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Effects of refined sugar and combination of light and agitation on yields and mycelial morphology of *Grifola frondosa* AM cultivated in submerged culture

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Abstract

Grifola frondosa or Maitake mushroom is well known for its medicinal value such as anticancer, antioxidant and immunomodulatory properties. This work aims to investigate the effects of refined sugar (referred as sucrose; a low-cost substrate) as an alternative carbon source and light combined with agitation conditions on the mycelial growth, polysaccharides production, and fungal morphology. The optimum sucrose concentration for *G. frondosa* AM fermentation was detected at 6% (w/v), producing high yields of biomass and bioactive polysaccharides. Cultivation of *G. frondosa* AM fungus in the absence of light with flask rotation at 120 rpm resulted in rapid growth of mycelium and β-glucans as high as 29% (w/w). Mycelial morphology in a flask shaking condition displayed various formations as filamentous to spherical shape, while at static condition it only exhibited mycelium-matted formation. Furthermore, cultivation of *G. frondosa* AM in the dark can alter the mycelial morphology from loosely long cylindrical to tight packing structure analyzed by scanning electron microscope (SEM).

Key words – Beta-glucan – Biomass – Dark – Endo/exopolysaccharides – Maitake mushroom – Mycelium – Sucrose

Introduction

Various edible mushrooms belonging to higher Basidiomycetes have gained popularity worldwide due to their medicinal values for human health (Elisashvili 2012, Bandara et al. 2019). *Grifola frondosa* (Dicks.) Gray or Maitake mushroom has increased in popularity for human consumption with health promotion, especially for lung and liver. The major bioactive compounds in Maitake mycelia are polysaccharides accounting for approximately 48% (He et al. 2017). Crude polysaccharides from *G. frondosa* exhibit several pharmacological properties such as anti-oxidation, anti-hyperglycemia, anti-inflammation, anti-tumor and immunomodulation through stimulating a host-mediated immune response (Kodama et al. 2005, Lee et al. 2010, Lin 2010, He et al. 2017). In that, it contains β-glucans accounting for 13% in the water-soluble polysaccharides, for example grifolan (β-1,3-glucan, 1,6 branches) and MD-fraction (β-1,6-glucan, 1,3 branches) involved in anti-tumor and immunomodulatory activities (He et al. 2017). The bioactive polysaccharides can be found in fruiting bodies, culture mycelium (intracellular polysaccharides or

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IPS) and culture broth (exopolysaccharides or EPS) in the submerged cultivation (Elisashvili 2012, He et al. 2017, Bandara et al. 2019)

Many recent studies have focused on finding optimal cultivation condition for each medicinal mushroom, especially in the liquid fermentation since the system facilitates the industrial potential (Lee et al. 2004, Shih et al. 2008, Tang et al. 2008, Chao et al. 2011). Several fermentation factors have been investigated and optimized such as nutrient composition, sugar concentration, temperature, culture pH, aeration and agitation rate, and special additives (Elisashvili 2012). The carbon source is a main parameter effecting on the production of mycelial biomass and bioactive compounds (Lee et al. 2004, Tang et al. 2008, Elisashvili et al. 2009, Chao et al. 2011). Agitation and aeration could improve *G. frondosa* GF 9801 mycelia and EPS production in a 25-L stirred fermenter (Cui et al. 2016). Cui et al. (2016) also reported that a change of *G. frondosa* GF 9801 morphology affected the production of biomass and polysaccharide content and compositions. Moreover, works on other fungal growth demonstrated the influences of light and darkness in the fungal metabolism, developmental and physiological processes, and secondary metabolite production (Babitha et al. 2008, Zhang et al. 2016). Cultivating *G. frondosa* in liquid fermentation had been done in a single-factor optimization, in other cases, not regulated several factors together (Kim 2003, Lee et al. 2004, Shih et al. 2008, Lin 2010, Cui et al. 2016). Hence, submerged cultivation of *G. frondosa* to sustain high yields at a lowest cost of carbon substrate with favored morphology is a great challenge for its success on commercialization.

In submerged culture, several studies revealed that valuable mushrooms prefer different types of growing condition and carbon sources such as glucose, sucrose, maltose, or lactose in biomass and polysaccharides productions (Lee et al. 2004, Tang et al. 2008, Elisashvili et al. 2009, Chao et al. 2011, Elisashvili et al. 2012). Glucose is an ordinary sugar used in submerged cultivation for various mushrooms including *G. frondosa* (Lee et al. 2004, Elisashvili 2012, Cui et al. 2016), but it is an expensive carbon substrate (49.5$ US/1 kg), as well as sucrose for microbiology grade. Our effort was to find an alternative carbon source for *G. frondosa* cultivation. To our knowledge, there was no report on using refined sugar as a carbon source in submerged fermentation for *G. frondosa*. Refined sugar is purified from sugarcanes in which mainly consisting of sucrose. Refined sugar is commercially available in the market and cheap (0.70$ US/1 kg). In addition, research on how light affects *G. frondosa* morphology and polysaccharide production is scarce. Thus, the effects of refined sugar (referred as sucrose) and combination of light and agitation on the production of *G. frondosa* mycelial biomass, bioactive polysaccharides (IPS, EPS, and β-glucan), and mycelial morphology were presented here for the first time.

Materials & Methods

Fungal material and seed culture

G. frondosa strain AM was purchased from Aloha Medicinals Inc. (Nevada, USA) and was deposited at the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand. Potato dextrose was used as a seed culture medium. The activated mycelium was maintained on the potato dextrose medium (agar or broth) at 25 ± 2°C under light intensity of 300 lux.

