.42	-	+	-
Streptomyces	5-SCR 19	44.07 ± 0.64	+	+	-
Streptomyces	5-SCS 12	21.15 ± 0.48	+	+	-
Streptomyces	1-SCS 6	27.08 ± 0.78	+++	+	-
Streptomyces	SCR 2	25.96 ± 0.56	+	+	-
Streptomyces	5-SCR 15	12.22 ± 0.50	+	+	-
Streptomyces	5-SCS 10	30.07 ± 0.34	+	-	-
Streptomyces	2-SCR 21	44.89 ± 0.76	+	-	-
Micromonospora	5-SCS 17	17.68 ± 1.50	+	-	-
Micromonospora	1-SCS 12	1.96 ± 1.23	++	-	-
Streptomyces	SCR 11	19.07 ± 0.11	+	-	-
Streptomyces	SCR 12	36.04 ± 0.02	+	+	-
Streptomyces	2-SCR 28	20.57 ± 0.05	+	+	-
Streptomyces	2-SCR 18	24.65 ± 0.61	+	-	-
Streptomyces	1-SCS 7	34.76 ± 0.92	-	-	-
Streptomyces	3-SCS 9	18.64 ± 0.17	+	+	-
Streptomyces	2-SCR 31	0	+	+	-
Streptomyces	5-SCR 22	0	+++	-	-
Streptomyces	1-SCS 3	42.61 ± 0.43	+	-	-
Micromonospora	5-SCS 11	19.23 ± 0.12	+	-	-
Streptomyces	5-SCS 35	0	+++	+	-
Streptomyces	5-SCS 9	3.54 ± 0.03	+	-	-
Streptomyces	2-SCR 5	4.87 ± 0.23	-	-	+
Streptomyces	5-SCS 39	19.89 ± 0.39	+	+	-
Micromonospora	5-SCS 1	18.32 ± 0.12	+	-	-
Streptomyces	2-SCR 43	3.97 ± 0.02	+	-	-
Micromonospora	5-SCS 4	22.56 ± 0.97	+	-	-
Micromonospora	5-SCS 31	3.33 ± 0.45	+	-	-
Micromonospora	3-SCS 7	17.32 ± 0.22	+	-	-
Streptomyces	SCR 3	17.36 ± 1.02	++	+	-
Streptomyces	SCR 5	100 ± 0.34	++	+	-
Streptomyces	1-SCR 2	21.54 ± 0.41	+	-	-
Micromonospora	5-SCS 3	18.02 ± 0.12	+	+	-
Streptomyces	5-SCS 38	34.16 ± 0.81	+	+	-
Micromonospora	5-SCS 30	18.54 ± 0.32	+++	-	-
Streptomyces	5-SCR 13	31.53 ± 0.15	+	-	-
Streptomyces	5-SCS 29	32.23 ± 0.32	+	-	-
Streptomyces	2-SCR 25	9.23 ± 0.89	+	-	-
Streptomyces	SCR 17	4.15 ± 0.14	-	-	-
Streptomyces	5-SCR 26	+	+	-	-
Streptomyces	SCR 7	24.21 ± 0.34	+	+	-
Streptomyces	SCR 10	21.59 ± 0.17	+	-	-
Streptomyces	2-SCR 4	65.34 ± 0.56	+	+	-
Streptomyces	2-SCR 29	0	+++	-	-
Micromonospora	1-SCR 5	21.45 ± 0.32	-	-	-
Streptomyces	1-SCR 10	20.13 ± 0.67	+	+	-

Note: -mean Negative
+ mean Low
++ mean Moderate
+++ mean High

Endophytic actinomycetes which isolated from mandarin orange plant in northern region of Thailand produced IAA in range of 1.4-140.38 µg/mL in liquid media containing L-tryptophan 2 mg/mL. The effective strain was classified as member of the genus *Nocardiopsis* with production of 62.23-222.75 µg/mL [29]. Furthermore, the application of IAA from effective strain found to stimulate growth of mandarin orange better than plant without microbial IAA. In addition, root length and root wet weight were also increased [29]. The report of IAA production from endophytic actinomycetes from sugar cane was rare whereas from other plant were frequently reported.

3.2 Siderophore production

The study of siderophore production was done on synthetic media containing Chrome azural S (CAS). Among 102 isolates, there are 93 isolates (91.2%) produced siderophore in solid media at various level (Table 1). This may implied that endophytic actinomycetes from sugarcane required iron for activity of cell. Moreover, those isolates produced siderophore more than that strain from jasmine rice but close to the production from soil actinomycetes. The number of siderophore producing endophytic actinomycetes was different from Rungin *et al* [30]. It is possible that type of vegetation affects to the distribution of siderophore producing actinomycetes.

Iron is an essential element for all life [31] because it has an important role in cell interaction related to DNA synthesis, respiration, and photosynthesis [32-33]. In general, iron was abundant in soil but availability was limited by iron fixation in form of insoluble FeOH. This form was unavailable for plant and microorganisms [34]. When microorganism leaves in iron deficient environment, they produced siderophore to dissolve ferric iron and control balance of iron [35-37].

Many bacteria can produce siderophore for example *Escherichia coli, Salmonella, Klebsiella pnuemoniae, Vibrio chlierae, Aeromonas, Aerobacter aerogens, Enterobacter, Yersinia, Mycobacterium* [38]. The types of siderophore produced from bacteria are hydroxamate catecholate salicylate and carboxylate. They are important in solubilization of iron. *Actinomycetes* also produced siderophore such as *Actinomadura madurae, Nocardia asteroids* and *Streptomyces griseus*. Most actinomycetes from soil and rhizosphere had abilities to produce siderophore. The effective genera were classified as *Streptomyces*. Siderophore from rhizobacterium affected on iron uptake of plant and effect to inhibit growth of plant pathogenic microorganisms [39].

