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Production of secondary metabolite E2.2 from *Phaleria macrocarpa* endophytic fungus

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ABSTRACT

Objective: To isolate new endophytic fungus from *Phaleria macrocarpa* (*P. macrocarpa*) that is able to produce E2.2 compound.

Methods: Endophytic fungi were isolated from *P. macrocarpa*. Morphological and molecular identification was done to determine the species of the endophytic fungus. High performance liquid chromatography was used to determine the ability of this fungus to produce E2.2 compound and to quantify the total yield of E2.2 from fungal fermentation. Fermentation process was optimized by observing suitable medium, pH and length of fermentation process. Phloroglucinol and gallic acid addition were examined to determine the effect of each compound on E2.2 production.

Results: One endophytic fungus was successfully isolated from *P. macrocarpa* plant. Morphological and molecular identification showed that it was a *Colletotrichum gloeosporioides* which belonged to Glomerellaceae family. This fungus showed highest production of E2.2 when incubated in potato dextrose broth with initial pH value of the medium at 5, and was incubated for 15 days. Phloroglucinol was found to better enhance E2.2 production.

Conclusions: *Colletotrichum gloeosporioides* found in *P. macrocarpa* plant is promising as a potential alternative source of E2.2.

1. Introduction

A new way of generating natural compound is from endophytic fungi. Endophytic fungi are fungi that colonize living plant tissue without causing any immediate, overt negative effects [1]. Mutualistic interaction between endophytic fungi and its host has generated a lot of interests regarding its broad potential in research. Several studies have shown that endophytic fungi are able to produce compounds that are similar to the secondary metabolites produced by its host [2]. Hence, endophytic fungus has high potential as a new source of bioactive compound.

Phaleria macrocarpa (*P. macrocarpa*) is a native Indonesian plant which has been used traditionally as herbal drink to treat many types of diseases such as cancer and diabetes [3]. Recent studies also showed that *P. macrocarpa* exhibited numerous different bioactivity. A study by Hendra *et al.* showed that this plant exhibited antioxidative, anti-inflammatory and cytotoxic activity [4]. Our previous study has successfully proven that *P. macrocarpa* has anti-proliferative activity against two types of cancer cells (MDA-MB-231 and MCF-7 human breast adenocarcinoma cell lines) [5]. To further ascertain our findings, we isolated numerous different compounds from *P. macrocarpa* and found that E2.2 compound was responsible for the anti-cancer activity of *P. macrocarpa* [6].

Although our previous study has suggested that the use of E2.2 compound from *P. macrocarpa* as a treatment of cancer is promising, use of natural resources in pharmaceutical production still faces many challenges such as limited source of raw material and unstable quality of raw material due to the environment [7]. A large amount of *P. macrocarpa* fruit is needed to produce standardized *P. macrocarpa* extract. Consequently, a new alternative method to obtain E2.2 compound efficiently and effectively is required. Therefore, the present study is focused on obtaining E2.2

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compound from endophytic fungi of *P. macrocarpa* as a novel source of E2.2 compound.

2. Materials and methods

2.1. Isolation and purification of endophytic fungi

Healthy *P. macrocarpa* plant was collected in August 2012 from West Java, Indonesia. Roots, stems and leaves of the plant were washed under tap water for 10–15 min and cut into small sizes around 1 cm × 1 cm before being surface-sterilized. Surface sterilization was done in four steps. First, samples were rinsed in tap water. Then, the samples were immersed in 70% alcohol for 1 min, followed by 5.3% NaOCl for 5 min, 70% ethanol for 30 s, and washed with sterile water until no trace of the previous solution was left.

Isolation process of endophytic fungi from *P. macrocarpa* was done using rose bengal chloramphenicol agar (BD Difco, New Jersey, USA). The surface-sterilized sample was placed in a Petri dish (Iwaki, Japan) containing the selective medium, and incubated for 1 week at 27 °C. After 1 week of incubation, all fungi isolates were transferred into potato dextrose agar (Oxoid, Hampshire, United Kingdom).

