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Use of Stimulatory Agents To Enhance the Production of Bioactive Exopolysaccharide from *Pleurotus tuber-regium* by Submerged Fermentation

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ABSTRACT: Fatty acids, organic solvents and surfactants were investigated for their stimulatory effects on the growth of fungal mycelium and production of exopolysaccharide (EPS) by submerged fermentation of an edible mushroom *Pleurotus tuber-regium*. Addition of 3.0 g/L Tween 80 at the late stage of exponential growth phase provided the best stimulatory effect on mycelial biomass and fungal EPS production with a 51.3 and 41.8% increase, respectively. The chemical structure of the EPS produced with addition of Tween 80 was found to be a glucomannan with similar monosaccharide composition and glycosidic linkages but a significantly lower molecular weight ($3.18 \pm 0.09 \times 10^6$ Da), compared to the control ($4.30 \pm 0.12 \times 10^6$ Da). These two EPS could significantly inhibit the growth of chronic myelogenous leukemia K562 cells in a dose dependent manner, with an estimated IC_{50} value of 43.7 and 47.6 μg/mL, respectively. The use of stimulatory agents to enhance production of bioactive fungal EPS can be applied in other fungal fermentation processes for enhancing production of useful metabolites.

KEYWORDS: exopolysaccharide, mushroom mycelium, *Pleurotus tuber-regium*, submerged fermentation, stimulatory agents

INTRODUCTION

Nowadays, mushrooms have attracted much attention as a functional food and as a source for the development of drugs and nutraceuticals. In many Asian countries, mushrooms such as lingzhi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*), and yiner (*Tremella fuciformis*) that have been collected, cultivated and used for hundreds of years are being evaluated as edible and medicinal resources.[1] Polysaccharides are the best known and most potent mushroom-derived substances with a number of medicinal properties. *Pleurotus tuber-regium*, an edible mushroom first discovered in Africa, is used as a functional food to promote health and longevity.[2] The nonstarch polysaccharides extracted from the sclerotium, mycelium and culture medium of *P. tuber-regium* have been demonstrated to have both immunomodulatory and direct cytocidal antitumor activities.[3−5] Commercial cultivation of the fruiting body and sclerotium of *P. tuber-regium* is rare, and the supply of this mushroom from the wild cannot meet the growing demand.[6] Because of the low biomass conversion and long harvest time, there is only limited success when cultivating the sclerotium of *P. tuber-regium* using solid compost in the laboratory.[7,8] Our previous work in growing the mycelium of *P. tuber-regium* in a defined medium by submerged fermentation has been found to be a simple, fast and efficient alternative method for producing exopolysaccharides (EPS) that have similar bioactivities as the polysaccharides isolated from this edible mushroom.[3,9]

Many factors may affect the production of mushroom mycelial EPS by submerged fermentation, including but not limited to carbon and nitrogen source, carbon-to-nitrogen ratio, mineral elements, oxygen supply, initial pH and temperature.[10−14] Hence, many investigations have been conducted on the optimization of submerged culture conditions. Recently, there is an increasing interest in the search for chemical agents to stimulate mushroom mycelial EPS production by submerged fermentation. These stimulatory agents include vegetable oil, fatty acids, and surfactants which were originally used as antifoam agents.[15,16] Many of them have been proved to be effective stimulatory agents in the production of useful metabolites in bacteria, fungi and medicinal mushrooms. It was reported that detergents improved the yield and quality of xanthan in cultures of *Xanthomonas campestris*, with 0.1 g/L Triton X-100 having a 1.5-fold increase in the production of the xanthan gum.[17] In another study, addition of 1.0% (v/v) methanol in cultures of *Aspergillus niger* GCB-47 doubled the yield of citric acid.[18] EPS production in *Ganoderma lucidum* was markedly increased with addition of safflower oil.[19] These stimulatory agents were presumed to mediate cell permeability by reorganizing the cell membrane and/or directly affecting synthesis of enzymes involved in the formation of target products.[20] Compared to the wide application in bacteria and other fungi, the use of chemical agents to enhance edible mushroom mycelial EPS production by submerged culture is a relatively new approach.

So far, the effects of fatty acids, organic solvents and surfactants on mycelial growth and EPS production by submerged fermentation of *P. tuber-regium* have not been reported yet. The objectives of this work were to evaluate the enhancement effects of these stimulatory agents on the production of mushroom mycelial biomass and EPS as well as to compare the physicochemical characteristics and biological activity of the EPS produced with and without the addition of stimulatory agents.

