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SCREENING AND CHARACTERIZATION OF ENDOPOLYSACCHARIDE FROM *Pleurotus pulmonarius* IN SUBMERGED CULTURE FERMENTATION

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ABSTRACT

The fermentation of Pleurotus pulmonarius (non-irradiated) and Pleurotus pulmonarius (irradiated) were carried out in Erlenmeyer flask 500 mL (working volume 250 mL) and screened for the presence of β-glucan (1, 3:1, 6). The biomass obtained was extracted using Modified Mizuno Method to get the endopolysaccharide. The endopolysaccharides of Pleurotus pulmonarius (irradiated) contained higher content of β-glucan (1, 3:1, 6) with 16.7 g/100g crude polysaccharide compared to the non-irradiated strain. The irradiated strain was chosen for further investigation. Fractionation of endopolysaccharide Pleurotus pulmonarius (irradiated) using column chromatograph yielded 7 fractions. The first fraction (F_1) contained high molecular weight fraction ~ 10^5 Da which potential immunomodulation characteristics. The Pleurotus pulmonarius (irradiated) was produced in a 5 L air-lift bioreactor. The highest biomass was obtained at air flowrate of 2 L/min, yielding productivity of 2.56 g/L.d.

Keywords: β-glucan, column chromatography, molecular weight, mushroom, submerged culture fermentation

INTRODUCTION

Production of β-glucan in submerged culture fermentation has not been extensively researched due to the fact that solid-state fermentation has been the main approach. The time taken to produce fruit bodies in solid state fermentation (SSF) often varies and especially for some medicinal mushrooms, the length tend to be longer. Submerged culture fermentation (SCF) has the advantage of producing higher quantity of mycelium, in a compact space, shorter incubation time and with less contamination (Bae et al., 2000; Choi et al., 2011). Paek et al. (2005) reported that the main types of bioreactor are mechanically stirred (stirred-tank bioreactor), pneumatically stirred (air-lift bioreactor) and non-stirred bioreactor (gas phase). Mechanically stirred bioreactor has few limitations such as requirement for high power and high shear stress. In pneumatically stirred bioreactor (air-lift), both functions of mixing and aeration are supplied by air-inlet to the system giving low shear stress, high biomass/yield with low input power requirement (Paek et al., 2001).

Several polysaccharides have been commercialized for clinical treatments of patients undergoing therapy, including schizophyllan, lentinan, grifolan, krestin and polysaccharide-K (PSK) (Zhang et al., 2007). Krestin which was derived from mycelium of *Trametes versicolor* has a molecular

weight of 1.0×10^5 Da, Lentinan from fruit body of *Lentinus edodes* with 5.0×10^5 Da and Sonifilan from broth of *Schizopyllum commune* with 4.5×10^5 Da (Mizuno, 1999; Ooi and Liu, 2000). Mushroom derived polysaccharides, mainly β-glucan have been used as source of therapeutic agents functioning by modulating animal and human response and inhibiting certain tumor growth (Ooi and Liu, 2000; Wasser, 2002; Zhang et al., 2007). The polysaccharides can reduce the side effects significantly when taken prior to and during radiotherapy/chemotherapy treatments (Smith et al, 2002). Other usage reported includes thickening and stabilizing agents in chemical industries and enhancing the skin's natural ability to heal and protect itself against infection (Lee et al., 2003).

For an anti-tumor application, in-vitro studies suggested that polysaccharide with large molecular weight can directly activate leukocytes, stimulating their phagocytic, possess antimicrobial activities, including the production of reactive oxygen and nitrogen intermediates. Intermediate molecular weight possess in vivo biological activities, but their cellular effects are less clear. Very short β-glucan (5000-10,000 Da) are generally considered inactive (Akramiene et al., 2007). Other reports also indicate high molecular weight glucans seem to be more effective than those of low molecular weight. (Mizuno, 1999; Shu and Lung, 2004). Thus our work focus on the production of high molecular weight glucan.

The use of irradiation has been proven as means to produce mutant of several edible mushrooms which has lead to commercially superior strains with higher productivity (Djajanegara and Harsoyo, 2000). In Malaysia, no researcher has reported any work on irradiated strain of *Pleurotus pulmonarius* in submerged culture fermentation. The objective of this research is to compare the β-glucan content from the irradiated strain and non-irradiated strain of *Pleurotus pulmonarius.* Then, the compound will be produced in air-lift bioreactor.