Carbon source and cultivation periods

Seed culture of *G. frondosa* strain AM was inoculated at 3% (v/v) and cultivated in 100 mL of fermentation medium (250-mL Erlenmeyer flask). The formulation of fermentation medium was developed and optimized in our laboratory. It consisted of 6 g/L yeast extract, 6 g/L peptone, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $MnSO_4 \cdot 5H_2O$, 10 g/L olive oil, and 30 g/L glucose or sucrose (refined sugar). Glucose was normally used as a carbon source in cell culture; therefore, it was used as a reference. Refined sugar was used to compare with glucose for the efficiency as an alternative carbon source. Refined sugar contains >99% sucrose (Mitr Phol, Thailand), therefore we referred as sucrose throughout. The cultures were grown at 25 ± 2°C under

light intensity of 300 lux for 21, 30, 60, and 90 days. Mycelial biomass was analyzed to find the optimal period of cultivation for further use in the next experiments.

Optimization of sucrose concentration

Seed culture of *G. frondosa* strain AM was inoculated at 3% (v/v) and cultivated in 100 mL of fermentation medium (250-mL Erlenmeyer flask) containing sucrose at a concentration of 2, 3, 4, 5, or 6% (w/v). Based on the optimization of cultivation period, cultivation at 30 days was the most promising period since daily biomass production rate was at the highest. Therefore, the cultures were grown at 25 ± 2°C under light intensity of 300 lux for 30 days and used in the analyses for biomass and bioactive polysaccharides.

Environmental conditions for *G. frondosa* growth

Seed culture of *G. frondosa* strain AM was inoculated at 3% (v/v) and cultivated in 100 mL of fermentation medium (250-mL Erlenmeyer flask) containing 6% (w/v) sucrose, optimal sucrose concentration. Light or darkness, with or without flask rotation at a speed of 120 rpm were applied in cultivation. The cultures with defining conditions were grown at 25 ± 2°C under light intensity of 300 lux for 30 days and used in the analyses for biomass, polysaccharides, and mycelial morphology.

Sample preparation and extraction

Samples were collected for mycelium and broth separately at the defining periods for each experiment. The mycelial biomass was lyophilized using ScanVac (CoolSafe 110–4, Denmark) for 48 h and was expressed as dry weight (DW) in g/L.

The lyophilized mycelium was used in extraction of intracellular polysaccharides (IPS). Sample was crushed into powder and extracted with hot water for 2 h at 121°C. The extract was filtered through a filter paper (Whatman No.1). The filtrate was then evaporated, reduced to half of its volume. It was then mixed with 95% ethanol at a ratio of 1:4 (v/v) and incubated at 4°C for 24 h. The precipitates were collected, lyophilized and stored at -20°C. The precipitates were further measured for IPS, expressed as mg/g, by conventional phenol-sulfuric acid method (Dubois et al. 1956).

The fermentation broth was centrifuged at 8000×g for 20 min. The collected supernatant was filtered through a 0.45 µm membrane filter and then evaporated, reduced to half of its volume. The concentrated filtrate was extracted for secreted polysaccharides with 95% ethanol at a ratio of 1:4 (v/v) and incubated at 4°C for 24 h. The total precipitates, expressed as EPS powder (g/L), were collected, lyophilized and stored at -20°C. The precipitates were further measured for exopolysaccharides (EPS), expressed as EPS (g/L), by conventional phenol-sulfuric acid method (Dubois et al. 1956).

Determination of bioactive polysaccharides

IPS and EPS contents were determined by phenol-sulfuric acid method using glucose as the standard and expressed as mg/g and g/L, respectively (Dubois et al. 1956). Beta-glucan content, expressed as % w/w, was analyzed using Yeast and Mushroom β-Glucan assay kit (Megazyme®, Ireland). The procedure was followed according to the manufacturer's instruction.

Morphological analysis using scanning electron microscopy (SEM)

Mycelial morphology of *G. frondosa* strain AM was analyzed using a scanning electron microscope (Hitachi SU8020, Japan). The samples were coated with platinum on Quorum sputter coater (Q150R ES, England) prior to the analysis.

Statistical analysis

All experiments were performed as a completely randomized design with at least three replications. The data were expressed as means ± standard deviations (n = 3). The results were

analyzed for statistical significance by analysis of variance (ANOVA) using Tukey's comparison or Student pair t' test at the significance level of 0.05 in JMP® program version 10.0.0 (SAS Institute Inc., USA).

Results

Effects of carbon source and cultivation time for *G. frondosa* AM mycelium growth

Carbon sources (glucose and sucrose, 3% w/v) affected on *G. frondosa* AM mycelium production in the submerged culture at various cultivation times. Mycelium yield increased with time in both carbon sources (Fig. 1). At days 21 and 30 of cultivation, there were no significant differences in mycelium biomass from the two carbon sources. However, at days 60 and 90 of cultivation, mycelium biomass in glucose was significantly higher than that in sucrose for about 9% and 12%, respectively.

Having screened for the appropriate cultivation time, it revealed that cultivation for 30 days in sucrose giving favorable yield as the mycelium production per day was high (0.212 ± 0.023 g/L·d) and was comparable to that in glucose (0.230 ± 0.012 g/L·d). Cultivation time for 30 days in sucrose gave a high productivity rate compared to 0.147 ± 0.005 g/L·d and 0.110 ± 0.004 g/L·d from days 60 and 90 of cultivation, respectively. Thus, 30 days of cultivation in sucrose was chosen to grow *G. frondosa* AM mycelium for further investigations.

