First Report of Plant Growth Promoting Endophytic Bacteria from Medicinal Invasive Plants (*Chromolaena odorata*)

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First Report of Plant Growth Promoting Endophytic Bacteria from Medicinal Invasive Plants (*Chromolaena odorata*)

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Abstract. *Ki rinyuh* (*Chromolaena odorata*) is one of invasive plants species in Indonesia with potency as traditional medicine. The purpose of this study was to verify the presence of endophytic bacteria symbionts with *Chromolaena odorata*, and to evaluate the plant-growth promoting properties of endophytic bacteria in producing IAA, producing hydrolytic enzymes (α-amylase, β-amylase, cellulase, chitinase, protease) solubilizing phosphate. Isolation of endophytic bacteria was carried out by surface sterilizing the samples of roots, stems, leaves with 70% alcohol and 2% sodium hypochlorite, followed by direct plating of organ parts (1-2 cm) on top of Trypticase Soy Agar (TSA) medium. Bacterial isolates were differentiated through morphological biochemical characterization. A total of 19 endophytic bacteria were successfully recovered from *Chromolaena odorata* roots, stems and leaves. Four isolates produced the highest IAA, namely BECA1 (109 ± 0.98 ppm), BECA5 (104.13 ± 0.32 ppm), BECA8 (104.13 ± 0.71 ppm) and BECB3 (83.29 ± 0.47 ppm). Three isolates exhibit the highest phosphate solubilization (+++) namely BECA5, BECA1, BECA8 after 4 days of incubation. Furthermore, BECB3 produced a considerable hydrolytic enzyme activities: β-amylase (+++), α-amylase (++), cellulase (+++), chitinase (++) and protease (+++) compared to other isolates. Our result may provide an insight upon the beneficial interaction by plant-growth promoting endophytic bacteria to support the invasiveness of the plant.

1. Introduction

Endophytic bacteria are microorganisms living in the internal tissues of plants without exposing any negative impacts, and are ascertained that diverse endophytic bacteria exhibit symbiotic relationship to plant host [1,2]. Many factors contribute to the diversity of endophytic bacterial communities in a plant tissue. Endophytic bacteria may be isolated from roots, leaves, stems, flowers, fruits, and seeds of various plant species and were successfully reported to produce several factors that increased plant growth, i.e. auxin, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, inorganic phosphate solubilization, phytohormone synthesis, nitrogen fixation, and siderophores [3-6]. Endophytic bacteria also live freely to produce hydrolytic enzymes to control its own niche against pathogenic microbes [7]. The potential of endophytic bacteria in stimulating plant growth has been reported from various plants [8,9].

Natural ecosystem has become a habitat for plants. In particular, invasive plants are part of the natural ecosystem. The existence of invasive plants may affect other plant species. Invasive plants can suppress or even eliminate native species [10,11,12] and alter the ecosystem functions [13-18]. The main reason why invasive plants tend to survive and overgrow their competitor plants may be supported by specific factors. Previous studies have reported that mutualistic system is an important factor to invasive trait [19,20]. The ability to compete with other plants increased when mutualistic associations occurred with microorganisms [21-25]. Improved protection by invasive plants defended its existence from the competition in habitat. The protection may be highly correlated with the variation of endophytic bacterial communities that live in these plant tissues. This community of endophytic bacteria will produce secondary compounds and hormones to outcompete other competitors [26,27].

Endophytic bacteria from invasive plants used for traditional medicines received special attention in this study. This background is supported by a survey report from the World Health Organization showing that nearly 80% of the world's population, especially developing countries, is highly dependent on traditional medicines in the form of plant extracts [28]. Invasive plants *Chromolaena odorata* is one of 75 important invasive plant species in Indonesia [29]. Previous research has reported that *Chromolaena odorata* can be used as a source of anticholesterol, antioxidant, antidiabetic, antibacterial [30-33].

The multifunction *Chromolaena odorata* becomes an interesting object to study due to the assumption upon endophytic bacteria which may stimulate plant growth. This study was the first report on endophytic bacteria from roots, stems and leaves of invasive *Chromolaena odorata* in North Sumatra by testing IAA activity, phosphate solubilization and production of hydrolytic enzymes (β-amylase, α-amylase, cellulase, chitinase and protease). Endophytic bacterial isolates will later be evaluated for its prospect in the application as biofertilizers and natural bioherbicides.

