

Production of Biomass by *Schizophyllum commune* and Its Antifungal Activity towards Rubberwood-Degrading Fungi

(Penghasilan Biojisim oleh *Schizophyllum commune* dan Aktiviti Antikulat ke atas Kulat Pereput Kayu Getah)

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ABSTRACT

Rubberwood is the most popular timber for furniture manufacturing industry in Malaysia. Major drawback concerned that rubberwood is very prone to attack by fungi and wood borers, and the preservation method using boron compounds exhibited hazardous effect to the workers. Fungal-based biological control agents have gained wide acceptance and *Schizophyllum commune* secondary metabolite played an important role in term of antifungal agent productivity. The effects of initial pH, incubation temperature and agitation on biomass production by *S. commune* were investigated under submerged shake culture. In this work, it was found that the synthetic medium with initial solution pH of 6.5 and incubated at 30°C with shaking at 150 rpm provided the highest biomass production. The biomass extract from *S. commune* was then applied onto the rubberwood block panel to investigate its effectiveness. The results showed that biomass extract at a concentration of 5 µg/µL could inhibit the growth of selected rubberwood-degrading fungi, such as *Lentinus sp.*, *L. strigosus* and *Pycnoporus sanguineus*.

Keywords: Antifungal activity; biomass production; effectiveness; rubberwood-degrading fungi; *Schizophyllum commune*

ABSTRAK

Kayu getah ialah kayu yang paling popular untuk industri pembuatan perabot di Malaysia. Kelemahan utama bagi kayu getah itu adalah serangan oleh kulat dan pengorek kayu dan kaedah pengawetan menggunakan sebatian boron menunjukkan kesan berbahaya kepada pekerja. Agen kawalan biologi yang berasaskan kulat telah mendapat penerimaan luas, dan metabolit sekunder *Schizophyllum commune* memainkan peranan penting dalam produktiviti agen antikulat. Kesan pH awal, suhu penderaman dan pengoncangan bagi penghasilan biojisim oleh *S. commune* telah dikaji di bawah kultur kelalang goncang. Dalam kajian ini, didapati bahawa medium sintetik dengan pH permulaan 6.5 dan dikultur pada 30°C dengan goncangan pada 150 rpm menghasilkan biojisim tertinggi. Ekstrak biojisim daripada *S. commune* kemudiannya telah ditindakbalaskan ke atas blok kayu getah untuk menguji akan keberkesannya. Keputusan menunjukkan ekstrak biojisim pada kepekatan 5 µg/µL berkeupayaan merencat pertumbuhan kulat pereput kayu terpilih, seperti *Lentinus sp.*, *L. strigosus* dan *Pycnoporus sanguineus*.

Kata kunci: Aktiviti antikulat; keberkesanan; kulat pereput kayu getah; penghasilan biojisim; *Schizophyllum commune*

INTRODUCTION

Nowadays, the Malaysian rubber industry is well-known internationally for its developed and progressive R&D programs that enabled the country to establish itself as the world leader in rubber production, processing and manufacturing technologies. In fact, the Malaysian furniture industry plays an important role at the international level since it is able to turn cheap and plentiful rubberwood timber into value-added products at a competitive price. Rubberwood has good overall wood-working mechanical properties and machining qualities for sawing, boring, turning, nailing and glaring, which are suitable for use in furniture making (Teoh et al. 2011). Unfortunately, the main drawback for rubberwood used in wood processing industry is the biodegrading problem by wood-degrading fungi.

And yet, an economical schedule for the industrial scale treatment of rubberwood using boron compounds in the form of disodium octaborate tetrahydrate ($\text{Na}_2\text{B}_8\text{O}_{13}\cdot 4\text{H}_2\text{O}$), disodium tetraborate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$) and boric acid (H_3BO_3) have been developed, particularly for indoor applications to protect from insect borers and fungi. Meanwhile, the highly toxic, but safe once fixed, copper-chromium-arsenic (CCA) preservatives are widely used in many countries due to their efficacy and cost effectiveness (Teoh et al. 2011). However, these compounds have become less popular due to their toxicity and hazardous effect to the workers (Zaidon et al. 2008). Therefore, it is necessary for rubberwood to be treated with appropriate preservatives for protection, particularly using natural resources.