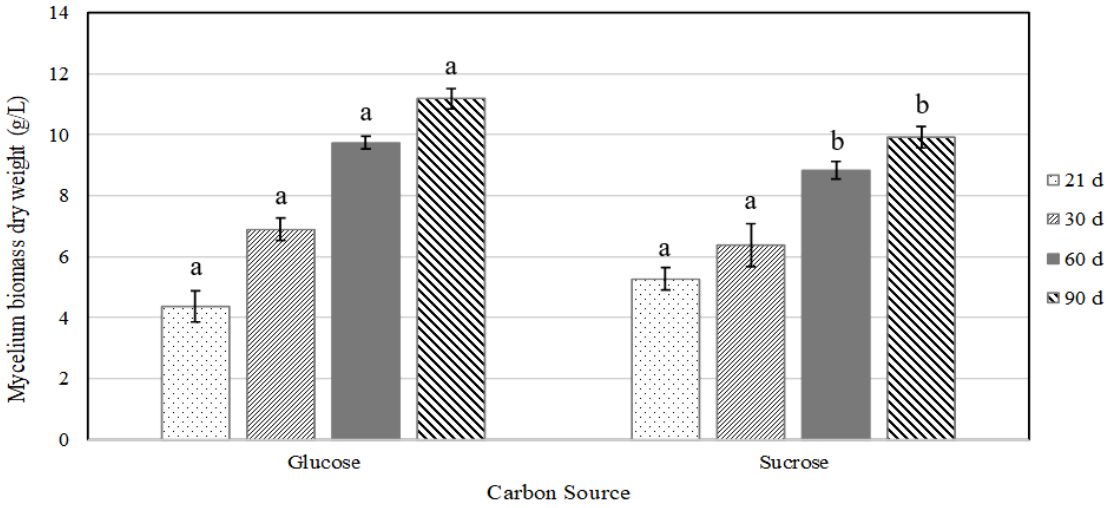

Fig. 1 – Dry weight of harvested *Grifola frondosa* strain AM mycelium from glucose or sucrose (3% w/v) at various cultivation times. Different letters on bars at the same cultivation time mean significantly different between two carbon sources according to t-test ($P \leq 0.05$) ($n = 3$).

Effects of sucrose concentration on *G. frondosa* AM biomass and bioactive polysaccharides

Sucrose was further used as the primary carbon source in the fermentation medium. To find the optimal sucrose concentration for mycelium biomass and bioactive polysaccharides such as EPS, IPS, and β-glucan, sucrose was applied in the *G. frondosa* culture at various concentrations ranging from 2 to 6 percent (w/v). At sucrose concentration of 4%, 5%, or 6% (w/v), mycelium biomass was not significantly different from each other, but they were significantly higher than biomass obtained from 2% and 3% sucrose (Fig. 2A). However, the EPS powder (total precipitates from culture broth) obtained from 6% sucrose was significantly higher than the others ($p<0.0001$), except the 2% sucrose.

The amounts of IPS from mycelium and EPS from culture broth, extracted from EPS powder, at various sucrose concentrations were investigated. For the IPS content, there was a no significant difference in yield among the sucrose concentrations of 4%, 5% and 6% (w/v), but they were

significantly higher than 2% and 3% sucrose ($p<0.0001$) (Fig. 2B). For the EPS content, it was found highest at 6% sucrose and was significantly different from other concentrations.

The content of beta-glucans was also observed in IPS from mycelium and in EPS from culture broth, extracted from EPS powder, at various sucrose concentrations. It demonstrates that β-glucans in both IPS and EPS increased with the increasing of sucrose concentration (Fig. 2C). Beta-glucans extracted from the mycelium grown in medium containing 5% or 6% (w/v) sucrose showed similar output with a no significant difference, but they were significantly higher than the others ($p<0.0001$). However, the highest content of β-glucans (24.3% w/w) was observed from EPS fraction obtained from culture broth containing 6% sucrose. It was significantly higher than in other EPS fractions.

