

SCREENING OF BIOACTIVE COMPOUNDS FROM BACTERIAL ENDOPHYTES ISOLATED FROM MICROPROPAGATED PLANTS

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Received: July 24, 2015; Revised: September 26, 2105; Accepted: February 16, 2016

Abstract

Bacterial endophytes are microorganisms inhabit in plant tissues. Additionally, they are able to produce effective bioactive compounds leading to apply in agricultural, industrial and medical fields. This study was carried on isolation of bacterial endophytes from rice and pineapple cultivated in micropropagation, identification of bacterial strains and evaluation for bioactive compounds production including antimicrobial activities, Indole-acetic-acid (IAA) production and phosphate solubilization ability. Fourteen isolates of bacterial endophytes were obtained for the present study. Antimicrobial activities against six strains of human pathogenic bacteria and phytopathogenic fungi were investigated. Three isolates of bacterial endophytes from pineapple were able to inhibit the growth of *Bacillus cereus* and *Salmonella typhimurium*. Then five bacterial endophyte isolates exhibited the growth inhibitory activity on *Aspergillus* sp., *Collectrichum* sp., *Campylocarpon fasciculare* and *Rhizopus nigricans*. Furthermore, evaluation of plant growth promoting activities including IAA production and the ability of phosphate solubilizing were examined. Results showed that all of sixteen bacterial isolates exhibited IAA production activity ranged from 0.38 µg/ml to 237.5 µg/ml nutrient broth supplemented with 200 µg/ml of tryptophan. Additionally, eight isolates of bacterial endophytes possessed the phosphate solubilization ability and the highest solubilization index was 2.8.

Keywords: Bacterial endophytes, bioactive compounds, micropropagation

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Introduction

Endophytes are microorganisms that inhabit inside plant tissues and do not cause any negative impact on host plant (Wilson, 1995). Recently, there are several researches have studied on bioactive compounds from bacterial endophytes. These compounds are varied from antimicrobial agents, antioxidant agents, anticancer agents and phytohormones (Pimental *et al.*, 2011). Therefore, there have been great attempts to apply these effective substances in agricultural, industrial and medical fields (Strobel and Daisy, 2003). Micropropagation is the large-scale plant cultivation in aseptic environment (George *et al.*, 2008). The contamination from bacterial endophytes occasionally occur (Cassells, 1997). This situation may partly effect to the system while this microorganisms act as chemical synthesizer within plant (Owen and Hundley, 2004). According to several studies, few researches about isolation of bacterial endophytes from micro-propagation such as corn root, grape, and strawberry were reported (Thomas, 2004; Dias *et al.*, 2008; Szilagyi-Zecchin *et al.*, 2014). Then various types of bacterial endophytes obtained, it might indicated the opportunity to explore novel sources of bioactive compound producer. Therefore, aims of this study were to isolate and identify of bacterial endophytes from micropropagation. The determination for bioactive compounds from bacterial endophytes was also carried out.

Materials and Methods

Isolation of Bacterial Endophytes from Plant Samples (Kumrapich, 2012)

Plant samples including rice and pineapple were obtained from plant tissue culture laboratory, Faculty of Science, Maejo University (Figure 1). They were divided into several parts such as leaves, roots and stems and section into small pieces. They were carried out surfaced sterilization with 70% ethanol for 1 min, then immersing in 6% Clorox and 75% ethanol for 5 min and 30 sec, respectively. In order to eliminate sterilizers,

pieces of samples were washed three times of sterile distilled water. After that they were absorbed access water on sterile tissue papers. To confirm complete sterilization, water obtained from the final surface sterilization process was spread on Nutrient Agar and observed for growth of bacterial colony. Surface sterilized plant samples were put on Nutrient Agar (NA) containing nystatin and incubated at 37°C for 24-48 h. After growth of bacterial endophytes was occur selected bacterial colonies were purified by cross streak on NA.

Identification of Bacterial Endophytes

Purified bacterial endophytes were examined for bacterial morphology. Additionally, DNA extraction and PCR reaction were performed. After that 16S rRNA sequence analyzing was carried on and examined with the NCBI nucleotide online database.