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■ MATERIALS AND METHODS

Inoculum and Culture Conditions. The culture of *P. tuber-regium* was obtained from Fungi Perfecti, LLC (Olympia, USA). Stock culture was maintained on potato dextrose agar (PDA) plate and subcultured periodically.

To prepare the inoculum, 10-day-old mycelia in PDA agar were transferred to the seed medium by punching out 25 pieces of 1 cm^2 of the agar with the sterilized cutter. The seed culture was incubated in a 250 mL conical flask containing 50 mL of seed culture medium for 4 days. Then shake-flask culture experiments were carried out in 250 mL conical flasks containing 100 mL of culture medium after inoculating with 10% (v/v) of the seed medium. The defined medium contained 30 g/L glucose, 4 g/L yeast extract, 1 g/L KH_2PO_4, and 0.6 g/L $Mg_2SO_4 \cdot 7H_2O$. The cultures were incubated at 30 °C in a rotary shaker incubator at 200 rpm.

Chemicals. A total of 13 stimulatory agents in 3 chemical categories were added into the culture medium at various concentrations on the first day of fermentation as shown in the table and figure captions. They include (1) fatty acids (linoleic acid, oleic acid, palmitic acid, stearic acid), (2) organic solvents (methanol, ethanol, hexane, chloroform, toluene), and (3) surfactants [Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 80 (polyoxyethylene sorbitan monooleate), CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), Triton X-100 (polyoxyethylene octyl phenyl ether)]. All the chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Determination of the Yield of *P. tuber-regium* Mycelia and EPS. Mycelia from different submerged fermentation systems were filtered through a Whatman #4 filter paper to separate the mycelia and culture medium. The mycelia obtained were washed twice with distilled water, and then the yield of mycelial biomass was determined gravimetrically after lyophilization. Four volumes of 95% ethanol were added to the mycelium-free culture medium, and overnight precipitation of EPS was allowed. The precipitated EPS were separated by centrifuging at 3838*g* for 15 min, and the supernatant was discarded. The yield of EPS was determined gravimetrically after lyophilization.

Carbohydrate and Protein Content of EPS. The carbohydrate and protein content of all the EPS obtained above were analyzed by the phenol−sulfuric acid method[21] and the modified Lowry method,[22] respectively.

Monosaccharide Composition. All the EPS isolated from the culture medium were subjected to sequential acid hydrolysis (12 M sulfuric acid for 1 h at 35 °C, and then 2 M sulfuric acid for 1 h in a boiling water bath). Alditol acetates of the neutral and amino sugars in the acid hydrolysate were prepared according to the method described by Blakeney et al.[23] with β-D-allose as the internal standard. Alditol acetates of the monosaccharides were quantified by an HP6890 series II gas chromatography, using an Alltech DB-225 capillary column (15 m × 0.25 mm i.d., 0.25 μm film) with the following oven temperature program: initial temperature, 170 °C; temperature rise at 2 °C/min to 220 °C and final hold for 15 min. The carrier gas was helium and detection was by flame ionization.

Molecular Weight (M_w) Determination. A TSK gel G5000 PW size exclusion column (30 cm × 7.5 mm i.d., Cat. No. 8-05764, Supelco) with a PWH Guard column (7.5 cm × 1.5 mm i.d., Cat. No. 8-06762, Supelco) were used to determine the M_w profile of EPS with and without addition of Tween 80 according to the profile given by a range of pullulan standards (6, 12, 24, 48, 112, 212, 404, 788 kDa; Shodex Standard P-82, Showa Denko). The flow rate of the eluent (0.2 M sodium chloride) was 0.8 mL/min and the temperature of the column was controlled externally at 25 °C. The samples and standards were dissolved in 0.2 M sodium chloride, filtered by the 0.45 μm Millipore filter, passed through the size exclusion column, and then were detected by a Waters 2414 refractive index (RI) detector. M_w of *P. tuber-regium* EPS was estimated from a calibration curve obtained from the retention time of pullulan standards against the log value of their molecular weights.