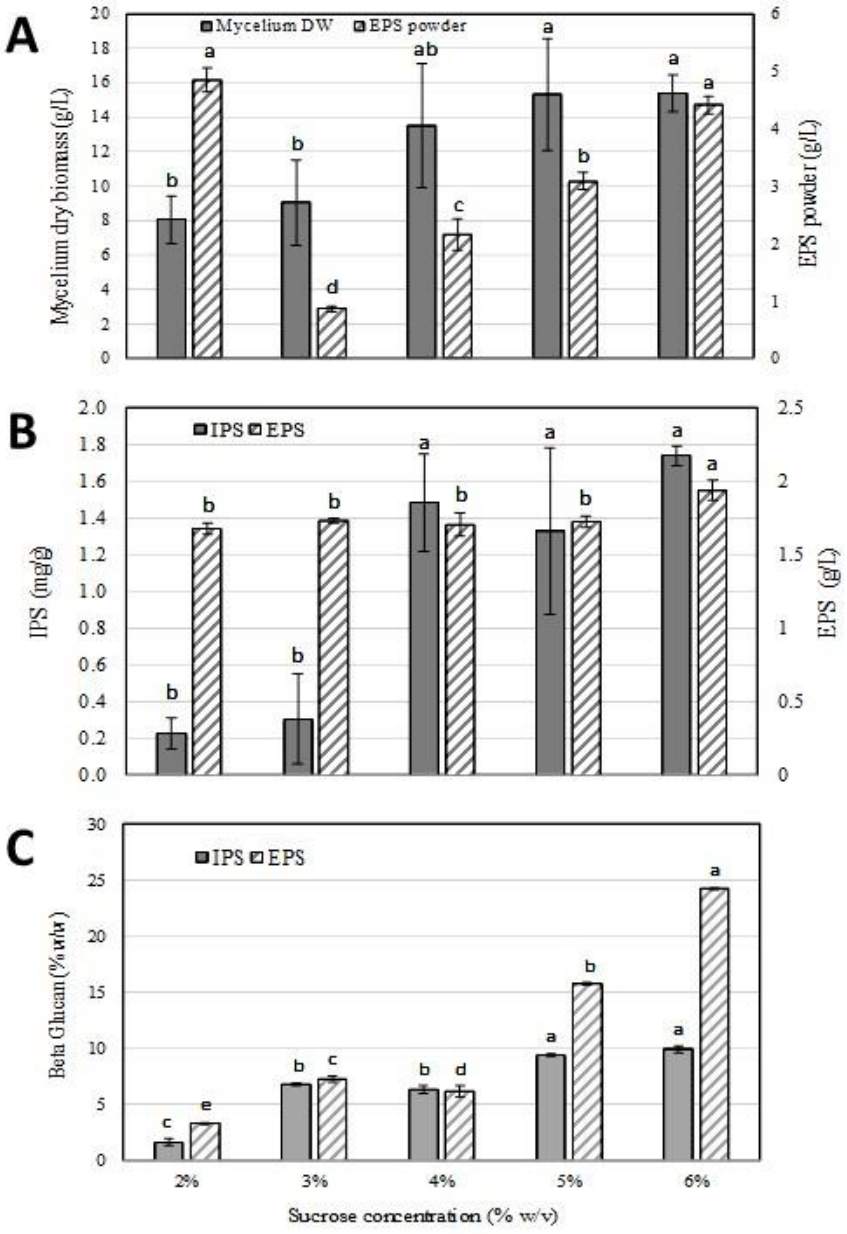

Fig. 2 – Yields from *Grifola frondosa* strain AM grown in fermentation medium containing sucrose at various concentrations: A dry weight of mycelium and total precipitates (EPS powder) from culture broth. B intracellular polysaccharides (IPS) from mycelium and exopolysaccharides (EPS) from culture broth. C β-glucans in the fraction of IPS and EPS. Different letters on bars in the same category mean significantly different according to ANOVA using Tukey's comparison ($P\leq0.05$) ($n = 3$).

Based on the observations, sucrose concentration at 6% (w/v) was optimal and provided satisfactory yields of mycelium biomass and bioactive polysaccharides (IPS, EPS, and β-glucan). Therefore, fermentation medium containing 6% sucrose was further used to observe whether different submerged culture conditions would affect the growth and the content of bioactive polysaccharides of *G. frondosa* strain AM.

Effects of light and agitation on *G. frondosa* biomass and bioactive polysaccharides

G. frondosa strain AM was grown in submerged culture with 6% sucrose at different conditions. The active mycelium were cultivated under light or darkness, with or without flask shaking for 30 days. Then mycelium biomass and bioactive polysaccharides were analyzed.

Overall, highest amount of both mycelium biomass and the EPS powder were obtained under darkness with shake condition and were significantly different from other conditions ($p < 0.0001$) (Fig. 3A). Mycelium significantly produced when the culture was grown in the dark, regardless of a flask shake. Significantly high amount of EPS powder was obtained from shaking conditions, regardless of light perception.