Evaluation for Bioactive Compounds Production

Antibacterial Activity

The antibacterial activities of bacterial endophytes against selected pathogenic bacteria were investigated by the modified method of Héchard *et al.* (2005). Bacterial endophytes and 5 animal pathogens (*Bacillus cereus*, *Escherichia coli*, *Enterobacter cloacae*, *Salmonella typhi* and *Shigella flexneri*) were grown in BHI at 37°C for 24 h. The nutrient agar was inoculated with 1% (v/v) of cultures of pathogens and poured on sterile plates. Bacterial endophytes were then pointed on solidified media and incubated at 37°C for 24 h. Inhibitory zone was observed and the ratio of diameter of clear zone and colony were calculated.

Antifungal Activity

Bacterial endophytes were initially grown in Brain Heart Infusion (BHI) at 37°C for 24 h. Bacterial cultures were dropped on Potato Dextrose agar (PDA) at one sites of plate. After that continuously dropped spore suspension of phytopathogenic fungi at the opposite site and incubated at room temperature for 5-7 days. Measurement of the diameter of fungal colony and the percentage of inhibition on phytopathogenic fungi were



Figure 1. Plant samples; Rice (Left) and Pineapple (Right)

Table 1. Identification of bacterial endophytes from rice and pineapple cultivated in plant tissue culture systems

Isolates	Bacterial Name	Sources
AC_R1_B1	<i>Bacillus pumilus</i>	Pineapple
AC_R1_BF2	<i>Bacillus amyloliquefaciens</i>	Pineapple
AC_R1_BY3	<i>Bacillus amyloliquefaciens</i>	Pineapple
AC_L1_B4	<i>Bacillus amyloliquefaciens</i>	Pineapple
RiceTIB1	<i>Klebsiella pneumoniae</i>	Rice
RiceTIB2	<i>Klebsiella pneumoniae</i>	Rice
RiceTIB3	<i>Enterobacter oryzae</i>	Rice
RiceTIB4	<i>Klebsiella pneumoniae</i>	Rice
RT1.2C	<i>Enterobacter asburiae</i>	Rice
RT3.1A	<i>Klebsiella pneumoniae</i>	Rice
RT9	<i>Enterobacter asburiae</i>	Rice
RT11	<i>Bacillus amyloliquefaciens</i>	Rice
RT12	<i>Bacillus amyloliquefaciens</i>	Rice
RT16	<i>Enterobacter asburiae</i>	Rice

carried out. This method was done by the modified techniques of Rabindran and Vidyasekaran (1996) and Zhao *et al.* (2010).

$$I = \left(\frac{C - T}{C} \right) \times 100$$

I = Percent inhibition

C = Radial growth of the pathogen in control

T = Radial growth of pathogen in treatment

Screening of IAA production

To prepare bacterial inocula, bacterial endophytes were grown in BHI at 37°C with 130 rpm shaking rate for 24 h. Bacterial cultures were standardized turbidity as No. 0.5 McFarland standard. 0.05 ml of cultures of bacterial endophytes were transferred to the fresh Nutrient Broth (NB) adding 200 µg/ml L-tryptophan and incubated at 37°C with same constant shaking rate for 48 h. Then bacterial

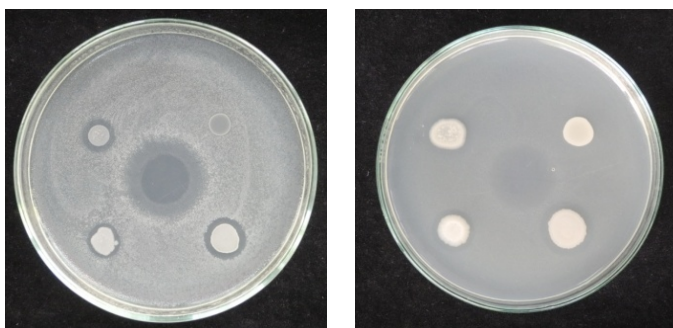


Figure 2. Inhibition zone of bacterial endophytes against *Bacillus cereus* (Left) and *Salmonella typhimurium* (Right)

Table 2. Antibacterial activity of bacterial endophytes

Isolates	Ratio of clear zone and colony sizes	
	<i>B. cereus</i>	<i>S. typhimurium</i>
<i>B. amyloliquefaciens</i> AC_R1_BF2	1.8±0.1 ^a	1.1±0.0 ^a
<i>B. amyloliquefaciens</i> AC_R1_BY3	1.5±0.1 ^{ab}	1.3±0.2 ^a
<i>B. amyloliquefaciens</i> AC_L1_B4	1.3±0.2 ^c	1.2±0.2 ^a

samples were centrifuged at 10000 rpm for 10 min and collected supernatants for determination of the auxin-like substance production (Glickmann and Dessaux, 1995).

