Original Article

# Physical and Chemical Fermentation Conditions Affect the Growth and Metabolite Production of Endophytic Fungi *Athelia rolfsii*

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## Abstract

**Background:** The effort to explore antimicrobial agents isolated from an endophytic fungus of *Coleus amboinicus* resulted in the finding of a potential aromatic compound having methoxy, hydroxyl, and methyl groups produced by *Athelia rolfsii*. This compound exhibited antibacterial activities with $IC_{50}$ values of <2 µg/mL against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Streptococcus mutans, and Pseudomonas aeruginosa*. This study was aimed to determine the effect of varying fermentation conditions (types of media, carbon sources, nitrogen sources, temperature, and pH) on biomass, total metabolites, and bioactive compound production. **Materials and Methods:** The fungal cultures were subjected to various treatment conditions and incubated for 12 days at 25°C 160 rpm followed by analyzing the biomass, metabolite, and bioactive compound production. **Results and Conclusion:** The study found that although the maximum total metabolite production was achieved in the Tryptic Soy Broth medium, both biomass and bioactive compound production accumulated at the highest amount in Potato Dextrose Broth and Sabouraud Dextrose Broth media. Adding 1% of different types of carbon sources did not significantly enhance biomass, total metabolite, and bioactive compound production. Three types of organic nitrogen sources used in this study did not significantly affect biomass and total metabolite production, but adding peptone produced the highest amount of bioactive compound. Supplementing inorganic source of nitrogen to the culture medium decreased the production. While pH 5.5 was found to be the optimum condition for total metabolite production, pH 6–7 resulted in higher productivity of the bioactive compound. The total metabolite production was best produced at 25°C, whereas higher temperatures were needed to get optimum bioactive and biomass production. This study found that the total metabolite production was 7.8 times higher when the culture was grown in PDB medium supplemented with 1% sucrose and 1% peptone and incubated at 27°C at pH 6.5; whereas a 15% increase was observed in the bioactive compound production.

**Keywords:** *Antimicrobial compound, Athelia rolfsii, biomass, endophyte, fermentation, total metabolite*

## Key Message

This study demonstrated the attempt for optimizing the production of a potential antimicrobial compound produced by an endophytic fungus *Athelia rolfsii*. The results showed that specific conditions must be defined to obtain optimum growth and metabolite production.

## Introduction

Endophytic microbes being able to produce secondary metabolites which are the same as the original plant or even better than the host plant are potential to be developed as a source of secondary metabolites capable of treating various types of diseases.[1,2] Despites their potency as sources of bioactive compounds, the metabolites produced by the endophytic fungi are strongly influenced by nutritional and environmental factors.[3] Many metabolites were degraded after the fungi were cultured alone or after a certain period of times. This may be caused by the differences in the environmental conditions after they are separated from their host. Strategies need to be done to maintain their production. Microbial secondary metabolites synthesis is influenced greatly by the type and concentration of the nutrients formulating the culture media. Among these nutrients, the effect of carbon has been widely studied and gave a great impact in research and industrial application.[4] The choices of carbon and nitrogen sources were found to significantly affect the production of cellulase enzyme by *A. terreus* and *Mucor plumbeus*.[5] pH and temperature effects were found to be the most influential factors for biomass and biopolymer concentrations, and then the optimized selection of nitrogen and carbon sources used during the fermentation of *Chryseobacterium indologenes* MUT.2 was also found to increase extracellular polymeric substances production, polymeric materials that



**How to cite this article:** Suharsanti R, Wahyuono S, Astuti P. Physical and chemical fermentation conditions affect the growth and metabolite production of endophytic fungi *Athelia rolfsii*. J Rep Pharma Sci 2022;11:85-91.