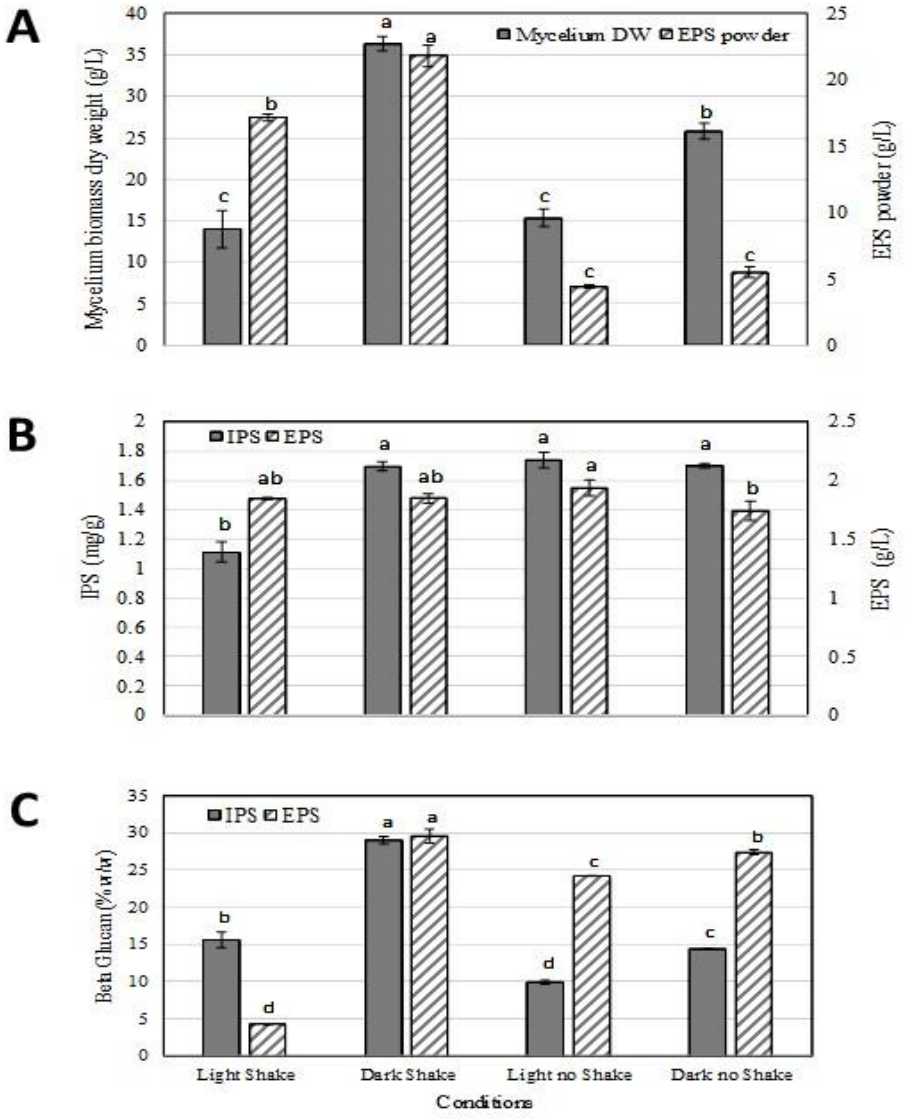

Fig. 3 – Yields from *Grifola frondosa* strain AM grown in fermentation medium containing 6% (w/v) sucrose at different cultivation conditions: A dry weight of mycelium and total precipitates (EPS powder) from culture broth. B intracellular polysaccharides (IPS) from mycelium and exopolysaccharides (EPS) from culture broth. C β-glucans in the fraction of IPS and EPS. Different

letters on bars in the same category mean significantly different according to ANOVA using Tukey's comparison ($P \leq 0.05$) ($n = 3$).

For the IPS content, there was a no significant difference in yield under 3 conditions (dark with shake, dark without shake, and light without shake), but they were significantly higher than that under the light with shake condition ($p<0.0001$) (Fig. 3B). For the EPS content extracted from EPS powder, flask shaking did not have an effect on yield regardless of light perception. EPS yield from culture grown under light condition was significantly higher than that grown in the dark when there was no flask rotation applied.

Investigation of beta-glucans in IPS and EPS fractions demonstrates that β-glucans significantly obtained at maximum from the culture grown in the dark with shake condition ($p<0.0001$) (Fig. 3C). The β-glucans in IPS fraction (mycelium) were significantly produced at a high amount from the shaking conditions, regardless of light perception. Moreover, β-glucans in EPS fraction (broth culture) were significantly obtained at high content when the culture was grown in the dark, regardless of a flask shake.

Effects of light and agitation on mycelial morphology of *G. frondosa* AM

There was a difference in mycelial characteristic visually observed in different cultivation conditions. The mycelium grown in the culture at static condition grew densely and mainly above the liquid media (Fig. 4A, B). In contrast, the mycelium grown in the culture with flask rotation mostly appeared in an agglomerated hairy pellets with core of circular shape and immersed in the media (Fig. 4C, D). The size of pellets under darkness was more uniform than those in light condition. There was a mix morphology of mycelium between matted filaments and pellets under darkness with flask shake (Fig. 4D). The morphology of *G. frondosa* AM mycelium under those conditions was also observed using SEM with magnification of 1000x (Fig. 5, left) and 3500x (Fig. 5, right). Without flask shaking conditions, mycelium cultivated under light (Fig. 5A) appeared thinner, loosen and longer in fiber length, compared to that in darkness (Fig. 5B). With flask shaking conditions, the mycelium looked denser and displayed tight–knit layers of hyphae with minimal loose fibers to be seen, especially in dark condition (Fig. 5C, D).