Phosphate Solubilizing Ability

Bacterial endophytes were grown in BHI at 37°C for 24 h. After that bacterial cultures were inoculated on National Botanical Research Institute Phosphate agar medium (NBRIP) at 37°C for 7 days. The diameter of halo and colony were measured and evaluated for phosphate solubilization index (Walpolá and Yoon, 2013).

Statistical Analysis

All data were analyzed by applying SPSS program. To compare of the significance of differences among means, they were subjected to the Dun-can's Multiple Range Tests at $p = 0.05$ and evaluated in triplicate.

Results and Discussion

Isolation of Bacterial Endophytes and Characterization of Bacterial Endophytes

Total 14 bacterial isolates were isolated from planlet samples including rice (*Oryza sativa*) and pineapple (*Ananas comosus*) from plant tissue culture laboratory, Maejo University. Fourteen isolates of bacterial endophytes were selected to characterize bacterial morphology by Gram staining tests. The identification of bacterial endophytes using 16S rRNA gene sequences analysis. The base sequencing results were compared to nucleotide online database at National Center for Biotechnology Information (NCBI) website. Results of bacterial characterization were shown in Table 1.

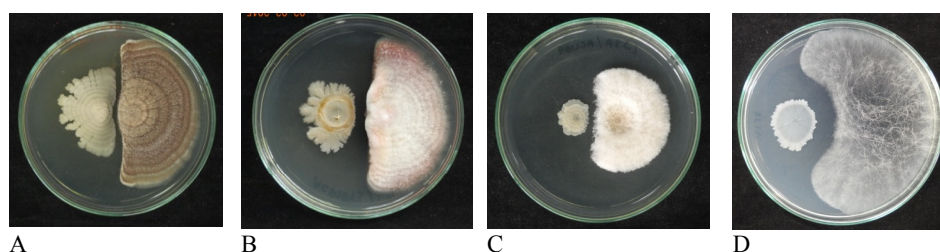


Figure 3. growth inhibition of 4 phytopathogenic fungi including *Aspergillus* sp. (A), *Campylocarpon fasciculare* (B), *Collectrichum* sp. (C) and *Rhizopus nigricans* (D)

Table 3. Growth inhibition of phytopathogenic fungi by *Bacillus amyloliquefaciens* strains

Strain	Percent of inhibition on growth of phytopathogenic fungi			
	<i>Aspergillus</i> sp.	<i>C. fasciculare</i>	<i>Collectrichum</i> sp.	<i>R. nigricans</i>
AC_R1_BF2	38.4±0.8 ^a	-	26.8±0.1 ^a	38.4±0.8 ^b
AC_R1_BY3	24.4±0.2 ^d	40.9±2.8 ^a	25.4±0.0 ^a	24.99±1.1 ^b
AC_L1_B4	22.5±0.6 ^c	28.7±2.5 ^b	23.7±1.2 ^{ab}	21.40±2.0 ^b
RT11	26.0±0.0 ^b	12.3±1.7 ^c	15.2±2.4 ^c	46.21±0.6 ^a
RT12	17.1±0.5 ^e	8.6±1.4 ^d	34.8±0.4 ^{bc}	46.03±0.9 ^a

Total of 14 bacterial isolates were isolated from micropropagated samples. Morphological examination revealed that bacterial isolates were categorized into 2 groups including 6 Gram-positive rod-shaped bacteria and 8 Gram-negative rod-shaped bacteria. Identification of bacterial endophytes by 16S rRNA gene sequence analysis were shown that bacterial isolates were identified and grouped into 3 genera including *Bacillus* spp., *Enterobacter* sp. and *Klebsiella* sp. related to several reports studied about isolation and identification of bacterial endophytes from rice (Zinniel *et al.*, 2002; Hardoim *et al.*, 2013; Hongrattipun *et al.*, 2014). To the best of our knowledge, there was no study reported about *Bacillus* sp. isolated from pineapple. Although the study of Abreu-Tarazi and co-workers. (2010) reported that *Pseudoxanthomonas* sp., *Stenotrophomonas maltophilia* and *Burkholderia cenocepacia* isolated from pineapple cultivated in plant tissue culture system.

