Bioprospecting of Bacterial Endophytes from *Curcuma aeruginosa*, *Curcuma xanthorrhiza* and *Curcuma zedoaria*as Antibacterial Against Pathogenic Bacteria

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Abstract. Bacterial endophytes live in plant tissue and produce secondary metabolites that potentially have antimicrobial properties. The purpose of this research was to determine the beneficial role of endophytic bacteria isolated from three different local plants namely, TemuIreng (*Curcuma aeruginosa*), Temulawak (*Curcuma xanthorrhiza*) and TemuPutih (*Curcuma zedoaria*) as the potent antibacterial agent against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae* and *Citrobacter freundii.* The study used descriptive method that includes isolation, identification of bacterial endophytes and their biological activity as antibacterial. A total of six endophytic bacteria have been successfully isolated and identified from the three different plants. They were identified as *Bacillus amyloliquefaciens* and *Bacillus cereus* from TemuIreng, *Bacillus amyloliquefaciens* and *Lysinibacillus sphaericus* from Temulawak, as well as *Bacillus sp.1* and *Bacillus sp.2* from TemuPutih. Antibacterial activity test showed that *B. amyloliquefaciens* from Temulawak exhibited 16 and 8 mm inhibition zone against MRSA and *K. pneumonia* bacteria, respectively. Whereas *Bacillus sp.1* and *Bacillus sp.2* from TemuPutih can only inhibit *K. pneumoniae* bacteria with 7 mm inhibitory zone.
Keywords: endophytic bacteria, antibacterial, *C. aeruginosa*, *C. xanthorrhiza*, *C. zedoa*

1. Introduction

Infectious diseases caused by bacteria are the most common type of illness experienced by humans, especially in developing countries, including Indonesia. Antibiotics are normally given to the patients in order to overcome the infection. However, nowadays, the pathogenic bacteria become more resistant due to massive application of these antibiotics in treating bacterial infection [1].

The misuse of antibiotics may cause resistance to the infectious pathogenic bacteria. Resistance occurs when the effect of antibiotic drugs become less effective against bacteria or when the bacteria are no longer sensitive to these drugs. The antibiotics resistance phenomenon is considered not only as an individual problem but also as worldwide problem. Thus, many countries try to overcome antibiotics resistance phenomenon. The World Health Organization (WHO) formulates 67 recommendations to control the increased resistance to antimicrobial drugs [2].

To date, researchers look for novel sources to obtain bioactive compounds, such as tissue culture, including searching for enzymes in plants tissue that play beneficial role in the formation of active compounds, transplant genes into bacterial cells, laboratory synthesis and utilize endophytic microbes found in plants tissue [3].

Endophytic bacteria are defined as the bacteria that live in plant tissues for a certain period of their life cycle. These bacteria can form colonies in plant tissues without harming the host. Moreover, several types of endophytic bacteria may be found in one plant tissue [4].

Microbial endophytes live symbiotically with the host and concomitantly produce particular secondary metabolites with their host plants [5]. Additionally, endophytic bacteria have beneficial role as secondary metabolite producers as contained in the host plants. Their ability to produce certain secondary metabolite compounds that similar to their host has been well documented. Therefore, development of active compounds that present in the host plants can be realized without exploiting the host plant, yet only by developing the microbes that associated with the plant [6].

Previous studies revealed that *Zingiberaceae* have medicinal properties such as anti-inflammatory, antitumor, anticancer, anti-hyperglycemia and antibacterial [7]. In Indonesia, several groups of these plants are commonly used as traditional medicine, for instance, TemuIreng (*C. aeruginosa*), Temulawak (*C. xanthorrhiza*) and TemuPutih (*C. zedoaria*).

TemuIreng contains curcuminoids, essential oils dan flavonoids that have antioxidant properties [8]. Sesquiterpene and monoterpeneare also contained in TemuIreng, which can be utilized as natural antibacteria [9]. Temulawak or the wild ginger has been studied for its vary biological activities such as antitumor, anti- inflammatory, antioxidant, hepatoprotective, and antibacteria. These biological activities are associated with the presence of bioactive compounds in form of curcuminoids and xantorhizol [10]. On the other hand, TemuPutih has many active compounds such as alkaloids, phenolics, flavonoids, saponins, glycosides, steroids and terpenoids [11].