**Received:** 29 Oct 2020
**Accepted:** 01 Feb 2022
**Published:** 29 Jun 2022

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**Access this article online**

**Website:** www.jrpsjournal.com

**DOI:**10.4103/jrptps.JRPTPS_136_20

**Quick Response Code:**

are widely used in food, textile, cosmetics, and pharmaceutical industries.[6,7] Supplementing elicitors were also reported to be able to maintain or increase fungal production of bioactive components. One example was addition of methyl jasmonate which was found to increase camptothecin yield when it was added in the culture medium of endophytic fungus Trichoderma atroviride LY357.[8] Similarly, water extract polysaccharides were found to be the most effective elicitor to enhance diepoxin zeta production in the liquid culture of endophytic fungus *Berkleasmium* sp. dzf12.[9]

Several physical factors also affect the growth and metabolite produced by the endophytic microbes.[10] Varying pH and temperature values had been shown to affect biomass and enzyme production in the culture of *A. fumigatus* LB-01-AP, a polygalacturonases producer.[11] The production of an antimicrobial agent by a soil mold *A. terreus* was reportedly influenced by pH and temperature of the fermentation culture.[12] Optimized fermentation nutrients and environments were also found to improve antimicrobial production of an endophytic fungus *Fusarium* sp. DF2 isolated from *Taxus wallichiana*.[13]

*Athelia rolfsii* is a soil-borne plant pathogenic fungus causing sclerotial blight diseases in many farmed and garden plants. This fungus was also used in biotechnology as a microbial tool to produce Scleroglucan (Sclg). The purified scleroglucan was reported to have considerable antibacterial and antioxidant activities with no cytotoxic effect on normal cell (W138) and tumor cell lines (HepG2 and PC3). This study also found that Sclg reduced the cytopathic effect of herpes simplex virus type-1 by 50% ($EC_{50}$) at 15 µg/mL and influenza virus (H5N1) at 50 µg/mL.[14] The previous study reported that an endophytic fungus *Athelia rolfsii* was isolated from *Coleus amboinicus* Lour. leaves, a medicinal plant used in Indonesian traditional medicine called Jamu. Semi-elucidation structure of the bioactive compound identified an aromatic compound having methoxy, hydroxyl, and methyl groups which showed potential antimicrobial activities against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Streptococcus mutans* with $IC_{50}$ values of <2 µg/mL.[15] This compound also showed cytotoxic activities against T47D breast cancer cell line ($IC_{50}$ of 174 µg/mL). However, the low yield of this compound limits its further development as antimicrobial and cytotoxic agents. The optimization process is highly important in every microbe to be able to produce metabolites on a large scale. This study will determine the effect of medium, sources of carbon, nitrogen, pH, and incubation temperature to the production of biomass, total metabolites, and bioactive compound production within fermentation medium of an endophytic fungus *Athelia rolfsii* isolated from *C. amboinicus* leaves. Considering the potency of the bioactive compound, this study is of importance to show the optimum conditions for its production by *Athelia rolfsii*, which was not reported previously.

## Materials and Methods

### Isolation and cultivation of endophytic fungi

The endophytic fungus was isolated from the leaves of *C. amboinicus* and maintained in Potato Dextrose Agar (PDA) medium for routine culture [Figure 1]. The fungus was identified as *Athelia rolfsii* based on the Internal Transcribed Spacer (ITS) fungal ribosomal DNA partial genetic analysis, as described previously.[15] To study the effect of cultural conditions (sources of carbon, nitrogen, and pH) on the growth, total metabolite, and antimicrobial agent production, the fungus was cultured in Potato Dextrose Broth (PDB) at 25°C and agitated at 160 rpm under batch fermentation as basal condition. The parameters were changed at one at a time according to the experimental design. Similarly, incubation temperature was manipulated as described in the flow chart [Figure 2].

### Biomass and total metabolite production

Upon each parameter change, mycelium was harvested by filtration using the Whatman filter paper that had been weighed previously. The residue on filter paper was dried at 80°C to a constant weight. The residue weight was considered as biomass in mg/100 mL of medium.[10]

The supernatant was partitioned with 25 mL of ethyl acetate three times. The ethyl acetate extract was collected and allowed to evaporate in an acid cabinet to dry. The ethyl acetate extract was weighed and considered as total metabolite in mg/100 mL of medium.[16]

### Selection of fermentation media

Different types of microbiological media were used in this study, namely, PDB, Czapek's Dox Broth, Sabouraud Dextrose Broth (SDB), Tryptic Soy Broth (TSB), and Nutrient Broth, which were purchased from Oxoid, UK. The fungal culture was inoculated in a 250 mL flask containing 100 mL medium, incubated at 25°C at 160 rpm for 12 days. The antimicrobial concentration was determined at the end of incubation. The best medium capable of producing highest amount of antimicrobial agent was used as basal medium for another parameter optimization. The experiment was conducted in triplicate.[3,10]

