Analysis of production kojic acid from endophytic fungi
Aspergillus flavus isolated from Annona squamosa leaves using an OSMAC Approach

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Abstract. An analysis production of kojic acid by endophytic fungi Aspergillus flavus from leaves of Annona squamosa has been undertaken using three different types of media including rice (Oryza sativa), sweet corn (Zea mays L.) and waxy corn (Zea mays ceritina). The fungi were extracted using ethyl acetate and quantitatively analyzed their content of kojic acid using HPLC. The extrapolation peak area of kojic acid’s content in each fungal medium to the regression equation of standard kojic acid resulted in the concentration of kojic acid in each extract 3.149% for rice media, 5.998% for sweet corn media and 2.226% for waxy corn media. Pure kojic acid from an extract of fungi grown on rice media was obtained from 90% DCM fraction by VLC fractionation. The structure was determined by means of NMR and HPLC analysis data.

1. Introduction

The discovery of Taxol (paclitaxol), a high valuable anticancer drug produced by the endophytic fungi Taxomyces andreana [1] and also other drugs by endophytic fungi such as antifungal agents’ griseofulvin from Penicillium griseofulvum [2] together with inhibitor of cholesterol biosynthesis lovastatin from Aspergillus terreus [3] have replaced the focus of the drugs discovery program from plants to endophytic microorganisms. Moreover, endophytic fungi that live in plant tissues are known to produce chemical compounds similar to those produced by their hosts. Several bioactive metabolites had been reported from endophytic fungi [4-6]. One of the bioactive fungal metabolites kojic acid was recently reported produced in large quantities by endophytic fungi Aspergillus flavus [6].

Kojic acid (5-hydroxy-2-hydroxymethyl-pyrone) has attracted much attention due to its wide application in cosmetic, medical, pharmaceutical, agricultural and food industries. It has been used as
an anti-inflammatory drug and painkiller in the medical sector and as a skin whitening agent, antioxidant and also as protection against UV radiation [7]. Most of kojic acid is produced by fungi from the genus *Aspergillus* spp. and *Penicillium* spp. [8] especially from the species *Aspergillus flavus* [9]. Several media have been used for production as kojic acid such as using cooked starch, yielded 0.25 g of kojic acid/g of corn starch [10].

One technique to expand and to increase the production of the secondary metabolite from endophytic fungi is by applying the concept of OSMAC (One Strain-Many Compound) including using different fermentation conditions to produce a variety of bioactive compounds [11]. This simple and effective approach can be undertaken by changing cultivation parameters such as media composition, aeration, pH, temperature, time of cultivation, cultivation vessel and addition of enzyme inhibitors [12].

Several reports have revealed that changes in the media of cultivation had an effect on the metabolite production of some endophytic fungi. For example, endophytic fungi *Chaetomium Chiversii* cultivated in liquid media and solid media showed different secondary metabolites profile [13]. Moreover, endophytic fungi *Fusarium tricinctum* cultivated in solid rice media added with vegetable and fruit juices caused an increase in the number of fusarielin J by 80 times [14]. Therefore, changing cultivation media may result in the changes profile and the production of secondary metabolites from endophytic fungi.

2. Experimental Method

2.1. Fungal material

Strain endophytic fungi *Aspergillus flavus* was isolated from *Annona squamosa* leaves. The fungus was identified by observing the morphological characteristics and by molecular biological protocol based on the sequence of the ITS region as described early [4]. The fungus was transferred onto a petri dish containing potato dextrose agar. A small portion of the fungus from the petri dish was transferred to the sterile solid rice (*Oryza sativa*), waxy corn (*Zea mays ceritina*) and sweet corn (*Zea mays L.*) media in L Erlenmeyer and incubated at room temperature for 30 days. After 30 days, the ethyl acetate was added into the culture and left for 2 days. The mixture was then filtered and the solvent was removed under vacuum.

