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Contributed Paper

Effect of Temperature on *Schizophyllum commune* Growth and 4H-pyran-4-one,2,3-dihydro-3, 5-dihydroxy-6-methyl- Production using a Bubble Column Bioreactor

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ABSTRACT

Schizophyllum commune, a basidiomycetes species, exhibited the anti-fungal properties by producing several bioactive compounds during secondary metabolism. One flavonoid fraction, named 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl- (DDMP), has received much attention for its antifungal activity toward wood-degrading fungi of rubberwood. In the present study, the effect of temperature on the growth and bioactive compound production by *Schizophyllum commune* was investigated in a bubble column bioreactor. Results revealed that the highest production of biomass (35.11±0.12 g/L), total flavonoids (1.328±0.007 µg QE/ mg sample), and DDMP (1.278±0.005 µg/mg sample) were achieved in the culture grown at 30 °C. The separation of crude extract was studied using a high performance liquid chromatography (HPLC) system equipped with a Zorbax XDB C18 column.

Keywords: *Schizophyllum commune*, total flavonoid, 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl- (DDMP), bubble column bioreactor, temperature, high performance liquid chromatography (HPLC)

1. INTRODUCTION

Screening of natural resources is an impressive tool for determining bioactive agents, in which those results showed that the available biodiversity of natural resources and the isolated of bioactive compounds can act as the potential leads for the development of clinically useful drugs [1,2]. According to current trends in the world, higher fungi have been widely applied in agriculture as a bio-control for pest management. Statistical data have showed that over 5,000 species belonging to approximately 1,200 genera of higher fungi have been reported from southern China [3]. Recently, fungi from the division basidiomycota have been shown to exhibit a rich and very diverse secondary metabolism. Additionally, mycelia and fruiting bodies of higher fungi are exposed to a number of predators or competitors, which can explain the production of antibiotics, insecticides, or feeding deterrents [4]. For example, Teoh *et al.* [5] reported that

Schizophyllum commune (basidiomycetes divisions) exhibited the anti-fungal properties by producing several bioactive compounds during secondary metabolisms.

Flavones, flavonoids, and flavonols are phenolic structure with one carbonyl group [6]. In general, these secondary metabolites are synthesized by plants in response to microbial infection and are often effective as antimicrobial agents against a wide array of microorganisms [7-9]. The mechanisms of antimicrobial flavonoids are the inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism [10]. Quercetin (3,3',4',5,7-pentahydroxy flavone) is one of the most common native flavonoids, which occurs mainly in glycoside forms such as rutin (5,7,3',4'-OH, 3-rutinose) [11]. On the other hand, Teoh *et al.* [12] revealed that a bioactive compound named 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP) consisted of flavonoid fractions has received much attention for its antimicrobial activity, particularly its antifungal activity toward the wood-degrading fungi of rubberwood.

Mushroom submerged fermentation is viewed as a promising alternative for efficient bioactive compounds production in order to guarantee a standardized product [13]. Recently, the bioreactor technology has been applied to the production of bioactive compounds through the secondary metabolites of microorganisms [14]. Bubble column bioreactors offer distinct advantages for some systems, particularly suitable for low-viscosity Newtonian broths [15]. Degaleesan *et al.* [16] revealed that, compared to other types of bioreactors, bubble column bioreactors provide a number of advantageous in both design and operation, such as high heat and mass transfer rates, compactness, and low operation and maintenance costs. Several researchers have studied on the utilization of bubble column bioreactors for biochemical processes such as fermentation, and the production of valuable products (*e.g.*, enzymes, proteins, and antibiotics) [17-19]. For example, Nanou *et al.* [20] found improved production of carotenes by *Blakeslea trispora* in a bubble column reactor.

Temperature is probably one of the most important environmental factors that determine fungal growth and product formation [21]. In research by Lee *et al.* [22], different optimum temperatures were used for the production of biomass and endopolysaccharide (10 °C), and exopolysaccharide (25 °C) for *Ganoderma applanatum* submerged cultivation. Both cell biomass and endopolysaccharide decreased as the culture temperature increased, suggesting that the mycelia accumulate polysaccharides in the cell at low temperatures. Most of the well-characterized higher fungi live best at temperature between 25 and 40 °C, although many thrive at high temperatures and others grow best (although slowly) at 0 to 15 °C. In fact, every organism has an optimum temperature for growth. As the generation time was increased, the temperature declined from that optimum [23].

