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EFFECT OF AGITATION SPEED AND CULTIVATION TIME ON THE PRODUCTION OF THE EMESTRIN PRODUCED BY *Emericella nidulans* MARINE FUNGAL

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Abstract

Emestrin, an epipolythidioxopiperazine (EPT), is bioactive secondary metabolite produced by the marine fungus *Emericella nidulans*. Emestrin is potential to be developed as anticancer agent. Our present study investigated the effect of the agitation speed and cultivation time on the production of the mycelial biomass and emestrin in *E. nidulans*. The fungal was cultivated in malt extract broth (MEB) medium with varying agitation speeds of 0, 50, 100, 150 rpm during 1,2,3 and 4 weeks of incubation at the temperature of 28 °C. Concentration of emestrin was determined by using high performed liquid chromatography (HPLC). The highest concentration of emestrin was found at static condition (0 rpm) cultivated for 1 week.

Keywords: emericella nidulands, emestrin, agitation speed, culvation time

1. Introduction

Marine-derived fungi have been intensively explored for their bioactive secondary metabolites. Fungal metabolites exhibited diverse biological activities such as anticancer, antimicrobial, antiplasmodial, antiinflammatory and antiviral agents (Xiong et al., 2009; Ingavat et al., 2011). One of marine-derived fungi that produce bioactive secondary metabolites is *E. nidulans*. In our research works this fungal species was isolated from the surface of marine ascidian *Aplidium longithorax* collected from Wakatobi Marine National Park, Southeast Sulawesi, Indonesia. Static culture of this fungus in MEB produced emestrin (Figure 1). The molecular weight of emestrin was 597.1105 $[M-H]^-$. Emestrin displayed anticancer activity against several cancer cell lines (Nursid et al., 2011; 2015).

Emestrin is a member of epipoly thiodioxo piperazines (ETPs) that belongs to a group of toxic secondary metabolites made only by fungi (Gardiner et al., 2005; Jiang & Guo, 2011). Emestrin A and B were originally isolated from the mycelial acetone extract of *Emericella striata* (Seya et al., 1985; Nozawa et al., 1987). Emestrin A, the first reported example of 15-membered macrocyclic ETP with strong antifungal activity, was biosynthetically derived from two molecules of phenylalanine and one molecule of benzoic acid (Jiang & Guo, 2011).

Submerged cultures of filamentous fungi are widely used to produce commercially important metabolites including many antibiotics and the cholesterol lowering drugs (statins) such as lovastatin ($C_{24}H_{36}O_5$, Mevinolin, Monacolin K and Mevacor™). In submerged cultures, fungi can be grown as broths of freely suspended mycelia and pellets or clumps (Metz & Kossen, 1977). In our previous study (Nursid et al., 2012), optimal media for emestrin production was MEB containing malt extract, yeast extract, and soya peptone. Production of secondary metabolites in sub merged cultures is influenced by many factors such as agitation and time of cultivation. Agitation is an important parameter for mixing the nutrient, mass and heat transfer. Agitation makes shear forces, causing

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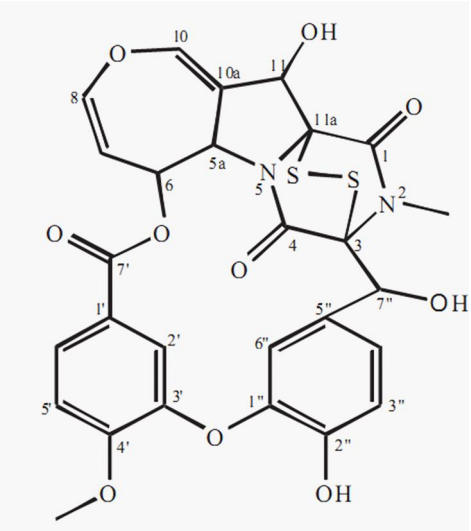

Figure 1. Chemical structure of emestrin.

morphological changes, variation in their growth and product formation, and also damage to the cell structure (Kim et al., 2003).

The purpose of this study was to determine the effects of agitation speed and cultivation time on the mycelial biomass and emestrin production by *E. nidulans* in submerged culture. The morphological features between the different agitation speed were also described.