EPS Linkage Analysis. The sugar linkages of the EPS with and without addition of Tween 80 were analyzed by methylation using the method described by Anumula et al.[24] followed by GC−MS of the partially methylated alditol acetate (PMAA) derivatives. In brief, methylsulfinyl methyl sodium was prepared from sodium hydride and dimethyl sulfoxide (DMSO). EPS that was insoluble in DMSO was premethylated by methylsulfinyl methyl sodium and methyl iodide prior to the normal methylation procedures. The PMAA samples were prepared from the methylated EPS by acid hydrolysis (2 M trifluoroacetic acid at 121 °C for 1 h), reduction of the hydrolyzed sugars by sodium borohydride, and acetylation by acetic anhydride. The PMAA samples were analyzed by gas chromatography (Agilent Technology, 6890N)−mass spectrometry (Agilent Technology, 5973N). The GC conditions were as follows: Alltech DB-225 capillary column (15 m × 0.25 mm i.d., 0.25 μm film); helium as carrier gas at a flow rate of 1.0 mL/min; initial oven temperature at 160 °C, followed by 4 °C/min rise to 220 °C and final hold for 15 min; injector temperature at 280 °C; interface temperature at 280 °C. The MS conditions were as follows: ion source temperature at 250 °C, ionization energy at 70 eV, detector voltage at 1.5 kV, and mass range from 50 to 350. Each PMAA was identified by matching its mass spectrum with the NIST/EPA/NIT database in the computer.

Cell Lines and Cell Viability Assay. Chronic myelogenous leukemia K562 and monkey normal kidney Vero cell lines were purchased from American Type Culture Collection (ATCC). The K562 cells were grown in IMDM medium, and Vero cells were grown in RPMI-1640 medium. Both media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin, incubating under an atmosphere of 5% CO_2 at 37 °C.

Cell viability of treated suspended K562 cells was measured by trypan blue dye exclusion method based on the ability of viable cells to exclude the dye. An amount of 2.5 × 10^3 cells were seeded in each well of a 96-well microplate. The EPS with and without addition of Tween 80 at final concentrations of 12.5, 25, 50, 100, 200, and 400 μg/mL were incubated with the cells for 72 h at 37 °C. The number of viable cells that excluded the trypan blue dye was counted using a hemacytometer. The treatment groups were compared with control groups in the absence of EPS. The result was expressed as inhibition ratio calculated as [(number of control cells − number of treatment cells)/number of control cells]. The value of IC_{50}, which is the concentration of EPS required to inhibit the K562 cells by 50% of the control level, was estimated from the plot.

Statistical Analyses. All statistical analyses were performed by using the software SPSS Statistics 17.0 (SPSS, Chicago, Illinois). All the data obtained were analyzed by one-way ANOVA, and tests of significant differences were determined by using Tukey multiple comparison or Student's *t* test at $p < 0.05$.

■ RESULTS AND DISCUSSION

Time Course Study. A time course study of mycelial biomass and EPS production in the submerged culture of *P. tuber-regium* is shown in Figure 1. There was almost no lag period because the mycelial cells were activated during the 4-day preinoculation seed culture period. After inoculation, a rapid increase of EPS concentration was observed from day 1 to day 6, which was in line with the exponential growth of mycelial cells (Figure 1). The EPS production increased with the mycelial cell growth and reached a maximum level of 0.73 g/L after 7 days of fermentation, while the maximum mycelial biomass production occurred at 6.86 g/L before entering the stationary phase. Based on these observations,

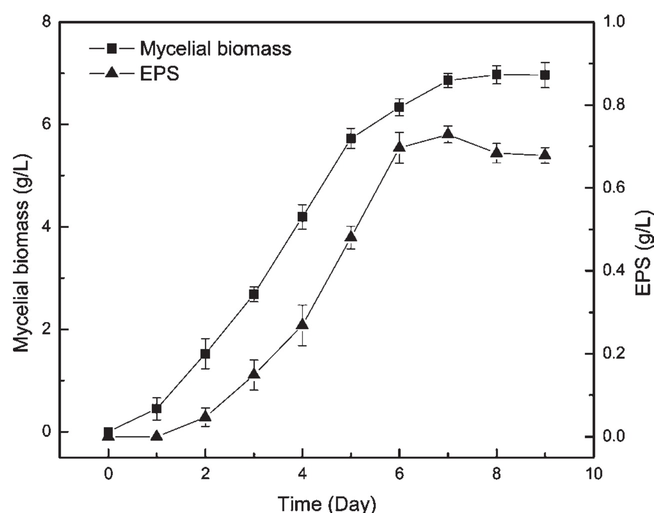

Figure 1. Time profiles of mycelial biomass and EPS productions in submerged fermentation of *P. tuber-regium*. (Values are mean ± standard deviation, $n = 3$.)