Recently, a total of 31 endophytic bacterial isolates was successfully obtained from ginger plants, which consist of 17 different genera [12]. Milliana, (2015) isolated endophytic bacteria such as *Actinomycesviscosu, Bacillus brevis*and *Pseudomonas stutzeri*from *C. xanthorriza*, which have antifungal properties against *Candida albicans.* Indeed, the diversity of endophytic bacteria in medicinal plants is tremendously high [13]. A total of 23 genera of bacteria were identified from the Zingiberaceae group (*Curcuma zedoaria*), which are collected from Bogor, West Java, Indonesia [14]. This study aims to characterize the endophytic bacteria from three different indigenous Zingiberaceae species. Additionally, isolation, identification of bacterial endophytes and their bioactivity test as antibacterial candidates will be evaluatedin order to obtain an insight on the prospect of these bacterial endophytes as natural antibacterial against detrimental pathogens.

2. Materials and methods

In this study, descriptive method was performed including isolation and identification of endophytic bacteria, as well as antibacterial activity test using Kirby-bauer method against *MRSA*, *K. pneumoniae* and *C. freundii.*

2.1 Isolation of bacterial endophytes

Isolation of endophytic bacteria was conducted using the modified method from Hung and Annapurna (2004). Briefly, plant samples were cut and cleansed with water [15]. Afterwards, samples were washed with sterile distilled water and soaked in ethanol 70% for 30 s and then in sublimate solution ($HgCl_2$) 0.1% for 2 min, rinsed with sterile distilled water prior to drying. Furthermore, dried samples were then smoothed and a total of 1 g was taken for serial dilution until 10^{-7}. The last three dilutions were placed into the petri dish containing *Nutrient Agar* (NA) medium and then incubated for 24 h at 37°C. Incubation from each species of different bacterial colonies was subcultured into a tilted agar medium.

2.2 Identification of bacterial endophytes

Identification of endophytic bacteria was conducted using equipment from VITEK 2 Compact BioMerieux. Briefly, the isolates of endophytic bacteria for identification were inoculated into reaction tubes containing 3 ml of sterile physiological NaCl with 0.5 McFarland turbidity.

2.3 Antibacterial activity test

Test on antimicrobial activity was performed using Kirby-bauer or disc diffusion method [16]. Shortly, pure bacterial isolates were inoculated in 3 ml of sterile physiological NaCl with 0.5 McFarland turbidity with 2x dilution (1.5×10^6 CFU ml^{-1}). Afterwards, a total of 0.1 ml of suspension was inserted into each sterile vial bottle containing disc paper, then incubated for 24 h at 37°C. Moreover, a total of 0.1 ml of tested bacteria (*MRSA*, *K. pneumoniae* and *C. freundiii*) with similar McFarland turbidity was flattened onto the agar surface. Later on, the discs paper containing bacterial endophytes were placed on the surface of the agar medium and incubated for 24 h at 37°C prior to measurement on the bacterial inhibition zone.

3. Results

3.1. Bacterial isolates

A total of 6 isolates of endophytic bacteria were successfully isolated from the three different species of ginger plants. Two isolates were obtained from TemuIreng (isolate codes: TI5 and TI7). Similar number of isolates was also found in Temulawak (TL2 and TL7) and in TemuPutih (TP3 and TP4). Macro- and microscopic observation of bacterial endophytes from 3 species of ginger plants is presented in table 1 and 2, respectively.

Table 1. Macroscopic characteristics of bacterial endophytes from three different species of ginger plants.

Samples type	Isolate code	Colony morphology				
		Form	Elevation	Color	Edge	Surface
TemuIreng	TI5	Amorphous	Flat	Milkish white	Jagged	Coarse
	TI7	Spheric	Convex	Milkish white	Flat	Smooth
Temulawak	TL2	Amorphous	Flat	White	Jagged	Coarse
	TL7	Amorphous	Convex	Yellowish white	Diverge	Smooth
TemuPutih	TP3	Amorphous	Flat	White	Jagged	Coarse
	TP4	Spheric	Convex	Milkish white	Jagged	Coarse

Table 2. Macroscopic characteristics of bacterial endophytes from three different species of ginger plants.

Isolate code	Cell form	Gram type
TI5	Bacillus	Positive
TI7	Bacillus	Positive
TL2	Bacillus	Positive
TL7	Bacillus	Positive
TP3	Bacillus	Positive
TP4	Bacillus	Positive

3.2. Bacterial endophytes identification

In this study, identification by using VITEK 2 Compact BioMerieux revealed that 6 endophytic bacteria were identified (table 3).

Table 3. Identification on endophytic bacteria from three different species of ginger plants.

Isolate code	Identification results
TI5	*Bacillus amyloliquefaciens*
TI7	*Bacillus cereus*
TL2	*Bacillus amyloliquefaciens*
TL7	*Lysinibacillussphaericus*
TP3	*Bacillus sp.1*
TP4	*Bacillus sp.2*

3.3 Antibacterial activity test

Test on antibacterial activity was performed to determine the potential antibacterial properties from endophytic bacteria in TemuIreng, Temulawak and TemuPutih. Antibacterial test for the identified endophytic bacteria is presented in figure 1.

