RESEARCH ARTICLE

Optimization of the isolation method of endophytic actinomycetes from *Psidium guajava* (L.) and *Ziziphus mauritiana*

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Abstract:

The endophytic microorganism population varies in plants and some of them are known to be able to produce bioactive compounds that can protect plants against pathogens. Therefore, the present study was designed to isolate the endophytic actinomycetes from *Psidium guajava* (*P. guajava*) and *Ziziphus mauritiana* (*Z. mauritiana*). The efficacy of two surface sterilization methods and two different incubation temperatures for isolation of actinomycetes were assessed ~~by~~ using low based media. Two procedures were used for surface sterilization, the procedure I (70% ethanol) and procedure II (99% ethanol) and the isolation plate was then incubated at 30°C and 37°C for one month. The procedure II of surface sterilization was observed more effective in eliminating the epiphytes and incubation at 37°C was found to be practical for the isolation of endophytic actinomycetes. Based on morphological characteristics, all seven isolates were identified with Streptomyces spp and were isolated from plants roots. From the seven isolates, 71.4% (n=5) were isolated from Z. mauritiana and 28.6% (n=2) from *P. guajava*. In conclusion, the isolation and optimization to enhance the growth of endophytes through surface sterilization and incubation temperature were described and the endophytic actinomycetes can potentially be harvested from ethnomedicinal plants.

Keywords: *Endophytic actinomycetes*, ethnomedicinal plant, incubation temperature, surface sterilization

1. INTRODUCTION

Endophytes are microorganisms that usually found in plants that can be either bacteria, fungi, or actinomycetes. These microorganisms are usually known for having a symbiotic association with the host plant where it can be producing plethora substances that provide protection and especially survival value to the plant [1]. Actinomycetes are defined as gram-positive bacteria with transitional forms between bacteria and fungi or known as filamentous bacteria. It is said that the actinomycetes have been used for drug discovery because it produces more than 10,000 bioactive compounds. Endophytic actinomycetes possess metabolites with a broad-spectrum activity where it can be used to treat multidrug-resistant pathogens, such as Methicillin-resistant Staphylococcus aureus (MRSA) [2]. Actinomycetes isolated from ethnomedicinal plants can produce a wide diversity of economic importance such as novel compounds that can be exploited in pharmaceutical industries [3]. In contemporary, there is a renewed attraction in these ethnomedicinal plants and a rising interest in producing more drugs derived from plant sources. Many current drugs mimic naturally occurring molecules or possess structures that are fully or partly derived from natural resources [4]. Nevertheless, perhaps it can be an alternative source for many therapeutics, in particular with recent failures of antibiotics against multi-drug resistant microorganisms [5].

Medicinal plants with established ethnomedicinal properties are promising candidates for the isolation of potent endophytic actinomycetes [6]. Even though acquiring the plants to be tested is relatively easy, only several types of plants are covered with actinomycetes. Actinomycetes are bacteria that possess characteristics like fungi so it might live well at 30°C or 37°C. The incubation temperatures for actinomycetes were found with optimum growth of endophytes at 30°C and 37°C. Isolation of endophytic actinomycetes has been the subject of many studies [7]. The diversity of actinomycetes depends on the isolation techniques used. Surface sterilization has been frequently reviewed on the proper way to effectively enhance the growth of endophytic actinomycetes. The selection procedure for surface sterilization is crucial to maximizing the growth of endophytes. If using a sterilization procedure that damaging the tissue it can affect the endophyte isolation [8].

Two ethnomedicinal plants have been selected for the present study which was *P. guajava* and *Z. mauritiana*. *P. guajava* has been known as guava that belongs to a family of *Myrtaceae*. *P. guajava* has been used in folk medicine and it is believed to have active components that help to treat several diseases [9]. This plant possesses several medicinal properties ranging from antimicrobial activity to anticancer properties. The main elements found in *P. guajava* are vitamins, tannins, phenolic, essential oils, flavonoids, sesquiterpene alcohols and terpenoid acids [10]. *Z. mauritiana*, also known as the Chinese date belongs to a family of *Rhamnaceae*. The compounds that derived from this plant possess antidiarrheal, antidiabetic and antifungal activity [11]. The leaves of this medicinal plant can be used to treat fever and asthma. Other than that, it also can be used as a poultice. The juice of root can be used to alleviate gout and rheumatism [12].