Schizophyllum commune is a species of basidiomycetes belonging to the Schizophyllaceae of Agaricales. This filamentous fungus secretes a neutral homoglucon that consists of a backbone chain of 1,3- β -D-glucopyranoses units linked with single 1,6-bounded β -D-glucopyranoses at about every third glucose molecule in the basic chain, known as 'Schizophyllan' (Rau et al. 1992). In fact, the schizophyllan is a non-ionic, water soluble homopolysaccharide consisting of a linear chain of β -D-glucopyranosyl groups produced during fermentation process. This polysaccharide has attracted attention in recent years in pharmaceutical industry as immunomodulatory, antineoplastic, and antiviral activities (Kumari et al. 2008). Recent research done by Teoh et al. (2012) reported that the biomass extract from *S. commune* inhibited most of the selected white rotting fungus with minimum inhibitory concentration (MIC) values ranging from 0.16-5 μ g/ μ L. Furthermore, it was found that this fungus possessed an antifungal activity with the presence of several interesting compounds, such as glycerine, 2(3H)-furanone, 5-heptyldihydro-, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP) and triacetin (Teoh et al. 2012).

Submerged liquid fermentation of macrofungi began slowly during the 1950s due to the fact that filamentous fungi are morphologically complex microorganisms. It is well known that mycelia cultivation with submerged fermentation technology can lead to a higher mycelia production in a more compact space and in less time with fewer chances of contamination (Guo et al. 2009). Perhaps, the mycelia growth rate has been shown to vary with the environmental conditions, such as initial solution pH, incubation temperature and agitation speed. Many fungi are able to grow at a range of initial pH values because they can regulate the medium acidity to levels that support their growth. In the case that pH is not automatically controlled, the pH change is observed during fungi growth caused by excretion of waste products (Ertola et al. 1995). On the other hand, temperature is another important environmental factor that determines the fungal growth as it affects other culture variables such as dissolved oxygen tension (DOT), rate of medium evaporation, pellet formation and product formation (Fazenda et al. 2008). Besides, agitation intensity is another notable variable that influenced the mixing and oxygen transfer rate in fungal fermentation, thus influencing the mycelia growth morphology. In fact, a higher speed might damage the mycelia structure, leading to morphological changes and thus, detrimental to the mycelia growth and product formation (Fazenda et al. 2008).

In short, the yield and productivity of mushroom mycelium vary widely, depending on the mushroom species, substrate used and environmental conditions. The work reported here was carried out to determine the physical conditions required for the mycelia growth of *S. commune* in submerged shake culture. In addition, attempts were also made to evaluate the effectiveness of antifungal agent from culture mycelia against rubberwood-degrading fungi.

MATERIALS AND METHODS

FUNGAL STRAIN

The locally isolated fungal strain, *Schizophyllum commune*, was obtained from Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. The stock culture was grown on malt extract agar (MEA) at 30°C and maintained on agar slants prior to subsequent studies.

MYCELIA SUSPENSION PREPARATION

Mycelia suspension was prepared by suspending mycelia discs from 7-d-old culture plates in sampling bottles containing sterilized water and 0.1% (v/v) Tween 80. The disc of 5 mm 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 water were vortexed for 5 min in order to homogenize the mycelia suspensions.

FERMENTATION CONDITION

A total of 10 mL (10% v/v) of the mycelia suspension was added to 90 mL medium in 250 mL Erlenmeyer flask containing the following composition: 18 g/L yeast extract, 10 g/L malt extract, 38 g/L glucose, 1 g/L KH_2PO_4 , 1 g/L K_2HPO_4 , 0.6 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2 g/L $(\text{NH}_4)_2\text{SO}_4$. The medium was autoclaved at 121°C for 15 min before transferring the mycelia suspension into the culture media. The culture was incubated at $30 \pm 2^\circ\text{C}$, pH6.5 in an incubator shaker at 200 rpm for 5 d. The culture broth was then harvested and centrifuged at $4000 \times g$ for 15 min. The residues (called biomass) was then dried and homogenized prior to extraction process.

Effect of physical conditions on *S. commune* growth under shake flask cultivation Optimization of the physical conditions on *S. commune* growth by employing one-factor-at-a-time (OFAT) techniques was carried out in 250 mL Erlenmeyer flask. In this work, the main three parameters studied were initial pH (4.5-8.5), incubation temperature (25-45°C) and agitation (0-450 rpm). In each experiment, one factor was changed, while holding the other factors fixed. All experiments were performed in triplicates.

EXTRACTION OF ANTIFUNGAL AGENT FROM BIOMASS

In this work, a batch solvent extraction process was carried out. Dried biomass (100 g) was boiled in 80% (v/v) methanol-water mixture in a ratio of 1 g: 20 mL for 48 h. The extraction solvent was then 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.

EFFECTIVENESS STUDY OF ANTIFUNGAL AGENT ONTO THE RUBBERWOOD BLOCK PANEL

Rubberwood-degrading fungi used The selected locally isolated wild species of rubberwood-degrading

fungi: *Lentinus* sp., *Lentinus strigosus* and *Pycnoporus sanguineus* were obtained from Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. The stock culture was grown on malt extract agar (MEA) at 30°C and maintained on agar slants prior to subsequent studies.

