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Pharmaceutical Microbiology and Biotechnology Cultural Conditions Affect the Growth of Endophytic Fungi *Aspergillus fumigatus* and Improve Its Total and Bioactive Metabolite Production.

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ABSTRACT

Exploration of bioactive compounds from endophytic fungi isolated from *Piper crocatum* Ruiz & Pav resulted in the finding of pyrophen as one of bioactive compounds against T47D cell. This study was aimed to optimize cultural conditions for the growth of endophytic fungi *Aspergillus fumigatus* strain KARVS04 and improve its metabolite production in batch culture system. The fungi produced optimum bioactive compound in Potato Dextrose Broth at 29º C, pH 5 supplemented with starch or fructose as carbon sources and sodium nitrate as nitrogen source, following 10 days of fermentation and increased bioactive compound productivity from 1.3% to 14.7%. The optimal total metabolite production, however, was obtained in Czapex Dextrose Broth. The strain produced higher total metabolite when it was grown at pH ranging 6 – 7. Optimum production was achieved in basal media supplemented with glucose as carbon sources and beef or peptone as nitrogen sources. However, no significant differences in productivity were observed by varying temperature of incubation. The optimal mycelial growth was achieved in Sabourod Dextrose Broth, with the maximum growth at 29^{o} C supplemented with glucose and yeast as carbon and nitrogen sources, respectively. Varying pH of basal medium showed no significant differences in biomass production.

Keywords: Endophyte, *Aspergillus fumigatus,* bioactive metabolite, fermentation

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INTRODUCTION

Endophytic fungi are considered valuable sources of metabolites' production having pharmaceutical importance. Many of commercially potential and bioactive metabolites including those against various cancer cell lines are produced by endophytic fungi [1-7]. Biosynthesis of secondary metabolites by microorganisms is greatly influenced by environmental factors. Nutrient deficiency or additions of inducer or reduced growth rate often generate signals which result in regulatory events controlling secondary metabolism [8,9]. Effective screening processes such as by manipulating physical and chemical factors which influence the growth of microorganisms and metabolite production may lead to better exploitation of commercially important microorganisms. Cultural conditions such as media components, temperature, salinity, pH, incubation period, agitation rate and inoculum size have been reported to influence metabolite biosynthesis [10-12].

Filamentous fungi Aspergilus have been reported to be the second highest endophytic fungi producing bioactive compounds against cancer such as Brefeldin A [13,14], monomeric naphtho-g-pyrones, TMC 256 A1 [15], nigerasterols A and B [16], 2,14-dihydrox-7-drimen-12,11-olide [17], gliotoxin [18] and l-arginine deiminase [19]. Previous study, we reported the finding of pyrophen, an amino acid-pyrone derivative isolated from endophytic fungi *Aspergillus* sp, which has ability to modulate cell cycle progression by inducing S-phase arrest [20]. However, the productivity of this compound is still low (1.3 mg in 100 ml fermentation media), limiting its potential exploration for anticancer agent. This study was aimed to increase the productivity of this compound by varying fermentation conditions including media, carbon source, nitrogen source, temperature, and pH.

MATERIAL AND METHODS

Materials

Potato Dextrose Agar (PDA), Dextrose, Czapek's Dox Broth (CDB), Sabourod's Broth (SB), Tryptic Soy Broth (TSB), Nutrient Broth (NB) were purchased from Oxoid. Glucose, starch, sucrose, fructose, maltose, beef extract, yeast extract, peptone, ammonium chloride, sodium nitrat (Merck). TLC Silica gel 60 F_{254}, n-hexane, ethyl acetate (Merck). Endophytic fungi *Aspergillus* sp isolated from *Piper crocatum* Ruiz & Pav (Culture collection of Pharmaceutical Biology Department, Faculty of Pharmacy, UGM). The endophytic fungi was identified as *Aspergillus fumigatus* Strain KARVS04 based on morphology of the culture and analysis of rDNA homology with DDBJ/DNA data Bank of Japan or NCBI.

Fermentation of culture in basal medium

The fungal were grown in potato dextrose agar for 7 days at room temperature. A hundred ml of potato dextrose broth in 250 ml conical flask were inoculated with 5 plug of mycelial in 5 mm diameter and incubated at RT for 14 days. The mycelial were harvested every two days, filtered using Whatman filter, dried at the oven at 50º C until a constant weight and the growth was expressed as mg from 100 ml of media. The filtrates were extracted using ethyl acetate thrice, evaporated using rotary evaporator under reduced pressure to yield ethyl acetate extract.