2.2. Kojic acid identification

The crude extracts and fractions were analyzed for the amount of chemical compound using HPLC with ACE 5 C18 column (150x4.6 mm id) using methanol as the mobile phase with a flow rate of 1 mL/min with detection at 235 nm and semi-preparative column Phenomenex Luna 5u C18 (250x10 ) with flow rate 1.5 mL/min. The EtOAc extract also subjected to vacuum liquid chromatography column eluted with the mixture of n-hexane : EtOAc (20, 40, 60, 80 and 100% n-hexane) and MeOH : DCM (20, 50, 70, 90 and 100% DCM). Each fraction was analyzed with HPLC. The structure of kojic acid was confirmed with the 1H NMR spectrum performed on Agilent Varian 500 MHz with methanol (MeOD) as the solvent.

3. Results and Discussion

The media used for cultivation has been known to affect the growth of endophytic fungi and thus can influence the production and the diversity of secondary metabolites [12-14]. In this study, rice was used as a source of carbohydrates together with local yellow corn (jagung kuning) (*Zea mays L.*) and waxy (bose) corn media (*Zea mays ceritina*) found in Kupang (Timor). Rice has sufficient nutritional content, soft texture and smooth shape and thus creating more room for fungi to multiply while corn has a higher protein content (9.5%) compared to rice (7.4%). Endophytic fungi were grown in solid rice, sweet and waxy corn media and incubated for 30 days [15].

Ethyl acetate crude extracts from three different media were analyzed for the content of kojic acid using HPLC (Figure 1-4). Intrapolation of peak area for the content of kojic acid in each cultivation...
media to the regression equation of standard kojic acid revealed that the concentration of kojic acid in each extract were 3.149% for rice media, 5.998% for sweet corn media and 2.226% for waxy corn media. From the results of the analysis, it was observed that the highest concentration of kojic acid was from the extracts of *A. flavus* grown on sweet corn media followed by rice and then waxy corn media. However, based on the weight of the extracts, the rice media produced a higher amount of kojic acid as the main metabolite. Based on the weight of extracts from each media, the amount of kojic acid was estimated around 0.5782 grams for rice, 0.20322 grams for sweet corn and 0.1583 gram for waxy corn.

Therefore, the *Aspergillus flavus* extract on rice media was further separated based on its level of polarity with VLC (Vacum Liquid Chromatography). The stationary phase used in VLC was silica gel G60 with a size of 70-230 mesh eluted in an increased gradient from nonpolar to polar using mixture of *n*-hexane: ethyl acetate and dichloromethane (DCM): methanol (MeOH). Pure kojic acid was obtained from 90% DCM fraction as shown in Figure 5 has the same retention time as the standard (Figure 1).

![Figure 1. Standard HLPC chromatogram of kojic acid 1 mg/mL](image1)

![Figure 2. HLPC chromatogram of *Aspergillus flavus* extract from rice media](image2)
Figure 3. HPLC chromatogram of *Aspergillus flavus* extract from yellow corn media

Figure 4. HPLC chromatogram of *Aspergillus flavus* extract from bose corn media.

Figure 5. HPLC chromatogram of isolated kojic acid from 90% dichloromethane fraction
The structure of pure kojic acid obtained from the 90% DCM fraction was confirmed by comparison with the standard via HPLC analysis at the same condition and also supported by the $^1$H NMR spectra, as shown in Table 1.

**Table 1.** $^1$H NMR Data of kojic acid in MeOD (500 MHz)

| Position | Chemical shifts, $\delta$ (ppm) |
|----------|---------------------------------|
|          | $^1$H-NMR                        | $^1$H-NMR$^{16}$     |
| 2        | -                               | -                    |
| 3        | 6.45 s, 1H                      | 6.52 s, 1H           |
| 4        | -                               | -                    |
| 5        | -                               | -                    |
| 6        | 7.95 s, 1H                      | 7.97 s, 1H           |
| 7        | 4.41 s, 2H                      | 4.43 s, 2H           |

4. Conclusion

Changes in media cultivation have led to the different chemical profiles of the endophytic fungi *Aspergillus flavus*. The use of local yellow corn and waxy corn media was able to expand the chemical profiles of *A. flavus*, but the production of kojic acid was better controlled using the rice media.

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