

Anticancer Activity of Polysaccharides Produced by *Pleurotus ostreatus* in Submerged Culture

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It has been known for many years that some compounds produced by edible mushrooms encompass anticancer activities. Most of production methods were based on cultivation of mushroom in solid medium. In the present study *Pleurotus ostreatus* mycelia were grown in submerged culture. The cultivation of fungal cells in submerged culture resulted in higher growth rate with better control of production process. The bioactive polysaccharides (both intracellular and extracellular) were extracted from culture by solvent repeated precipitation. The polysaccharide structure was determined by examining NMR, IR spectra and the primary structure of the polysaccharide was mainly glucan. The ¹³C NMR spectral pattern indicated the polysaccharides are highly branched with mainly 1→3 and 1→6 linkage. The results of *in vitro* anti cancer studies demonstrate that this type of polysaccharides possesses anticancer activity against human oesophageal cancer cell line. Moreover, in the course of *in vitro* studies, mushroom polysaccharides showed anti-tumour activity and also considered to be biological response modifier because of their mechanism of action through stimulation of the immune system. The polysaccharide activity is especially beneficial in clinics when used as an adjuvant with chemotherapy to decrease its side effect. This work describes production process of anti cancer compound(s) by mushrooms and suitable for pharmaceutical industries.

1. Introduction

Mushrooms have been eaten and appreciated for their flavour, economical and ecological values, and medicinal properties for many years. Mushrooms

are abundant sources of a wide range of useful natural products with biological activities (Lorenzen and Anke, 1998; Wasser, 2002; Mao and Zhong, 2004). Much interest in biotechnological methods for the production of microbial polysaccharides has been generated for applications in pharmaceuticals industries (Kuo *et al.*, 1996, Liu *et al.*, 1997). However, most of polysaccharides with various physiological activities frequently originated from fungi especially mushrooms. Moreover, mushroom has some other medical applications for the treatment of diseases such as hypoglycaemia (Yuan *et al.*, 1998; Kiho *et al.*, 1994; Yang *et al.*, 2002). In spite of most researches deal with polysaccharides extracted from the fruiting bodies, other has studied extracellular polysaccharides (EPS). The EPS term is used to describe polysaccharides found outside the cell or which are free within the surrounding medium. The production of EPS from mushroom in submerged culture is interesting because of several advantages over the conventional method using fruit body extraction. In submerged culture, the growth rate of fungal cell is higher and the product requires only relatively simple purification steps (Cavazzoni and Adami, 1992; Jong and Birmingham, 1992). Moreover, some wild mushrooms are not able to be cultivated in traditional way and able only to grow in submerged culture using enriched culture medium under controlled cultivation conditions. Recent studies of EPS production from mushrooms demonstrated their significant anticancer activity (Kim *et al.*, 2001; Ng 1998).

Pleurotus ostreatus is an edible mushroom belonging to the family Basidiomycetes. This fungi is one of the five main edible fungi cultivated worldwide and display easy adaptation to different growth conditions in submerged cultures (Marquez-Rocha *et al.*, 1999; Bae *et al.*, 2000; Rosado *et al.*, 2003).

In this context, the current study reports some results for cell growth and EPS production kinetics during cultivation of *P. ostreatus* in stirred tank bioreactor. The produced polysaccharide was extracted and completely identified. Further studies of its anticancer activity were also done.

2. Materials and Methods

2.1. *Microorganism and cultivation conditions*

Basidiomycetes fungus *Pleurotus ostreatus* NRRL 366 was kindly provided by the agriculture research service Peoria, USA. The strain was maintained and

reactivated monthly in Petri dishes containing a sterile solid potato dextrose agar medium (PDA, Oxoid, UK). Cells were incubated at 28°C for 14 days and stored in a refrigerator at 5°C.

2.2. *Medium for cell growth and EPS production in bioreactor*

The medium used in bioreactor experiments was composed of [g l⁻¹]: glucose, 20.0; KH₂PO₄, 0.46; K₂HPO₄, 1.0; MgSO₄×7H₂O, 0.5; peptone, 2.0 and yeast extract, 2.0. Glucose was sterilized separately and added to the cultivation medium before inoculation; pH was adjusted to 5.5 after sterilization.