Selection of culture media

In order to obtain the optimum growth and production of metabolite, the culture were growth in different microbiological media namely Czapek's Dox Broth (CDB), Sabourod's Broth (SB), Tryptic Soy Broth (TSB), Nutrient Broth (NB) and Potato Dextrose Broth (PDB). The biomass accumulation and metabolite production in each medium were determined after 10 days of incubation.

Effect of carbon and nitrogen sources

The effect of carbon and nitrogen sources on mycelial growth and metabolite production was carried out by adding 1% of each carbon and nitrogen resources to basal medium separately. Various carbon sources such as glucose, starch, sucrose, fructose, maltose and nitrogen sources such as beef extract, yeast extract, peptone, ammonium chloride, and sodium nitrat were used in this study. Each flask containing 100 ml of PBD

was inoculated with 5 plugs of 5 mm in diameter of fungi grown previously in PDA for seven days. Following 10 days of incubation in RT, the biomass, total and bioactive metabolite production were determined. The experiment was conducted in three replicates for each carbon and nitrogen sources.

Effect of pH

The optimization of pH condition for *A. fumigatus* strain KARVS04 fermentation was carried out by adjusted pH of basal medium at range of 5 – 7. The strain of *A. fumigatus* (5 plugs of 5 mm in diameter) was inoculated into 100 ml of PDB media and the culture was incubated for 10 days at RT. Dry mycelial weight and metabolite production was determined at the end of fermentation. Three replicates were used for each pH value.

Effect of incubation temperature

The fungal strain (5 plugs of 5 mm in diameter) was inoculated into 100 ml of basal medium at various temperatures ranging from 25 – 30° C at a difference of 1° C. The culture was incubated for 10 days, prior to determination of mycelial dry weight and metabolite production. Three replicates were used for each temperature.

Bioactive metabolite determination

Bioactive concentration in each treatment was determined using TLC densitometer. Following TLC using n-hexane:ethyl acetate (1:9) as mobile phase and Silica gel 60 F_{254} as stationary phase, the separated bioactive compound was subjected to scanning using TLC-3 scanner (Camag) at optimum wave length using isolated bioactive compound as standard.

Data analysis

The statistical data analysis was conducted using SPSS by comparing different treatments using one way ANOVA followed by t-test using threshold for significance 5%.

RESULTS AND DISCUSSION

Results

Fermentation of fungi and bioactive metabolite profiles

Results in this study revealed that the endophytic fungi *A. fumigatus* strain KARVS04 reached maximum growth at 12 days of incubation (Figure 1). However, total metabolite production tended to decrease and level up following 10 days of incubation. Therefore, the experiments were carried out for 10 days of incubation.

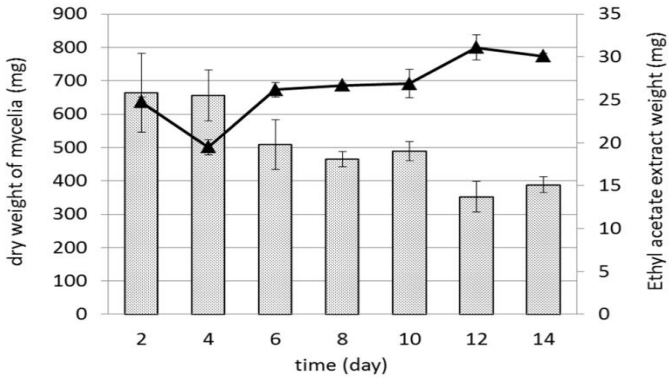

Figure 1. Mycelial growth and total metabolite production of *A. fumigatus* strain KARVS04.
The data plotted are means ± SE of three replicates. ▒ = ethyl acetate extract, ▲= dry weight of mycelia.

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Optimization of culture media

Attempts to study the cultural media optimum for the mycelial growth and metabolite production were conducted by growing the culture at various types of medium. The fungi grew better in Sabourod's Broth media (848 ± 65.4 mg), however the total metabolite production was higher in Czapek's Dox Broth media (56.2 ± 3.4 mg) (Figure 2). Interestingly, although total metabolite production in Potato Dextrose Broth media was lesser than other medias, the bioactive content analysis within the extract revealed the highest production (5.2 ± 1.8%). Hence, optimization of different cultural and environmental parameters was conducted using PDB media.

