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Production of Endopolysaccharides from Malaysia's Local Mushrooms in Air-Lift Bioreactor

Shaiful Azuar Mohamad[1*], Mat Rasol Awang[1], Rusli Ibrahim[1], Choong Yew Keong[2], Mohd Yusof Hamzah[1], Rosnani Abdul Rashid[1], Sobri Hussein[1], Khairuddin Abdul Rahim[1], Fauzi Daud[3], Aidil Abdul Hamid[3], Wan Mohtar Wan Yusoff[3]

[1]Agrotechnology and Biosciences Division, Malaysian Nuclear Agency, Bangi, Malaysia
[2]Herbal Medicine Research Centre, Institute For Medical Research, Kuala Lumpur, Malaysia
[3]Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Malaysia
Email: *azuar@nuclearmalaysia.gov.my

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Abstract

Four local mushroom species, viz. *Auricularis polytricha*, *Lentinus edodes*, *Agrocybe sp* and *Pleurotus flabellatus* were grown under submerged culture and screened for endopolysaccharides. The fermentation was done in 250 ml working volume Erlenmeyer flask and the fermentation curves for all species were established. *Pleurotus flabellatus* has the highest rate of biomass production at the rate of 0.180 g/L/day, at 10 days hence chosen for further investigation. Two additional media, viz. Mushroom Complete Media (MCM) and Yeast Malt (YM) were selected to be compared with potato extract(PE) media used initially. MCM media produced the highest biomass productivity at the rate of 0.311 g/L/day. *Pleurotus flabellatus* biomass was extracted using modified Mizuno method and the endopolysaccharide obtained was tested for *β*-glucan. The yield of *β*-glucan was 7.70 ± 1.11 g/100g. The polysaccharides were purified using column chromatography to yield four fractions. The fourth fraction F_4, gave the highest molecular weight at 3.058×10^6 Dalton (11.8%) and 1.282×10^4 Dalton (88.2%). The mushroom, *P. flabbelatus* was cultured using air-lift bioreactor, and the highest productivity was obtained at air-flowrate 2 L/min, yielding 2.25 g/L/day. The yield of biomass against substrate used (glucose consumption) $Y_{b/s}$ was 0.78 g/g.

Keywords

Submerged Culture Fermentation, Mushroom, *β*-Glucan, Column Chromatography, Molecular Weight

*Corresponding author.
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1. Introduction

β-glucan obtained from mushrooms have been used as source of therapeutic agents functioning by modulating animal and human response and inhibiting certain tumor growth [1]-[3]. The mushroom derived polysaccharides can reduce the side effects significantly when take prior to and during radiotherapy/chemotherapy treatments [4]. Several polysaccharides including schizophyllan, lentinan, grifolan, krestin and polysaccharide-K (PSK) have been commercialized for clinical treatments of patients undergoing therapy [2]. Several reports about commercial products showed that Krestin which was derived from mycelium of *Trametes versicolor* had a molecular weight of 1.0×10^5 Dalton, Lentinan from fruit body of *Lentinus edodes* with 5.0×10^5 Dalton and Sonifilan from broth of *Schizopyllum commune* with 4.5×10^5 Dalton [3] [5]. This paper will focus on screening of *β*-glucan for local mushrooms grown under submerged culture fermentation.

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 less contamination [6] [7]. The air-lift bioreactor will be used to compare biomass production in shake flasks.

β-glucan from local mushrooms will contribute to the development of the local industry if the productivity of SCF can be improved. This can be achieved by ensuring the productivity of the mycelium related to the endopolysaccharides production to produce at least 5% w/w of endopolysaccharides with different media.

2. Methodology

2.1. Biological Materials

The mushroom strains were collected by the Bioprocess Group, Agrotechnology and Biotechnology Division, Malaysian Nuclear Agency. The strains were maintained on potato-dextrose-agar (PDA) and subcultured every 3 months. Four local species of mushrooms tested were *Auricularia polytricha*, *Lentinus edodes*, *Agrocybe sp* and *Pleurotus flabellatus* due to its availability at the Nuclear Malaysia (NM) Mushroom Culture Collection and various reports showed the presence of *β*-glucan for all species.