### Effect of supplementary carbon and nitrogen sources

Various sources of carbon were used to study the growth, total metabolite, and antimicrobial agent production. One percent of glucose, fructose, sucrose, maltose, and starch (Merck, Darmstadt, Germany) was added separately into 250 mL flask containing 100 mL PDB medium.[3,10] Similarly, different types

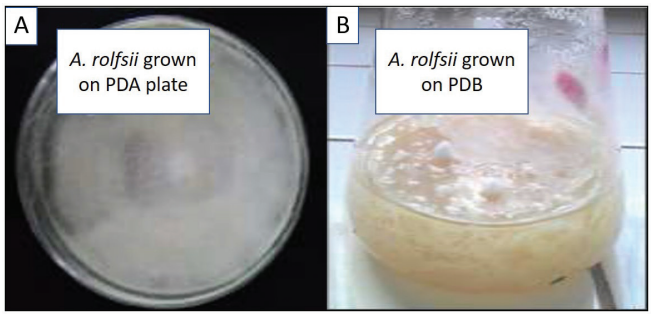


**Figure 1: The growth of *Athelia rolfsii*. Growth on PDA at day 7 (A) and PDB at day 12 at 25°C, five plugs of (A) on PDB agitated at 160 rpm without adding any variations/basal medium (B)**

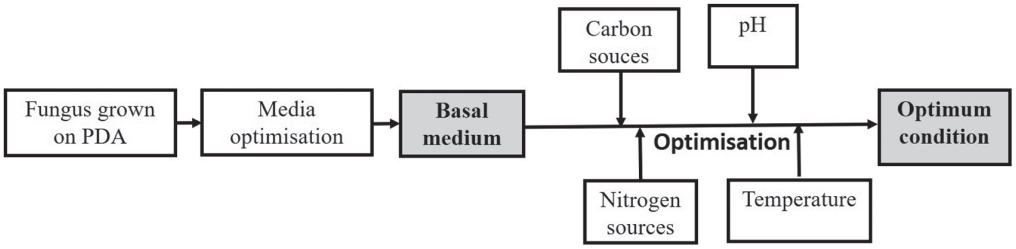


**Figure 2: Flowchart for optimizing condition for *Athelia rolfsii* growth, metabolite, and bioactive compound production**

of organic nitrogen sources (beef extract, yeast extract—Oxoid, UK) and inorganic sources of nitrogen ($NH_4Cl$, $NaNO_3$—Merck, Darmstadt, Germany) were added separately to the PDB medium. Five plugs of fungal strain were added to each flask, and the cultures were incubated for 12 days at 25°C at 160 rpm. Biomass, total metabolite, and antimicrobial agent production were determined at the end of fermentation. The experiment was conducted in triplicate.[10,17]

### Effect of pH and temperature

To determine the most suitable pH for biomass, total metabolite, and antimicrobial agent production, an experiment was conducted by adjusting the pH of the PDB medium at different levels in the range of 5–7.[10] Following addition of five plugs of fungal strain, the culture flasks were incubated for 12 days at 25°C at 160 rpm. The effective temperature for biomass, total metabolite, and antimicrobial agent production was also determined by varying 1°C interval ranging from 25°C to 30°C. Culture flasks containing five plugs of fungal strain in the PDB medium were incubated for 12 days at a predetermined temperature at 160 rpm, and biomass, total metabolite as well as antimicrobial agent production were determined at the end of incubation. The experiment was conducted in triplicate.[18,19]

### Determination of antimicrobial agent concentration

The concentration of antimicrobial compound within ethyl acetate extract was determined by thin layer chromatography (TLC) densitometry. Twenty milligrams of ethyl acetate extract were dissolved in 1 mL mixture of methanol: chloroform (1: 1 v/v). Ten microliters of extract solution was sprayed onto TLC plate using Linomat 5 (CAMAG) ($n$ = 3). The isolated antimicrobial agent was used as standard control. This standard control was obtained from the previous study which showed white crystalline powder (melting point of 175.21–175.90ºC) and exhibited 100% level of purity as determined by liquid chromatography analysis with retention time of 2.2 min.[15] Following the development of the TLC plate (stationary phase = silica gel F254; mobile phase = chloroform: methanol: glacial acetic acid (2:1:0.15 v/v) (Merck, Darmstadt, Germany), the plate was scanned at the maximum wavelength using TLC Scanner 3 (CAMAG).[16] The concentration of the antimicrobial agent within the samples was calculated based on the equation made using the standard curve of isolated antimicrobial agent, and the concentration was designated as % of antimicrobial agent within ethyl acetate extract.