2.2. Identification of E2.2-producing endophytic fungi

E2.2-producing endophytic fungi were identified based on its morphological and molecular characteristics. The fungi were cultivated in Petri dish containing potato dextrose agar (Oxoid, Hampshire, United Kingdom) and incubated for 1 week at 27 °C. After 1 week of incubation, macroscopic (colony appearance) and microscopic (mycelia) characteristics were examined. Molecular identification was done using nucleotide sequencing of internal transcribed spacer (ITS) region of rRNA. DNA preparation and DNA sequencing were done using standardized method by Molecular Biology Service species barcoding from 1st Base (Singapore). Sequences obtained were then submitted to BLAST and NCBI to determine the strain of the fungi.

2.3. Fermentation and extraction

Fungal isolate was cultivated and incubated for 7 days to obtain the desired secondary metabolites. One plug (1 mm × 1 mm) of mycelia was inoculated into 100 mL of potato dextrose broth (PDB) (BD Difco, New Jersey, USA) and incubated in an Innova® 40/40R Shaker Incubator (New Brunswick Scientific Co., Inc., USA) (Percival Scientific, USA) at 27 °C, 150 r/min. After incubation, mycelia and fermentation broth were separated using a 0.2 μm filter (Merck Millipore, Darmstadt, Germany). The fermentation broth was then extracted two times with equal volume of ethyl acetate (liquid–liquid extraction). The resulting ethyl acetate phase was then collected and evaporated under pressure until crude extract was obtained. After fermentation broth was extracted and crude extract was obtained, the extract was dissolved in methanol and filtered through 0.22 μm membrane filter (Iwaki, Japan) prior to high performance liquid chromatography (HPLC) analysis. HPLC analysis was done using SunFire C18 column 4.6 mm × 150 mm, 5 μm (Milford, USA). Methanol: 0.01% acetic acid was used as the mobile phase, flow rate was 1.0 mL/min, detection wave length was 320 nm, and injection volume was 20 μL.

2.4. Optimization and quantification of E2.2 production

In order to obtain highest concentration of E2.2, optimization of fermentation condition was done. The fungi were incubated in two different medium with different incubation time and pH. All fungi isolates were incubated in PDB (Hampshire, United Kingdom) and malt extract broth (Hampshire, United Kingdom). The cultures were incubated in 4 different pH (4, 5, 7 and 8.5). The cultures were also incubated in different incubation time (4, 12, and 15 h). After incubation, E2.2 concentration was measured using HPLC.

2.5. Effect of phloroglucinol and gallic acid on E2.2 production

The effects of phloroglucinol and gallic acid on the production of E2.2 compound were determined by adding 0.1 mg/mL of the substance in the fermentation medium. Each culture was then incubated for 7 days at 27 °C, 150 r/min. After incubation, E2.2 concentration was measured using HPLC.

3. Results

3.1. Isolation and purification of endophytic fungi

One fungal isolate was successfully isolated and purified which we later referred to as KP11.

3.2. Identification of E2.2-producing endophytic fungi

Early identification was done by examining the morphological characteristic of the fungus. Figure 1 shows the morphological character of the isolated fungus. Based on morphology analysis shown in Figure 1, the fungus could be described as filamentous fungi with white and orange colored velvety colony. It had conidia as vegetative spore and septate hyphae.

Figure 2 shows phylogeny tree of the isolated fungus based on neighbor-joining analysis compared to other similar fungi strains. Based on this analysis, this fungus was identified as *Colletotrichum gloeosporioides* (*C. gloeosporioides*) with 99% similarity.