2. Material and Methods

2.1. Fungal Material

E. nidulans was from a culture collection of Biotechnology Laboratory, Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, Jakarta, Indonesia. This strain was isolated from the surface of marine ascidia *A. longithorax* collected from Wakatobi Marine National Park, Southeast Sulawesi, Indonesia. The stock culture was preserved in the mixture of 20% glycerol and 80% malt exract agar (v/v) and stored in deep freezer at -80 °C.

2.2. Fungal Cultivation

E. nidulans was initially grown on malt extract agar (MEA) medium in a petri dish and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized cutter. The seed culture was grown in a 250 ml flask containing 100 ml MEB medium consisting of 3 g yeast extract, 3 g malt extract, 5 g peptone, in sea water with salinity of 10 ppt). The cultures were agitated at 0 rpm, 50, 100, and 150 rpm for 1, 2, 3, and 4 weeks at the temperature of 28 °C. All experiment were performed in triplicate.

2.3. Extraction

Mycelium harvested from the culture and filtered with filter paper (Whatman no. 41). Fungal mycelium dried by using freeze-drying then weighed. Extraction of secondary metabolites from dried mycelium was carried out by using 50 ml of methanol (MeOH) : methylen chloride ($CHCl_2$) (1:1, v/v) three times. Extraction of secondary metabolites from broth culture was performed three times with ethyl acetate (3 x 100 ml). The MeOH-$CHCl_2$ and combined EtOAc solution was concentrated by vaccum rotary evaporator and dried by using vaccum concentrator. Mycelium and broth crude extracts was weighed to obtain the yield.

2.4. Analysis of Emestrin

Analysis of emestrin in the mycelium and broth extract was carried out by using high performance liquid chromatography (HPLC, Shimadzu). The HPLC condition was set up as follows: mobile phase was acetonitrile 15% in water (gradient 40 minutes), C_{18} column 150 x 2.0 mm (Phenomenex), photo diode array (PDA) detector; column temperature was 30 °C; injection volume was 10 µl with flowrate was 0.2 ml/ minutes. Emestrin concentration was calculated with standard curve of emestrin in µg/g crude extract.

3. Result and Discussion

3.1. Pellet Morphology

Agitation speed of liquid culture of *E. nidulans* caused differences in mycelium morphology. At the agitation speed of 50 rpm, mycelium of *E. nidulans* remains on the surface of medium, only a little

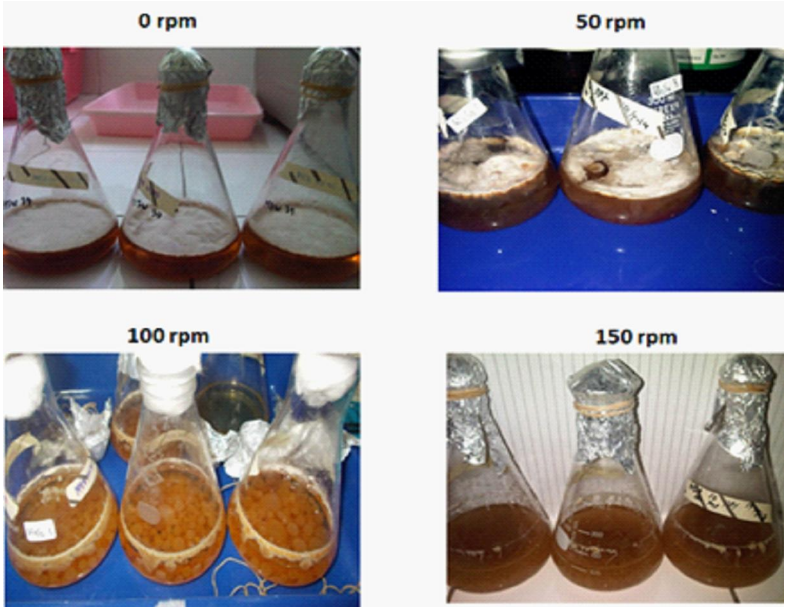

Figure 2. Mycelium morphology of *E. nidulans* cultivated at 0, 50, 100, and 150 rpm agitation speed.