### Statistical analyses

The data obtained from each fermentation variation were analyzed statistically with different tests. The variations include media type, carbon source, nitrogen source, salinity variation, pH, and incubation temperature. Data with normality and homogeneity tests were followed by parametric analysis of variance and Tukey's *post hoc* or non-parametric analysis of Kruskal–Wallis and Mann–Whitney. Tukey's *post hoc* test was carried out with a 95% confidence level (significance <0.05). Data were statistically analyzed for each group that came from three replicates and had omitted contaminated results.[20,21]

## Results

### Selection of basal medium

Although PDA was used for routine culture maintenance, further characterization of media suitable to use as basal medium needs to be explored. The study was conducted to compare different types of medium used to obtain optimum production of the bioactive antimicrobial compound. Five different media were used to ferment endophytic fungus *Athelia rolfsii* under same conditions. Among five cultural media, PDB and SDB were found to be the best media for antimicrobial production with 43.0% and 36.8% productivity, respectively [Figure 3]. Similarly, PDB and SDB were found to be the best media for biomass production, achieving 544.4 and 625.1 mg/100 mL, respectively. It is interesting to note that the total metabolite production reached 28.8 mg/100 mL when the fungal strain was cultured in TSB medium, and this was the highest compared with other types of media. In this study, PDB was chosen as basal medium and used for optimizing other growth parameters.

### Effect of carbon sources

The fungal strain was grown on PDB medium supplemented by 1% glucose, sucrose, fructose, maltose, and starch, respectively. This study showed that both biomass and total metabolite production did not significantly vary among the treatments [Figure 4]. Similarly, the bioactive compound production did not significantly differ, although sucrose and starch were found to be able to induce higher production attaining 31.5% and 28.8%, respectively.

### Effect of nitrogen sources

To investigate the effect of nitrogen sources on the biomass, total metabolite, and bioactive compound production, three

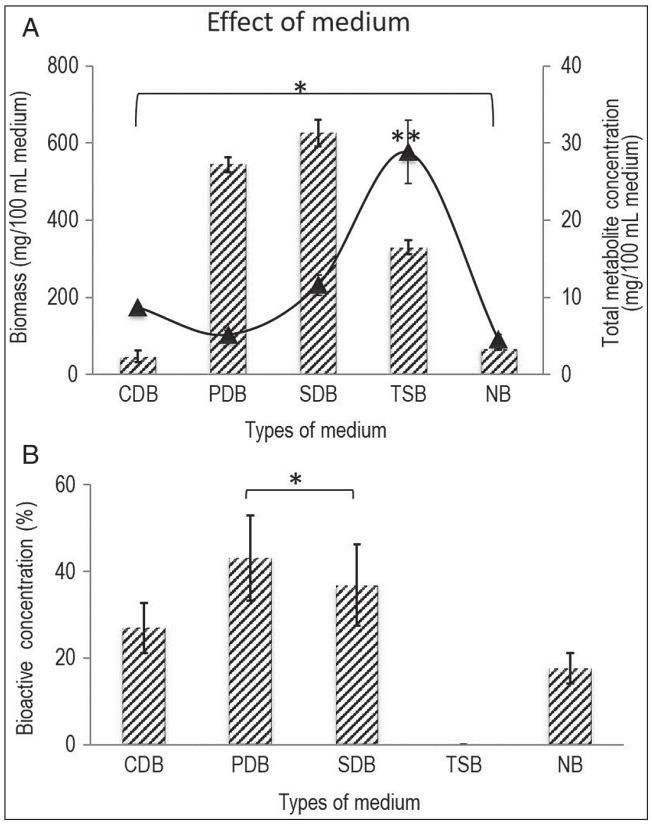


**Figure 3: Optimization of types of medium for *Athelia rolfsii*. Optimization for biomass and total metabolite production (A). *There was no significant difference. **There were significant differences. Optimization for bioactive compound production (B). *There was no significant difference**