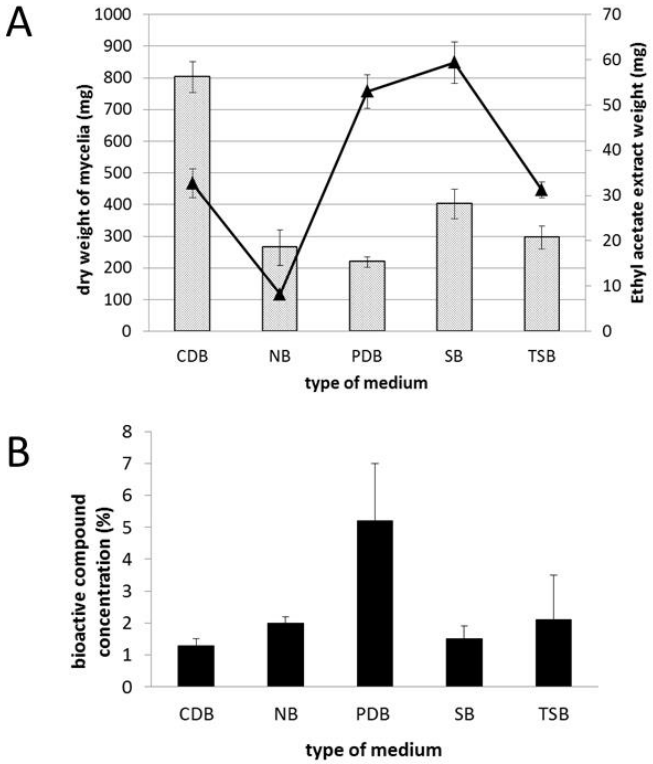

Figure 2. The growth and total metabolite production extracted with ethyl acetate (A) and bioactive compound (B) produced by endophytic fungi *A. fumigatus* strain KARVS04 cultured at different type of media. Czapek's Dox Broth (CDB), Nutrient Broth (NB), Potato Dextrose Broth (PDB), Sabourod's Broth (SB) and Tryptic Soy Broth (TSB). The data plotted are means ± SE of three replicates. ▒ = ethyl acetate extract, ▲= dry weight of mycelia, ▉ = bioactive compound.

Effect of carbon and nitrogen resources

The fungi grew in all carbon sources used in this study, but reached maximum growth (825.0 ± 10.9 mg) with glucose followed by fructose (818 ± 54.5 mg) as supplements. Highest production of total metabolite was achieved under glucose supplementation reaching 37.6 ± 6.1 mg (Figure 3). However, bioactive metabolite was produced in higher concentration when the strain was grown in PDB media supplemented with starch (14.6 ± 1.8 %) followed by fructose (13.1 ± 2.9%). Various types of nitrogen sources affected the growth, total and bioactive metabolite production. Maximum growth of the strain was achieved in PDB media supplemented with yeast (1026.9 ± 18.5 mg) (Figure 4). Whilst the strain produced higher total metabolite when it was incubated in PDB media supplemented with peptone (37.2 ± 9.7 mg) or beef (36.4 ± 12.3 mg), highest bioactive metabolite concentration was achieved in media supplemented with sodium nitrate (14.7 ± 1.4%).

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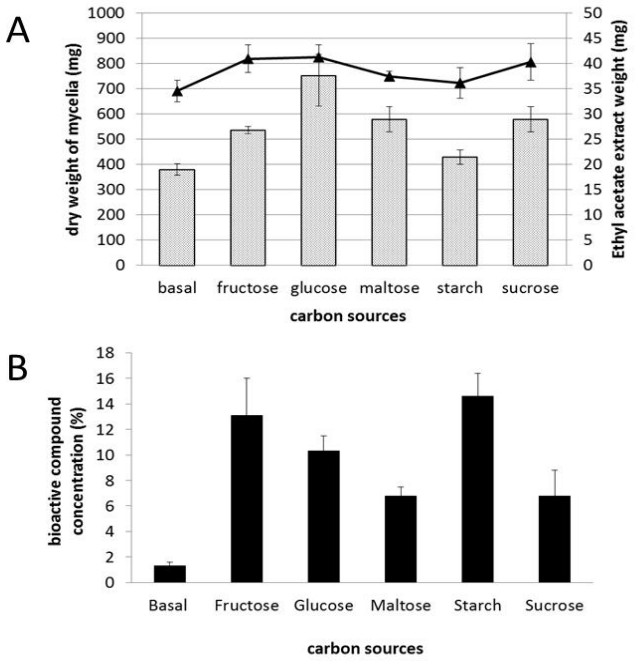

Figure 3. The growth and total metabolite production extracted with ethyl acetate (A) and bioactive compound (B) produced by endophytic fungi *A. fumigatus* strain KARVS04 cultured at different type of carbon sources. The data plotted are means ± SE of three replicates. ▒ = ethyl acetate extract, ▲= dry weight of mycelia, ▉ = bioactive compound.