Treatment of rubberwood block panel The rubberwood block treating procedure was carried out according to ASTM D4445 (2003). In this work, the 5 µg/µL antifungal agent solution was prepared and poured into beakers containing the rubberwood block specimens. Similarly, the control specimen was treated with distilled water. After 5 min, the solution was poured out and the beakers were tightly covered and stored overnight. This would allow the draining of excess solution and time for the fungicides to be deposited onto the wood before inoculation. After overnight storage, the specimens were placed into prepared Petri dishes and inoculated with selected rubberwood-degrading fungi. They then were incubated at room temperature for 2 weeks.

RESULTS AND DISCUSSION

EFFECT OF PHYSICAL CONDITIONS USING ONE-FACTOR AT-A-TIME (OFAT) METHOD

Effect of initial pH Figure 1 shows that *S. commune* grew fairly well in acidic, neutral and alkaline environments. It was observed that the tested fungus grew well in slightly acidic condition with initial pH 6.5 with maximum biomass production (31.6±0.1 g/L). Beyond this pH, the fungus growth decreased. According to a few researchers, various species of mushrooms, such as basidiomycetes and ascomycetes, provided good mycelia growth under moderately or slightly acidic pH during submerged cultivation (Adebayo-Tayo et al. 2011). In fact, most natural environments having pH values between 5-9 and most organisms had their optimum in this range. This proved that most fungi especially *S. commune* preferred slightly acidic more than neutral medium culture for their growth. The results in agreement with the study done by Fasidi and Akwakwa (1996), who reported that acidic pH

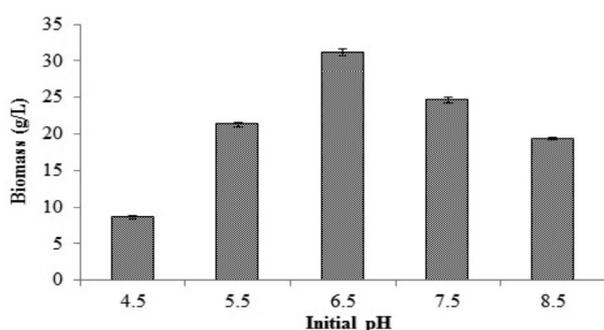


FIGURE 1. Effect of initial pH on growth of *S. commune* in submerged shake culture

value of 5.5-6.5 could provide good growth for *Pleurotus tuber-regium*. However, about 8.6±0.1 g/L of biomass was produced at pH 4.5 in this work, which suggesting that very moderate/strong acidic alkaline environments were inhibitory to the fungus growth. Therefore, the optimum initial pH value of 6.5 was used for further work.

Effect of incubation temperature Temperature is one of the important environmental factors for growth of filamentous fungi. Figure 2 shows that the highest biomass (31.9±0.1 g/L) was produced at 30°C. This might be due to the suitability of the tested fungus at this range of temperature (26-35°C) (Fazenda et al. 2008). Beyond this point, the growth of *S. commune* decreased. This observation agrees well with the study done by Gbolagade et al. (2006), who reported that growth of basidiomycetes, *Lentinus subnudus*, was inhibited at extremely low and high temperature and the denaturation of fungal internal structure at higher temperature. As a result, a moderate temperature of 30°C was used for further work.

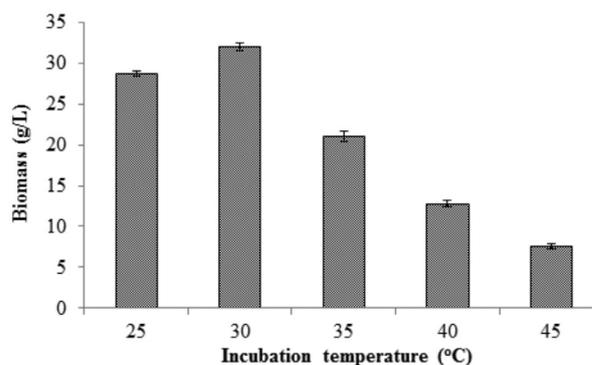


FIGURE 2. Effect of incubation temperature on growth of *S. commune* in submerged shake culture