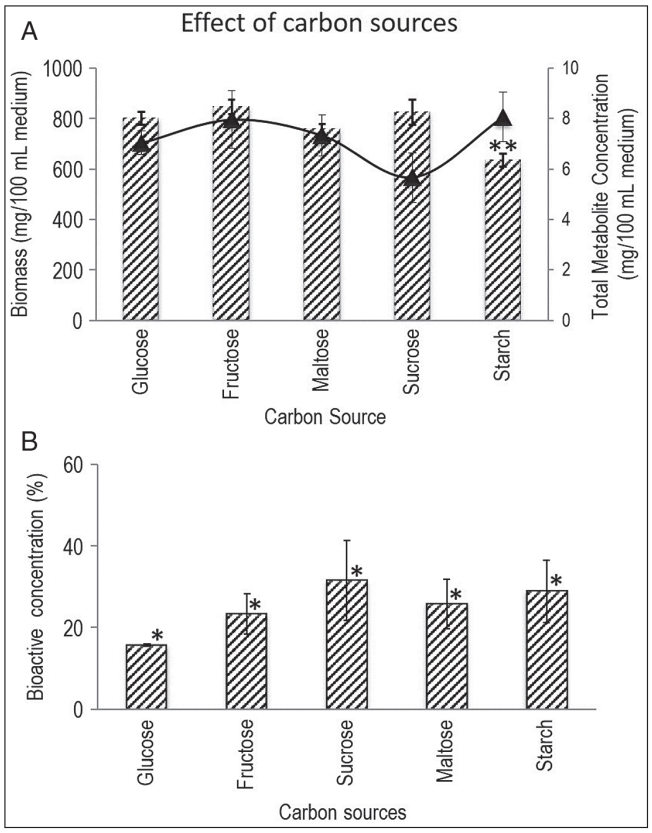


**Figure 4: Effect of different carbon sources. Effect on biomass and metabolite production (A). **There were significant differences in all groups for biomass. Effect on bioactive compound production (B). *There was no difference between groups for bioactive concentration**

types of organic nitrogen sources, namely, beef extract, yeast extract, and peptone, and two types of inorganic nitrogen sources ($NH_4Cl$ and $NaNO_3$) were examined. Adding three different types of organic nitrogen sources did not give any significant differences in fungal growth, but slightly higher total metabolite production was observed upon supplementation of the beef extract (16.5 mg/100 mL medium) [Figure 5]. In this study, $NH_4Cl$ and $NaNO_3$ suppressed the growth and total metabolite production of *Athelia rolfsii*. The antimicrobial agent, however, was best produced when peptone was added to the fermentation culture reaching 12.8%.

### Effect of pH and incubation temperature

Biomass production upon growing *Athelia rolfsii* at pH ranging from 5 to 7 did not vary between treatments [Figure 6]. However, the total metabolite production was the highest when the fungus was grown at pH 5.5 (9.2 mg/100 mL medium). On the contrary, at this pH, the antimicrobial productivity was found to be minimum. Better production of the antimicrobial agent was observed when the culture was fermented at pH above 6, reaching 46.8% at pH 6.5. Maximum growth of *Athelia rolfsii* was recorded at incubation temperature 30°C (519.8 mg/100 mL) [Figure 7]. Less growth was observed at 27°C when this temperature was found to be optimal for antimicrobial agent production (53.2%). The total metabolite production, however, was best when the fungus was grown at 25°C (19.0 mg/100 mL). Attempts to combine optimum conditions for antimicrobial agent production were made by growing *Athelia rolfsii* in the PDB medium supplemented with 1% sucrose and peptone and incubated at pH 6.5, 27°C, 160 rpm for 12 days. Antimicrobial agent concentration at the optimum conditions increased by 15% when compared with that in the PDB medium. It is interesting to note that 7.8 times increase of the total metabolite was observed [Figure 8]. The TLC profile of ethyl acetate extract under UV detection was altered upon medium optimization.