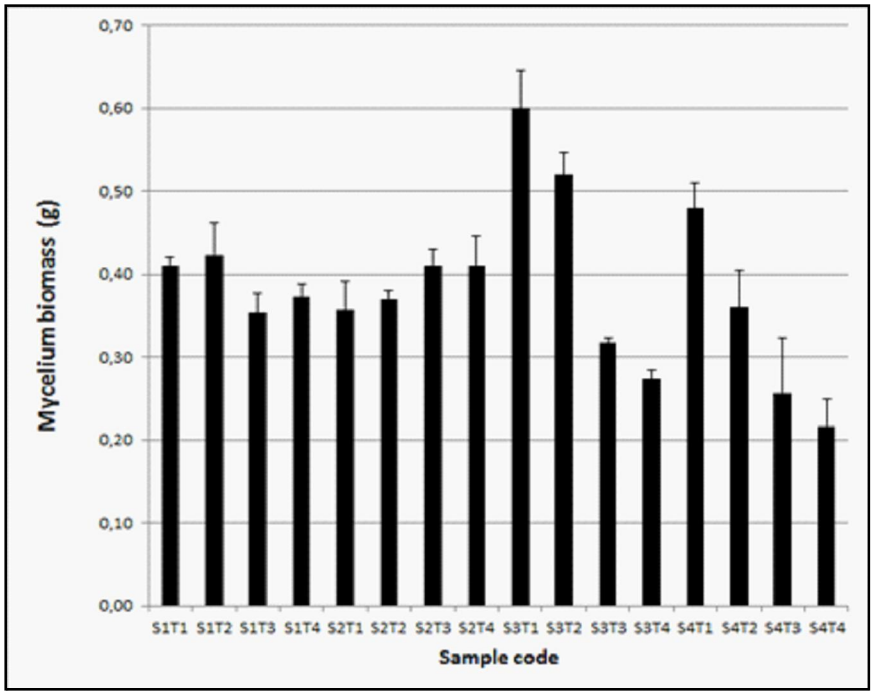

Figure 3. Effect of agitation speed and cultivation time on mycelium biomass (± standard deviation)

Note: S1T1: 0 rpm 1 week, S1T2 = 0 rpm 2 weeks, S1T3 = 0 rpm 3 weeks, S1T4 = 0 rpm 4 weeks, S2T1 = 50 rpm 1 week, S2T2 = 50 rpm 2 weeks, S2T3 = 50 rpm 3 weeks, S2T4 = 50 rpm 4 weeks, S3T1 = 100 rpm 1 week, S3T2 = 100 rpm 2 weeks, S3T3 = 100 rpm 3 weeks, S3T4 = 100 rpm 4 weeks, S4T1 = 150 rpm 1 weeks, S4T2, 150 rpm 2 weeks, S4T3 = 150 rpm 3 weeks, S4T4 = 150 rpm 4 weeks.

mycelium submerged in the culture media. In this state, mycelium still sporulated on the surface of medium. However, at agitation speed of 100 and 150 rpm, mycelium morphology transformed into spherical and submerged in the culture media. Diameter of pellets at 100 rpm were 40 - 60 mm whereas pellets diameter at 150 rpm were smaller (10 - 20 mm). In static condition (0 rpm), all mycelium sporulated on the surface (Figure 2). The specific growth morphology of fungal mycelium obtained under given conditions

depends on several factors including the fungal strain, the method of initiation of culture (e.g. spores, pellets, dispersed mycelium), the nature of the growth medium, and the hydrodynamic regime in the bioreactor (Lopez et al., 2005). There was a significant correlation between the speed of agitation and morphology hypa including tannase enzyme production (Purwanto et al., 2009). In the production of exopolysaccharide from *Grifola fondosan* fungi, mycelium morphology was greatly influenced by pH, aeration and hydrodynamic conditions during fermentation process (Lee et al., 2004).

The highest mycelial biomass was obtained from cultivation using the agitation speed of 100 rpm for one week and the lowest was using agitation treatment of 200 rpm for four weeks (Figure 3). The former was likely due to the optimum nurient and oxygen distribution in the flask, however, at speed of 150 rpm, biomass growth (pellet) was disturbed by shear forces. Agitation intensity was a critical point influencing biomass production (Kim et al., 2003). Agitation resulted in a better contact with air (namely oxygen) in order to increase biomass (Noraziah & Izyani, 2012). *Aspergilus niger* mycelial growth optimally at the agitation speed of 100 rpm, however tanase production optimally occured at the speed of 130 rpm (Purwanto et al., 2009). Mycelial growth depends on nutrient availability in the media. Starch, sucrose, maltose, glucose, and galactose are the carbon sources that are often used in submerged culture, while pepton is the nitrogen source.