Thus, the present study is aimed to determine the effect of temperature on the production of bioactive compounds (total flavonoid and DDMP) by *Schizophyllum commune* in a bubble column bioreactor. The separation and purification of DDMP from the crude sample was also performed.

2. MATERIALS AND METHODS

2.1 Fungal Strain Used

A locally isolated *Schizophyllum commune* was obtained from the Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia

(FRIM), Kepong, Malaysia. Stock cultures were grown on malt extract agar (MEA) at 30±2 °C and maintained on agar slants prior to subsequent studies.

2.2 Mycelia Suspension Preparation

The mycelia suspension was prepared by suspending mycelia discs from 7-d-old culture plates in sampling bottles containing sterilized distilled water, and 0.1 %(v/v) polysorbate 80 (Tween® 80). A disc of 5 mm in diameter was cut on the mycelia mats of the agar plate using a sterilized cork borer. A total of 10 discs for every 100 mL sterilized distilled water were vortexed for 5 min in order to homogenize the mycelia suspensions. The mycelia suspension was then standardized according to 1.0 McFarland standard turbidity prior to subsequent used.

2.3 Medium Preparation for Bench-scale Fermentation

The medium components were optimized in shake flask level as published by Teoh *et al.* [5] and the optimized parameters were used for bench scale fermentation. Briefly, the fermentation medium (pH 6.78) contained in g/L of 18.74 yeast extract, 10 malt extract, 38.65 glucose, 1 KH_2PO_4, 1 K_2HPO_4, and 0.59 $MgSO_4 \cdot 7H_2O$.

2.4 Bubble Column Bioreactor Study

The bioreactor used in this study was fabricated by Fermentec (Malaysia), as illustrated in Figure 1. It was 500 mm in length, and had a 50 mm internal diameter, with a 1.5 L working volume. The top of the column was closed with a rubber stopper. For dispersion of the air bubble, a porous membrane with 50 mm in diameter and with a 3 mm thickness incorporated with pore sizes 16 to 40 µm was used in the bottom part of the system. This column was connected to a Biostat-B control panel board supplied by Sartorius (Malaysia). The bioreactor was equipped with a digitally controlled pH electrode, temperature probe, and polarographic dissolved oxygen (pO_2) electrode. The airflow rate was measured using a rotameter, and the temperature was maintained through the double jacket design of the column. Silicone-based antifoam (Witeg GmbH, Germany) was used to control the formation of foam, while the pH of the culture broth during fermentation was controlled by the automatic addition of 2M NaOH and 2M HCl.

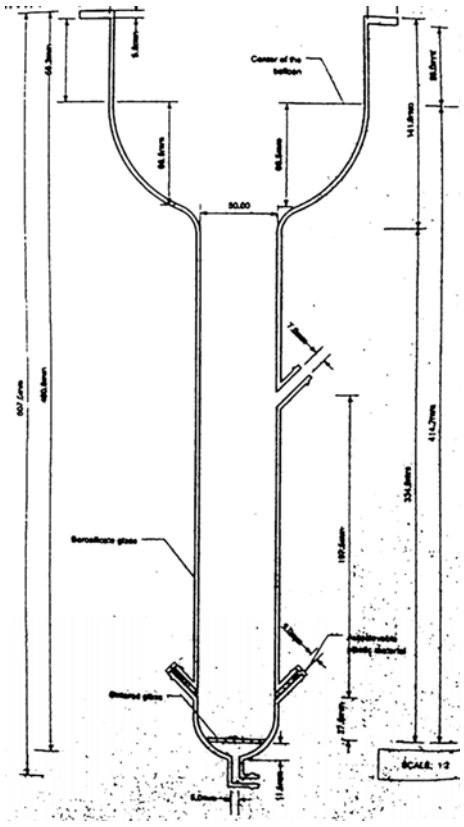

Figure 1. Schematic diagram of 2 L bubble column bioreactor.

2.5 Effect of Temperature on Growth and Bioactive Compound Production by *Schizophyllum commune*

The effect of temperature on growth and the production of bioactive compound by *S. commune*, the change in dissolved oxygen

(DO), and the glucose concentration profile of the fermentation broth were investigated under varying temperature controls ranging from 25 to 60 °C.