## Discussion

Microbial metabolite biosynthesis is tightly regulated by mechanisms which control its production so that sometimes it is present at undesirably low level. In the case of metabolites possessing bioactivity importance for therapy or industrial application, strategies need to be done to increase the yield. Designing an appropriate medium and determining the conditions for cultivation are of prime importance for improving the yield.[22] Physical or chemical factors reportedly influenced the microbial growth and production of bioactive metabolites.[6,16,17,23] The results presented in this study agreed with previous reports, showing that the type of medium

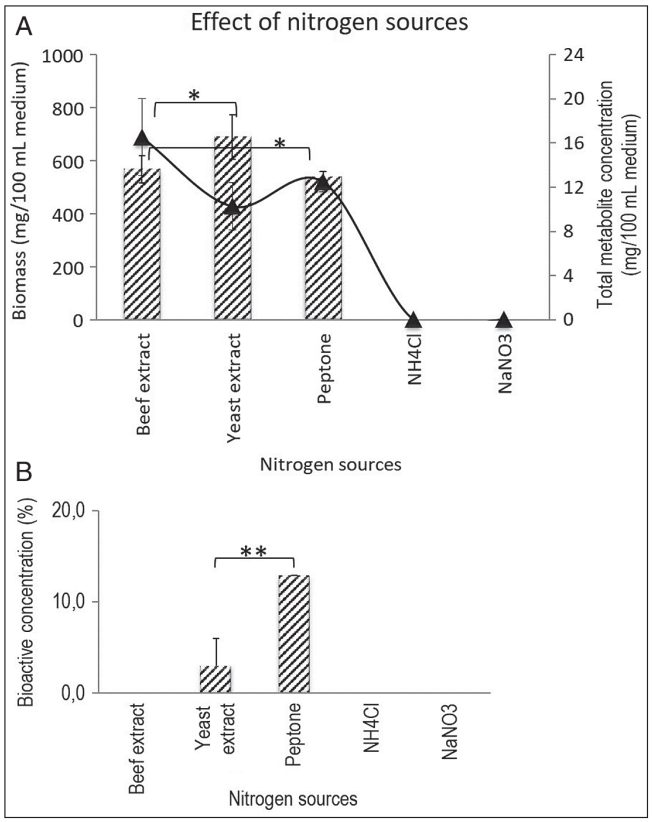


**Figure 5: Effect of different nitrogen sources. Effect on biomass and metabolite production (A); effect on bioactive metabolite production (B). *There were no significant differences and **there were significant differences**

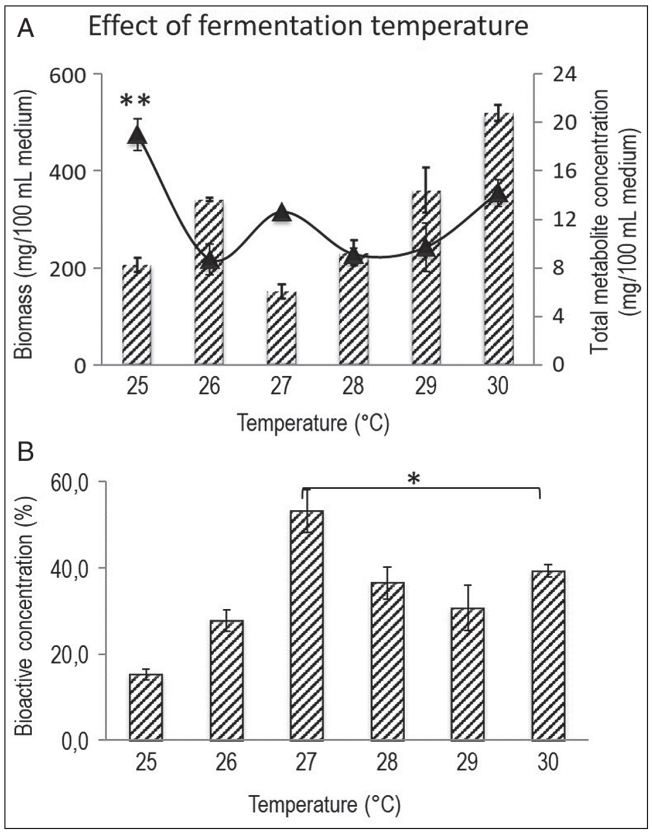


**Figure 7: Effect of fermentation temperature. Effect on biomass and metabolite production by *Athelia rolfsii* (a). **There were significant differences in all groups on total metabolite. Effect on bioactive compound production (B). *There were no significant differences between groups**