2.3. *Inoculum preparation and bioreactor cultivation*

Pleurotus ostreatus was initially grown on PDA medium in a petri dish for 14 days at 28°C. The surface mycelia with spores were harvested in sterile saline solution and used as inoculum. Cells were gently homogenized to prevent the formation of large aggregates in submerged culture. Inoculum for bioreactor was in form of 50 ml (mycelium-spore) suspension with optical density (O.D.) of 1.0 at 600 nm. Cultivation was carried out in a 3 L stirred tank bioreactor Bioflow III (New Brunswick Scientific Co., New Brunswick, NJ, USA) with a working volume of 2,0 L. Agitation was performed using a three 4-bladed rushton turbine impellers ($d_{i(\text{impeller diameter})} = 65 \text{ mm}$; $d_{t(\text{tank diameter})} = 135 \text{ mm}$, $d_i d_t^{-1} = 0.48$) at 400 rpm. Aeration was performed by filtered sterile air [1 v/vxm]. Dissolved oxygen concentrations were analyzed by polarographic electrode (Ingold, Germany). Foam was suppressed, when necessary, by the addition of silicon antifoam reagent (Fluka, Switzerland).

2.4. *Analysis*

2.4.1. *Sample preparation and cell dry weight determination*

During cultivation in bioreactor, aliquots (in form of 10-15 ml) of the culture were taken from the bioreactor vessel through a sampling system. Samples

were collected in pre-weighed centrifugation tube of 15 ml (Falcon, USA), centrifuged at 4°C with 5000 rpm for 20 min. Supernatant was frozen at -20°C for sugar and EPS determination. The cell pellets were washed twice by distilled water, centrifuged again and dried in oven at 60°C for determination of cell dry weight.

2.4.2. Determination of glucose

Glucose was determined in the fermentation media by enzymatic method using a glucose determination kit (Glucose kit Cat. No. 4611, Biocon Diagnostic GmbH, Burbach, Germany).

2.4.3. Extraction of polysaccharide from mycelial culture

After sample centrifugation, the resulting culture filtrate was mixed with equal volume of absolute ethanol, stirred vigorously and kept overnight at 4°C. The precipitate exo-biopolymer was centrifuged at 10.000g for 20 min. discarding the supernatant (Bae *et al.* 2000). The precipitate of pure EPS was washed separately with ethanol, acetone and ethyl ether then lyophilized.

2.4.4. IR spectrometry

Finely ground solid polysaccharide samples compressed with potassium bromide (KBr) into the form of a thin tablet then placed directly into the sample beam of the spectrometer and the absorption of the spectrum was measured.

2.4.5. ¹³C NMR spectrometry

The experiment was done according to the method described by Barrett-Bee *et al.* (1982). The polysaccharide sample 50 mg was dissolved in one ml of concentrated dimethyl sulphoxide (DMSO) by ultrasonic for 10 minutes. The solution was introduced into a procession ground tube then subjected to measurement.

2.5. Cytotoxicity Determination

2.5.1. Cell culture

Oesophageal cancer cell line was routinely maintained at 37°C, 5% CO₂. Cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin.

2.5.2. Crystal violet assay

Initial screening of mycelial polysaccharide for cytotoxic activity was carried out by means of crystal violet staining of treated cells (Saotome *et al.*, 1989). Oesophageal cancer cells were plated (1,500 cell/well) in 96 well tissue culture plate. After 24 hour incubation, polysaccharide was added in different concentrations (50-1000µg ml⁻¹). Following 48 hours incubation, observation of cell number and morphology were made and the plates were then processed for staining. Media were discarded; the plates allowed to dry and 100 µl absolute Methanol was applied to each well for 10 minutes. Methanol was discarded and replaced with staining solution for 20 minutes. Plates were rinsed with water and 100 µl of water was added to each well for 1 hour. The water was discarded and replaced with further 100 µl water. Plate was read at 595 nm on micro-plate reader.