2.2. Screening for High Biomass Species

The stock cultures of the species were transferred into the petri dish with PDA as the medium. Then 1 cm of the agar plate culture, was cut with a sterilized cutter and tranferred into a 500 ml Erlenmeyer flask containing 250 ml of media incubated using orbital shaker at 50 rpm, at room temperature of 25˚C. The composition of media consists of potato extract (100 g/l) and glucose (30 g/l). The biomass was collected after 4, 6, 8, 10, 12, 14 and 16 days to obtain fermentation curves for all species.

2.3. Screening of Media

Based on literature, the media used were Mushroom Complete Media (MCM) which consists of 20 g/l glucose, 2 g/l meat peptone, 2 g/l yeast extract, 0.46 g/l KH_2PO_4, 1 g/l K_2HPO_4, and 0.5 g/l $MgSO_4 \cdot 7H_2O$. Yeast Malt (YM) consists of 10 g/l glucose, 3 g/l yeast extract, 3 g/l malt extract, and 5 g/l meat peptone. The fermentation curves of these media were compared to the initial media used.

2.4. Characterization

2.4.1. Hot Water Extraction to Produce Endopolysaccharides

The biomass (100 g) produced was extracted to obtain the endopolysaccharides using modified Mizuno method [8], 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.

2.4.2. Endopolysaccharide and *β*-Glucan Determination

The endopolysaccharide was tested using Mushroom and Yeast Beta Glucan Assay Procedure (Megazyme International Ireland Limited, 2008). The total beta 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 followed by enzymatic hydrolysis using amyglucosidase and invertase, then filtration 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.

2.4.3. Column Chromatography

The endopolysaccharides obtained from the extraction process were fractionated using Toyopearl DW-65F in column chromatography. Toyopearl DW-65F was diluted in phosphate buffer (0.05 M sodium dihidrogen phospate, 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 endopolysaccharides sample obtained from the packed column were collected every 4 min and tested using phenol sulphuric acid test and its absorbance was measured at 490 nm.

2.4.4. Endopolysaccharides Molecular Weight Determination

Average weight of endopolysaccharides, M_w, 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 × 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 [9]. 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.

2.5. Production in Air Lift Bioreactor (Submerged Culture Fermentation)

The 250 ml of mycelia biomass (500 ml shake flask) in MCM media was transferred to a 2.5 L working volume air lift bioreactor (5 L total volume) 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.

3. Results

3.1. Screening for High Biomass Species

The fermentation curves for all species were plotted and shown in **Figure 1**.

Figure 1 shows mycelia growth profiles in submerged culture fermentation for the four species selected using potato extract as the crude media. The biomass collected ranged from approximately 0.1 g to 0.7 g. As the fermentation duration increased, more media were consumed to produce more biomass. *P. flabellatus* produced the most consistent rate and highest biomass production whilst *L. edodes* species showed the lowest biomass production rate. The production rate of *Agrocybe sp* was slightly lower than *P. flabellatus* whilst the rate of *A. polytricha* was initially low but increased at the end of fermentation. The most consistent production was by *P. flabellatus*, with the highest rate of biomass production at 0.180 g/L/day, at the fermentation duration of 10 days.