2. Materials and methods

2.1. Isolation of endophytic bacteria

Roots, stems and leaves of invasive plant *Chromolaena odorata* were collected from Sicike-cike National Park (02°39″N 098°23″E, elevation: 1,383 m), North Sumatra, Indonesia. Fragments of root, stem and leaf (±1-2 cm) were washed under flowing tap water. Surface sterilization was performed by dipping fragments into solutions as follows: 70% EtOH (3 min), NaOCl (5 min), distilled water, 70% EtOH (1 min), distilled water. Fragments were dried aseptically using filter paper. Fragments were further cut into smaller segments and placed on top of Tryptic Soy Agar (TSA). Samples were incubated at 37ºC for 16-18 hr. Colonies grown on media were immediately transferred into Nutrient Agar (NA) supplemented with Ketoconazole (0.3 g/100 mL).

2.2. Screening of plant growth promoting properties by endophytic bacteria

2.2.1. IAA production

Determination of IAA quantity by endophytic bacteria was based on colorimetry method or color changes using Salkowsky reagent [34]. One hunderd µL of overnight culture (OD_{600}=0.5) was inoculated into 100 mL Nutrient Broth (NB) containing 0.2% L-tryptophan (w/v) and incubated for 5 d at 28°C±3°C. Culture was centrifuged at 10,000 rpm for 30 min at 4°C. Supernatan was mixed with 2 mL Salkowsky (0.1 M $FeCl_3$ solution + 400 mL of concentrated H_2SO_4 + 580 mL of distilled water). Mixture was gently homogenized and incubated in dark condition for 30 min until formation of pinkish red color. IAA concentrations were estimated using spectrophotometer under wavelength A_{530} along with blank solution without bacterial culture. Standard curve of IAA was made previously by following the same procedure using various concentrations: 0, 30, 60, 90, 120 and 140 ppm (Figure 1).

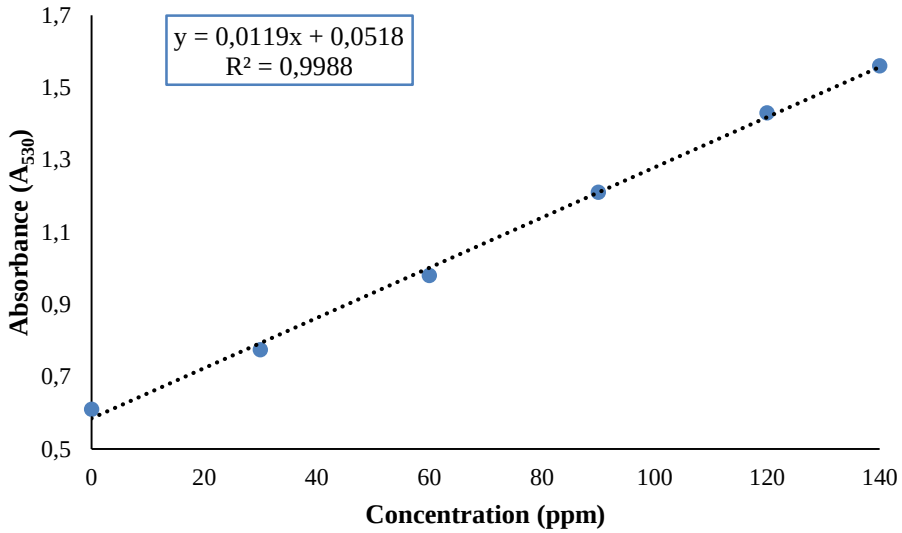


Figure 1. Standard curve of IAA

2.2.2. Phosphate solubilization

A loopful of bacteria was streaked into Pikovskaya agar [35] with minor modifications (g/L): glucose (10.0), $(NH_4)_2SO_4$ (0.5), $Ca_3(PO_4)_2$ (5.0), NaCl (0.3), $MgSO_4.7H_20$ (0.3), KCl (0.2), $MnSO_4.4H_2O$ (0.03), $FeSO_4.7H_2O$ (0.03), yeast extract (0.5), agar (15.0), in 1,000 mL distilled water. Culture was incubated at 28°C±3°C for 24 hr. Clear zones formed around colonies indicate a positive result of phosphate solubilization.