3.3. Fermentation and extraction

The *C. gloeosporioides* was cultured in PDB and incubated for 14 days. After 7 days of incubation, fungi mycelia have formed and the medium appeared darker. The extracted fermentation broth was dark brown. Crude extract of the fermentation broth was examined with HPLC to determine whether the fungal isolate was able to produce E2.2. The HPLC
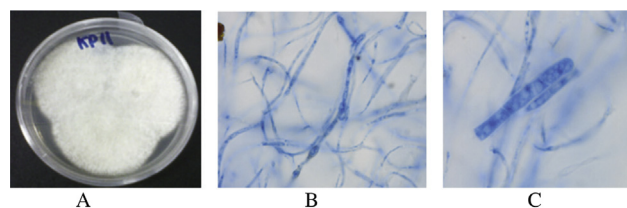

Figure 1. Morphological characteristics of KP11 (A) and microscopic morphology of KP11 (B and C).
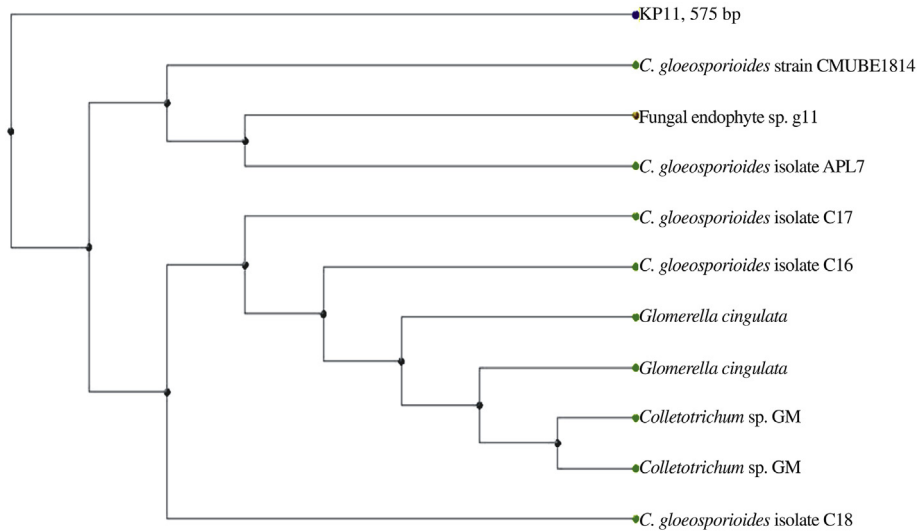
Figure 2. Phylogeny tree of KP11 based on ITS sequences of ribosomal DNA.
C. gloeosporioides: *Colletotrichum gloeosporioides*.

result showed that the fungal isolate turned out to be an E2.2-producing endophytic fungus (Figure 3).

3.4. Optimization and quantification of E2.2 production

Table 1 shows the optimum fermentation condition for the *C. gloeosporioides* isolate. The result suggested that E2.2
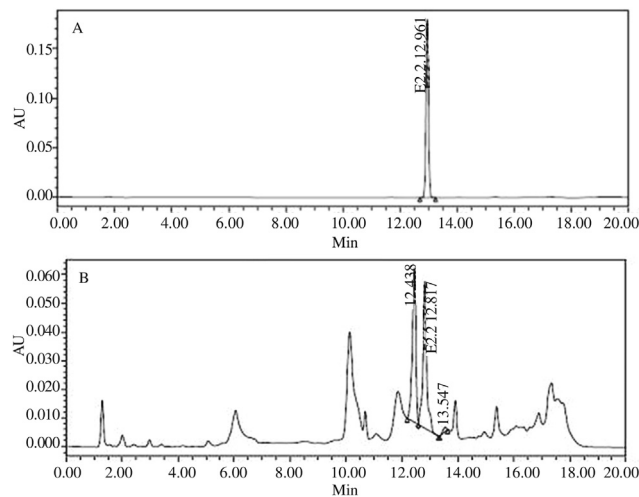

Figure 3. HPLC profile of E2.2 compound (A) and fermentation broth of KP11 (B).

production was optimum in starch-rich medium. Acidity did not cause significant difference in E2.2 production, although highest yield of E2.2 was obtained in pH 5. The result also suggested that longer incubation time resulted in higher concentration of E2.2.