2.6 Extraction of Biomass

The culture broth obtained after the fermentation process was harvested and centrifuged at 4,000 *g* for 15 min. The biomass was then dried and homogenized before the extraction process. Dried biomass (100 g) was boiled in 80% (v/v) methanol-water mixture in a ratio of 1 g: 20 mL for 48 h. Then, the extraction solvent was separated via filtration and the filtered extract was evaporated using rotary evaporator. The extract obtained (called biomass extract**)** was then dried and kept at 4 °C for further analysis.

2.7 Analytical Method

2.7.1 Determination of biomass

The procedure was carried out using the method of Branco *et al.* [24], with slight modification. The biomass harvested at different time interval was homogenized at 50 Hz for 6 min. According to Banerjee *et al.* [25] the mycelia biomass can be analyzed using a spectrophotometer after fragmentation of the mycelia by homogenization. Furthermore, sensitivity of this method depends on the extent of fungal hyphal fragmentation achieved during the homogenization procedure; short homogenization periods (≤ 6 min) are sufficient for the required sensitivity. Then, the biomass concentration was determined by optical density (OD) measurements at 600 nm, in a spectrophotometer (XMA 1200V). The OD values were correlated with biomass concentration by means of a calibration curve.

2.7.2 Determination of glucose concentration

The glucose concentration was estimated based on the Dinitrosalicylic acid (DNS) method as described by Ghose [26].

2.7.3 Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) was measured spectrophotometrically by an aluminium chloride colorimetric assay reported by Ordonez *et al.* [27], with slight modification. A 100 mg sample (biomass extract) was diluted with 85 % ethanol, and 0.5 mL of the diluted sample was then pipetted into 0.5 mL of 2 %(v/v) $AlCl_3$ ethanol solution (2 g $AlCl_3$ in 100 mL ethanol). Ethanol was used as blank in this study. The absorbance was then measured at 420 nm after 30 min at room temperature. A yellow color indicated the presence of flavonoids. The flavonoid content was expressed as micrograms of quercetin per milligram of sample (μg QE/mg sample).

2.7.4 Analysis using UV-visible spectrophotometer for determination of DDMP

In this analysis, DDMP was determined using the method of Cechovska *et al.* [28]. Because commercial DDMP was not available on the market, the concentration of this compound was determined using a UV-Vis spectrophotometer (Evolution 201). Norfuraneol, a pentose-derived analogue of DDMP, which has similar electrochemical properties-half-wave potential at range 0.30 to 0.33 V, was used as a calibration standard for the quantitation of DDMP.

2.7.5 Separation and purification of DDMP

The concentration of DDMP was measured by high performance liquid chromatography (HPLC) (Agilent Series 1200) system equipped with a column (Zorbax XDB C18, 150 × 4.0 mm, 5 μm size). The

mobile phase was a mixture of deionized water and acetonitrile in a ratio of 95% to 5%, at a flow rate of 1 mL/min. The analysis was conducted at 30 °C. A 20 µl sample was diluted with ethanol and injected into the HPLC. The DDMP peak was detected within a retention time of 3.5 to 4.5 min.

3. RESULTS AND DISCUSSION

3.1 Effect of Temperature on Growth and Bioactive Compounds Production

In this study, the temperature was controlled using double-jacketed glass equipped with a water inlet and outlet attached to the bubble column bioreactor. The effect of temperature on the growth of *S. commune* was studied at various temperatures of 25, 30, 40, 50, and 60 °C, with fixed aeration rate of 4 L/min, and an initial glucose concentration 38.64 g/L with controlled pH at 6.78. Figure 2(a) shows that the growth rate of *S. commune* was enhanced as the temperature increased from 25 to 30 °C, but it gradually reduced as temperature was increased from 40 to 60 °C. The highest production of *S. commune* biomass (35.11±0.12 g/L) was attained in the culture grown at 30 °C. This high level of biomass was about four times greater than that at 60 °C. This showed that *S. commune*, a mesophilic type of fungus, grew well in the temperature range 20 to 45 °C. In this study, the optimum temperature for *S. commune* growth was 30 °C. Similar trends were also obtained for the production of TFC and DDMP (Figure 2(b&c)). According to Fazenda *et al.* [21], small variations in temperature could greatly reduce the fermentation productivity. Increasing in temperature might result in higher metabolic rate, but decreased the solubility of dissolved oxygen in the medium.