2.3. Screening of hydroltic enzymes produced by endophytic bacteria

2.3.1. α- /β-amylase

Hydrolytic activity of α-/ *β*-amylase was screened by streaking a loopful of bacteria into Starch Agar (SA) with composition (g/L): soluble starch (10.0), yeast extract (1.0), NaCl (18.0), agar (15.0), in 1,000 mL distilled water. Culture was incubated at 28°C±3°C for 24 hr. Plates were flooded with Iodine's solution (1 g iodine dissolved in 2% KI solution) and left for 10 min [36]. Clear zones formed around colonies indicate a positive result of α-amylase activity. Activity of *β*-amylase was measured using same procedure by reducing soluble starch composition to 5.0 g.

2.3.2. Protease

Hydrolytic activity of protease was screened by spotting a bacteria colony into Skim Milk Agar (SMA) with composition (g/L): peptone (4.0), yeast extract (1.0), skim milk (12.0), NaCl (18), agar (15.0), in 1,000 mL distilled water. Culture was incubated at 28°C±3°C for 24 hr. Clear zones formed around colonies indicate a positive result of protease activity.

2.3.3. Cellulase

Hydrolytic activity of cellulase was screened by streaking a loopful of bacteria into Bushnell Has Medium (BHM) with composition (g/L): CarboxylMethyl-Cellulose (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), NH_4NO_3 (1.0), $FeCl_3 \cdot 6H_2O$(0.05), NaCl (18.0), $CaCl_2$ (0.02), agar (15.0), in 1,000 mL distilled water [37]. Culture was incubated at 28°C±3°C for 96 hr. Plates were flooded with 0.3% Congo red solution and left for 20 min. Plates were further washed with 1M NaCl to observe clear zones around colonies which indicate a positive result of cellulase activity [38].

2.3.4. Chitinase

Hydrolytic activity of chitinase was screened by spotting a bacterial colony into Colloidal Chitin Agar (CCA) with composition (g/L): Na_2HPO_4 (6.0), KH_2PO_4 (3.0), NH_4Cl (1.0), NaCl (0.5), yeast extract (0.05) agar (15.0), colloidal chitin 1% (w/v) in 1,000 mL distilled water. Culture was incubated at 28°C±3°C for 96 hr. Clear zones formed around colonies indicate a positive result of chitinase activity. Preparation of colloidal chitin was based on previous study [39].

3. Results

3.1. Morphological characteristics of isolated endophytic bacteria from C. odorata

Endophytic bacteria was isolated from surface sterilized fragment of roots, stems and leaves of *C. odorata* (Figure 2). Thirteen bacterial isolates were obtained from this isolation effort. Morphological characteristics of isolates were characterized by observing typical colony morphologies (Table 1). Microscopic examination revealed that most isolates were rod-shaped bacteria.

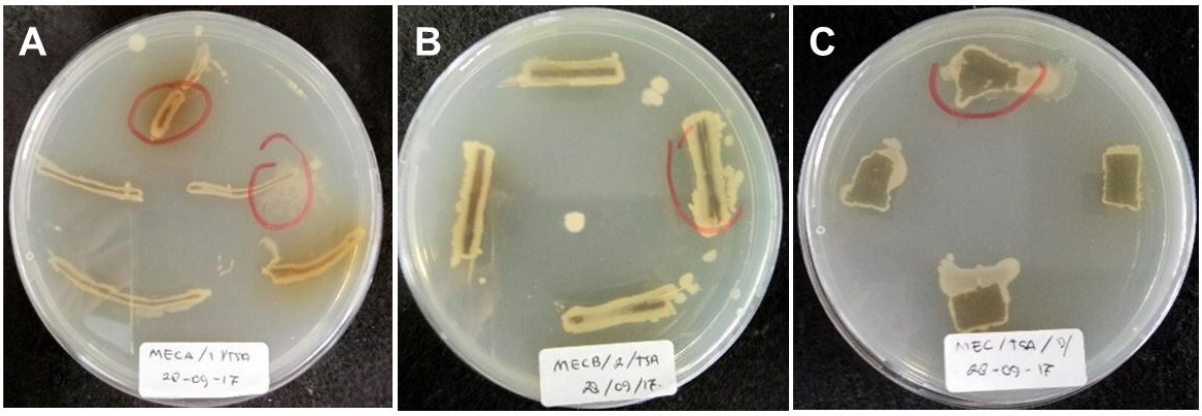


Figure 2. Appearance of bacterial colonies growing from fragment parts of *Chromolaena odorata,* A. Root, B. Stem, C. Leaf