3.2. Emestrin Detection and Concentration

Emestrin concentration was detected by HPLC based on the resulting peak retention time and UV characteristics. Emestrin had been isolated from the previous work (Nursid et al., 2011). In that work, standard emestrin was detected at 17.302 min (Figure. 4). Detection of emestrin was also conducted based on the UV absorption of the emestrin at λ_{max} 202 nm. Based on the retention time and UV characteristics, emestrin was detected in the mycelial extract.

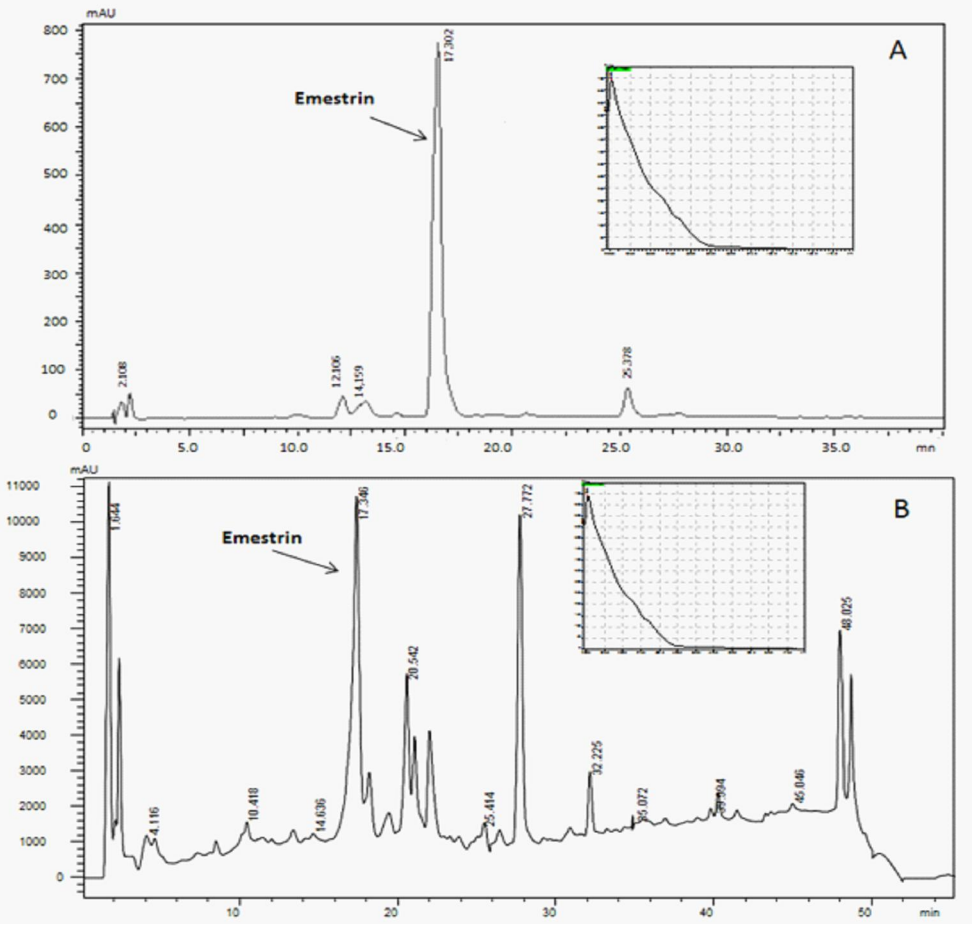

Figure 4. Emestrin standard chromatogram (A, insert: emestrin UV absorption) and emestrin contained mycelial crude extract chromatogram (B).