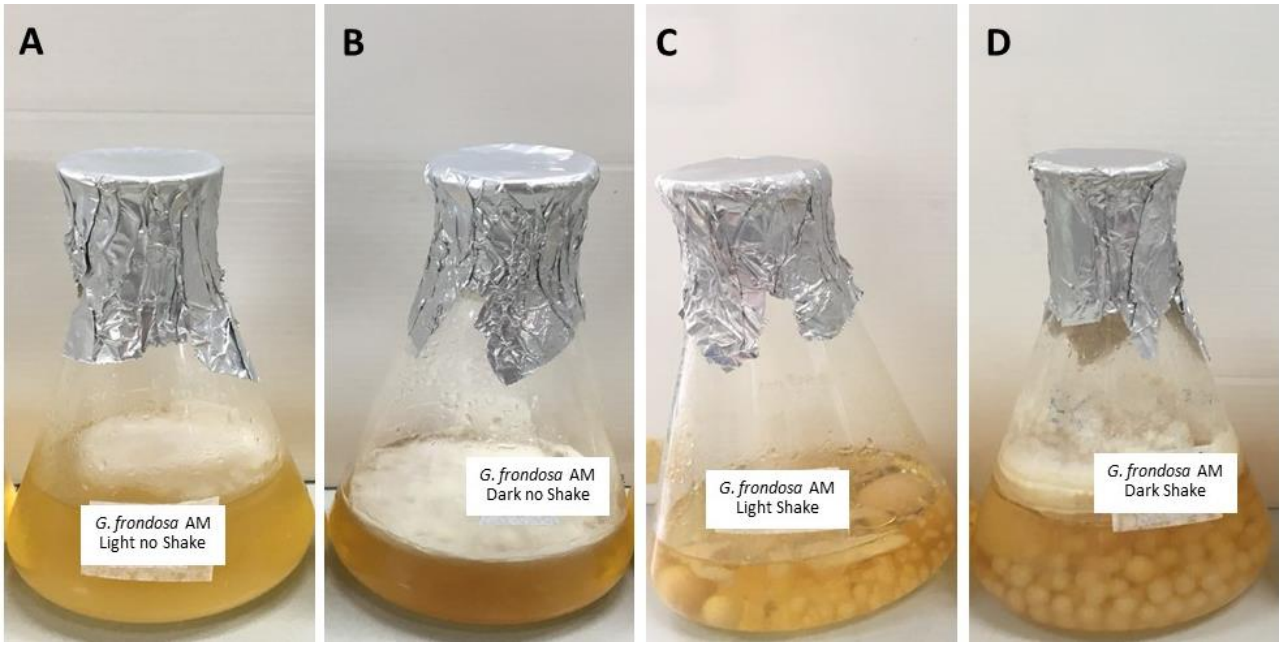

Fig. 4 – Mycelial morphology of *Grifola frondosa* strain AM cultured in fermentation medium containing 6% (w/v) sucrose in submerged conditions for 30 days: A light without shake. B dark without shake. C light with shake. D dark with shake. Images were captured in the laboratory setting.

Discussion

Cultivation period

For optimization of the cultivation period, submerged culture of *G. frondosa* AM for 30 days in either glucose or sucrose provided sufficient carbon for mycelial growth with a high productivity rate at 0.230 ± 0.012 g/L·d and 0.212 ± 0.023 g/L·d, respectively (Fig. 1). Mycelial biomass from refined sugar was slightly lower but not significant than that from glucose, but it would allow for a much less operational cost (at least 62.5 times in a cost reduction per 1 kg of sugar). Growing further longer to 60 days or 90 days in fermentation culture would increase the mycelial biomass, but the productivity rate per day was lower compared to 30 days cultivation. Mycelial biomass in glucose was higher than that in sucrose at days 60 and 90. It could be explained by that glucose is readily available for nutrient uptake since it is a monosaccharide. In contrast, sucrose is a disaccharide composed of glucose and fructose. It might need a breakdown by invertase before utilization in the forms of glucose-6-phosphate or fructose-6-phosphate in the cells (Chao et al. 2011, Patyshakuliyeva et al. 2013). However, mycelial growth of *Rigidoporus ulmarius* can use glucose or sucrose as the carbon source without any differences in biomass for 49 days of cultivation (Chao et al. 2011).

Kim (2003) cultured *G. frondosa* (KACC51146) on the sucrose-asparagine (SA) medium consisting of 20 g/L of glucose. The study revealed that the mycelial dry weight of *G. frondosa* reached the maximum at day 21, while polysaccharide was at the highest peak at day 18 of cultivation. However, both biomass and polysaccharide constantly produced until day 30 from its peak. Our finding agreed with this work in the fact that cultivation of *G. frondosa* AM in medium consisting of either glucose or sucrose for 30 days provided favorable productivity rate of mycelial growth and polysaccharide production (Fig. 2). Moreover, several studies indicated that yields from medicinal mushrooms usually at their peaks in less than 30 days of cultivation in a small scale or stirred-tank fermenter since sugar depletion would prevent the EPS production and mycelial growth (Elisashvili 2012).

Sucrose concentration

At 30 days of cultivation, it allowed mycelia to grow and to synthesize bioactive polysaccharides in a dose-dependent manner of sucrose concentrations (2%-6% w/v) in most cases. Increased amount of sucrose seems to stimulate the fungal growth to the maximum point at 4% w/v sucrose and crude polysaccharides (EPS powder) to the maximum point at 6% w/v of sucrose (Fig. 2A). IPS production was corresponded with the growth of mycelium, as well as the EPS content was obtained highest at 6% w/v (Fig. 2B). Furthermore, increased content of the β-glucans was observed in both IPS and EPS fraction with the increasing of sucrose concentration. *G. frondosa* AM mycelia could produce and secrete as high as 24% w/w of β-glucan, which could be found at 6% w/v sucrose (Fig. 2C). Elisashvili et al. (2012) concludes from several studies that when the amount of carbon source is sufficient for mycelia production, it would produce abundant exopolysaccharides. However, too much of sugar in the media might prevent cell growth due to high osmotic pressure in some species. For example, at 4% w/v glucose inhibited the growth of *Lentinus edodes* and *Pleurotus tuberregium* (Elisashvili et al. 2012). Our finding suggested that *G. frondosa* could tolerate the osmotic pressure from sucrose at the concentration as high as 6% w/v. To note that *G. frondosa* AM is well responding to the increased sucrose concentration, it would be interesting to observe the invertase activity in the future.

Several studies showed that the fungal growth and polysaccharides production improved when the carbon source concentrations increased. Tang et al. (2008) reported that sucrose was a suitable carbon source for the Chinese truffle (*Tuber sinense*) in the submerged fermentation. The cell growth and polysaccharides (EPS and IPS) production was increasing in the culture containing 2% to 8% w/v of sucrose, but constantly produced when sucrose exceeded 8% w/v. They also mentioned that the cell growth could be obtained higher (in a reduced productivity rate) in the culture containing sucrose concentrations at 9.5–12.5% w/v, but the cultivation period has to be

extended. Chao et al. (2011) reported that increasing sucrose concentration from 0.5% to 2% w/v would enhance mycelium of Basidiomycete *Rigidoporus ulmarius* to synthesize polysaccharides with the high molecular weight (>600 kDa), including the β-glucans with medicinal functions. Our finding agreed with these works to the viewpoint that yields were correlated with the level of sugar, but at the different range of concentrations. This suggested that cell growth and polysaccharides production could be influenced by sugar at preferable concentration for each type of mushrooms. In our case, 6% w/v of sucrose was suitable for *G. frondosa* AM the most.