3.2. Screening of Media

Figure 2 shows the mycelial growth profile for *P. flabellatus* using two additional media obtained from the lite-

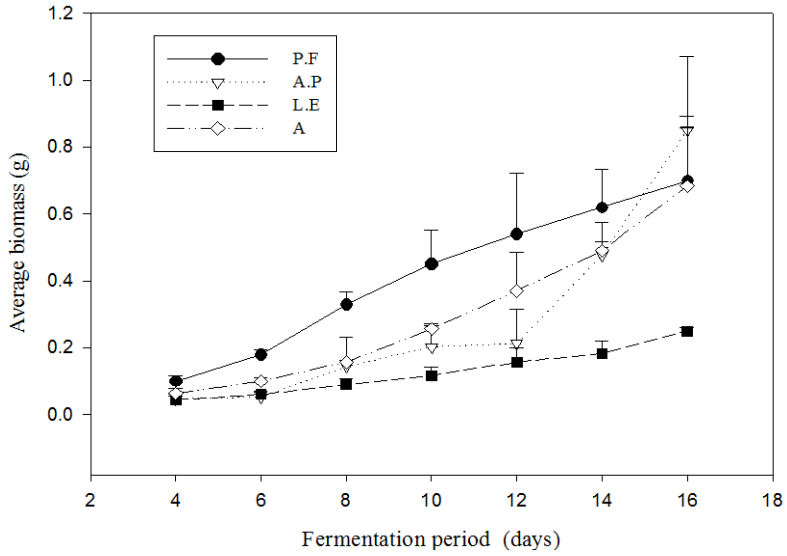

Figure 1. Fermentation curves for *Agrocybe sp*, *Auricularia polytricha*, *Lentinus edodes*, and *Pleurotus flabellatus*.

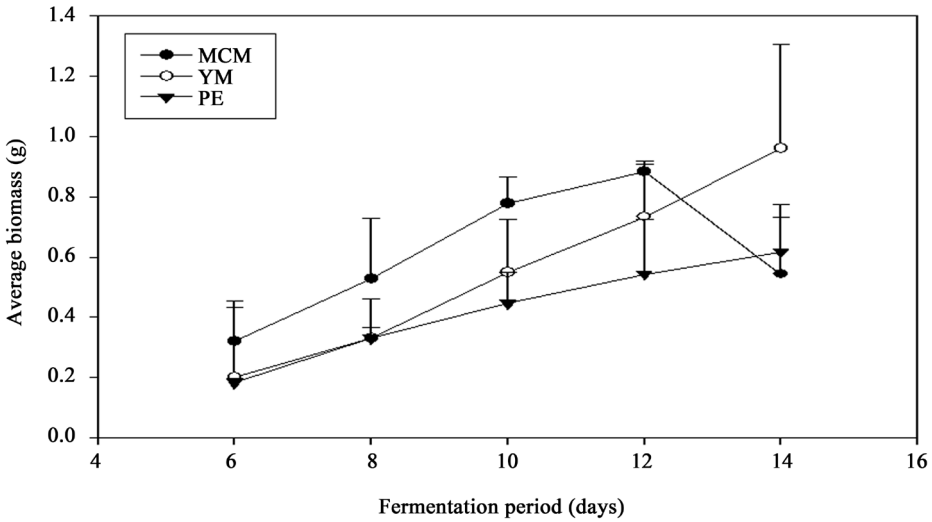

Figure 2. The fermentation curve of *P. flabellatus* using different media compared to the potato extract media.

rature compared to the initial potato extract media used. The two media produced more biomass compared to the crude media of potato extract. YM produced the most consistent rate but the production rate for MCM was higher than YM up to day 12. The calculation for each media and duration is shown in **Table 1**.

From the table, the highest production rate was 0.311 g/L/day using media MCM, and again the fermentation period of 10 days. Using this media the production rate increased by 72.7%. Hence, MCM was chosen for subsequent experiment.

3.3. Characterization of Polysaccharides

3.3.1. *β*-Glucan Determination

After the extraction process using Modified Mizuno method, 100 mg of polysaccharide from the mycelium of *P. flabellatus* sample was used to test the presence of beta glucan using the beta glucan assay kit (**Table 2**). 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. The total glucan in the biomass was 17.54 ± 2.91 g/100g whilst beta glucan yield was 7.70 ± 1.11 g/100g. No publication has reported this finding for the species studied.