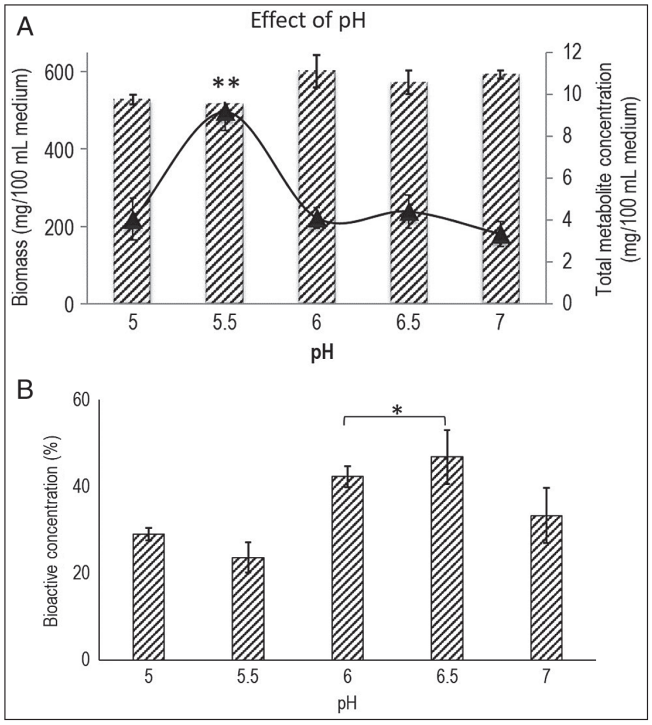


**Figure 6: Effect of different pH values. Effect on biomass and metabolite production by *Athelia rolfsii* (a). **There were significant differences in all groups on total metabolite. Effect on bioactive compound production (B). *There were no significant differences between groups**

determined biomass and bioactive metabolites production.[18,19,24] Oxalic acid but not mycelial growth was produced at the highest yield when *Sclerotium rolfsii* SC64, another strain of *Athelia rolfsii*, was grown in PDB,[30] similar to our research finding that the antimicrobial compound was best produced in PDB or SDB. The presence of dextrose as source of carbon in both media may be beneficial for mycelial growth and bioactive metabolite production as the fungal strain was maintained in PDA prior to culture in broth media, suggesting less transition adaptation.

In this study, both biomass and total metabolite production were not influenced by the type of carbon sources. A slight increase in antimicrobial agent production was observed upon addition of sucrose and starch, although it was not significantly different. This is similar to the study reported found that supplementation of wheat bran with various carbon sources (1% w/w) did not show any significant increase in the production of lovastatin.[25] Sucrose has been reported as preferable source of carbon for scleroglucan biosynthesis by *Sclerotium rolfsii*, branched exopolysaccharide importance for pharmaceutical formulations and therapeutic values.[16,26-28] Nitrogen, like carbon, is an essential element for fungal functional as well as structural purposes. In this study, it was found that sources of nitrogen are not equally good for mycelial growth. Obvious effect was seen upon addition of inorganic nitrogen sources, which exhibited inhibitory effect,