MATERIALS AND METHODS

Biological Materials

The non-irradiated and irradiated mushroom strains were obtained from the Agrotechnology and Biotechnology Division, Malaysian Nuclear Agency. The strains were maintained on potato-dextrose-agar (PDA) and subcultured every 3 months. Two local strains of mushrooms were investigated, which were *Pleurotus pulmonarius* (non-irradiated) and *Pleurotus pulmonarius* (irradiated).

Hot Water Extraction to Produce Endopolysaccharides

The biomass (100 g) was extracted to obtain the crude endopolysaccharides using modified Mizuno method (Mizuno et al., 1992), involving hot water extraction for at least 2 h, filtration, concentration process and centrifugation. The supernatant was added to absolute ethanol (ratio 1:1) and kept overnight before lyophilization to get the polysaccharides.

Screening for High β-Glucan Content Strains (β-Glucan Determination)

The endopolysacharide was tested using Mushroom and Yeast β-Glucan Assay Procedure (Megazyme International Ireland Limited, 2008). The total β-glucan was obtained by hydrolysing the sample in concentrated HCl (37% v/v, ~10 M), followed by neutralization with KOH (2 M) and filtration with Whatman GF/A glass fibre filter paper before enzymatic hydrolysis by exo-1,3 β glucanase and β-glucosidase. The α-glucan was obtained after the sample was hydrolysed with 2 M KOH and followed by enzymatic hydrolysis using amyglucosidase and invertase and then filtered with Whatman No.1 filter paper. Both reactions above were reacted with Glucose Oxidase and Peroxidase (GOPOD) before measurement using UV Spectrophotometer at 510 nm. The total glucan and α-glucan were obtained from the test done. The amount of β-glucan was obtained by subtraction of α-glucan from the total glucan.

Column Chromatography

The endopolysaccharides obtained from the extraction process was fractionated using Toyopearl DEAE 650M in column chromatography according to IMR (Institute for Medical Research, Malaysia) protocol. Toyopearl DEAE 650 M was diluted in phosphate buffer (0.05 M sodium dihydrogen phosphate, 0.05 M of disodium hydrogen phosphate, and 0.1 M sodium chloride in 1 L of deionized water) and packed in a column. The fractions of polysaccharides sample obtained from the packed column were collected every 2 min 30 sec was tested using phenol sulphuric acid test and its absorbance was measured at 490 nm.

Molecular Weight Determination

Average weight of endopolysaccharides was determined by GPC-MALLS (Gel permeation Chromatography-Multiangle Laser Light Scattering). The GPC system comprised an Agilent G1310A pump (Agilent Tecnologies, Santa Clara, USA), an Agilent G1329A auto-injector with an injection loop of 100 µL and a Wyatt 986 refractometer (Wyatt Technology, Santa Barbara, USA). The MALLS apparatus has a Wyatt Dawn-Heleos II laser photometer (Wyatt Technology, Santa Barbara, USA) equipped with a K5 flow cell and a He–Ne laser operating at k = 632.8 nm. An aqueous SEC column: Shodex OHpak SB-806 HQ (8.0 mm x 300 mm) (Showa Denko, Kawasaki, Japan) was used for the analysis.

The mobile phase consisted of a filtered (0.22 µm) phosphate buffer (0.05 M sodium dihidrogen phospate, 0.05 M of disodium hydrogen phosphate, and 0.1 M sodium chloride in 1 L deionized water) solution obtained using ultrapure water. The flow rate was 0.5 mL/min and analyses were performed at room temperature. The samples were dissolved in phosphate buffer solution and filtered (0.45 µm) to eliminate dust particles. The MALLS instrument was placed directly after the GPC columns and before the refractive index detector (DRI). Prior to measurements, a Dawn apparatus was calibrated using HPLC grade toluene and normalized using a 20 nm polystyrene latex standard (Thermo Scientific, Fremont, USA) in phosphate buffer solution. The performance of the HPSEC-MALLS system was checked with monodisperse pullulan of various molecular weights. A dn/dc value of 0.148 for β-glucan was used at wavelength 490 nm (Young and Castranova, 2005). Data were collected from the DRI and MALLS and evaluated with the ASTRA software 5.3.4.14. Since β-glucans are polydisperse polysaccharides, average weights were compared. Results were estimated using second-order Zimm model.