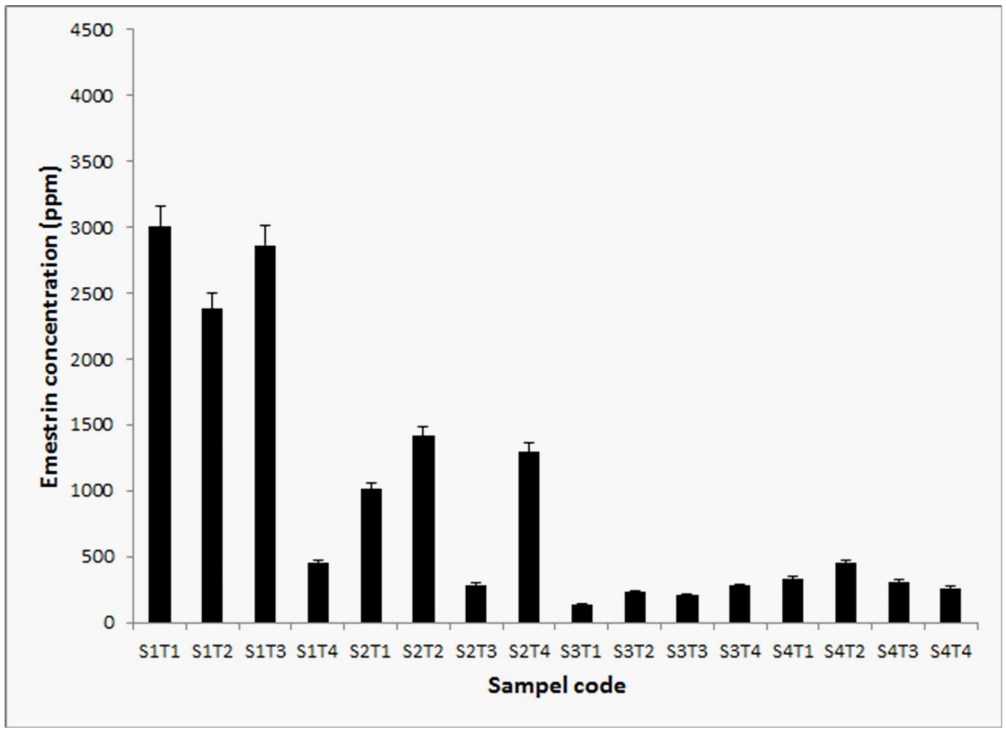

Figure 5. Emestrin concentration in the mycelium extract (± standard deviation).

Note: S1T1: 0 rpm 1 week, S1T2 = 0 rpm 2 weeks, S1T3 = 0 rpm 3 weeks, S1T4 = 0 rpm 4 weeks, S2T1 = 50 rpm 1 week, S2T2 = 50 rpm 2 weeks, S2T3 = 50 rpm 3 weeks, S2T4 = 50 rpm 4 weeks, S3T1 = 100 rpm 1 week, S3T2 = 100 rpm 2 weeks, S3T3 = 100 rpm 3 weeks, S3T4 = 100 rpm 4 weeks, S4T1 = 150 rpm 1 weeks, S4T2, 150 rpm 2 weeks, S4T3 = 150 rpm 3 weeks, S4T4 = 150 rpm 4 weeks.

According to the emestrin standard curve, we calculated emestrin concentration in every agitation speed and cultivation time treatment. The highest emestrin concentration was obtained from *E. nidulans* mycelial that was cultivated at static condition (without agitation) (Figure. 5) and followed by agitation treatment of 50 rpm.

The highest emestrin concentration was obtained in the static condition, while the highest biomass was obtained in agitated culture at 100 rpm for one week. The result showed that emestrin production does not depend on biomass quantity. Some active compound resulted from explorative study on fungi was produced in static condition. Anti-leukemia active compound of sorbicilactone was produced in lab scale from a static cultur of *Penicillium chrysogenum* (Bringmann et al., 2007). So it was with cytotoxic compound of scopularide that was produced static culture of fungi *Scopulariopsis brevicaulis* (Tamminen et al., 2014). Our results suggested that, for emestrin production in large scale, it is necessary to improve culture conditions, such as mixing, aeration, and nutrient source optimation. Therefore, in certain level, agitation is needed for emestrin production in large scale.

Generally, in submerged cultures, it is necessary to agitate the culture broth in order to obtain good mixing and thereby promote heat and mass transfer (Gong & Zhong, 2005). However, agitation also creates shear force. The shear stress exerted by the impeller blades in an agitated fermenter usually reduces and modifies the mycelium pellet growth in terms of diameter, circularity and compactness, and the accumulation of metabolites (Liu et al., 2012).

4. Conclusion

The highest mycelial biomass of the marine fungus *E.nidulans* was obtained from cultivation using agitation speed of 100 rpm for one week, while the highest emestrin concentration was obtained in the static fungal culture.

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