3.3.2. Column Chromatography and Molecular Weight Determination

From the column chromatography, the value of absorbance from each fractions obtained from phenol sulphuric acid test were plotted against the number of bottles collected at 4 min interval from the column as shown in **Figure 3**. Samples from bottle 6 - 14, 15 - 21, 25 - 37 and 38 - 60 were combined to give fraction F_1, F_2, F_3 and F_4, respectively, to be analyzed further.

The four fractions were run in GPC-MALLS to determine the molecular weight. The F_4 has the highest molecular weight with two possible molecular weight 3.058×10^6 Dalton (11.8%) and 1.282×10^4 Dalton (88.2%). Other fractions indicated a lower molecular weight in the range of ~10^3 Dalton.

3.4. Production in Air-Lift Bioreactor

Table 3 showed the biomass and productivity in the air-lift bioreactor. The highest productivity of biomass in air-lift bioreactor with 2.5 L working volume of *Pleurotus flabellatus* is 2.25 g/L/day at volume per volume per min (vvm) 0.8. The air inlet flow rate did not seem to affect the productivity of the biomass very much. The yield of biomass against substrate used (glucose consumption) $Y_{b/s}$ was 0.78 g/g.

4. Discussion

The mushroom species *P. flabellatus* was chosen due to its consistency and highest production rate of mycelium at the rate of 0.180 g/L/day. For the media screening, MCM was chosen with highest productivity at the rate of

Table 1. The production rate calculation for different media.

Fermentation days	MCM (g/L/day)	YM (g/L/day)	PE (g/L/day)
6	0.213 ± 0.090	0.133 ± 0.156	0.122 ± 0.01
8	0.263 ± 0.100	0.165 ± 0.065	0.165 ± 0.018
10	0.311 ± 0.036	0.220 ± 0.070	0.179 ± 0.040
12	0.294 ± 0.008	0.244 ± 0.061	0.181 ± 0.061
14	0.155 ± 0.066	0.274 ± 0.099	0.176 ± 0.032

Table 2. The yield of *β*-glucan from crude extract of *P. flabellatus*.

Species	Total glucan (mg/100mg)	*α*-glucan (mg/100mg)	*β*-glucan (mg/100mg)
Mycelium of *P. flabellatus*	17.54 ± 2.91	9.84 ± 3.82	7.70 ± 1.11

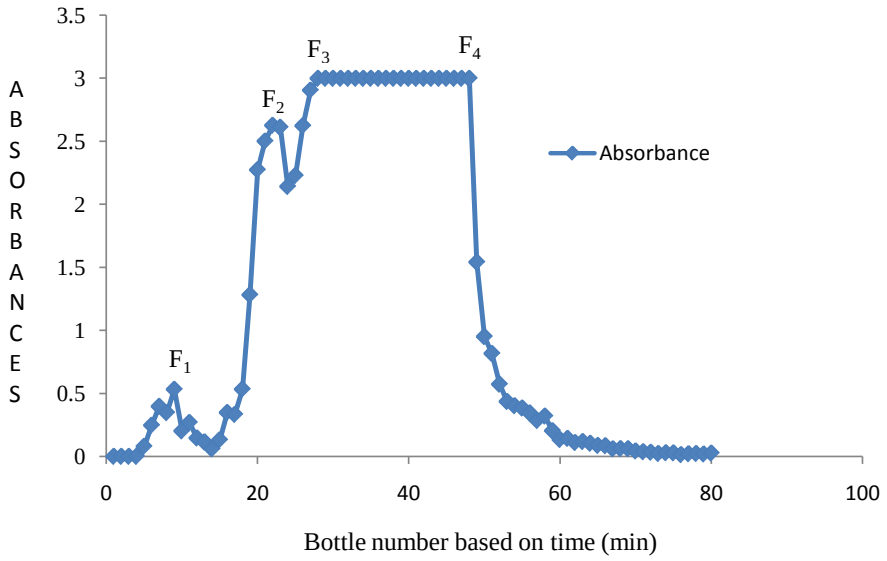

Figure 3. The reading of phenol sulphuric acid test from column chromatography of endopolysaccharides from *Pleurotus flabellatus*.