Effect of agitation Agitation is another important factor in the fermentation process since it would increase the amount of dissolved oxygen in the cultivation medium and influence the growth morphology of filamentous fungus. Figure 3 illustrates the effect of agitation on the morphological characteristic of *S. commune* in submerged cultivation. Typical branched hyphae were observed in the static flask culture (Figure 3(a)). When the flask was agitated from 50-250 rpm, aggregation of fungus mycelium occurred which formed spherical, compact masses of hyphae pellets (Figure 3(b) until 3(d)). This could be due to an aggregation process which occurred between the small clumps and through entanglement of spores in the hyphae. In fact, this pelleting helps to maintain low viscosity of medium and high aeration efficiency (Barberel & Walker 2000). Meanwhile, the size of pellets formed tends to reduce as the agitation speed increased from 50 to 250 rpm (Figure 3(b) until 3(d)). Agitation damaged the mycelia pellets, in which fragmentation of the hyphae from the outer surface of the pellet could cause in hyphal fragments and served as

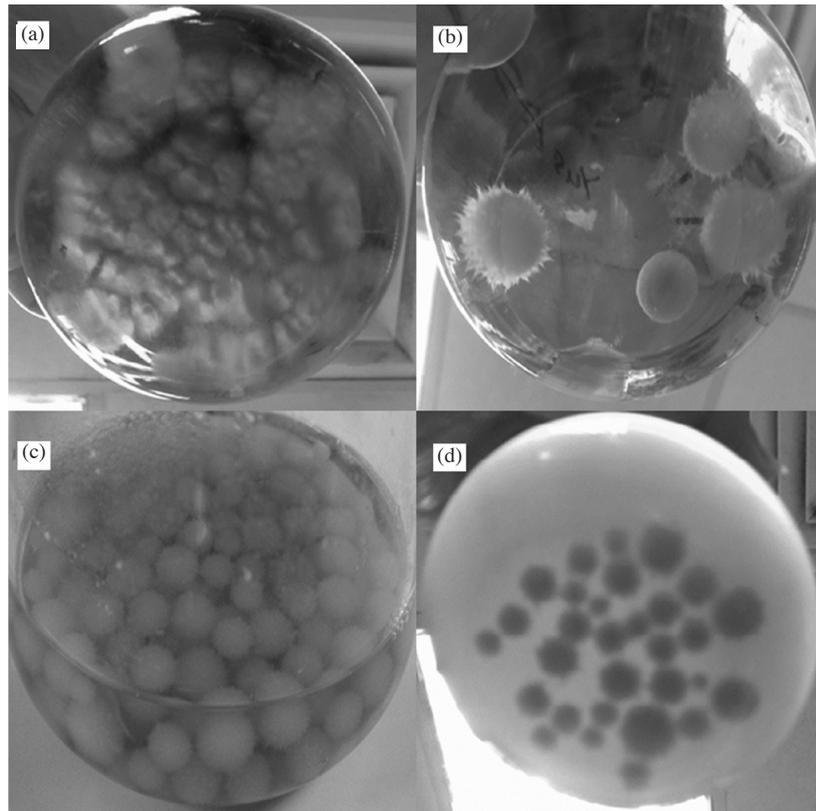


FIGURE 3. *S. commune* growth morphology during submerged cultivation under different shaken conditions: (a) 0 rpm, (b) 50 rpm, (c) 150 rpm, and (d) 250 rpm

new centres for biomass growth, or it caused the hyphal damaged accompanied with released of cytoplasm (Znidarsic & Pavko 2001).

Figure 4 evaluates the effect of agitation on growth of *S. commune* in submerged shake culture using a single factor experiment. The results showed that the biomass increased sharply with increased in agitation speed from 0 to 50 rpm. In contrast to an optimum agitation speed of 100 rpm for mycelia growth of *Aspergillus niger* in submerged cultivation by Purwanto et al. (2009), the highest biomass attained in this work was at 32.8 ± 0.2 g/L when the agitation speed was 150 rpm. Beyond this

level, a reverse trend was observed (Figure 4). It has been known for many years that oxygen has toxic effects on aerobically growing microorganisms, mainly due to the threat arising from reactive oxygen species (ROS) (Bai et al. 2003). In this work, more oxygen dissolved into the culture medium at high agitation and hence resulted in higher DOT. Consequently, the tested fungus might be exposed to potential oxidative stress caused by enhanced flux of ROS throughout the mycelium and thus inhibiting the growth. Therefore, agitation speed of 150 rpm was used for further study.