2.5.3. Detection of immune stimulation activity of Mushroom polysaccharides

Peripheral blood mononuclear cells (PBMC) were isolated from healthy individual by Ficoll-Hypaque (Sigma, St. Louis, MO, USA) gradient centrifugation. The purified cells was cultured at 1.0×10⁶ cell ml⁻¹ in RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 25mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma), 4mM L-glutamine (Cambrex), 100 U of penicillin and 100 µg streptomycin (Cambrex) and 10% FBS (GEBSCO, BRL, USA). PBMC were stimulated with 200, 100, 80, 40, and 20 µg ml⁻¹, of each of *P. ostreatus* mycelia exo-polysaccharids. All samples were assayed in triplicates. A positive control culture was included, where PBMC was stimulated with 2 µg ml⁻¹ Phytohemagglu-

tinin-L (PHA, Sigma). Proliferation was determined after incubation for 3 days at 37°C, 5% CO₂, and 95% humidity, by addition of 201 of BrdU labelling reagent (Roche, Penzerg, Germany) in the last 2 hours of the culture. The labelled cultures were harvested and the BrdU uptake was determined using Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche) following the manufacturer instructions. Data were presented as stimulation index (SI), where proliferation is considered positive if SI is ≥ 2 .

3. Results and Discussion

3.1. *Cell growth and EPS production in stirred tank bioreactor*

Cultivations were carried out in 3 L stirred tank bioreactor to investigate the kinetics of cell growth and EPS production. As shown in figure (1), cells grew exponentially during the first 216 h with growth rate of 0.009 [h⁻¹] and reached a cell mass of 4.7 g l⁻¹. After this time, cell mass kept more or less constant for the rest of cultivation time. During this phase, the glucose consumption rate was 0.054 [g l⁻¹ h⁻¹] and reduced to 0.021 [g l⁻¹ h⁻¹] as the cells entered the stationary growth phase. Thus, glucose was not the growth limiting substrate in this process. On the other hand, EPS production started after 48 h and produced with rate of 0.0063 [g l⁻¹ h⁻¹], reaching the maximal value of about 1.6 g l⁻¹ after 312 h. Thus, the production of EPS was not stopped as the cells entered the stationary phase. The data of dissolved oxygen [DO] clearly demonstrate also that during the exponential growth phase there was significant drop in DO value and increased again at the early stationary phase.

During exponential growth phase, the pH decreased gradually and reached about 3.5 after 144 h and increased with very low rate reaching only 3.8 at the end of cultivation. This drop in pH is assumed to be responsible for growth limitation in culture since neither carbon nor oxygen limitations were observed in this culture. The decrease in pH of culture medium during *Pleurotus* growth might be probably due to production of organic acids as suggested by Rajarathnam *et al.* (1992).

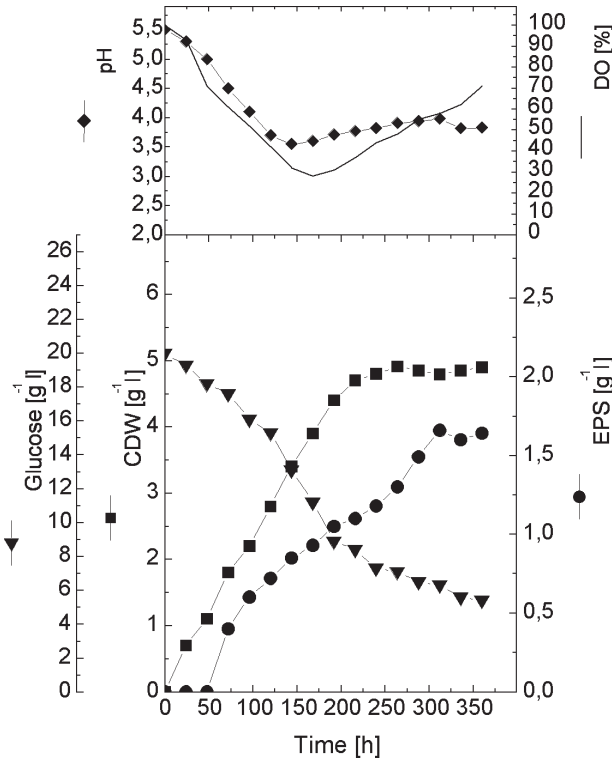


Figure 1. Cell growth and EPS production during submerged cultivation of *P. ostreatus* in 3 L stirred tank bioreactor.

3.2. Structure of isolated polysaccharide

The infrared spectrum of mycelial polysaccharide in Figure 2 indicates the presence of hydroxyl group which lies in the region between 3200-3600 cm while the band at 2900 is characteristic to C-H group as well, the IR spectrum shows finger print at 860 cm which lies in the anomeric region indicating the presence of a beta glucosidic bond. On the other hand, ¹³C NMR spectrum of mycelial polysaccharide exhibited signals at different resonance which indicated that the basic structure of the isolated polysaccharide is glucan with different linkages mainly 1-3, 1-6 (Figure 3).

