ORIGINAL ARTICLE

Optimization of Temperature and Fermentation Media in the Production of Secondary Metabolites by Endophytic Sporothrix sp and it's activity Against Candida albicans from Dahlia Tubers (Dahlia variabilis)

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ABSTRACT

Background: Endophytic fungi Sporothrix sp isolated from dahlia plants have been shown to produce secondary metabolites which have bioactivity as antifungi, but their production is not optimal. In this study the optimization of production was carried out with media modification and fermentation temperature.
Aim: To find the optimal temperature and the best fermentation media in producing secondary metabolites.
Methodology and result : The inhibitory power of secondary metabolites produced against Candida albicans is determined by agar disc diffusion method. There are 2 media variations, namely: (1) Huang et al media with peptone, Na CMC and (2) Huang et al media with Na CMC, ammonium sulfat variation. Temperature variations have four types, that is 10, 27, 37, 40℃. Observations were conducted for 20 days every 5 days on both the media and for four temperatures. Data were analyzed by A nova multi variate (α = 0.05). The antifungal activity was seen by the amount of inhibitory power minimum that appeared with the concentration of fungal fermentation extract 5% b/v. Analysis statistically have significanted on inhibitory power minimum is shown at 37°C giving 23.5 ± 0.70 mm result on the 20th day of variation media Huang et al with Na CMC and peptone.
Conclusion : The secondary metabolites produced function as an antifungal Candida albicans. UV-Vis measurements showed absorption at 229nm wavelengths and 272 nm showed the presence of conjugated double bonds and the characteristics of terpenoids. The IR spectrum measurement results show the absorption band on the N-H amine group at a wavelength of 3300-3000 cm-1, N-H flexural vibration extends in the area of 1650-1580 cm-1, for the amine can also be seen in the area 910-665 cm-1.
Keywords: Dahlia Tubers (Dahlia variabilis), Endophytic Fungus of Sporothrix sp , Candida Albicans,

INTRODUCTION

Indonesia is a tropical country that has a high level of rainfall. The humid environment is an ideal environment for fungus growth. Dermatophysis is one of skin diseases that arise in humans because of the humid clime. People often use anti-fungal drugs to cure infectious diseases cause by fungi. The use of chemical as synthesis drugs not only kills the fungus it self, but also negatively affects humans as the wearers as it accelerates the emergence of resistant and deadly normal species present on the human. Therefore, it is necessary alternative anti-fungal drugs are safe and derived from natural ingredients.

Secondary metabolites from plants have problems from limited plant numbers and live cycle from long plants (Prihatiningtias, 2007). Using endophytic fungi from plant tissues is another alternative to being able to produce the same secondary metabolites produced by the host plant (Strobel, 2003). Secondary metabolite compounds produced by endophytic microorganisms was done by fermentation methods, filled by temperature factor, pH, aeration, agitation and media composition (Pratiwi, 2008). Temperature is important at the growth of fungi, if it is less or exceeds the minimum or maximum temperature, the fungus will not be able to live. (Ganjar, 2006).

Secondary metabolites contained by varieties of flowers and leaves of dahlia plants are flavonoids, terpenoids, and phenols. The methanol extract of red flowering dahlia tubers showed activity to inhibit the growth of Escherichia coli, Basillus subtilis, Staphylococcus aureus, Candida utilis and Penicillium sp. In the previous research, anti fungal activity has been tested and the determination of minimum inhibitory concentration (MIC) of n-hexane extract and methanol and its fractions from dahlia tubers flowering red against fungi causing skin disease is Candida albicans and Microsporum gypseum (Pratiwi, 2008).

This study aims to find the optimal temperature and the best fermentation media from fermented fungus extract Sporothrix sp. To be able to produce secondary metabolites that function as anti-fungal Candida albicans.

MATERIALS AND METHODS

This medium is used as a fermentation medium for the production of antimicrobial compounds. The composition of the media can be seen in table.1. All ingredients were weighed and dissolved in 100 ml of aquadest for starter and 150 ml for production medium, then sterilized by autoclave at 121°C for 20 min. The media were incubated for one day to see no contamination of the media. Huang et al media variations were performed by substituting carbon and nitrogen sources with Na CMC and peptone, Na CMC and ammonium sulphate, as seen in Table 1.

Table 1: Composition of Media Variations Huang *et al*.