Antibacterial Activity

Antibacterial activities of bacterial endophytes were determined by spot-on-lawn method and inhibition zone of bacterial endophytes were shown in Figure 2. Results were shown that from total 14 isolates, 3 isolates were able to produce antibacterial agent against *Bacillus cereus* and *Salmonella typhimurium* as shown in Table 2

In this study, antibacterial activities of bacterial endophytes were evaluated by spot-on-lawn. From 14 isolates, 3 isolates of *B. amyloliquefaciens* (AC_R1_BF2, AC_R1_BY3 and AC_L1_B4) were exhibited antibacterial activities against *Bacillus cereus* and *Salmonella typhimurium*. The research of Sahu and co-workers (2014) stated that *Bacillus* sp. were able to inhibit growth of several animal pathogens and it may imply that antibacterial agent from *B. amyloliquefaciens* was bacteriocin-like substance (Lisboa *et al.*, 2006).

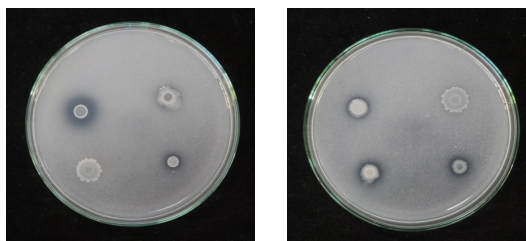


Figure 4. Phosphate solubilizing ability of bacterial endophytes

Table 4. IAA production of bacterial endophytes

Bacterial isolates	IAA content ($\mu\text{g/ml}$)
<i>Bacillus pumilus</i> (AC_R1_B1)	6.3 ± 0.0^e
<i>Bacillus amyloliquefaciens</i> (AC_R1_BF2)	5.7 ± 0.0^e
<i>Bacillus amyloliquefaciens</i> (AC_R1_BY3)	10.4 ± 0.0^e
<i>Bacillus amyloliquefaciens</i> (AC_L1_B4)	7.5 ± 0.0^e
<i>Klebsiella pneumonia</i> (RiceTIB1)	237.0 ± 7.5^a
<i>Klebsiella pneumonia</i> (RiceTIB2)	230.0 ± 6.1^a
<i>Enterobacter oryzae</i> (RiceTIB3)	6.0 ± 1.7^e
<i>Klebsiella pneumonia</i> (RiceTIB4)	177.0 ± 7.5^b
<i>Enterobacter asburiae</i> (RT1.2C)	45.5 ± 6.6^d
<i>Klebsiella pneumonia</i> (RT3.1A)	76.4 ± 0.2^c
<i>Enterobacter asburiae</i> (RT9)	56.8 ± 6.9^d
<i>Bacillus amyloliquefaciens</i> (RT11)	0.7 ± 0.5^e
<i>Bacillus amyloliquefaciens</i> (RT12)	0.4 ± 0.4^e
<i>Enterobacter asburiae</i> (RT16)	72.3 ± 6.6^c

Antifungal Activity

Antifungal activities of bacterial endophytes were determined by Dual culture method. The growth inhibition of four phytopathogenic fungi by endophytic bacteria were presented in Figure 3. Five isolates were able to inhibit growth of phytopathogenic fungi including *Aspergillus* sp., *Campylocarpon fasciculare*, *Collectrichum* sp. and *Rhizopus nigricans* as shown in Table 3.

From antifungal activity test, dual culture method was employed. This technique was studied of antagonistic activity of one species against the other species (Pereira *et al.*, 2003). Results showed that *B. amyloliquifaciens* (AC_R1_BF2, AC_R1_BY3, AC_L1_B4,

RT11 and RT12) were able to suppress fungal growth. The antifungal mechanism might cause the impact on plasma membrane permeability of pathogenic fungi (Haraguchi *et al.*, 1999). *B. amyloliquefaciens* are able to produce antifungal agent such as surfactin, iturin and fengycin (Athukorala *et al.*, 2009).

Currently, biological control from bacterial endophytes are widely attracted a lot of attentions because they possess high efficiency and low toxicity applied in agricultural field. Additionally, they are beneficial in agricultural works because they are able to promote plant growth by direct and indirect mechanism (Glick, 2012).