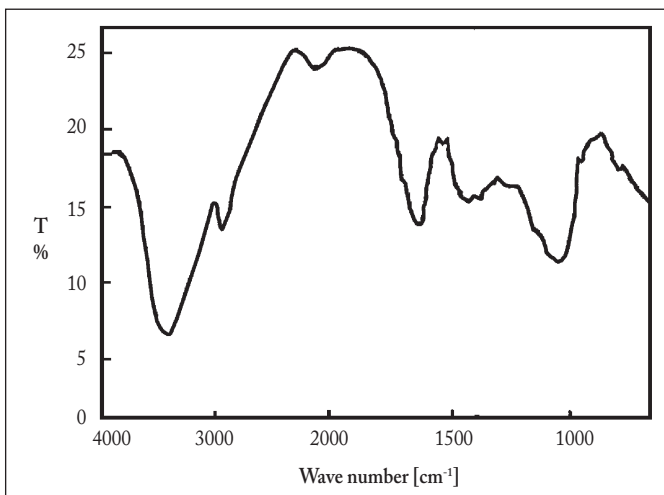


Figure 2. IR spectra of EPS produced by *P. ostreatus* in submerged culture.

The NMR spectral features of mycelial polysaccharide of *P.ostreatus* are very similar to polysaccharides isolated from different types of mushrooms such as *Lentinus edodes*, *Grifola frondosa* and *Ganoderma tsugae* (Mizuno *et al.*, 1992; Zhuang *et al.*, 1992; Zhang *et al.*, 1994). Although, there are significant differences in the relative intensities of peaks, reflecting the differences in the extent of branching.

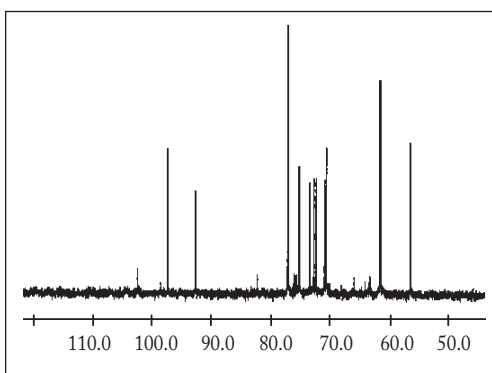


Figure 3. NMR spectra of EPS produced by *P. ostreatus* in submerged culture

3.3. Antitumour activity of mycelial polysaccharide

Polysaccharide isolated from mycelial culture broth was tested at different concentration and the results of crystal violet assay are presented as absorbance at 595 nm of treated and untreated cells (figure 4). In principle, an active compound could cause a decrease in cell number and hence ab-

sorbance with increasing drug concentration. The results indicate that the polysaccharide at 250, 500 and 1000 $\mu\text{g ml}^{-1}$ have a direct cytotoxic effect against oesophageal cancer cell line. While doses of 50 and 100 $\mu\text{g ml}^{-1}$ showed to have no cytotoxic effect on the cancer cell line. These data agrees with the data previously reported indicating that antitumour mushroom polysaccharides such as lentinan and schizophyllan showed no direct cytotoxicity to tumour cell lines *in vitro* (Aoyagi *et al.*, 1994). On the other hand, polysaccharide krestin and polysaccharopeptide PSP isolated from *Coriolus versicolor* mushroom had direct cytotoxicity to a wide range of tumor cell lines (Tsukagoshi *et al.*, 1984 and Yang *et al.*, 1992). We realize that that the polysaccharide did not fully enter into solution and sometimes even could increased absorbance reading because the precipitate contributed to the absorbance for this reason all cells were carefully examined microscopically prior to staining to determine the presence of precipitates and also to estimate cell density.