Table 1**.** Morphological characteristics of endophytic bacteria from *Chromolaena odorata*

Isolate code	Plant organs	Colony Morphology				Cell morphology	
		Form	Edge	Elevation	Colour	Shape	Gram staining
BECD1	Leaves	*Circular*	*Lobate*	*Raised*	White	Diplo	+
BECD2	Leaves	*Circular*	*Lobate*	*Flat*	Yellowish white	Bacilli	-
BECD4	Leaves	*Circular*	*Entire*	*Flat*	White	Bacilli	+
BECD3	Leaves	*Circular*	*Lobate*	*Raised*	White	Cocci	-
BECB1	Stem	*Circular*	*Entire*	*Raised*	Cream	Cocci	+
BECB2	Stem	*Irregular*	*Undulate*	*Flat*	Milkish white	Bacilli	+
BECB6	Stem	*Irregular*	*Lobate*	*Umbonate*	Yellowish white	Bacilli	+
BECA6	Root	*Circular*	*Undulate*	*Flat*	White	Diplobacilli	-
BECA5	Root	*Irregular*	*Undulate*	*Raised*	Milkish white	Bacilli	+
BECA7	Root	*Circular*	*Entire*	*Flat*	Milkish white	Bacilli	+
BECA1	Root	*Irregular*	*Entire*	*Raised*	White	Bacilli	+
BECA8	Root	*Irregular*	*Entire*	*Umbonate*	White	Bacilli	-
BECB4	Stem	*Circular*	*Undulate*	*Raised*	Milkish white	Diplobacilli	+
BECA9	Root	*Irregular*	*Entire*	*Raised*	White	Bacilli	-
BECA10	Root	*Circular*	*Undulate*	*Raised*	Cream	Bacilli	+
BECB3	Stem	*Irregular*	*Entire*	*Raised*	Yellow	Bacilli	-
BECA3	Root	*Circular*	*Undulate*	*Flat*	White	Cocci	+
BECB7	Stem	*Irregular*	*Entire*	*Raised*	Cream	Bacilli	+
BECA4	Root	*Irregular*	*Undulate*	*Flat*	Cream	Bacilli	+

Biochemical characteristics of isolates were characterized by determining positive resuls from biochemical reaction (Table 2). Most isolates were grouped into gram positive bacteria while six isolates, namely BECD2, BECD3, BECA6, BECA8, BECA9 and BECB3 were the only gram negarive bacteria.

Table 2. Biochemical characteristics of endophytic bacteria from *Chromolaena odorata*.

Isolate Code	Plant organs	Starch utilization	Gelatine hydrolysis	Citrate utilization	Motility test	Triple Sugar Iron Test			
						Butt	Slant	H_2S	Gas
BECD1	Leaves	-	+	-	+	Yellow	Yellow	-	+
BECD2	Leaves	-	+	+	+	Pink	Pink	-	-
BECD4	Leaves	-	-	+	+	Yellow	Yellow	-	-
BECD3	Leaves	-	-	+	-	Yellow	Yellow	-	+
BECB1	Stem	-	+	+	-	Yellow	Yellow	-	+
BECB2	Stem	-	+	+	-	Pink	Pink	-	-
BECB6	Stem	-	-	-	-	Yellow	Yellow	-	-
BECA6	Root	-	+	-	-	Yellow	Yellow	-	-
BECA5	Root	+	+	-	-	Yellow	Yellow	-	-
BECA7	Root	-	-	-	-	Yellow	Yellow	-	-
BECA1	Root	+	-	+	+	Yellow	Yellow	-	-
BECA8	Root	+	+	-	-	Yellow	Yellow	-	-
BECB4	Stem	+	+	-	-	Yellow	Yellow	-	-
BECA9	Root	-	+	-	-	Yellow	Yellow	-	-
BECA10	Root	+	+	-	-	Yellow	Yellow	-	-
BECB3	Stem	+	+	-	-	Yellow	Yellow	-	-
BECA3	Root	-	+	-	-	Yellow	Yellow	-	-
BECB7	Stem	-	+	-	-	Yellow	Yellow	-	-
BECA4	Root	-	+	-	-	Yellow	Yellow	-	-

3.2. IAA production

The results obtained four potential isolates with the highest IAA quantities namely BECA1 with 109±0,98 ppm, followed by BECA5 104,13±0,32 ppm, BECA8 104,13±0,71 ppm, and BECB3 83,29±0,47 ppm (Table 3). Visualization of IAA production by endophytic bacteria confirmed the high concentration of IAA through pinkish or red color formation.