As can be seen in Figure 2(a), the first 24 h was a lag phase for *S. commune* growth in the bubble column bioreactor, and the biomass obtained during this period was relatively similar for all the temperature tested. After that, a growth exponential phase was observed and maintained with stationary phase until 120 h. On the other hand, the TFC profile (Figure 2(b)) was similar to the *S. commune* growth profile, but yet the TFC was not considered as the growth associated product because it was normally secreted during secondary metabolism. The TFC profile actually had a longer lag phase for the first 52 h as compared to the growth profile, but it was not so obviously seen in Figure 2(b) prior to the exponential growth phase. Alternatively, it can be clearly seen that the DDMP profile had a longer lag phase, until 64 h, after which a sharp growth phase was observed, with the maximum yields achieved after 72 h for all temperatures tested. As highlighted by Jia *et al.* [29], the growth and secondary metabolites output were a coupled action process, as the production yield was influenced by the cell growth, secretion of secondary metabolites, and the rise in secondary metabolites yield.

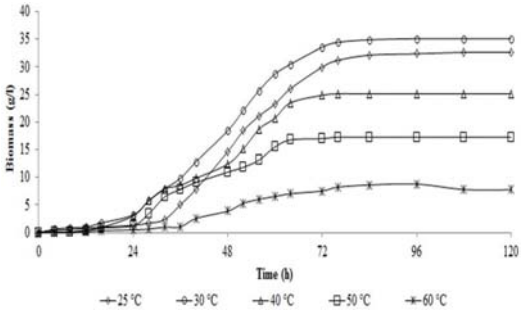

Figure 2(a). Effect of temperature on the growth by *Schizophyllum commune* in a bubble column bioreactor.

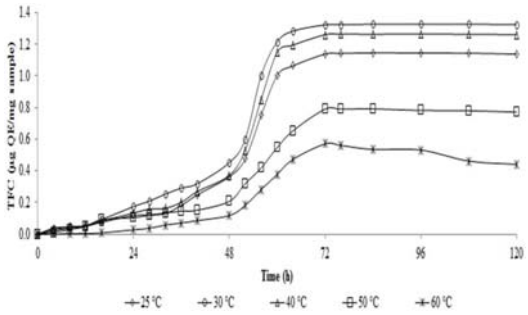

Figure 2(b). Effect of temperature on the total flavonoid content obtained by *Schizophyllum commune* in a bubble column bioreactor.

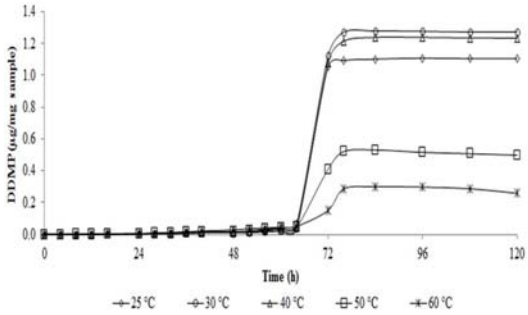

Figure 2(c). Effect of temperature on the DDMP production by *Schizophyllum commune* in a bubble column bioreactor.

3.2 Effect of Temperature on the Profile of Glucose Consumption and Dissolved Oxygen Level

Figure 3(a) represents the dynamic changes in glucose consumption by *S. commune* at different temperatures. An increase in the temperature from 25 to 60 °C led to an improvement in the utilization of all glucose available. However, at the end of cultivation, glucose still remained at 10 to 20 g/L in the culture medium for cultures at temperatures from 25 to 40 °C. As shown in Figure 3(b), the decline of all curves illustrated the decrease of dissolved oxygen content in the bioreactor. The DO levels from 50 and 60 °C were reduced to around 15 to 18 % at the end of the fermentation. Such a condition could be the result of higher oxygen consumption by the cells. In fact, the concentration of DO in the bioreactor fell rapidly during the exponential growth phase of *S. commune* fermentation process. This might be due to a higher growth rate of the cells coupled with the rapid increased of biomass concentration and also the production of TF and DDMP in this period. This is in agreement with a typical batch culture, where the dissolved oxygen concentration decreased in the media along with the growth time until the microorganism reached the stationary phase [30].