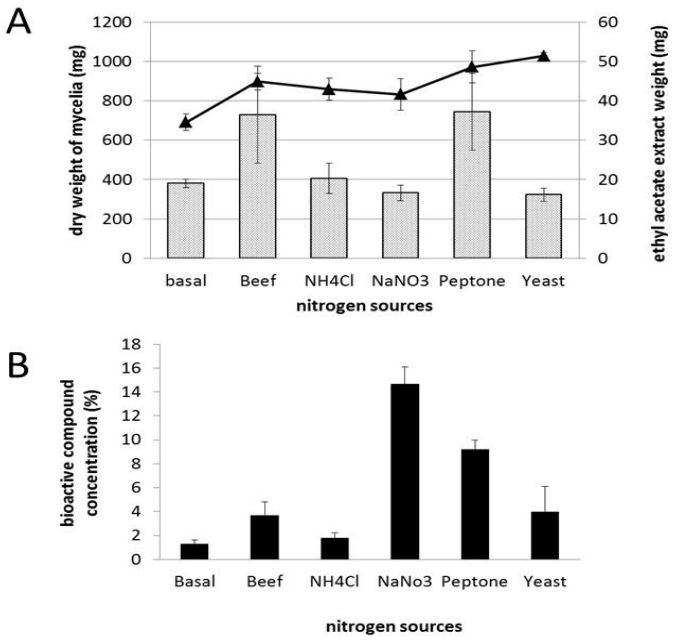

Figure 4. The growth and total metabolite production extracted with ethyl acetate (A) and bioactive compound (B) produced by endophytic fungi *A. fumigatus* strain KARVS04 cultured at different type of nitrogen sources. The data plotted are means ± SE of three replicates. ▒ = ethyl acetate extract, ▲= dry weight of mycelia, ▉ = bioactive compound.

Effect of pH

In this study we found that the mycelial growth did not show any significant differences when the strain was incubated at pH 5 – 7 (Figure 5). However, total metabolite production was higher when it was incubated at pH 6 – 7, ranging from 25.4 ± 4.1 mg at pH 7 to 29.2 ± 1.3 mg at pH 6. Interestingly, bioactive metabolite production reached maximum at pH 5 (12.2 ± 1.2%).

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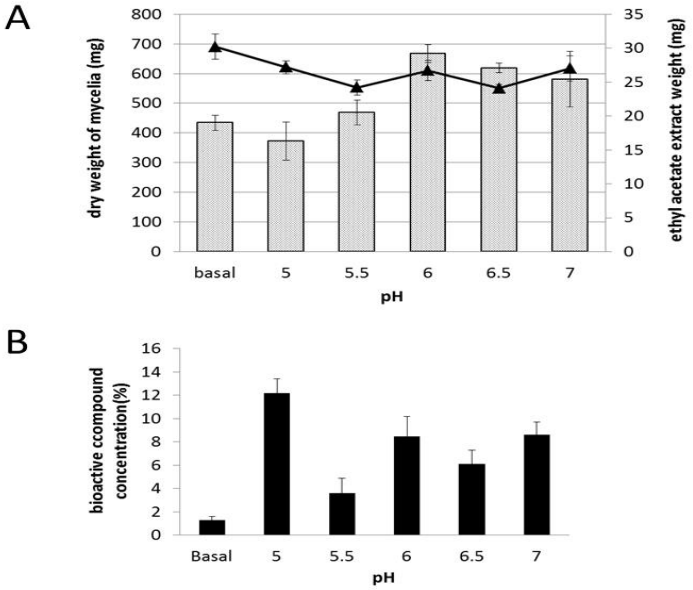

Figure 5. The growth and total metabolite production extracted with ethyl acetate (A) and bioactive compound (B) produced by endophytic fungi *A. fumigatus* strain KARVS04 cultured in PDB media at different pH level. The data plotted are means ± SE of three replicates. ▒ = ethyl acetate extract, ▲= dry weight of mycelia, ▉ = bioactive compound.