Table 5. The ability of the bacterial endophytes to solubilize insoluble phosphate by the solubilization index (SI)

Bacterial isolates	Solubilization index
<i>Bacillus pumilus</i> (AC_R1_B1)	2.4±0.03 ^{cd}
<i>Klebsiella pneumonia</i> (RiceTIB1)	2.3±0.06 ^{cd}
<i>Klebsiella pneumonia</i> (RiceTIB2)	2.4±0.02 ^{cd}
<i>Klebsiella pneumonia</i> (RiceTIB4)	2.3±0.2 ^{cd}
<i>Enterobacter asburiae</i> (RT1.2C)	2.2±0.0 ^d
<i>Klebsiella pneumonia</i> (RT3.1A)	2.8±0.5 ^a
<i>Enterobacter asburiae</i> (RT9)	2.5±0.1 ^{bc}
<i>Enterobacter asburiae</i> (RT16)	2.5±0.1 ^b

Screening of IAA Production

Evaluation of IAA production was determined by colorimetric method of (Glickmann and Dessaux, 1995). All of 14 isolates were able to produce IAA in NB containing 200 µg/ml tryptophan. Results were presented in Table 4.

Phytohormone production is one of significant direct approaches to stimulate plant growth. Other direct approach is alteration of unavailable nutrients including phosphorus, nitrogen and iron into more facilitated form (Nutaratat *et al.*, 2014). These microbes exhibit indirect mechanism by suppression of plant pathogen growth and lead to be phytopathogenic resistance (Basak and Biswas, 2009; Hao *et al.*, 2011; Panhwar *et al.*, 2012). From total 14 bacterial isolates, all of them exhibited IAA production activity when cultured in nutrient broth supplemented with 200 µg/ml of tryptophan. Amounts of IAA content were ranged from 0.38-237 µg/ml. The highest amounts of IAA were synthesized by bacterial endophytes *Klebsiella pneumoniae* (RiceTIB1) isolated from rice. Phetcharat and Duangpaeng (2012) reported that bacterial endophytes isolated from organic rice were able to produce IAA more than 10 µg/ml. Therefore, it might indicate that bacterial endophytes from rice provide high efficiency to synthesis IAA. According to report of Sang and co-workers (2014) which studied about *Klebsiella pneumonia* isolated from Korean rice possessed IAA production activity, there

was no report stated about IAA production from *Enterobacter asburiae* isolated from rice. Though Zakria *et al.* (2008) have studied about bacterial endophytes *Enterobacter asburiae* from sugarcane can synthesize high amount of IAA.

Phosphate Solubilizing Ability

To screen for phosphate solubilizing ability of bacterial endophytes, spot-on-lawn technique was employed and phosphate solubilizing ability of bacterial endophytes (Clear zones) were presented in Figure 4. Results showed that 8 isolates possessed phosphate solubilizing ability and the highest solubilization index 2.8 as shown in Table 5.

From solubilization index of bacterial endophytes *Klebsiella pneumoniae* (RT3.1A) and *Enterobacter asburiae* (RT16) possessed phosphate solubilizing ability. There was associated with study of Kiriya and co-workers (2011) that *Klebsiella pneumoniae* shown phosphate solubilizing activity and *Enterobacter asburiae* PSI3 can solubilize 77 mg/l rock phosphate (Gyaneshwar *et al.*, 1999). Mechanism of phosphate solubilization in phosphate solubilizing bacteria conducted through organic acids and acid phosphatase production (Ahmed *et al.*, 2009). Several phosphate solubilizing bacteria including *Bacillus*, *Pseudomonas* and *Enterobacter* were able to effectively increase phosphate uptake in plant, enhance growth of plant and yield of crops.

Conclusions

This study was aimed to search for novel sources of bioactive compounds from bacterial endophytes. Endophytes are microorganism which live in plant tissues and do not cause any harmful effects on host plant (Wilson, 1995). Several studies reported about production of bioactive compounds from endophytes. These microorganisms are able to synthesis various kinds of substances including antimicrobial agents (Yu *et al.*, 2010), antioxidant agents (Liu *et al.*, 2009), anticancer agents (Li *et al.*, 2012) and phytohormones (Jasim *et al.*, 2014). Therefore, it may imply that endophytes are the promising source of bioactive compounds which provide high yield production and high efficiency. Plant tissue culture system as known as micropropagation is unique plant cultivation system that aseptically culture plant in synthetic medium. One of the contaminations from micropropagated system comes from bacterial endophytes (Cassells, 1997). Many reports studied about isolation of bacterial endophytes from micropropagated plants such as potato, grape, banana and papaya (Pereira *et al.*, 2003; Thomas, 2004; Akello *et al.*, 2007; Thomas *et al.*, 2007). Although bacterial endophytes may affect to the micropropagated system, they are known as bioactive compound producer within plants. Therefore, the more bacterial endophytes isolated, the higher potential bioactive compound synthesizers founded. Consequently, these beneficial microorganisms may apply in several fields such as agriculture, industry, and medicine.

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