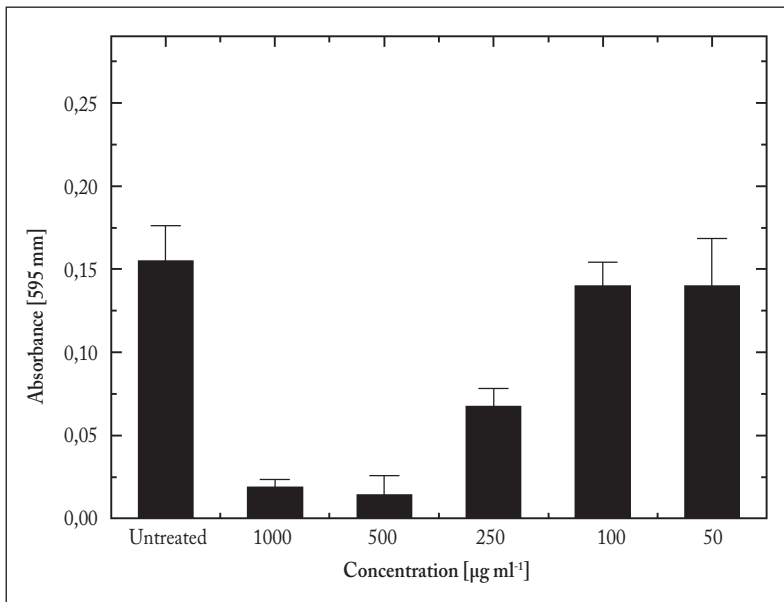


Figure 4. Cytotoxic activity of EPS of mushroom cells on Oseophygus cancer cell line.

3.4. Immunostimulatory effect of EPS

Several studies indicate that some natural mushroom (basidiomycete) products showed to have immuno-potentiating properties which generate considerable interest as possible pharmacological tumoricidal activity (Fujimiya *et al.*, 1998). Also it was reported that the (1→3)-branched (1→6)- β -glucan, termed D-Fraction, extracted from the fruit body of the maitake mushroom (*Grifola frondosa*) can enhance the activity of immunocompetent cells such as macrophages, helper T cells, and cytotoxic T cells to attack tumour cells (Kodama *et al.*, 2002). These findings support our data which is presented in figure 5 and showed that EPS of the mushroom strain *P. ostreatus* can stimulate proliferative response of normal PBMC in a dose independent manner.

4. Conclusion

The results in this work clearly indicated that mycelial exo-polysaccharide (EPS) isolated from *P. ostreatus* culture broth seems to have anticancer activity that is host mediated and cytotoxic. Further *in vivo* experiments will be carried out to ascertain the anti-tumour effect. Although the mode of cytotoxicity of the polysaccharides is still unknown, attempts will be made to examine and investigate the direct inhibition of RNA, DNA and protein synthesis as well as induction of specific functions of the immune system including macrophages, T-cells and natural killer cells. The confirmation of the curative effect of polysaccharides to cancer would be of great interest both to the nutraceutical industry and to the medical field. These facts give a basis to opinion of many researchers in the field that sustainable development of mushroom and their products in the 21st century can become a non-green revolution in natural products medicine.

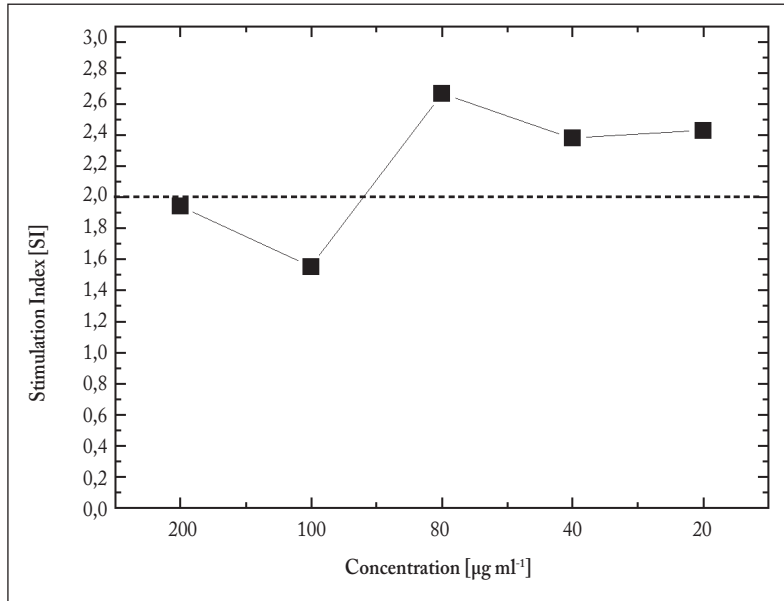


Figure 5. Stimulatory effect of different concentration of *P. ostreatus* EPS on normal PBMC. Data were presented as stimulation index (SI), where proliferation is considered positive if SI is ≥ 2 .

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