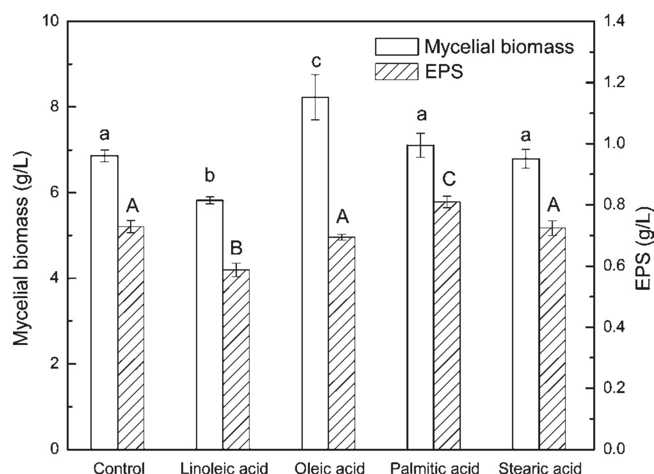

Figure 2. Effect of various fatty acids on mycelial biomass and EPS productions in submerged fermentation of *P. tuber-regium*. The concentration of fatty acids was 1.0 g/L. Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, $n = 3$) having different lowercase and uppercase letters, respectively, have significant difference (ANOVA Tukey's test; $p < 0.05$).

all subsequent submerged fermentation experiments were carried out for a maximum of 7 days.

Effect of Fatty Acids. To accelerate mycelial growth of some medicinal mushrooms such as *Ganoderma* species, plant oils have been proved to be an effective stimulatory agent.[19,25] The extent of enhancement depends on the types and composition of fatty acids present in plant oils. Pure fatty acids were used as potential stimulatory agent in the present study. The effect of fatty acids on mycelial biomass and EPS productions was studied by applying various fatty acids at 1.0 g/L into the culture medium (Figure 2). Among all the fatty acids studied, addition of palmitic acid and stearic acid did not have any significant effect on mycelial growth compared to the control. Oleic acid showed a strong stimulatory effect on mycelial biomass with a significant ($p < 0.05$) increase of 20.0%. In contrast, linoleic acid significantly ($p < 0.05$) inhibited the mycelial growth (Figure 2). The present results were consistent with the previous results,[16] in which mycelial growth of a medicinal mushroom *Cordyceps militaris* was remarkably increased by oleic acid and drastically suppressed by linoleic acid.

All the fatty acids used in the present study were the major components in the lipid of the mycelium of other *Pleurotus* species, *P. ostreatus* produced by submerged fermentation (palmitic acid 35.0%; stearic acid 5.5%; oleic acid 22.0%; linoleic acid 37.5%).[26] Therefore, the stimulatory/inhibited effect of these fatty acids on mycelial growth might be due to the partial incorporation into the fungal cell membrane thereby increasing/decreasing the uptake efficiency of nutrients from the culture medium. The relationship between the effect of these fatty acids on mycelial growth and the lipid composition of the mycelium of *P. tuber-regium* would be worthy of further study.

On the other hand, the effect of fatty acids on EPS production in the submerged fermentation of *P. tuber-regium* had no correlation with the effect on mycelial growth. Oleic acid, which increased the mycelial biomass by 20.0%, had no stimulatory effect on EPS production (Figure 2). In contrast, palmitic acid improved EPS production by 11.1% although it had no significant influence on mycelial growth. Linoleic acid suppress both mycelial growth and EPS production in *P. tuber-regium*, and this was in agreement with the results of Yang et al.,[19] in which EPS production by a medicinal mushroom *Ganoderma lucidum* was inhibited by linoleic acid.

Effect of Organic Solvents. The use of organic solvents is known to be a relatively effective method to increase cell permeability, which may enhance the release of EPS from the mycelial cells.[18,27]

The influence of five different organic solvents (methanol, ethanol, hexane, chloroform and toluene) on mycelial growth and EPS production in *P. tuber-regium* was studied by applying 0.1% (v/v) of individual solvents into culture medium. It was found that all the organic solvents, particularly chloroform and toluene, inhibited mycelial growth (Figure 3). When chloroform and toluene were added into the medium, the concentration of the corresponding mycelial biomass was only 53.1% and 62.4%, respectively, compared to that of the control. This result indicated that the addition of these organic solvents had a detrimental effect on cell growth, thereby significantly reducing the concentration of mycelial biomass.