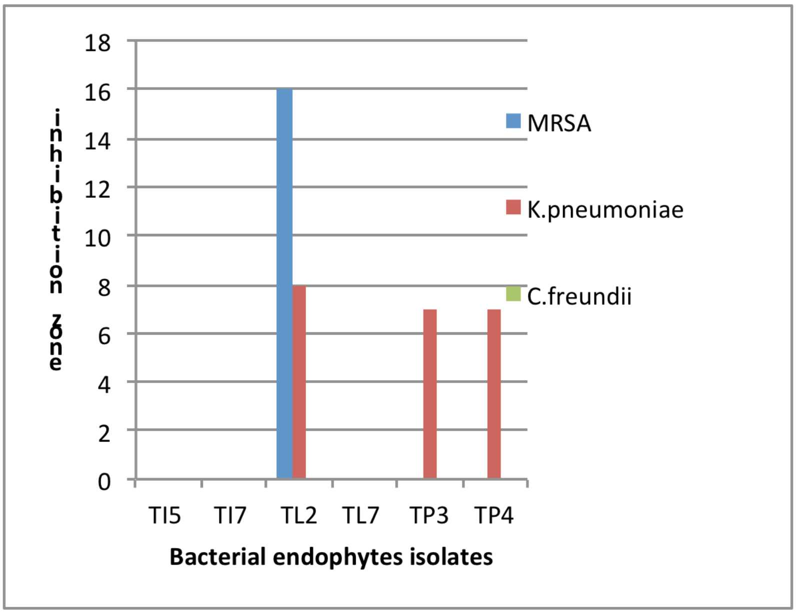

Figure 1. Antibacterial activity from six different bacterial endophyte isolates against MRSA, *K. pneumonia* and *C. freundii.*

Our antibacterial test revealed that the growth of MRSA and *K. pneumonia* were inhibited by theendophytic bacteria from Temulawak and TemuPutih Antibacterial test showed that only isolate of endophytic bacteria *B. amyloliquefaciens* from Temulawak that was able to inhibit the growth of MRSA with 16 mm of inhibition zone diameter or approximately 78% of inhibition zone diameter produced by Amoxicillin 20 µg (9 mm). Additionally, antibacterial activity test from the endophytic bacteria demonstrated that each isolates have different ability in inhibiting the growth of *K. pneumoniae.* This result is supported by the vary diameter zone produced by *B. amyloliqufaciens* from Temulawak, which is 8 mm or 20% of diameter zone produced by Amoxicilin 20 µg (10mm). Whereas isolates bacterial endophytes *Bacillus sp.1* and *Bacillus sp.2* resulting 7 mm of diameter zone or 30% of the similar dose of Amoxilin. In contrast, endophytic bacterial isolates of *B. amyloliqufaciens* and *B. cereus* from TemuIreng as well as isolates *L. sphaericus* from Temulawak did not produce inhibition zone or could not inhibit the growth of *MRSA*, *K. pneumonia* and *C. freundii*bacteria. Bacterial endophytes isolate TI5 is a similar bacterium to that of TL2, which is *B. amyloliquefacien.* Our test also revealed that no inhibition zone appeared from the endophytic bacterial isolates of TemuIreng, Temulawak and TemuPutih on the growth of *C. freundii*.

4. Discussion

Bacterial endophytes identification to our knowledge, *B. amyloliquefaciens*is a gram-positive rod-shaped bacterium that is closely related phylogenetically to *Bacillus subtilis*. Both species have many similar homologous genes. Indeed, *B. amyloliquefaciens* has a peritrichous flagella that allows for motility. The optimum temperature for bacterial growth is between 300C and 400C. Similar to other *Bacillus* species, *B. amyloliquefaciens* forms endospores that allow them to survive for long periods of time [17].

As to *B. amyloliquefaciens, B. cereus* is a gram-positive bacterium with bacillus shape, aerobic, motile and dispersed in soil, plant and food. Several species can be utilized as animal probiotics to reduce pathogenic bacteria such as *Salmonella* in the gut and cecum [18]. The bacteriocin isolated from *Bacillus* bacteria is derived from soil and plant tissue [19]. Moreover, *siderophores* with various antibacterial spectrums is *aminopolyol,* which is an antibiotic produced by *B.cereus* isolate from soil [20].