Composition	Weight or Volume
NaCMC	72 g
NaNO3	7,2 g
KH2PO4	2,4 g
Pepton or Amonium	2,4 g
KCl	1,2 g
MgSO4.7H2O	1,2 g
FeSO4.7H2O	0,024 g
Aquadest	2,4 L

A total of 50 ml of *Sporothrix sp* inoculums spores (5%) b/v were inoculated into 1 liter of each production medium (Huang *et al*., 2007 media variations) of Huang *et al* media variations with Na CMC and peptone, Huang *et al* variation with Na CMC and ammonium, then incubated for 20 days at 10ºC, 27ºC (room temperature), 37ºC and 40ºC (water bath temperature), dishwater with a speed of 150 rpm. On day 5, 10, 15 and 20 days, an anti microbial test was performed. Fermented mushroom cultures were taken and centrifuged at 5000 rpm for 20 minutes to separate supernatant and mass of cells, the fermentation supernatant was filtered using a Millipore syringe filter of 0.2 µm, this obtaining a crude extract of the dahlia endophytic fungus. Rough extract of *Sporothrix sp.* Endophytes is what will be used for antifungal tests. The results of the largest inhibitory test of this endophytic fermented fungal extract will be compared with the positive control of ketoconazole.

Culturing of pathogenic fungi: Candida albicans isolates from tilted agar were transferred to new aseptic PDA media and incubated for 4x24 hours. Subsequently inoculated into Sabouroud Dextrose Broth (SDB) medium and incubated for 48 hours. Mushrooms are readily available for antifungal testing if OD has reached 0.08 / 0.1 (equivalent to 107CFU / mL). If OD is greater than 0.1 then dilution is performed using a 0.85% NaCl solution.

Anti fungal test : Inoculum of pathogenic fungi (OD 600nm 0.1) equivalent to 107 CFU / mL (Martin, 2011) was inoculated as much as 1 mL into a test tube containing a liquid PDA medium as much as 15 mL (temperature 50oC) and divortex, then poured into a petri dish and left to solidify. Each of the 50 µL sterile rough sterile extracts was dripped onto sterile disc paper (6 mm in diameter) and allowed to dry. The positive control used was Ketoconazole concentration 30 µg and the negative control used was sterile fermentation medium. Then the disc paper is placed on a PDA medium containing Candida albicans. Subsequently petri dishes were incubated at room temperature. The clear zone diameter around the disc paper is measured after incubation for 2x24 hours.

Analysis of secondary metabolite compounds from endophytic fungi Sporothrix sp extract with UV-Vis and Infra Red Spectroscopy. Results of the largest inhibitory test were examined for secondary metabolites produced by Sporothrix sp. Endophytic fungus with Gas chromatography (QC-MS) method. Weigh the extract of fermented fungus Sporothrix sp as much as 100 mg, dissolve in 1 ml methanol, and inject as much as 1 µl into QC-MS.

Data analysis: Data were analyzed by A nova multi variate (α = 0,05). Anti-fungal activity is seen with the amount of inhibitory power that appears with fermentation extract concentration of 5% w/v fungus.

RESULT

Endophytic fungus Sporothrix sp of dahlia bulbs on tilted agar medium began to grow on the fifth day and shaped like white cotton. The next day the endophytic fungus shows a cloudy color on the fermentation medium. Sporothrix sp endophytic fungus implanted on production media variations of Huang et al (pepton and ammonium) on the fifth day indicates the discovery of white hyphae and fungi turbidity.

The results were obtained from 2x repetition with variation of Huang et al (pepton and ammonium), with observation for 2 days, the inhibitory effect was greater at 37°C, day 20 and on variation of Huang et al media with Na CMC and pepton.

The result of comparison of the influence of temperature variation and media variation in fermentation and can be seen with the amount of inhibitory power which can be seen in figure 1. On day 15 and 20 at 37°C with Huang et al variation media with Na CMC and peptone larger compared with the variation media Huang et al ammonium and Na CMC, which is 21 ± 1.41 mm and 23.5 ± 0.70 mm with ketoconazole as a positive control 6.3 ± 0 mm. In variation media Huang et al with Na CMC and Ammonium gave only 11 ± 0 mm and 13 ± 0 mm inhibition at 37°C with ketoconazole resistor of 6.4 ± 0 mm. In the picture below can be seen comparison of inhibitory power results of each variation of Huang et al media use

Table 2. Resistance Result of endophytic fungus *Sporothrix sp* to *Candida albicans* (Mean ± SD)