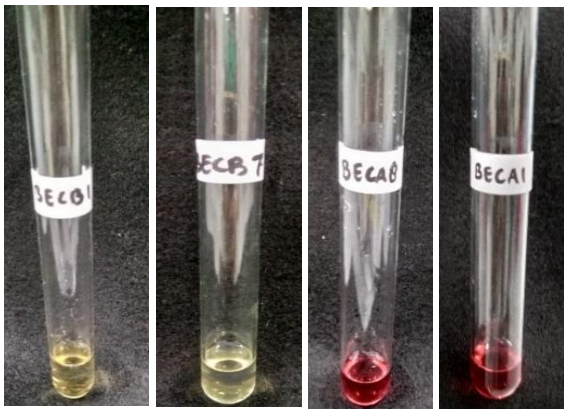
3.3. Phosphate solubilization

The results obtained three positive results by isolates BECA1, BECA5 and BECA8. The three isolates produced a considerably strong hydrolytic activity as shown by a clear zone around colony (Figure 3). One isolate, BECB3 was categorized as moderate phosphate solubilizer while isolates, BECD1, BECB4, BECA9 and BECA10 were considered as weak phosphate solubilizers (Table 4).

Figure 3. Phosphate solubilization activity of endophytic bacteria as shown through clear zone (A) around colony of (B) BECA1 and none of BECD2

Table 3. Production of IAA by the endophytic from *Chromolaena odorata*.

Isolate code	Plant organs	IAA concentration (ppm)	Color formation
BECD1	Leaves	18,17±0,83**	
BECD2	Leaves	10,94±1,09**	
BECD4	Leaves	3,97±0,21*	
BECD3	Leaves	8,59±0,45*	
BECB1	Stem	2,45±0,30*	
BECB2	Stem	5,81±0,22*	
BECB6	Stem	6,4±0,53*	
BECA6	Root	16,82±0,29**	
BECA5	Root	104,13±0,32***	
BECA7	Root	25,39±1,26**	
BECA1	Root	109±0,98***	
BECA8	Root	104,13±0,71***	
BECB4	Stem	19,93±1,02**	
BECA9	Root	12,12±0,93**	
BECA10	Root	38,84±0,31**	
BECB3	Stem	83,29±0,47***	
BECA3	Root	13,04±0,98**	
BECB7	Stem	3,71±0,42*	
BECA4	Root	9,76±0,77*	



*IAA production < 5 ppm; **IAA production 10-50 ppm; ***IAA production > 50 ppm (*=low, **=medium, ***=high)

3.4. Hydrolytic enzymes

The result of hydrolytic enzymes activity by endophytic bacteria is presented on Table 4. Stem isolate namely BECB3 and root isolate, BECA8 produced positive results to all tested hydrolytic enzymes assay. Majority of isolates produced protease, except isolate BECD3, BECD4 and BECA4.

4. Discussion

Recently, the medicinal plant, *Chromolaena odorata* with local name *ki rinyuh* is considered as an important invasive plant species in Indonesia [29]. Plants may have special adaptations to survive, in specific of invasive plant species which can alter the soil biota community to help invasive plant growth facilities [40] known as the "soil-plant feedback hypothesis" [41]. This hypothesis refers to the strong mutual relationship [42] by eradicating competitors in habitat [43] or opposing other biota which confer benefits to native plants in the ecosystem [44]. Invasive plants may possess distinctive physiological and biological components related to endophytic bacteria. The theory then supported our study on discovering any plant growth promoting properties displayed by endophytic bacteria in *C. odorata* as the first report from invasive alien plant species.

The study successfully recovered 19 isolates of endophytic bacteria from the root, stem and leaf of *C. odorata* which were differentiated based on their morphology, biochemical properties and gram grouping. The root accounts for the most isolates of endophytic bacteria. This is assumed to be directly correlated since complex interaction among organisms occur especially endophytic bacteria which enter the tissue through rhizospheric region.