There is a limited report on the isolation of endophytic actinomycetes from these ethnomedicinal plants, *P. guajava* and *Z. mauritiana* using water agar, even though these plants were well-known for their use in traditional medicine. Besides, the proper method to grow these endophytic actinomycetes on surface sterilization and incubation temperature remain uncertain because the endophytes usually hard to be cultivated as they require more specific conditions. Therefore, the present study was done to investigate the effectiveness of surface sterilization procedures and the suitable incubation temperature to grow the endophytes.

2. MATERIALS AND METHODS

2.1. Plant Materials

Leaves, stem, and root of *P. guajava* were collected from Taman Putra (2°13'15.8" north latitude and 102°16'00.6" east longitude), while *Z. mauritiana* were collected from Taman Delima Raya (2°13'41.3" north latitude and 102°16'28.9" east longitude), Melaka Tengah. Both plants were identified by Dr. Richard Chung Cheng Kong (Ph.D., Taxonomist, FRIM). The plant materials were labeled and stored in ziplock bags and kept at 4°C.

2.2. Surface Sterilization Procedures

Two surface sterilization procedures were performed to eliminate epiphytes from the plant materials. For procedure I, the samples were immersed in 70% ethanol, followed by 3.125% sodium hypochlorite (NaOCl) and rinsed with sterile distilled water [13]. For procedure II, the samples were then immersed in 99% ethanol, followed by 3.125% NaOCl, washed in 99% ethanol and finally rinsed with sterile distilled water [14]. The efficacy of surface sterilization techniques was validated by aseptically rolling surface sterilization plant tissue onto nutrient agar. The plate was incubated at 37°C overnight.

2.3. Isolation Of Endophytes

The plant leaves were crushed using mortar and pestle. The bark of the stems and roots were removed and then carefully excised into 1.0 cm long. All samples were placed on water agar and incubated at 30°C and 37°C. The incubation period was done for four weeks and the plates were observed every day. The actinomycetes colonies were subcultured on nutrient agar and stored in 20% glycerol solution at -80°C before use.

2.4. Macromorphology And Micromorphology Observation

The isolates of the actinomycetes colonies were identified based on their macromorphologies such as aerial, substrate mycelia and pigmentation. The micromorphology of the actinomycetes was observed using Gram staining. Characteristics of the spore-bearing hyphae and spore chain were determined under a light microscope with 100x magnification.

3. RESULTS AND DISCUSSION

The efficacy of surface sterilization procedure on the growth of actinomycetes observe within the four weeks (Table 1). The growth of endophytes was observed only in the second week for the procedure I, while for procedure II the growth of endophytic actinomycetes observed throughout the four weeks. The validity of surface sterilization for the procedure I cannot be accepted as the growth of epiphytes was detected in almost all sterile plates. In procedure II, contamination was observed starting on week two and increased with a longer incubation period. Contamination of epiphytic microorganisms can disturb the isolation process of pure endophytes on water agar (isolation plates). The growth of endophytic actinomycetes was characterized by the white ivory and wrinkled form while epiphytic contamination can be distinguished clearly at the plate where it surrounds the tissue materials (Figure 1).

Observation on isolation plates after one day of incubation was done where contamination occurred in all plates. The contamination rate was increased with the lengthened incubation period. Surface sterilization of plant material for isolation of endophytic actinomycetes using the procedure I was found to be not effective to eliminate epiphytic microorganisms on the plant surface. The contamination also showed that 70% of ethanol used in this study has a lack of activity against spore-forming bacteria [15]. Besides, the media agar used in this study was absent in antibacterial and antifungal agents which were failed to inhibit the epiphytes growth. However, the use of antibiotics in media agar might inhibit actinomycetes along the way with other bacteria [18]. NaOCl was thought to be an effective disinfectant agent against many bacteria, but the contamination occurred showing that some fungus and bacteria might survive probably due to resistance towards this disinfectant [8][16].

In procedure I, the concentration used quite low with 3.125% NaOCl. A previous study on the efficacy using sterilizing procedures using three sterilization agents including sodium hypochlorite, hydrogen peroxide, and chlorine gas, found that NaOCl was not effective against contaminants causing the seed germination rate remains low [17].