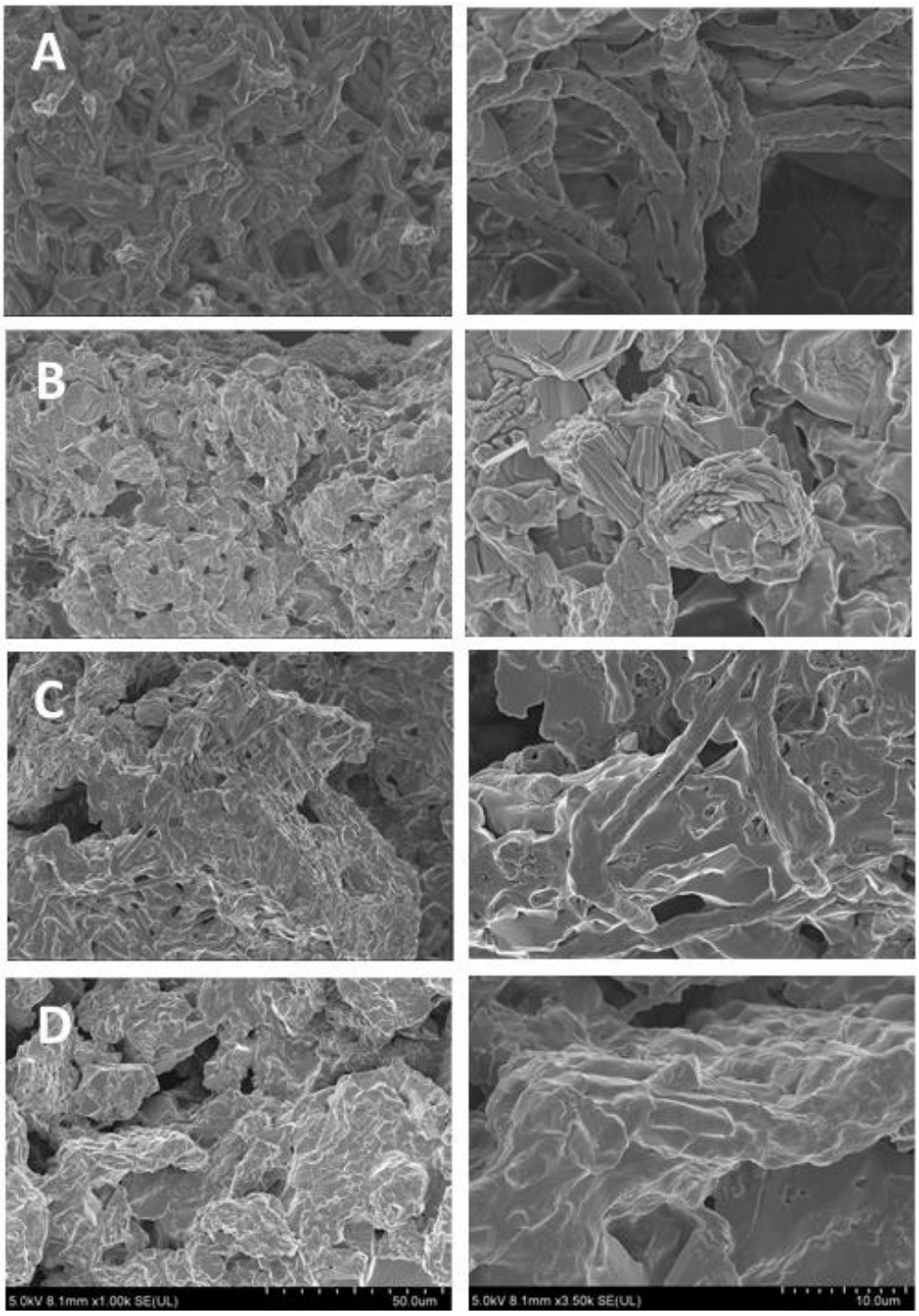

Fig. 5 – Mycelial morphology of *Grifola frondosa* strain AM cultured in fermentation medium containing 6% (w/v) sucrose in submerged conditions for 30 days: A light without shake. B dark without shake. C light with shake. D dark with shake. Images made by scanning electron microscopy (SEM) at magnification of x 1000 (left) and x 3500 (right).

Light and agitation

Recent studies indicate that light and agitation could improve the production of biomass and important metabolites and change the fungal characteristic. Flask shaking was usually applied during the cultivation of medicinal mushrooms in many studies while optimizing on the other factors, but with the single-factor experiment (Shih et al. 2008, Tang et al. 2008, Zhang et al. 2016). In this study, a combination of light and agitation clearly have effects on yields and mycelial morphology. Overall, *G. frondosa* AM grown in darkness with flask shake was positively induced especially for the cell growth and β-glucan production (Fig. 3A, C). The differences of biomass and EPS powder between the growing conditions (Fig. 3A) may be the results of the growth response of mushroom to the particular condition itself and from the level of polysaccharides and other substances precipitated in the crude polysaccharides. Mycelium biomass is mainly comprised of polysaccharides in the fungal cell wall (Fesel & Zuccaro 2016) and other macromolecules (e.g. proteins and lipids) and small molecular substances (e.g. secondary metabolites), while EPS powder could be substances (e.g. nutrients in the media, biomolecules and metabolites secreted from the fungal) presented in the culture broth (Elisashvili 2012). These molecules may also co-precipitate and present in the matrix during the extraction (Zhu et al. 2014).