Table 4. Hydrolytic enzyme activities of endophytic bacteria from *Chromolaena odorata*

Isolate code	Plant organs	PO_4 solubilization	β-amylase	α-amylase	Cellulase	Chitinase	Protease
BECD1	Leaves	+	-	-	-	-	+
BECD2	Leaves	-	+	-	+	-	+
BECD4	Leaves	-	-	-	-	-	-
BECD3	Leaves	-	-	-	-	-	-
BECB1	Stem	-	-	-	+	-	+
BECB2	Stem	-	+	-	+	-	+
BECB6	Stem	-	-	-	++	-	+
BACA6	Root	-	-	-	+	-	+
BECA5	Root	+++	++	+	++	-	+
BECA7	Root	-	+	-	-	-	-
BECA1	Root	+++	++	+	+	-	-
BECA8	Root	+++	++	+	+	++	+
BECB4	Stem	+	++	+	++	-	+
BECA9	Root	+	-	-	-	-	+
BECA10	Root	+	++	+	-	-	+
BECB3	Stem	++	+++	++	+++	+	+++
BECA3	Root	-	-	-	-	-	+
BECB7	Stem	-	++	-	-	-	+
BECA4	Root	-	-	-	-	-	-
Subtotal	Root = 9 Stem= 6 Leaves = 4	Root = 5 Stem = 2 Leaves 1	Root = 5 Stem = 4 Leaves = 1	Root = 4 Stem = 2 Leaves = 0	Root = 4 Stem = 5 Leaves = 0	Root = 1 Stem =1 Leaves = 0	Root = 6 Stem = 6 Leaves = 2
Total	19	8	10	6	9	2	14

- none;+ weak reaction; ++ moderate reaction; +++ strong reaction

Complete screening methods are conducted to endophytic bacterial isolates to analyze the important of relationship between endophytic bacteria and invasive plants. Endophytic bacteria directly help plant growth through hormone production, especially Indole Acetic Acid (IAA) and phosphorus mobilization. In addition, growth promotion exhibit by endophytic bacteria may also be supported through synthesis of antimicrobial activity, ammonia production and synthesis of hydrolytic enzymes or antibiotics against competing organisms[45].

The presence of potential IAA producers and phosphate solubilizers isolated from root region may indicate that plant root system is harbored first by endophytic bacteria to initiate plant growth of host [46,47]. Phosphorus is needed by plants for growth and development, but it is limited due to poor solubility in soil [48]. Endophytic bacteria are known to increase plant growth by dissolving and mobilizing phosphate [49]. In this study, not all bacterial isolates showed their potential to produce IAA and dissolve phosphate. As a result, the highest ability of BECA1 isolates produced IAA of 109 ± 0.98 ppm followed by BECA5 isolates with 104.13 ± 0.32 ppm and both isolates were prominent in dissolving phosphate.

The results also revealed that dominant bacterial isolates were gram positive bacteria. The finding may due to our limited sampling efforts and environmental factors affecting bacteria assemblages. Gram positive bacteria are known to vary greatly within host cells (tissue / organ plants), starting from the production of pigments, spores and secondary metabolites. This indicates that a large number of Gram-positive bacteria are obtained from *C. odorata*. Production of IAA from endophytic bacteria originating from plant roots is similar to previous study reporting the highest IAA production by isolate TUB5 was 36.6 ± 3.1 ppm with considerable phosphate solubilization activity [50].

Hydrolytic enzymes produced by endophytic bacteria are important properties for optimal colonization process in plant organs initiated through plant roots. Colonization of endophytic bacteria originates from the lateral roots penetrating epidermal tissue, cortex, endoderm, perisicle layer, naturally used as a highway for bacteria to enter phloem vessels and xylem which transport

photosynthesis (phloem), nutrients and water (xylem) [51]. The mutual relationship between *C. odorata* and its symbionts are seemed to be mostly controlled and regulated by endophytic bacteria residing in internal tissue of roots, yet enhancing the invasiveness of *C. odorata* in habitat. In this study, isolates BECA1 and BECA5 may be studied further for their potential as biofertilizers in agricultural field.

5. Conclusion

The first report on endophytic bacteria isolated from invasive medicinal plant, *Chromolaena odorata* has revealed 19 bacterial isolates in which tested for their plant growth promoting properties. The mutualistic relationship may be evaluated in detail by identifying single isolate or even microbial consortium exerting potential traits to *C. odorata* invasiveness. Further investigations are needed to uncover the complex interaction between invasive plant species and its microbial symbionts as one unity to thrive in competing environment.

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