Production in 5-L Air Lift Bioreactor (Submerged Culture Fermentation)

The 250 ml of mycelia biomass (500 ml shake flask) in MCM media was transferred to a 5 L air lift bioreactor (working volume 2.5 L) aseptically. The flow rates were varied from 0.5 L/min to 2.0 L/min. (vvm 0.2 to 0.8). The mycelia produced from the submerged culture fermentation were freeze-dried until constant weight. The mycelial biomass dry weights obtained were plotted against air flow rate inlet. The fermentation was done in 6 days (except for the condition at 0.8 vvm which only need 3 days for the mycelium to occupy the bioreactor).

RESULTS AND DISCUSSION

β-Glucan (1, 3:1, 6) Content Determination

Table 1 showed the content of β-glucan (1, 3:1, 6) for endopolysaccharides of non-irradiated and irradiated *Pleurotus pulmonarius*. The total glucan obtained from both non-irradiated and irradiated strains of *Pleurotus pulmonarius* were approximately a quarter of the crude endopolysaccharides. However the irradiated strain has lower content of α-glucan at 7.0 g/100g compared to the non-irradiated strain with α-glucan content at 19.00 g/100g. Thus the endopolysaccharides of *Pleurotus pulmonarius* (irradiated) has higher content of β-glucan (1, 3:1, 6) at 16.7 g/100g crude polysaccharide compared to the *Pleurotus pulmonarius* (non-irradiated) at 9.75 gm/100 gm. Thus, *Pleurotus pulmonarius* (irradiated) was chosen for further investigation.

Table 1: The content of β-glucan (1, 3:1, 6) from *Pleurotus pulmonarius* (irradiated) and *Pleurotus pulmonarius* (non-irradiated) in 100 gm of crude endopolysaccharide

Endopolysaccharides	**Total Glucan (g/100 g)**	**α-Glucan (g/100 g)**	**β-Glucan (g/100 g)**	**Ratio β/α**
Pleurotus pulmonarius (irradiated)	23.7	7.00	16.70	2.39
Pleurotus pulmonarius (non-irradiated)	28.75	19.00	9.75	0.51

The endopolysaccharides of *Pleurotus pulmonarius* (irradiated) has higher ratio of β/α glucan at 2.39 compared to *Pleurotus pulmonarius* (non-irradiated) at 0.51. The changes in the content of α-glucan and β-glucan for the *Pleurotus pulmonarius* (irradiated) may be attributed by the changes in its DNA structure caused by γ-irradiation. The function of β-glucan is to support the fungal cell shape whilst α-glucan contributes to cell wall integrity (Nik Ubaidillah et al., 2015). The content of β-glucan (1, 3:1, 6) is comparable to Ahmad et al. (2014) which reported that β-glucan in endopolysaccharides of *Lentinus squarrosulus* was 11.36 ± 0.27 g/100g, for the hot water extract and Mohamad et al. (2015) which reported that the endopolysaccharides from *Pleurotus flabellatus* was 7.70 ± 1.11 g/100g crude polysaccharide.

Column Chromatography and Molecular Weight Determination

Figure 1 showed the absobance of endopolysaccharide *Pleurotus pulmonarius* (irradiated) using column chromatography. Fractionation was done and the value of absorbance from each fraction collected was obtained from phenol-sulphuric acid test. The absorbance values were plotted against the number of bottles collected at 2 min 30 sec interval from the column. Samples from bottle 1-11, 12-15, 19-23, 50-53, 54-56, 57-58 and 59-61 were combined to give fraction F1, F2, F3, F4, F5, F6 and F7 respectively, to be analyzed further.