When considering the production of IPS and EPS, the content may seem comparable among the samples (Fig. 3B), but it is to be cautioned here that this was normalized by weight of total mass (Fig. 3A) of each condition before polysaccharide quantification. The content would significantly show different level of the total IPS and total EPS between the growing conditions. Moreover, different cultivation modes (i.e. carbon source, aeration, agitation) can influence fungal growth behavior, specifically cell wall alteration (Nemcovie & Farkas 2001, Ibrahim et al. 2015). The cell wall is a dynamic structure that can adapt to morphological changes as evidence from SEM analysis (Fig. 5). Aguilar-Uscanga & François (2003) reported an overall biomass reduction while discovered that chitin content increased two-to threefold more in *Saccharomyces cerevisiae* cultivated in shake flasks compared to bioreactor. The impact from collision caused cell wall composition shifted towards a higher chitin content, which can contribute to the strengthen of the cell wall due to shear forces. Even though the overall amount of IPS seems coincidentally unimpacted by this phenomenon, there were actual changes in the quantity of β-glucans, as evidence in Fig. 3C. It is reasonable to assume that amount of chitin might be altered as well (not measured). However, an increase in β-glucan production is more desired due to its medicinal value. It is also interesting to note here that the yield of mycelium in dark conditions (with or without shaking) are higher than that in light conditions. This is in agreement with the denser and compact mycelia morphology from dark conditions (Figs 4, 5). This phenomenon will be very beneficial to operational cost when upscaling the production.

Fungi use light as an environmental signal for growth and development and secondary metabolite production, but not as a source of energy. They have perception mechanisms for broad spectrum of lights (i.e., UV, blue, green, and red/far-red light) (Idnurm & Heitman 2005). To date, there has been no report on the effect of light on the growth of *G. frondosa*. The possible mechanism of effect of light on the growth of mushroom could be various depending on the mushroom species. Shrestha et al. (2006) observed that light might have displayed some degree of inhibitory effects on the growth rate of *Cordyceps militaris* mycelium as the dark incubation led to a more rapid growth and more mycelium density on agar plate when compared to the light incubation. It has been documented that light can control the nutrient uptake in fungi. For example, light could inhibit the uptake of an essential nutrient such as glucose in *Aspergillus ornatus*, resulted in a decreased metabolism and inhibition of cell growth (Tisch & Schmoll 2010). There are a number of studies shown that light also controls other metabolic processes, such as altered the amount of carbohydrates, but it could be varied from species to species. For example, polysaccharides biosynthesis in *Penicillium isariiforme* is induced, but a decrease of glycogen content is observed in *Trichoderma reesi* upon cultivation in light (Tisch & Schmoll 2010). Nemcovie & Farkas (2001) reported that the activity of enzymes involved in biosynthesis of cell wall components (chitin and β-glucan) of *T. viride* is also altered due to the light. An increase in

specific activity of β-1,3-glucan synthase by 130% and a decrease in specific activity of chitin synthase by 50% was observed when compared to dark controls. It was found that the cell walls accumulate β-1,3-glucan by 35-50% more while the content of chitin remained essentially constant. Thus, morphological changes could be seen due to the alteration of cell wall components that determine the shape of the cell wall (Nemcovie & Farkas 2001).

Morphology of the fungal could determine the target product. The mushroom mycelia can appear in different shapes such as pellet, clump, or filament depending on the culture conditions and environmental parameters including agitation speed. Agitation promotes the mass transfer of nutrients, heat, and oxygen (Shih et al. 2008, Tang et al. 2008, Elisashvili et al. 2012). Our finding is in agreement with several studies in regards to agitation, which helps *G. frondosa* AM produced more biomass and bioactive polysaccharides (Fig. 3). However, agitation may damage filamentous formation and cause sticky pellets to agglomerate resulted by shear forces, as seen in our study (Fig. 4). Shear forces can create stress on cell structures by cell collision, therefore it can cause the morphological changes of the fungal and yield formation (Fig. 5). Ibrahim et al. (2015) studied the effect of agitation speed at 50-250 rpm on the mycelial morphology and pectinase production of *Aspergillus niger* HFD5A-1 in the flask system. They reported that high intensity of agitation can reduce the freely mycelia formation, but increased the pectinase activity. Mycelial mat was formed at static condition while increased in agitation speed resulted in clumps to spherical pellets. From SEM analysis, the mycelia shape was cylindrical and branched in static condition, but changed to tight packing structures at the agitation speed of 100 rpm (Ibrahim et al. 2015). Thus, it is probably due to the shear forces have some positive effects on the enzymes that possibly change the cell wall components, thereby caused the morphological change of the fungal, as observed in the present study.

Conclusion

The productivity rate of *G. frondosa* AM mycelia growth in fermentation media decreased with cultivation time. The optimal growth condition for *G. frondosa* AM in submerged cultivation was 6% (w/v) sucrose grown in the dark at the flask agitation speed at 120 rpm. *G. frondosa* AM cultivation in darkness can change the mycelial morphology from loosely filamentous to a tight-packing shape. Higher amount of bioactive polysaccharides (i.e. β-glucans) might be achieved by increasing the concentration of sucrose and agitation speed, but the operational cost, osmotic pressure, and shear stress effect should be in considerations.

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