The immersion time of the plant materials in 70% ethanol was 5 minutes in the procedure I and only 1 minute in procedure II. Ethanol is known as a powerful sterilizing agent but also extremely phytotoxic. Plant material should be exposed to

ethanol only in seconds or minutes. Treating it with longer periods might damage the plant tissue and may cause less growth of endophytes in the procedure I. The procedure II able to isolate seven endophytic actinomycetes compare with the procedure I only one endophyte was isolated throughout the four weeks.

Table 1. The efficacy of surface sterilization procedures on the growth of actinomycetes

Type of plants	Part of plants	Week / Surface sterilization procedure							
		First week		Second week		Third week		Fourth week	
		I	II	I	II	I	II	I	II
P. guajava	Leaf	NG	NG	NG	NG	NG	NG	NG	NG
	Stem	NG	NG	NG	NG	NG	NG	NG	NG
	Root	NG	NG	NG	NG	NG	NG	NG	NG
Z. mauritiana	Leaf	NG	NG	NG	NG	NG	NG	NG	NG
	Stem	NG	NG	NG	NG	NG	NG	NG	NG
	Root	NG	G	G	G	NG	G	NG	G

G-Growth, NG- No growth

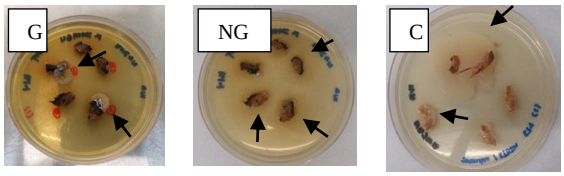

Figure 1. The efficiency of surface sterilization on the growth of endophytic actinomycetes and epiphytic contamination on water agar (Isolation plate). Arrow mark indicating Growth (G), contamination (C) and no growth (NG)

The present study indicated that seven endophytic actinomycetes with the strain of *Streptomyces* sp were successfully isolated. 71.4% (n=5) of isolates came from *Z. mauritiana* and 28.6% (n=2) from *P. guajava*. All endophytes came from the root of the plants. Actinomycetes present in the rhizosphere can be transferred easily to plant roots as the roots are the site of water and nutrient intake. Although the water agar as a low based nutrient media was used for subculturing the actinomycetes, the isolation of endophytes still can be done due to the efficacy of the pre-treatment sample by using surface sterilization.

The identification of endophytic actinomycetes was done on two weeks old plate based on the International Streptomyces Project (ISP). The seven isolates were identified with Gram-positive flexuous, spira, straight or rectus [19]. Several strains of Streptomyces sp. are an important producer of drug molecules because it exhibits structural and functional diversity. During the early years, the majority of the antibiotics were discovered from this kind of species [5]. In this study, all isolated actinomycetes were identified as Streptomyces sp. by morphological characteristics. Streptomyces is one of the dominant genera that been isolated as endophytic actinomycetes [3, 20].

The actinomycetes were observed growth at 37°C and no growth at 30°C (Table 2). Actinomycetes can grow in an extreme environment, where it characterized by acidic or alkaline pH, high temperatures, salinity, and high radiation environment or low levels of nutrients. Two types of thermophilic actinobacteria were strictly thermophilic and moderately thermophilic. Strictly thermophilic actinobacteria can grow in the temperature range between 37 and 65°C [21]. This might be the reason why in this study the actinomycetes grow at 37°C instead of 30°C. This reason can be supported by previous work where the incubation temperatures of 28°C, 37°C, and 45°C are considered optimal for isolation mesophilic, thermotolerant, and moderately thermophilic actinobacteria [22].

Table 2. Number of endophytic actinomycetes isolated at different incubation temperature

Number of endophytic actinomycetes	Incubation temperature	
	30°C	37°C
P. guajava	-	2
Z. mauritiana	-	5
Total	0	7

4. CONCLUSION

In this study, the isolation and optimization to enhance the growth of endophytes through surface sterilization and incubation temperature were described and endophytic actinomycetes were successfully harvested from *P. guajava* and *Z. mauritiana*. However, the properties of these actinomycetes are yet to be known. Therefore, further study on bioactivity of these actinobacteria compounds might be beneficial to the community. We should know the ability of the isolates on how it confers protection against pathogens to confirm the beneficial properties of these microorganisms.

ACKNOWLEDGEMENTS

This project was supported by IRMI, UiTM under the LESTARI grant scheme (600-IRMI/DANA 5/3/LESTARI (0069/2016)). The authors wish to acknowledge the Faculty of Health Sciences, UiTM Selangor, Puncak Alam Campus for providing necessary facilities to conduct the study.

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