To our knowledge, *L. sphaericus* is a rod-shaped positive gram bacterium, which is mandatory aerobic, non-motile and grows at pH between 5-10. This bacterium can survive at the temperature between 20 - 45° C. Biochemical test demonstrated that *L. sphaericus* give negative results for citrate, malonate, nitrate reduction, ONPG and Proskauer test, but positive for catalase and arginine test. This bacterium can't ferment carbohydrates including arabinose, glucose, mannitol, sucrose and trehalose [21].

In this study, identification on bacterial endophytes isolates TP3 and TP4 was unidentified. This because Vitek is an automatic bacterial indentification tool that based on biochemical characteristics that stored in Vitek's database. Therefore, in certain case, Vitek also has limited bacterial database. According to [22], these unidentified bacteria by Vitek apparatus indicate that there were more than 3 species that have similar biochemical test pattern or none of the species that have such test patterns contained in the database [22].

Antibacterial activity test revealed that endophytic bacteria from Temulawak and TemuPutih inhibit the growth of MRSA and *K. pneumonia*. Whileour antibacterial test showed that only isolate of endophytic bacteria *B. amyloliquefaciens*from Temulawak that was able to inhibit the growth of MRSA. According to Pan *et al.* (2009), a biosubstance that can produce inhibitory zone diameter between 10-20 mm is categorized as strong sensitive bioactive [23].

The prospect of endophytic bacteria *B. amyloliquefaciens* is considerably promising since it has better activity than Amoxicillin 20 µg. This implies that the natural antimicrobial compound from *B. amyloliquefaciens* from Temulawak is more efficient in inhibiting the growth of pathogenic bacteria compared to synthetic antibiotics.

Several strains of *B. amyloliquefaciens* are known to be able to produce several lipopeptide such as surfactin, fengycin and bacillomycins D that can inhibit *Fusariumoxysporum* and polyketides, for instance, bacillaene, difficidindanmacrolactin that can also inhibit *F. oxysporum* [24,25]. In addition, the peptide plantazolicin A, B and cyclic amyocyclic peptide produced from these bacteria also inhibit *B.subtilis* [26].

Our antibacterial activity test from the endophytic bacterial isolates demonstrated that each isolates have different ability in inhibiting the growth of *K. pneumonia.* Compared to that of Amoxilin, our findings imply that the antibacterial activity of these isolates can be classified as semisensitive (less sensitive) [23]. In addition, endophytic bacterial isolates of *B. amyloliqufaciens* and *B. cereus* from TemuIreng as well as isolates *L. sphaericus* from Temulawak did not produce inhibition zone or could not inhibit the growth of *MRSA*, *K. pneumoniae*and *C. freundii*bacteria. This fact supports the hypothese that endophytic bacterial isolates have different ability to produce secondary metabolites that can suppress the growth of pathogenic bacteria. According to Ruby (2011), the environmental conditions in which host plants grow, plant species and the age of plants will influence the population and the profile of the associated endophytic bacteria in it [27].

Antimicrobial compounds from several strains of *B. cereus* have been successfully isolated, and they are able to inhibit bacterial growth, ie. bacteriocin and *aminopolyol* antibiotics from *B. cereus* originated from soil. A study from Imsande *et al.* (2010) showed that the penicillinase content obtained from *B.cereus* serves to lower penicillin levels in patients with penicillin [19,28].

As compared to other tested bacteria, *C. freundii*appeared to be resistant to all bacterial endophyte isolates but not to Amoxicilin. This result confirms the study from Sagita [29] who reported that several strains of *C. freundii*show resistance to amoxic antibiotics, as well as the antibiotics group such as cefotaxime, cefotaxidine and ceftriaxone.

5. Conclusion

The present study demonstrates a total of six species endophytic bacteria were isolated from the three different ginger species. There were two identified isolates from TemuIreng (*B. amyloliquefaciens* and *B. cereus*), Temulawak (*B. amyloliquefaciens* and *L. sphaericus*) and TemuPutih (*Bacillus sp.1* dan*Bacillus sp.2*). Bacterial endophytes isolate *B. amyloliquefaciens* originated from Temulawak can inhibit the growth of MRSA, with 16 mm diameter inhibition zone and categorized as strongly sensitive against. In contrast, this isolate is less sensitive to *K. pneumoniae* with only 8 mm of diameter inhibition zone. Additionally, *Bacillus sp.1* and *Bacillus sp.*2 can inhibit the growth of *K. pneumoniae* with 7 mm inhibition zone and categorized as the less sensitive. Our findings also suggest that further research on characterization of growth curve from endophytic bacteria is required to identify the growth phase when the secondary metabolites are produced. Furthermore, secondary metabolite isolation from bacterial endophytes is also required to obtain the high value product, for instance, as natural antibacterial against pathogen.

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