Table 3. The productivity of *Pleurotus flabellatus* biomass production in air-lift bioreactor.

Air inlet flowrate (L/min)	vvm	Mycelium biomass (g)	Reducing sugar (g/L)	Productivity (g/L/day)
0.5	0.2	20.40 ± 1.98	10.69 ± 1.71	2.04 ± 0.20
1.0	0.4	22.00 ± 2.12	8.30 ± 0.61	2.20 ± 0.21
1.5	0.6	20.85 ± 1.63	10.49 ± 2.36	2.09 ± 0.16
2.0	0.8	22.50 ± 4.10	8.49 ± 1.19	2.25 ± 0.41

0.311 g/L/day. In a similar study for *Ganoderma resinaceum* in 250 ml shake flask using MCM medium, the biomass production rate obtained was 0.333 g/L/day [10]. Another study indicated that the maximum biomass produced for *Pleurotus sajor caju* in shake flask was 6.5 g/l in 10 days (0.650 g/L/day) using deproteinized whey, diammonium phosphate and yeast extract as fermentation medium [11].

For the *β*-glucan content using assay kit by Megazyme, a paper reported that the *β*-glucan in endopolysaccharides of *Lentinus squarrosulus* was 11.36 ± 0.27 (%w/w) for the hot water extract in submerged culture fermentation [12]. Another paper reported the *β*-glucan content obtained from the fruit body of *G. applanatum*, *T. versicolor*, *L. edodes*, and *G. lucidum* to be 16.0, 33.4, 41.2 and 41.4 g/100g, respectively using dry weight of dialyzed crude extract [13]. This researcher used higher purity of crude extract using dialysis technique.

The high molecular weight in the order of 10^6 with 3.058×10^6 Dalton (11.8%) and 1.282×10^4 Dalton (88.2%) obtained from *P. flabellatus* indicated this compound has the potential to be explored for anti-tumor as reported by Akramiene and coworkers [14] regarding the application of high molecular weight *β*-glucan. Another report showed that the insoluble glucan obtained from yeast separated using size exclusion chromatography also has two peaks with molecular weight of 1×10^6 Da (1% of total mass) and 1.5×10^4 Da (99% of total mass) [15].

For the production of biomass using air-lift bioreactor, the value of 2.25 g/L/day reported in this experiment is in the same range as reported by Cho and coworkers [16]. The report showed that for *Tremella fuciformis* in 5 L airlift bioreactor, the maximum dry weight obtained was 10.30 g/l at days 5 (productivity 2.06 g/L/day). For the same species using stirred-tank bioreactor, the cell dry weight obtained was 8.83 g/L (productivity 1.77 g/L/day) [16].

In another study using stirred tank fermentor, the productivity of *Pleurotus sajor-caju* biomass was 0.648 g/L/day, with 3 L working volume, agitation speed of 150 rpm, and aeration rate of 2 vvm [17].

5. Conclusions

The species *P. flabellatus* has the highest biomass productivity (0.180 g/L/day) with potato extract as the crude media. Enhanced biomass productivity (0.311 g/L/day) was achieved with MCM. The yield of beta glucan from submerged culture fermentation of *P. flabellatus* was 7.70 ± 1.11 g/100g. The productivity of biomass in airlift bioreactor was 2.25 g/L/day and approximately 12.5 times higher compared to the initial value. The fourth fraction F_4 gave the highest molecular weight with 3.058×10^6 Dalton (11.8%) and 1.282×10^4 Dalton (88.2%).

The *β*-glucan (1.3:1.6) from *Pleurotus flabellatus* species has the potential to be produced in submerged culture fermentation at a higher productivity. The quantity and quality of the *β*-glucan can be purified further and tested for its effectiveness towards anti-tumor application. The high molecular weight produced from this research can be analyzed for a single compound and determine its exact molecular structure. The air-lift bioreactor can be custom-made and produced at a cheaper price locally.

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