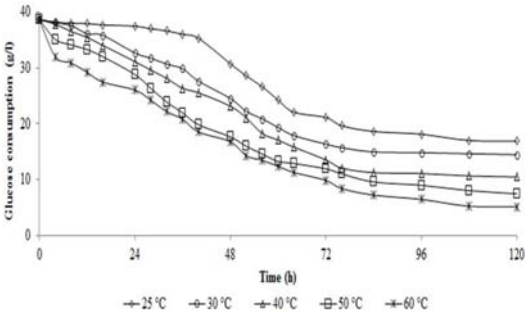

Figure 3(a). Effect of temperature on glucose consumption by *Schizophyllum commune* in a bubble column bioreactor.

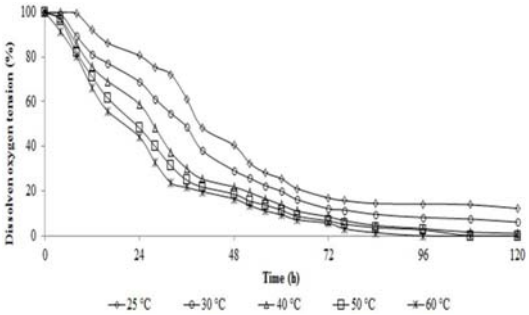

Figure 3(b). Effect of temperature on partial dissolved oxygen by *Schizophyllum commune* in a bubble column bioreactor.

3.3 Separation and Purification of DDMP from *Schizophyllum commune* Crude Extract

To precisely determine the presence of DDMP in the methanol extract of *S. commune*, a purification process was carried out using a HPLC. After several trial and error analyses, it was found that the column Zorbax XDB C18 (150 mm × 4.0 mm, 5 µm) incorporated with mobile phase (0.02 M KH_2PO_4, acetronitrile) was able to separate DDMP from its matrix. In the present study, the separation mechanism was at reversed phase chromatography, which depending on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand at stationary phase. Figure 4 describes the HPLC chromatogram for both DDMP purified from a sample, and standard DDMP. It was found that the DDMP concentration was more than 100 ppm from 10 mg/µL *S. commune* biomass extract. Thus, the valuable bioactive compound, DDMP, can be obtained through the secondary metabolite of *S. commune* using a bubble column bioreactor.

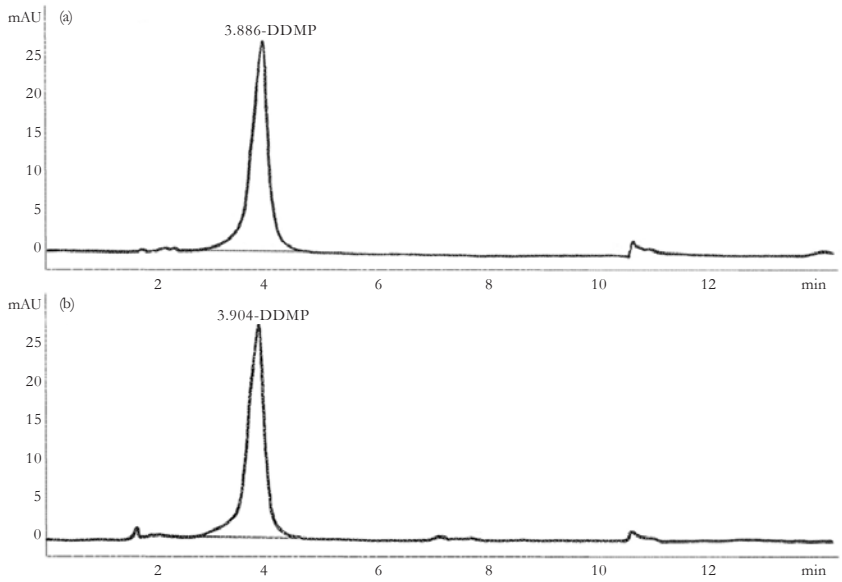

Figure 4. HPLC chromatogram for (a) a purified DDMP from a sample (RT: 3.886 min), and (b) a standard DDMP (RT: 3.904 min).

CONCLUSION

In the present study, a 1.5 L bubble column bioreactor was chosen to investigate the growth and production of bioactive compounds by *Schizophyllum commune*. Considering the effect of temperature, the highest biomass and total flavonoid contents, as well as DDMP production, were achieved at 35.11 g/L, 1.328 µg QE/mg sample, and 1.278 µg/mg sample, respectively. Then, the purified DDMP can be obtained via the separation using high performance liquid chromatography (HPLC) under certain operating conditions.

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