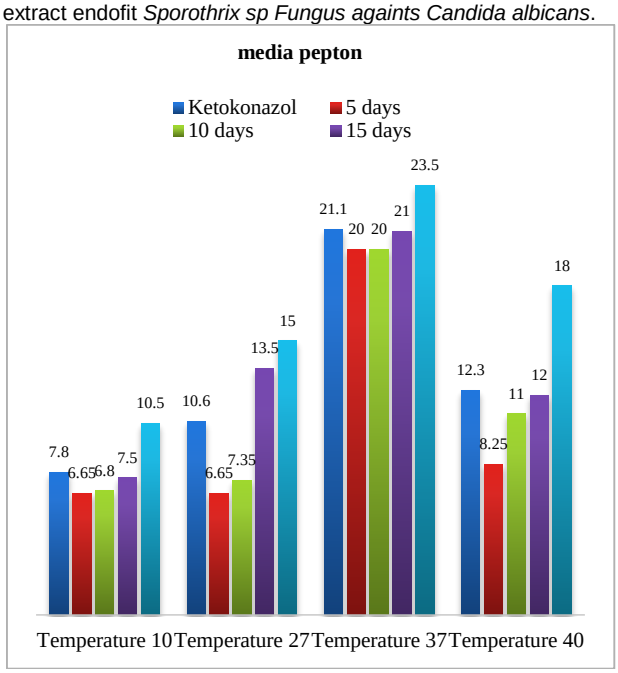
		Diameter Hampers (mm)			
Media	**temperature**	**Day 5**	**Day 10**	**Day 15**	**Day 20**
Peptone	10°C	6.65±0.07	6.8±0.28	7.5±0.70	10.5±2.12
	27°C	6.65±0.07	7.35±0.91	13.5±2.12	15±1.41
	37°C	20±0	20±0	21±1.41	23.5±0.70
	40°C	8.25±2.12	11±0	12±0	18±0
Ammonium	10°C	6.3±0	6.4±0	8.5±2.12	8±1.41
	27°C	6.3±0	6.3±0	6.6±0	6.5±0
	37°C	6.5±0	6.6±0	11±0	13±0
	40°C	6.4±0	6.55±0.07	6.6±0.14	6.55±0.07
Ketokonazol		6.2±0	6.2±0	6.3±0	6.4±0

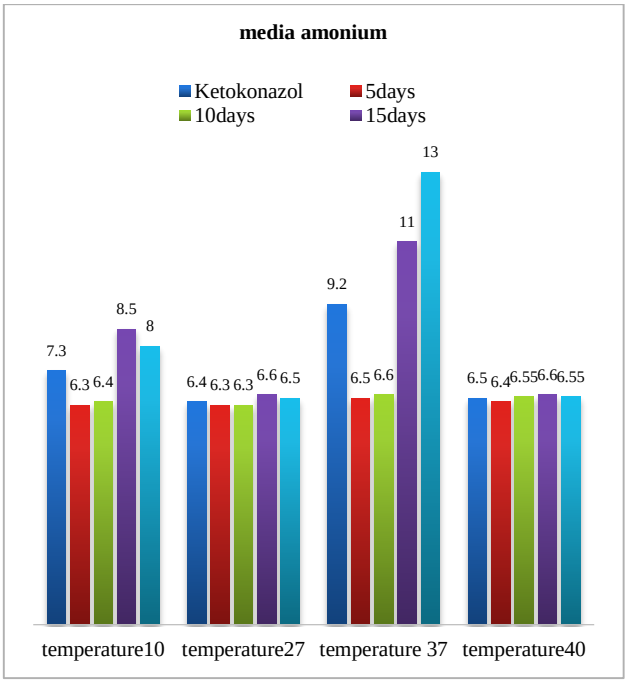
Information : 5 = fermentation H-5, 10 = fermentation H-10, 15 = fermentation H-15, 20 = fermentation H-20, (+) = control positif fungus (ketokonazole), (-) = control negatif fungus (media steril fermentation).

Table 2. Results of measurements of endophytic fungi within 5 days.

Results Measurement of Optical Density Temperature Variation Temperature *Sporothrix sp*					
MEDIA	**TEMPERATURE**	**DAY 5**	**DAY 10**	**DAY 15**	**DAY 20**
AMONIUM	10°C	1.394	1.79	1.472	1.427
	27°C	1.133	1.281	1.207	1.171
	37°C	1.243	0.733	1.293	1.271
	40°C	1.239	1.223	1.258	1.267
PEPTON	10°C	1.448	1.347	1.358	1.448
	27°C	1.653	1.27	1.668	1.349
	37°C	1.515	1.593	1.795	1.569
	40°C	1.384	1.4	1.399	1.395

Picture 1. Comparison of the inhibitory diameter of fermentation extract endofit *Sporothrix sp Fungus againts Candida albicans*.





DISCUSSION

Fermentation is one of the media productions of antimicrobial compounds from microorganisms. Endophytic fungus *Sporothrix sp* growth that can be seen in Table 2 below. At the end of the stationary phase, secondary metabolites (antimicrobials) are produced by microorganisms (Pelczar, *et al*.1986) .This is because secondary metabolites are usually synthesized at a fixed population-a number that grows equal to the number of dead. If in a fermentation medium some of the nutrients begin to be depleted then produced secondary metabolites. The limited nutrients cause the accumulation of secondary metabolite enzyme inducer and release of genes for secondary metabolite synthesis (Pratiwi, 2008).