The use of chloroform and toluene had a detrimental effect not only on the mycelial growth but also on EPS production, and this was not consistent with the previous results, in which these two organic solvents increased EPS production even though mycelial growth of *Collybia maculate* TG-1 was reduced.[27] This result suggests that the choice of cell-permeabilizing agent is species-specific and related to the composition of the cell wall and its membrane. In contrast, it is noted that the addition of methanol and hexane significantly ($p < 0.05$) increased EPS production by 17.7% and 15.5%, respectively, although they decreased mycelial biomass (Figure 3). The stimulatory effect of methanol addition on mushroom EPS production was similar with a previous study by Haq et al.,[18] in which methanol increased citric acid production by submerged culture of *Aspergillus niger* GCB-47. Based on the present results and those previously reported, it is thought that the increased production of mycelial EPS is a secretory function of organic solvent, which acts by modifying the outer cell wall structure, thereby facilitating the release of EPS from the inside of mycelial cells. However, use of organic solvents as stimulating agent for EPS production should be carefully

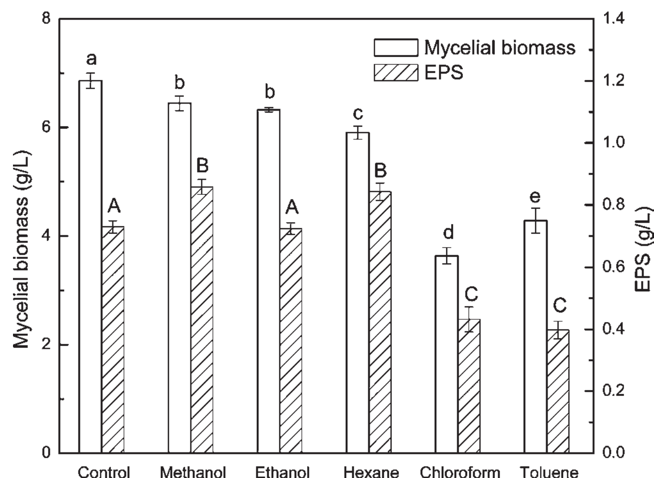

Figure 3. Effect of various organic solvents on mycelial biomass and EPS productions in submerged fermentation of *P. tuber-regium*. The concentration of organic solvents was 0.1% (v/v). Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, $n = 3$) having different lowercase and uppercase letters, respectively, have significant difference (ANOVA Tukey's test; $p < 0.05$).

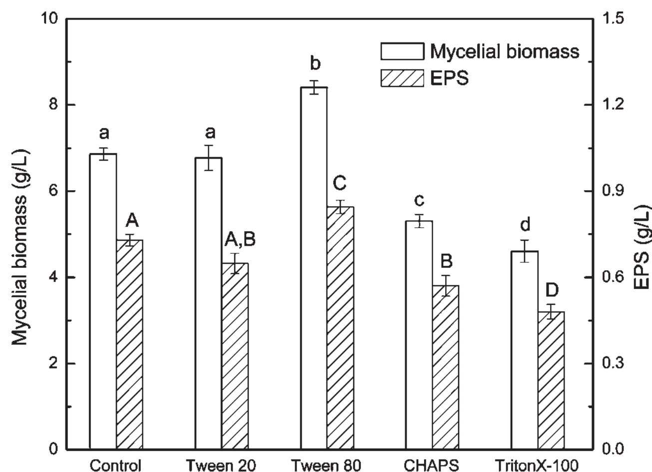

Figure 4. Effect of various surfactants on mycelial biomass and EPS production in submerged fermentation of *P. tuber-regium*. The concentration of surfactants was 1.0 g/L. Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, $n = 3$) having different lowercase and uppercase letters, respectively, have significant difference (ANOVA Tukey's test; $p < 0.05$).

controlled as cells may be subjected to lysis or destruction of the intracellular components by nonpolar solvents like chloroform and toluene. In general, the toxicity of chloroform and toluene on mycelial cells of *P. tuber-regium* may be much greater than that of methanol, ethanol and hexane, resulting in an apparent drop in the mycelial biomass and EPS production. Further studies on using methanol and hexane, which both had shown stimulatory effect for EPS production in submerged fermentation of *P. tuber-regium*, are required.