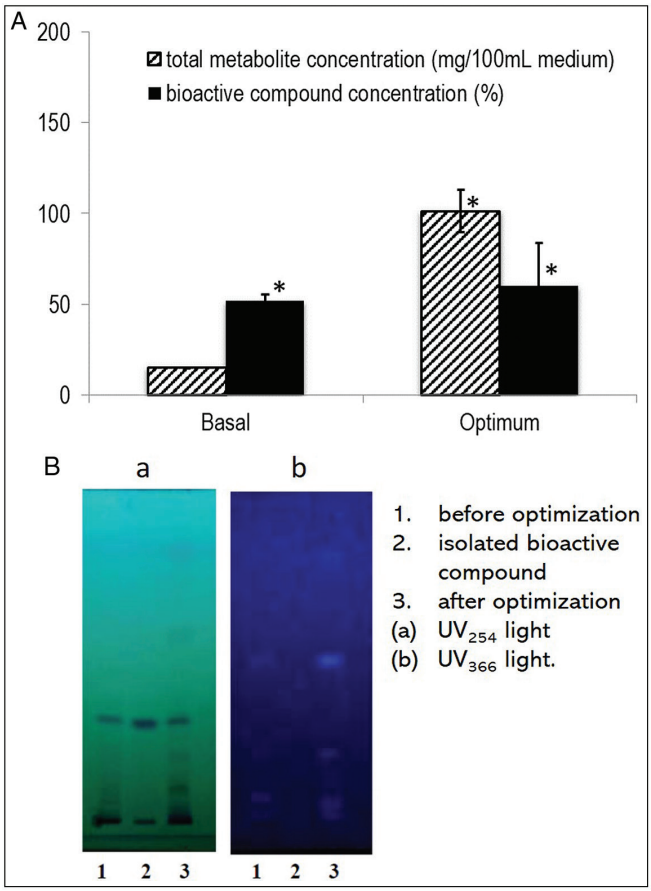


**Figure 8: Total metabolite and bioactive compound production (A) and TLC profiles of the ethyl acetate extract (B). Stationary phase mobile phase = silica gel F254; stationary phase = chloroform:ethyl acetate:glacial acetic acid (2:1:0.15 v/v)**

similar to the production of lovastatin from *Meyerozyma guilliermondii*.[25] The fungal strain favors organic nitrogen sources for mycelial growth and total metabolite production. Best antimicrobial agent production, however, was seen upon peptone supplementation, indicating that its production was subjected to metabolic regulation by this source of nutrients. Other study revealed that *S. rolfsii* mycelial growth varied among different treatments of nitrogen sources and peptone recorded to reach maximum growth.[29] This study indicated that optimizing composition of media may affect certain metabolite production.

The optimal range of initial pH values for oxalic acid accumulation is 4.0–6.0, with an optimum pH of 5.0 (9.83 mM). Optimal range of initial pH values for mycelium growth isolate of *Sclerotium rolfsii* SC64 was 3.5–6.0, with an optimum pH of 5.0 (0.1240 g).[30] pH of culture media is one factor important for metabolism and determining biosynthesis of secondary metabolites. Fungal pH regulatory system controls metabolites, enzymes, and membrane protein production, so that they are in the pH environments which ensure their physiological function. Fungal adaptation to pH variations is also critical for their survival and pathogenicity.[31] The present study revealed that maximum growth was found at 30°C whereas lower temperature was needed for total metabolite (25°C) and antimicrobial agent production (27°C). The finding is similar to that reported by Tang *et al.*[30] who found the optimum temperatures of *Sclerotium rolfsii* mycelial dry weight and virulence were at 30°C and 27°C, respectively, for its bioactive metabolite oxalic acid and echinacoside yield.[32] Combining all the parameters, optimum conditions for biomass production could be achieved when the fungus was grown in PDB medium supplemented with fructose and yeast extract and incubated at pH 6 at a temperature of 30ºC. The total metabolite production was optimum when the fungus was fermented in the PDB medium with the addition of starch and beef extract, at pH 5.5 and at a temperature of 25ºC. The fungus showed best bioactive compound production when it was fermented in PDB medium containing sucrose and peptone and incubated at pH 6.5 at a temperature of 27ºC.

This study demonstrated that components within culture media affected the growth and secondary metabolite production in fungi. The differences in total metabolite and antimicrobial agent production under specific conditions indicated that some nutrients may be inducing or inhibiting secondary metabolite. Some conditions may affect the biosynthetic pathway of certain metabolites but not others. Therefore, combining all parameters at a time was not necessary to induce the antimicrobial agent. In the present study, TLC profiles of metabolites within ethyl acetate under UV detection exhibited differences before and after optimization. Similar finding was reported in which variations in *Streptomyces sparsus* VSM-30 secondary metabolite profiles were observed under different culture conditions.[33]

## Conclusion

The investigation presented in this study showed that the antimicrobial agent produced by *Athelia rolfsii* was greatly affected by cultural conditions. While it was optimal for bioactive compound production, the conditions were not the same for total metabolite or fungal growth. Choosing suitable combination of parameters are of importance to achieve the optimum results. Growing fungi in PDB medium supplemented with 1% sucrose and 1% peptone and incubating at 27°C at pH 6.5 resulted in 7.8 times higher in total metabolite production, and these conditions increased bioactive concentration up to 15%. Further study is needed to determine other factors such as elicitors to optimize bioactive compound production.

### Acknowledgments

The authors thank the Indonesian Ministry of Research and Technology/National Innovation Agency for providing the funding under UGM PDUPT project.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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