3.5. Effect of phloroglucinol and gallic acid on E2.2 production

Figure 4 shows the effect of phloroglucinol and gallic acid addition on the fermentation broth visually. It is clearly seen that phloroglucinol or gallic acid addition resulted in less visible mycelia growth and also the darker fermentation broth obtained. Therefore, gallic acid and phloroglucinol should not be added more than 0.1 mg/mL as it would be toxic to the fungi's growth.

HPLC analysis was done to investigate the effect of phloroglucinol and gallic acid on E2.2 production by *C. gloeosporioides*. The results showed that both phloroglucinol and gallic acid enhanced the amount of E2.2 produced by *C. gloeosporioides*. In the control medium, *C. gloeosporioides* produced 665.72 µg/L of E2.2. However, in the presence of 0.1 mg/mL phloroglucinol or gallic acid, this endophytic fungus was able to produce more than two fold of E2.2 compound compared to the control. Phloroglucinol addition resulted in 2032.53 µg/L of E2.2 compound whereas gallic acid addition resulted in 1736.989 µg/L of E2.2 compound.

Table 1

The effect of some fermentation conditions on E2.2 concentration.

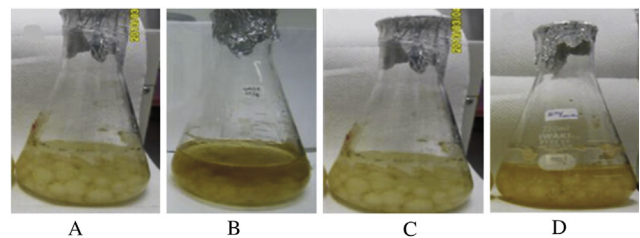| Condition		E2.2 concentration (µg/L)
Growth medium	PDB	31.8133
	MEB	11.4187
Initial medium pH	4	1818.0100
	5	1887.6200
	7	1838.5500
	8.5	1800.9100
Incubation time	4	34.9167
	12	638.3670
	15	665.7170

MEB: Malt extract broth.

Figure 4. The effect of phloroglucinol and gallic acid addition into fermentation medium.
A: No phloroglucinol addition; B: Addition 0.1 mg/mL of phloroglucinol; C: No addition of gallic acid; D: Addition of 0.1 mg/mL of gallic acid.

4. Discussion

Endophytic fungi have been known to produce compounds which are similar to its plant host [8]. In this research, we use endophytic fungi from *P. macrocarpa* isolated using rose bengal agar to produce E2.2 compound. Rose bengal is often used for fungi isolation since it contains chloramphenicol as antibiotic to inhibit bacterial growth. The presence of rose bengal also helps the coloration of fungi colony, hence making the isolation process easier.

The initial endophytic fungi isolation process on *P. macrocarpa* plant resulted in 28 endophytic fungi isolates. However, at the end of the purification step, there is only 1 remaining isolate that is successfully isolated and preserved. According to Arnold *et al.*, the diversity of endophytic fungi in a host plant is highly influenced by the characteristic of its host and growth condition or spatial factor of the host plant [9]. Moricca *et al.* also reported that symbiotic relation between endophytic fungi and its host plant is controlled by the genes of both the fungi and the host plant, and is modulated by environmental factors [10]. The different condition between its natural habitat and laboratory condition might be the cause of limited number of isolated endophytic fungi from *P. macrocarpa* plant as it fails to adapt to different environmental condition.

After we have obtained the endophytic fungus isolate, we identified this fungus based on its morphology and DNA sequence. The results showed that KP11 was *C. gloeosporioides*, a common endophytic fungus that was present in a wide range of host plants. Several studies stated that this fungus may also be a plant pathogen [11,12].

Different studies have shown that *C. gloeosporioides* was found in several different plant hosts and therefore it is not host-specific. However, *C. gloeosporioides* in different plants produce different metabolites specific to its host. For example, *C. gloeosporioides* that was isolated from *Cryptocarya mandiocanna* is able to produce antifungal compound [13]. *C. gloeosporioides* from *Piper nigrum* was found to produce piperine [14]. Furthermore, *C. gloeosporioides* from *Forsythia suspense*, *Artemisia mongolica* and *Tectona grandis* were able to produce phyllirin, colletotric acid, and taxol respectively [15–17]. In this study, we have successfully isolated *C. gloeosporioides* that is able to produce E2.2 compound found in *P. macrocarpa*.