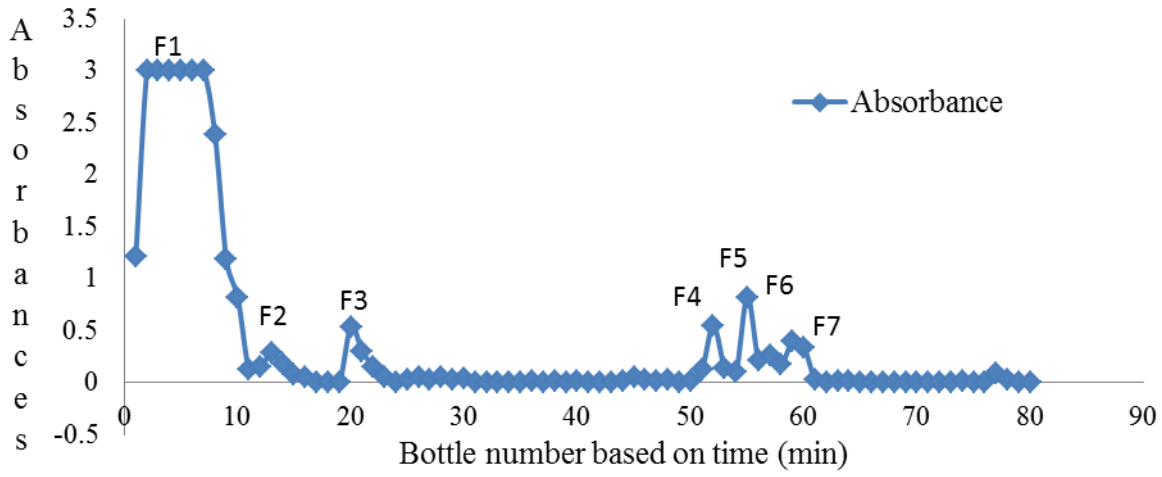

Figure 1: The absorbance using phenol-sulphuric acid from column chromatography of *Pleurotus pulmonarius* (irradiated) endopolysaccharides collected every 2.5 minutes

The seven fractions were run in GPC-MALLS to determine the molecular weight. The F_1 has the highest molecular weight estimated to be ~ 10^5 Da. Other fractions indicated a lower molecular weight in the range of ~ 10^4 Dalton (F_2) or non-detectable for the chosen column.

The endopolysaccharides of *Pleurotus pulmonarius* (irradiated) yield 7 fractions from column chromatography but only the first fraction, F_1 has the highest molecular weight ~ 10^5 Da. Thus, the first fraction F_1 has the potential immunomodulation characteristics as reported by various sources (Akramiene et al., 2007; Carbonero et al., 2012; Mizuno, 1999; Lin and Yang, 2006; Shu and Lung, 2004). Mohamad et al. (2015) reported the fraction with highest molecular weight obtained from *Pleurotus flabellatus* has two peaks with molecular weight of 3.058×10^6 Da (11.8%) and 1.282×10^4 Da (88.2%).

Production in Air-Lift Bioreactor

Table 2 showed the biomass productivity of *Pleurotus pulmonarius* (irradiated) in 5 L air-bioreactor (working volume 2.5 L) at different air-inlet flowrate. The highest biomass productivity was 2.56 g/L.d achieved at air-inlet flowrate of 2 L/min (vvm 0.8). The yield of biomass against substrate used (glucose consumption) $Y_{b/s}$ was 0.87 g/g at maximum productivity conditions.

Table 2 : The biomass productivity of *Pleurotus pulmonarius* (irradiated) in the air-lift bioreactor 5 L (working volume 2.5 L) and initial glucose concentration 20 g/L

vvm	Duration (days)	Mycelium Biomass (g)	Reducing Sugar (g/L)	Glucose Consumed (g)	Productivity (g/L.d)	Yield b/s
0.2	6	18.34	11.38	21.55	1.22	0.85
0.4	6	12.37	13.53	16.18	0.82	0.76
0.6	6	17.77	11.75	20.63	1.18	0.87
0.8	3	19.18	11.20	22.00	2.56	0.87

The productivity of *Pleurotus pulmonarius* (irradiated) in air-lift bioreactor obtained from this experiment at 2.56 g/L.d, has shown that its performance was comparable to other studies using a similar system but higher than that using stirred tank system. Mohamad et al. (2015) reported the productivity of *Pleurotus flabellatus* mycelium in 5 L air-lift bioreactor was 2.25 g/L.d at air-flowrate 2 L/min. In contrast to that using stirred tank bioreactor, Kim et al. (2002) reported that, productivity of *Pleurotus sajor-caju* biomass was 0.648 g/L.d, in 3 L working volume bioreactor with agitation speed of 150 rpm and aeration rate of 2 vvm. For the species of *Tremella fuciformis*, Cho et al. (2006) reported that the productivity of biomass in 5 L airlift bioreactor was 2.06 g/L.d which was higher by 16% compared productivity in stirred tank bioreactor at 1.77 g/L.d.

CONCLUSIONS

The *Pleurotus pulmonarius* (irradiated) contained higher content β-glucan (1, 3:1, 6) and was chosen for production in air-lift bioreactor. The endopolysaccharide of *Pleurotus pulmonarius* (irradiated) was produced in air-lift bioreactor with the highest productivity at 2.56 g/L.d. The endopolysaccharide showed the presence of β-glucan (1, 3:1, 6) with high molecular weight with potential immunomodulation characteristics.

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