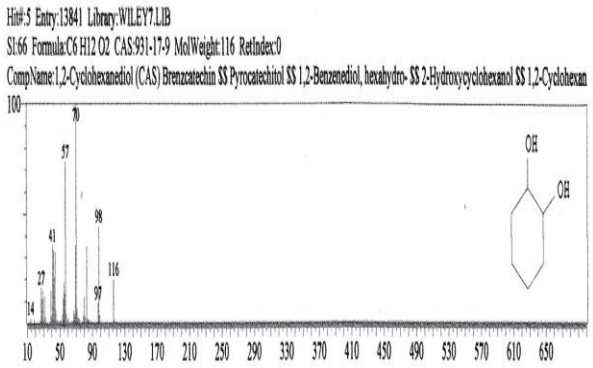
In fermentation of endophytic fungus, stirring with shaker at 150 rpm. Stirring (agitation) aims to increase the supply of oxygen in the medium and increase heat exchange so that the temperature distribution becomes homogeneous across the substrate. The medium used in the fermentation is the Huang et al variation media where the carbon source is replaced by Na CMC and its nitrogen source is replaced by peptone and ammonium, this is because the longer the carbon chain or the more complex the carbon source, the microbe will produce secondary metabolites. The complexity of a medium is a critical condition because the microbes will produce secondary metabolite compounds to sustain their life (Kumala et al, 2006).

The medium should contain nutrients for growth, energy sources, cell substance constituents and fermentation product biosynthesis. Carbon and nitrogen are important elements in fermentation due to microbial cells or fermentation products in the form of secondary metabolites composed of these components (Pratiwi, 2008). The presence of endophytic fungal extract activity is caused by Na CMC in Huang et al media variation which is a complex carbon source because the carbon must be broken down first into sucrose and sucrose will be first hydrolyzed into glucose and fructose, so it can be used as a source of nutrition by endophytic fungi.

The temperature of 20°C-37°C is the optimal temperature for the growth of endophytic fungi especially Sporothrix sp. If the temperature fermentation falls from the optimum temperature it will decrease the growing speed of the endophytic fungus, so if the temperature is extreme or too high from the optimal temperature will cause death in the endophytic fungus. In this study, the optimum temperature of endophytic fermentation of Sporothrix sp fungi was 37°C, with 23.5 ± 0.70 mm inhibitions, on day 20 with variation medium of Huang et al pepton and Na CMC.

On the 20th day, secondary metabolites are generated more visually than the value of inhibitory power in Candida albicans, this is supported by optical density examination on the 20th day the number of endophytic fungi that grow decreased from 1,795 to 1,569. The results of the analysis on the 20th day of endophytic fungi in the fermentation process occurred stationary phase.

According to Fitriyah.D, 2013, Endophytic fungus Sporothrix sp. produce secondary metabolite compounds by conducting qualitative and positive tests containing flavonoid compounds. Secondary metabolite results are examined using the QC-MS method or gas chromatography. The resulting secondary metabolites serve as an antidote to Candida albicans. The secondary metabolites found as anti-fungi in Candida albicans are 1.2 Cyclohexanediol Brenzcathechin Pyrocatechitol 1,2 Benzenediol. (QC-MS method). The chemical structure of the compound can be seen in Figure 2 below.



Data were analyzed by A nova multi variate (α = 0,05). Anti-fungal activity is seen with the amount of inhibitory power that appears with fermentation extract concentration of 5% w/v fungus. The result of statistically significant analysis on minimum inhibitory was shown at 37°C gave 23.5 ± 0.70 mm result on the 20th day of variation media Huang *et al* with Na CMC and pepton.

Picture 2. The in hibitory diameter of extract endophytic Sporothrix sp fungi againt Candida albicans on temperature 37°C with Huang et al media variation Na CMC and pepton

CONCLUSION

Secondary metabolite production of endophytic fungus Sporothrix sp can be done by optimizing fermentation environment factors such as temperature and growth media. Data were analyzed by A nova multi variate (α = 0,05). Anti-fungal activity is seen with the amount of inhibitory power that appears with fermentation extract concentration of 5% w/v fungus. The result of statistically significant analysis on minimum inhibitory is shown at 37°C giving 23.5 ± 0.70 mm results on the 20th day of variation media Huang et al with Na CMC and peptone. The resulting secondary metabolites function as antifungal Candida albicans. UV-Vis measurements showed absorption at 229nm wavelengths and 272 nm showed the presence of conjugated double bonds and the characteristics of terpenoids. The IR spectrum measurement results show the absorption band on the N-H amine group at a wavelength of 3300-3000 cm-1, N-H flexural vibration extends in the area of 1650-1580 cm-1, for the amine can also be seen in the area 910-665 cm-1. This can be used as an antifungal drug. Seeing the potential produced by endophytic fungus Sporothrix sp needs further research to optimize other fermentation environment factors.

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