Effect of Surfactants. Addition of surfactants into the culture medium may be an effective strategy to increase the yields of mycelial cells and EPS in fermentation processes since this strategy was proved to be successful in the bacterial fermentation, in which addition of surfactants could increase the yield of xanthan in cultures of *X. campestris*.[17] To study the effects of surfactants in submerged fermentation of *P. tuber-regium*, four surfactants (1.0 g/L) were applied in the cultivation and the results of cell growth and EPS production are shown in Figure 4. It is interesting to find that, with the addition of two surfactants of the Tween series, Tween 80 showed a strong stimulating effect on both cell growth and EPS production while Tween 20 did not exhibit any favorable or detrimental effect. This result was similar to a previous study which had shown that the shorter carbon chain (12 carbon side chain in Tween 20 vs 18 carbon side chain in Tween 80) of surfactants exhibited a higher diffusion rate in cell wall and thus might damage the cell membrane or interact with other cellular biomolecules, decreasing cell vitality.[28] The maximum mycelial biomass and EPS production obtained with addition of 1.0 g/L Tween 80 reached 8.41 and 0.84 g/L, respectively, which accounted a significant enhancement ($p < 0.05$) of 22.6% and 15.0% in biomass and EPS, respectively (Figure 4). It is assumed that this stimulatory effect is likely due to the action between surfactant and cell membrane. In theory, surfactants are amphiphilic, containing both hydrophobic groups and hydrophilic groups. The fungal cell membrane also consists primarily of a layer of amphiphilic phospholipids. Hence, the surfactants might partially be incorporated into the fungal cell membrane thereby increasing the uptake efficiency of nutrients from the culture medium. In this way, nutrient absorption to cell surface can be greatly enhanced through the aid of surfactant.[29] Galindo et al.[17] also suggested one mode of the mechanism of the surfactant is by affecting mass transfer either by changing the surface film resistance or the hydrodynamics in the cell membrane.

In contrast, addition of CHAPS and Triton X-100 showed significant inhibition ($p < 0.05$) on the cell growth and EPS production of *P. tuber-regium* (Figure 4). This suggested that these two surfactants displayed higher toxicity and lower biocompatibility to the mycelial cells than Tween 20 and Tween 80. The present results were consistent with those reported by Nascimento et al.,[30] in which the addition of 1.0% (v/v) Tween 80 to a *Candida lipolytica* culture medium increased cellular viability, with maximum stimulation of protease secretion.

Based on the above results, use of palmitic acid, methanol, hexane and Tween 80 as additives could stimulate the EPS production in *P. tuber-regium*. Among these, Tween 80, which is a permitted food additive (E433), is safer than the other three chemical agents when used in food application. Therefore, Tween 80 was selected as the stimulatory agent in the subsequent experiments.

Effect of Concentration of Tween 80. The effect of different concentrations of Tween 80 on mycelial biomass and EPS production in submerged culture of *P. tuber-regium* is shown in Table 1. Production of mycelial biomass and EPS increased significantly ($p < 0.05$) as the concentration of Tween 80 increased from 0.5 to 3.0 g/L, beyond which no significant increase was observed. Higher concentration (5.0 g/L) of Tween 80 resulted in the generation of excessive foam, which has a detrimental effect not only on sterile environment but also on mass and heat transfer process in submerged culture.[31] Accordingly, the maximum amounts of mycelial biomass (9.26 g/L) and EPS (0.93 g/L) were obtained when 3.0 g/L Tween 80 was added to the medium (Table 1). This also corresponded to a significant increase ($p < 0.05$) of 35.0 and 27.4% in the production of mycelial biomass and EPS, respectively, when compared with the control.

Table 1. Effect of Concentration of Tween 80 on Mycelial Biomass and EPS Production in Submerged Fermentation of *P. tuber-regium*[a]

concn of Tween 80 (g/L)	mycelial biomass (g/L)	EPS (g/L)
0	6.86 ± 0.14 a	0.73 ± 0.020 a
0.5	7.24 ± 0.16 b	0.80 ± 0.017 b
1.0	8.41 ± 0.15 c	0.84 ± 0.024 b
3.0	9.26 ± 0.12 d	0.93 ± 0.016 c
5.0	8.95 ± 0.09 d	0.91 ± 0.014 c

[a] Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, $n = 3$) within each column having different lowercase letters have significant difference (ANOVA Tukey's test; $p < 0.05$).

Table 2. Effect of Addition Time of Tween 80 on Mycelial Biomass and EPS Production in Submerged Fermentation of *P. tuber-regium*[a]

addition time	mycelial biomass (g/L)	EPS (g/L)
control	6.86 ± 0.14 a	0.73 ± 0.020 a
1st day (24 h)	9.26 ± 0.12 b	0.93 ± 0.016 b
3rd day (72 h)	9.91 ± 0.13 c	0.94 ± 0.018 b
5th day (120 h)	10.38 ± 0.25 d	1.03 ± 0.018 c

[a] The concentration of Tween 80 was 3.0 g/L. Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, $n = 3$) within each column having different lowercase letters have significant difference (ANOVA Tukey's test; $p < 0.05$).