HPLC was used to measure E2.2 concentration in the crude extract of *C. gloeosporioides* KP11 fermentation broth. Several peaks were seen in the HPLC result, which indicates that KP11 produces other compounds aside from E2.2. According to Tan and Zou, the ability of endophytic fungi to produce certain bioactive compounds originally characteristic of the host might be related to genetic recombination of the endophytic fungi with the host plant which occurs in evolutionary time [18]. Since *P. macrocarpa* contains a wide range of compounds, we assume that *C. gloeosporioides* KP11 has evolved to produce several different compounds similar to the compounds produced by *P. macrocarpa*.

In order to achieve optimum E2.2 production by *C. gloeosporioides* KP11, suitable fermentation condition must be applied during the fermentation process. Optimization was carried out on medium, pH, and length of incubation time. Table 1 shows the optimum fermentation condition for KP11. The result showed that PDB was more suitable for KP11 fermentation medium compared to MEB. In PDB, E2.2 concentration was almost three fold of the E2.2 concentration in MEB. This might be due to higher content of carbon source in PDB compared to MEB. Liquid state fermentation is known to depend mostly on soluble sugar in its medium for source of fermentation substrate which means that sugar content in the media highly affects the fermentation process [19]. A study by Desmukh *et al.* showed that compared to other carbon sources, growth of *C. gloeosporioides* was at its highest when incubated in a starch-rich medium [20]. PDB has a higher starch content compared to MEB, which explains the higher E2.2 concentration in PDB.

Table 1 also shows that E2.2 concentration increased from pH 4 to 5, and then gradually decreased from pH 7 to 8.5 where the optimum pH was at 5. This result is similar to a study by Drori *et al.* on external pectate lyase secretion by *C. gloeosporioides* [21]. The study showed that pectate lyase production increases when the pH medium increases from 4 to 6 suggesting that there is an ambient pH signal transduction pathway in *C. gloeosporioides*.

Longer incubation time also showed higher yield of E2.2. The simple explanation to this is longer incubation time resulted in higher accumulation of E2.2 compound in the medium. Therefore, compared to 4 and 12 days, 15 days of incubation showed higher E2.2 concentration. Further study is needed to determine at what stage of growth the KP11 fungus stop producing E2.2.

Aside from optimization of growth medium, we also tried to enhance E2.2 production by adding phloroglucinol and gallic acid into the growth medium at the beginning of fermentation process. As previously shown in the results, phloroglucinol and gallic acid addition to the fermentation broth appeared to increase E2.2 production by KP11. The increase in E2.2 concentration might be due to the similar structure of E2.2 and phloroglucinol or gallic acid. E2.2 compound is known to be a benzophenone which is comprised of two benzene rings. Both phloroglucinol and gallic acid have a benzene structure. Because of the similar structure, it seems that phloroglucinol and gallic acid can be used as a precursor in E2.2 synthesis. Use of artificial precursor to increase production of a desired metabolite has been used in several experiments. One example is the use of tryptophan as a precursor for the production of indolediterpenoid [22].

Furthermore, phloroglucinol has been related to the synthesis of benzophenone although there is still no definite pathway. There are some studies that suggest phloroglucinol supports benzophenone synthesis in shikimate pathway whereas other studies suggest that benzophenones are derived from phloroglucinol [23]. This relation between phloroglucinol and benzophenone synthesis might explain the increased E2.2 production after phloroglucinol addition.

In light of our discovery, *C. gloeosporioides* KP11 endophytic fungus is potential to be a new source of E2.2 compound. This method provides a more rapid, efficient, and controllable way of producing E2.2. However, further study is required to optimize the production of E2.2 compound in larger scale with this method.

C. gloeosporioides KP11 found in *P. macrocarpa* plant is promising as a potential alternative source of E2.2 compound.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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