Rungin et al. isolated endophytic actinomycetes from Thai jasmine rice. There are 56% of isolates produced siderophore stimulating growth of jasmine rice directly [30]. Most of siderophore produced from actinomycetes was secreted to fix iron out side cell. *Streptomyces* also produced siderophore same as other microorganisms. The major siderophore from *Streptomyces* was trihydroxamate siderophore as known in the name of desferrioxamine [40-42] and griseobactin [43]. *Rhodococcus* and *Nocardia* produced a novel heterobactin such a kind of siderophore [44-45].

3.3 Phosphate solubilizing activity

Phosphate solubilizing activity of endophytic actinomycetes were determined on Pikovskaya medium containing insoluble phosphate ($Ca_3(PO_4)_2$). Among 102 isolates, 52 isolates (51%) showed phosphate solubilizing activities (Fig.1A). The percentage of phosphate solubilizing endophytic actinomycetes from this study was similar to actinomyces from soil and rhizosphere [46]. Various *Streptomyces* were reported as the most potential phosphate solubilizing actinomycetes [46-47]. In addition, aquatic sediment was reported as a source of *Streptomyces* with potential phosphate solubilizing [48]. Phosphate solubilizing actinomycetes could promote mycelium growth and spore germination of mycorrhizal fungi in co-cultivation [47].

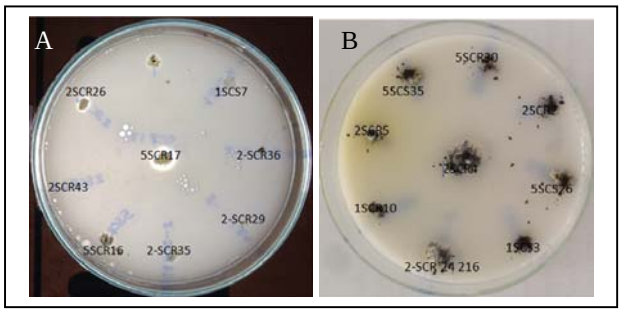

Figure 1. Phosphate solubilizing activity (A) and Leonardite solubilizing activity (B) of endophytic actinomycetes on Pikovskaya medium containing $Ca_3(PO_4)_2$ as insoluble phosphate after incubated at room temperature for 5 days

Phosphorus is a macro nutrient that essential for plant growth. Actually, phosphorus was abundant in soil in a form of unavailable for plant because it was easily fixed with some metal ion and clay mineral in soil. This process led to phosphorus deficient of plant and necessary to add phosphorus fertilizer into soil. The applications of phosphate solubilizing microorganisms cooperate with phosphate chemical fertilizer was successes to increase yield and sweetness of sugar cane [49]. The addition of rock phosphate during compost making led to the solubilizing of more available phosphate in compost. The advantage of phosphate solubilizing is environmental friendly [50]. Phosphate solubilizing microorganisms were found in various environment such as soil, rhizosphere and marine [48], [50], [51-53]. The effective fungi is mycorrhizal that association with plant roots. However, the application of mycorrhizal fungi was limited due to the used of fungicide and access of phosphate fertilizer. Now a day, the study on phosphate solubilizing actinomycetes was wildly spread.

The phosphate solubilizing mechanisms of microorganisms were operated by two ways, first is occurred by the activity of enzyme phosphatase and the second is by activity of organic acids [48].

3.4 Leonardite solubilizing activity

All of isolated endophytic actinomycetes, it was found that only isolate 5-SCR 27 was able to solubilize leonardite as shown in Fig. 1B. Leonardite was a low quality oxidized coal usually found in lignite mine which was originated from decomposition of lignite coal. Leonardite insoluble in water and major components were fulvic acid, humic acid and humin. Humic acid content in leonardite was considered as valuable substance as soil amendment. It was rich in negative functional group that can storage plant nutrient in form of anion and slow release for plant. Furthermore, humic acid can improve physical, chemical and biological properties of soil.

According to structure of leonardite, the core structure consist of aromatic core which was difficult to attack by microorganisms. That why the number of leonardite solubilizing actinomycetes was small. The leonardite solubilizing microorganisms was first reported by Cohen and Gabriele [54] by the secretion of dark brown droplet from low quality coal by *Coriolus versicolor* and *Poria monticola*. Later, various microorganisms were found to solubilized leonardite such as *Candida* sp. ML113, *Penicillium waksmanii*, *Sporothrix* sp. *Asergillus* sp. [55] *Bjerkandera adusta*, *Poria placenta*, *Fomes annosus* [56] and including *Streptomyces* sp. [57].

4. Conclusions

Endophytic actinomycetes were isolated from sugar cane in Thailand. The biological activity of IAA, siderophore production, phosphate solubilizing and leonardite solubilizing were determined. Among 102 isolates, there are 46, 93, 52 and 1 isolate showed the activity of those, respectively. The result indicated there are diverse activity of endophytic actinomycetes. Only fifteen isolates showed three biological activities. The potential strain was SCR5 with shown three tested biological activity and produced the highest amount of IAA of 100 µg/ml.

It this very interesting that endophytic actinomycetes from sugar cane play an important role on biological activity that gives a benefit for plant in many ways. They can supply a nutrient in form of available phosphate for plant, support plant growth from IAA production and indirect control of plant pathogenic infection from the production of siderophore. Our further study will focus on the optimum condition bioactive compounds production and effect on plant production in field.

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