Effect of incubation temperature

The endophytic fungi *A. fumigatus* strain KARVS04 grew better when it was incubated at 29º C (769 ± 18.3 mg) followed by RT (728 ± 4.2 mg) (Figure 6). However, gradual decrease in total metabolite production was observed with the lowest production was seen at higher temperature of 30º (11.4 ± 0.8 mg). Analyzing bioactive metabolite concentration revealed that maximum production was achieved at 29º C, reaching 9.5 ± 0.7%.

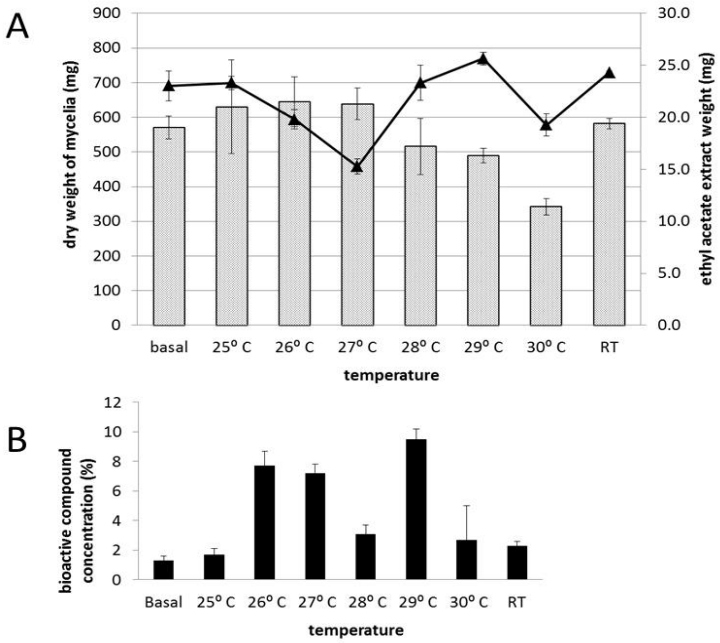

Figure 6. The growth and total metabolite production extracted with ethyl acetate (A) and bioactive compound (B) produced by endophytic fungi *A. fumigatus* strain KARVS04 cultured in PDB media at different temperature of incubation. The data plotted are means ± SE of three replicates. ▒ = ethyl acetate extract, ▲= dry weight of mycelia, ▉ = bioactive compound.

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Thin Layer Chromatography

TLC analysis of the extract obtained from culture medium supplemented with 1% starch and 1% sodium nitrate, incubated 10 days at pH 5 and temperature of 29º C showed difference in metabolite content and concentration (Figure 7). More substances and increased concentration were found when it is observed under UV_{256} and UV $_{366}$ lamp. Major additional components were seen at hRf of 20, 68 and 88.

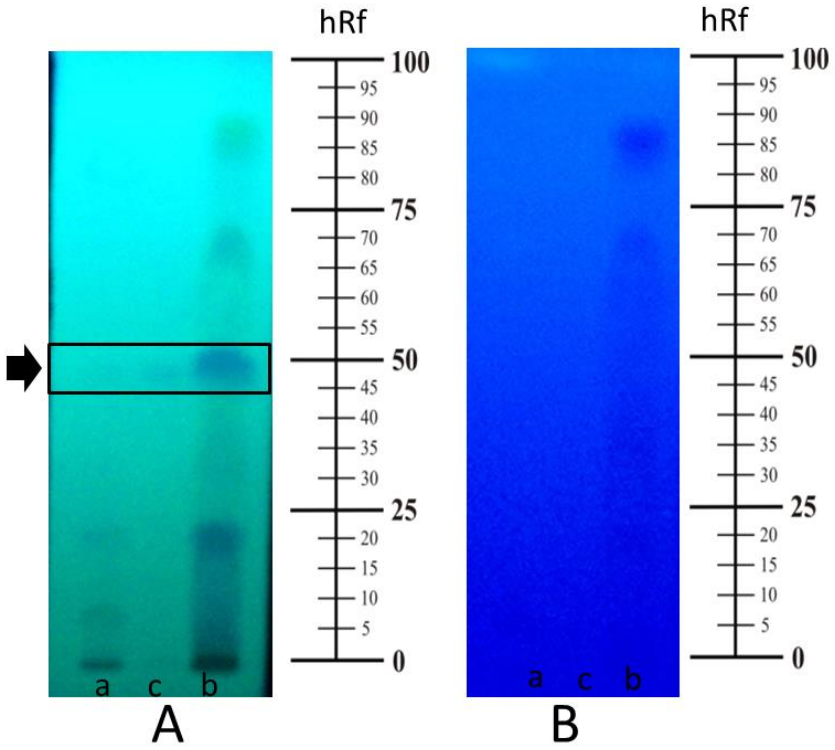

Figure 7. TLC profile of ethyl acetate extract. *A. fumigatus* culture was grown in basal medium (a) and PDB media supplemented with 1% starch and 1% sodium nitrate, incubated 10 days at pH 5 and temperature of 29º C (b), as detected using UV_{254} (A) and UV_{366} lamp. Isolated bioactive compound (pyrophen) was used as standard (c). Black arrow indicated the location of bioactive compound. Stationary phase: silica gel 60 F_{254}. Mobile