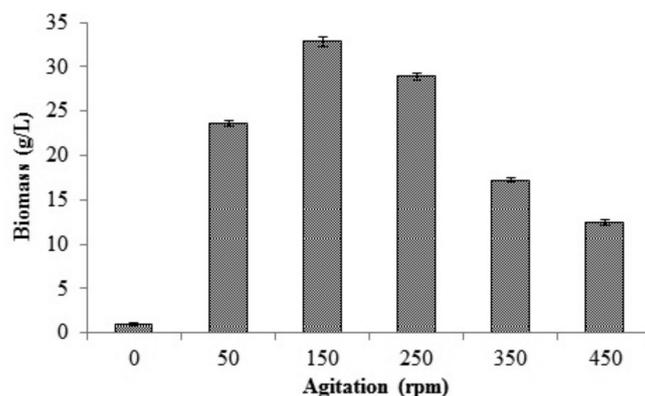


FIGURE 4. Effect of agitation on growth of *S. commune* in submerged shake culture

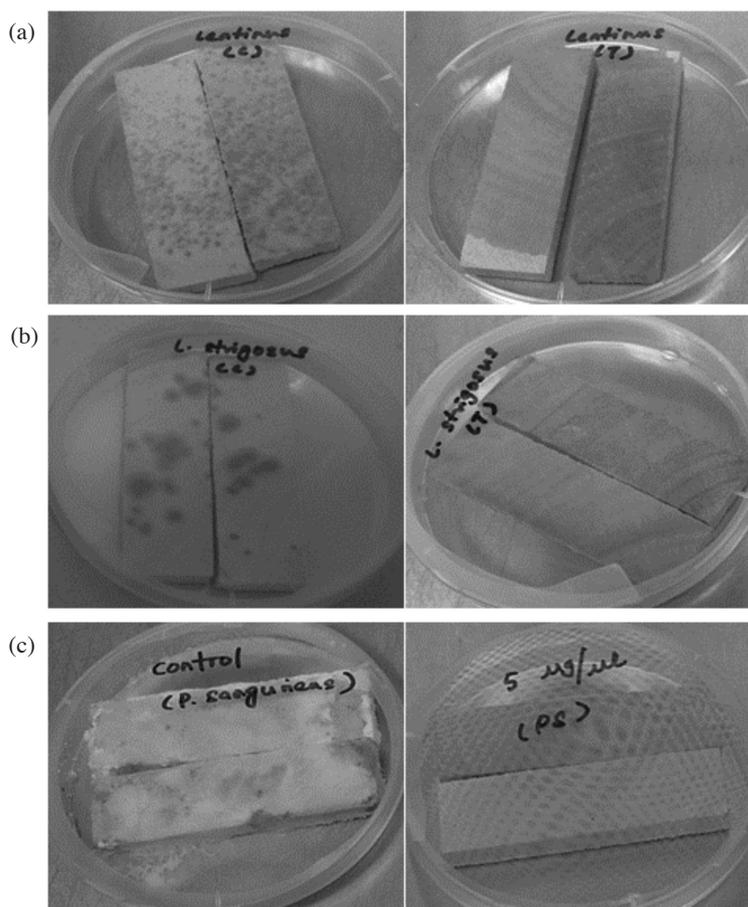


FIGURE 5. Effect of *S. commune* biomass extract (5 µg/µL) against (a) *Lentinus* sp. (b) *L. strigosus* and (c) *P. sanguineus* on the rubberwood block panel

EFFECTIVENESS STUDY OF ANTIFUNGAL AGENT ONTO THE RUBBERWOOD BLOCK PANEL

S. commune is known as potential producer of different metabolites including antifungal substances, in which the oxidized shizophyllan possesses antimicrobial activity against a broad range of bacterial species with the concentration as an important factor (Kumari et al. 2008). In this work, the effect of *S. commune* biomass extract at concentration of 5 µg/µL against selected rubberwood-degrading fungus: *Lentinus* sp., *L. strigosus* and *Pycnoporus sanguineus* on the rubberwood block panel was studied and result after 2 weeks of incubation is portrayed in Figure 5. Obviously, there was significantly change on the untreated rubberwood block (control) surface as the tested rubberwood-degrading fungus growth which can be easily seen. Meanwhile, there was no growth observed on the rubberwood block treated with 5 µg/µL of biomass extract. Furthermore, Figure 5(c) illustrates that *P. sanguineus* was very prone to attack the rubberwood with its fast growth rate as compared to *Lentinus* sp. (Figure 5(a)) and *L. strigosus* (Figure 5(b)).

CONCLUSION

It was found that *S. commune* yielded the highest growth in the synthetic medium with initial solution pH6.5 and

incubated at 30°C, 150 rpm. This slow growing filamentous fungus produced antifungal agent of which its effectiveness was carried out on the rubberwood block panel. No growth was observed on the treated rubberwood block surface, thus indicating that the *S. commune* biomass extract at concentration of 5 µg/µL could inhibit the growth of selected rubberwood-degrading fungi.

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