Effect of Addition Time of Tween 80. To better understand the effect of Tween 80 on the mycelial growth and EPS production, 3.0 g/L Tween 80 was added on the first, third and fifth days of cultivation, which represented the initial, intermediate and late stages of the exponential growth phase of the mycelia. Addition of Tween 80 at the late stage of the exponential growth phase had resulted in a significant increase in both mycelial growth and EPS production compared with the early growth stages (Table 2). Nascimento et al.[30] had reported the addition of Tween 80 to a culture medium of *C. lipolytica* would increase cellular viability. Accordingly, it is reasonable to assume that Tween 80 not only prolonged the viability of mushroom mycelia but also resulted in a higher EPS production. The optimum results were achieved when 3.0 g/L Tween 80 was added to the culture medium on the fifth day of the fermentation, corresponding to 51.3 and 41.8% significant increase ($p < 0.05$) in mycelial biomass and EPS production, respectively (Table 2).

Effect of Tween 80 on the EPS Composition. The carbohydrate and protein content of EPS from *P. tuber-regium* grown with and without addition of Tween 80 is shown in Table 3. There was no significant difference ($p > 0.05$) between carbohydrate and protein content of EPS from cultures with and without addition of Tween 80. The EPS had about 82% carbohydrate and 12% protein content. In other *Pleurotus* species, polysaccharides are bound with proteins or peptides as a polysaccharide−protein or −peptide complex which showed potent antitumor activity.[32] For example, *Pleurotus sajor-caju* produced protein-containing polysaccharides (76% carbohydrate, 24% protein) which showed 90.8% tumor inhibition of Sarcoma 180 in mice.[33]

The monosaccharide composition analysis indicated that the EPS of *P. tuber-regium* consisted of mainly mannose and glucose. However, the EPS composition did not significantly change irrespective of addition of Tween 80 (Table 3).

Table 3. Carbohydrate, Protein Content, Monosaccharide Composition and Molecular Weight of EPS with and without Addition of Tween 80 (3.0 g/L Added on the 5th Day)[a]

	control	with addition of Tween 80
carbohydrate content (%)	81.5 ± 2.4 a	82.2 ± 2.0 a
protein content (%)	12.0 ± 0.3 b	12.5 ± 0.2 b
monosaccharide composition (%)		
mannose	56.6 ± 1.2 c	57.5 ± 1.7 c
glucose	43.4 ± 1.2 d	42.5 ± 1.7 d
mol wt (Da × 10^6)	4.30 ± 0.12 e	3.18 ± 0.09 f

[a] Data (mean value ± standard deviation, $n = 3$) within each row having the same lowercase letters have no significant difference (Student's *t*-test; $p > 0.05$).

Table 4. GC−MS Data for Partially Methylated Alditol Acetates of EPS Produced with and without Addition of Tween 80 in Submerged Fermentation of *P. tuber-regium*[a]

	molar ratio		
partially methylated sugar	with Tween 80	without Tween 80	linkage type
3,4-Me_2-Man*p*	1.0	1.0	→2,6)-Man*p*-(1→
1,3,4-Me_3-Man*p*	1.5	1.5	→6)-Man*p*-(2→
2,3,4,6-Me_4-Glc*p*	1.5	1.5	Glc*p*-(1→

[a] Me: methyl residues. Man*p*: mannopyranosyl residues. Glc*p*: glucopyranosyl residue.

It was found that M_w is very important to the bioactivity of the mushroom polysaccharides. Polysaccharides having high M_w usually exert biological responses by receptors presented on the cell surface. From the size exclusion chromatogram results, *P. tuber-regium* produced a large M_w EPS with high homogeneity (data not shown). Moreover, the addition of Tween 80 in culture medium affected the M_w of EPS (Table 3). The EPS produced by *P. tuber-regium* with addition of Tween 80 had a signifcantly ($p < 0.05$) lower M_w ($3.18 \pm 0.09 \times 10^6$) than that produced without addition of Tween 80 ($4.30 \pm 0.12 \times 10^6$). Fungal polysaccharides having M_w higher than 2×10^5 are shown to have potent antitumor activity.[34] Therefore, the biological activities of high M_w EPS of *P. tuber-regium* produced with and without addition of Tween 80 were investigated in the last section of this study.