DISCUSSION

Aspergillus fumigatus has been reported to produce various metabolites having anti-cancer properties [19,21]. Additionally, endophytic *A. fumigatus* from several plants was also found to produce bioactive compounds against cancer cell lines [5,22]. Previous investigation in our lab found an amino acid-pyrone derivative, namely pyrophen, isolated from endophytic fungi *A. fumigatus* strain KARVS04. Although limited studies reported its potential as anticancer agent, our study showed its cytotoxic activity against T47D cell [20]. However, its low of productivity limited its exploration for anti-cancer agents. This study reported the attempts to increase biomass, total and bioactive (pyrophen) metabolite production from endophytic fungi *A. fumigatus* strain KARVS04 by optimizing the chemical and environmental conditions of fermentation.

The choice of fermentation medium is one of the key successes of microbial fermentation for industry. The components within cultural medium such as carbon and nitrogen sources, trace elements, vitamins, pH and salt concentration as well as physical aspects such as aeration and agitation, have been reported to affect microbial growth and its metabolite production [23,24]. In this study Potato Dextrose broth was found to be the best medium for the production of pyrophen. This media has been reported to be used for production of various bioactive metabolites such as tropolone [25], cladospolide D [26], asperaldin [27], and 5-Hydroxyramulosin [2]. The different of PDB compared to other media is that PDB contain starch while others are not.

Cultural medium components such as carbon and nitrogen sources may affect metabolite production in fungi [28]. Carbon or nitrogen sources which are quickly metabolized will increase the mycelial growth but it potentially inhibits secondary metabolite production [9,29]. In this study, media with high glucose or fructose resulted in faster growth of *A. fumigatus* strain KARVS04 and the culture with glucose supplementation produced highest total metabolite. Interestingly, media supplemented with starch showed highest bioactive metabolite production followed by fructose. These results indicated that simple carbon sources are important for both fungal growth and total metabolite production and the bioactive component (pyrophen) required more complex or slowly metabolized carbon sources for its production. On the other hand, medium supplemented with yeast as nitrogen source showed the highest growth but with low total metabolite production. Maximum bioactive metabolite, however, was achieved in media with sodium nitrate as nitrogen source. This study suggested that organic complex nitrogen source is preferable for biomass production [30] and a simpler inorganic nitrogen source like sodium nitrate is required for optimum bioactive compound, an amino acid – pyrone derivative, pyrophen production. Nitrogen is known as one of major component of complex macromolecule within living organism. It is an essential component of amino acid required for biosynthesis of various bioactive compounds [31].

The pH of medium determines the growth and secondary metabolite production within microorganism. It may affect the synthesis of the secondary metabolites by directly affecting the cell or by modulating enzyme activity, intermediate production or solubility [32]. In present study we found that *A. fumigatus* strain KARVS04 grew in all pH level, however, total metabolite production tended to be higher in pH value of 6 – 7. Interestingly, lower pH level is required for optimum bioactive metabolite production. These results suggested that acidic medium is the best for production of pyrophen. Similar result had been reported earlier for the production of acidic protease by *A. fumigatus* [33].

Environmental factors such as temperature of incubation may impact the growth of microorganisms and its metabolite production [32]. There was gradual decrease of total metabolite production in cultural medium incubated from 25 - 30º C. The production dropped dramatically when it was incubated at 30º C indicating lower temperature of incubation required. However, at temperature of 29º C seemed to be optimum for both the mycelial growth and bioactive metabolite production.

CONCLUSION

In this study we observed that maximum production of biomass, total metabolite or bioactive metabolites by *A. fumigatus* strain KARVS04 were achieved at different conditions. This study demonstrated that pyrophen production could be enhanced by modifying carbon and nitrogen resources as well as optimizing pH and temperature of incubation. The production increased ~10 times higher in modified medium. More components were observed in ethyl acetate extracts indicating its potential exploration for other bioactive substances having pharmaceutical values.

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