Methylation analysis using GC−MS analysis of the EPS produced with and without addition of Tween 80 both showed the presence of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-mannitol, 2,5,6-tri-*O*-acetyl-1,3,4-di-*O*-methyl-mannitol, and 1,5-di-*O*-acetyl-2,3,4,6-di-*O*-methyl-glucitol, in a molar ratio of about 2:3:3 and 2:3:3, respectively (Table 4). The mannose/glucose ratio of the EPS found in methylation (1.67: 1) (Table 4) was comparable to those found by GC analysis of monosaccharide (1.30−1.35: 1) (Table 3). Results of GC−MS analysis of EPS indicated the presence of [→2,6)-Man*p*-(1→], [→6)-Man*p*-(2→], and [Glc*p*-(1→] in the EPS produced by submerged fermentation of *P. tuber-regium* irrespective of addition of Tween 80. It seems that the EPS was mainly consisted of a mannan having a (1→6)-linked main chain of mannopyranosyl residues, almost all of which were branched at *O*-2 with side chain containing two

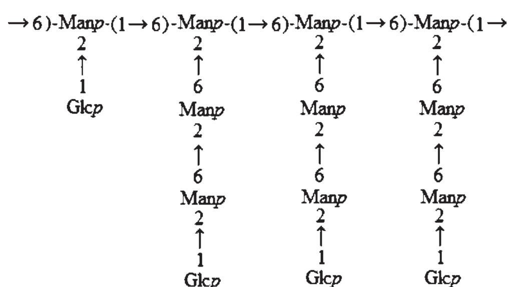

Figure 5. A proposed repeating unit of EPS produced by submerged fermentation of *P. tuber-regium*.

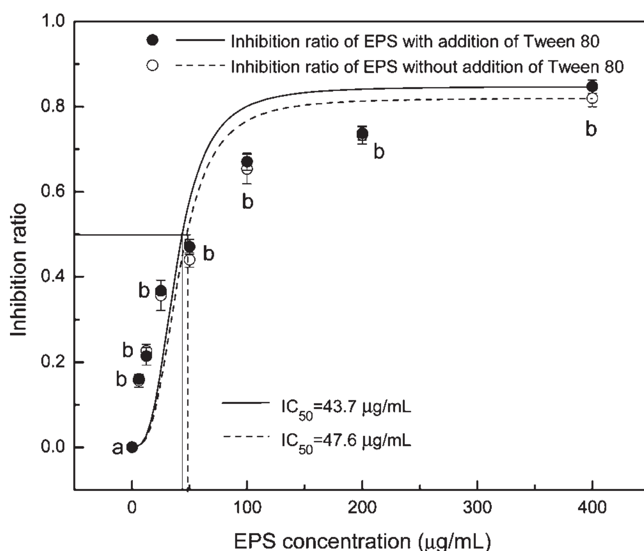

Figure 6. Effect of EPS with and without addition of Tween 80 on the growth of K562 cells. The K562 cells were incubated with EPS at concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 μg/mL for 72 h. Results are expressed as mean value ± standard deviation, $n = 5$. Different letters represent significant difference between the number of cells in the control group and the treatment group by Student's *t*-test ($p < 0.05$).

[→6)-Man*p*-(2→] residues and a terminal glucopyranosyl residue. A proposed partial structure of the repeating unit for EPS of *P. tuber-regium* is shown in Figure 5. Such highly branched polysaccharide should be very soluble in water which may facilitate its biological activities through molecular interaction with cells. As reported by Rosado et al.,[35] two different EPS were found in *Pleurotus ostreatoroseus*: a mannan having a main chain of (1→6)-linked mannopyranosyl residues, almost all of which were branched at *O*-2 with side chains of different lengths of mannopyranosyl units, and a (1→4)-linked galactan. Therefore, it seems that the chemical structures of EPS among different *Pleurotus* species are not the same and could be very diversified.

Effect of EPS on K562 Cell Viability. EPS, both with and without addition of Tween 80, could significantly inhibit ($p < 0.05$) the growth of K562 cells in a dose dependent manner, with an estimated IC_{50} value of 43.7 and 47.6 μg/mL, respectively (Figure 6). Moreover, no cytotoxic effect of both EPS at concentration up to 400 μg/mL could be found on the normal monkey Vero cells (data not shown), suggesting that their cytotoxic effects were preferential against tumor cells only. EPS extracted from the culture medium of *P. tuber-regium* had also been demonstrated to have direct cytocidal antitumor activities toward another leukemic cell HL-60 with a much higher IC_{50} value of 300 μg/mL in our previous study.[3]

Based on the above results, addition of Tween 80 could significantly increase mycelial growth and EPS production in submerged fermentation of *P. tuber-regium*. The structural composition and bioactivity of the EPS produced by addition of Tween 80 was not significantly different from those of the control except of the M_w. Hence, the strategy of supplementation of stimulatory agents in this study can be applied in other fungal fermentation processes for enhancing production of extracellular metabolites. Detailed mechanistic study of Tween 80 on the enhancement of EPS production is underway.

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