MYCELIAL AND EXO-POLYSACCHARIDES PRODUCTION BY SUBMERGED CULTURE OF THE EDIBLE MUSHROOM *PLEUROTUS* SPECIES

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Abstract

Submerged cultures of four *Pleurotus* species, a popular mushroom cultivated in Egypt, was used for production of bio-active protein-bound polysaccharides. The maximum concentration of mycelial biomass approximated 10 gl^{-1} with 2.76 g/l^{-1} protein in *Pleurotus florida* after 14 days incubation in shake flask culture. The exopolysaccharide (EPS) production after the same incubation period was higher in *P. sajor-caju* and *P. florida* (1.4 g/l^{-1}) than *P. ostreatus* and *P. erengii* (0.8 g/l^{-1}). The main components of the isolated polysaccharides were glucans as shown by the NMR spectra. The electrophoretic pattern indicates that the separated polysaccharides are bound to proteins and the molecular weight ranges from 10 to 200 KD. The polysaccharide showed a notable proliferation activity for peripheral blood mononuclear cells (PBMC).

Key words: mushroom, polysaccharide, *Pleurotus*, submerged culture, mycelial biomass.

INTRODUCTION

Mushrooms have long been attracting a great deal of interest in many areas of food and biopharmaceuticals. They are regarded as popular or effective medicines used to treat various human diseases such as hepatitis, hypertension, hypercholesterolemia and gastric cancer (Lee *et al.*, 1994).

Microbial polysaccharides have received special attention due to their wide industrial application (Franz, 1989). In particular, several groups of polysaccharides from edible or medicinal mushrooms are attractive because they have potent biological and pharmacological activities, including immunostimulating antitumour, and hypoglycaemic activity (Franz, 1989 and Borchers *et al.*, 1999).

Submerged culture, in contrast to cultivation on solid media, gives rise to potential advantages of higher mycelial production in a compact space and shorter time with lesser chances of contamination (Yang and Liau, 1998). Moreover, the mycelial biomass can have various uses, which is an advantage as far as fermentation is concerned because the process residue is reduced (Maziero *et al.,* 1999). Possible uses of this biomass are food or feed in the form of protein supplement or source of lipids. It can also be used for the extraction of flavours and other metabolites, such as enzymes and polysaccharides (Jong and Birgmingham, 1993). A recent utilization of fungal biomass is for wound healing. Chitin, that has a healing capacity, is already in the fibrous form when extracted from the fungal cell wall (Hamlyn and Schmidt, 1994).

The aim of the present study was to investigate the possibility of producing exopolysaccharide and fungal biomass using four different species of *Pleurotus* in submerged culture. A chemical study of the produced EPS was performed as well.

MATERIALS AND METHODS

Microorganisms **:** *Pleurotus* spp. used in this study were *P. sajor-caju* STCPI-27, *P. erengii* ATCC 36047, *P. ostreatus* NRRL 2366 and *P. florida* STCPI-10. The stock cultures were maintained on potato dextrose agar (PDA) slants. Slants were incubated at 25°C for 7 days then stored at 4°C.

Liquid culture media **:** Composition of the fermentation medium was as follows gl^{-1}: Peptone 1.0, yeast extract, 2.0, K_2HPO_4, 1.0, $MgSO_4.7H_2O$, 0.2, $(NH_4)_2SO_4$, 5.0 and glucose 39.0, pH 6.0 (Cavazzoni and Adami, 1992). The flask culture experiments were performed in 250-ml flasks containing 100 ml of the fermentation medium. This medium was inoculated with two 5 mm discs cut from the periphery of a 7-day old fungal culture grown on PDA medium in Petri-dishes. Incubation was performed at 25°C on a shaker at 180 rpm for 14 days. Culture broth was separated by filtration and the fungal biomass was washed with distilled water, lyophilized and weighed.

Analytical methods **:** The fungal biomass was analyzed for total nitrogen using microKjeldahl method as described in the A.O.A.C. (1995), and the total crude protein was calculated.

In order to precipitate the exopolysaccharides, the culture broth was centrifuged at 6000 xg for 10 min and the resulting supernatant was filtere mixed with equal volume of absolute ethanol, stirred vigorously and left overnight at 4°C (Bae *et*

al., 2000). Both the precipitated top and bottom fractions of the polysaccharides were collected together, then lyophilized and the weight of the polymer was estimated.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out according to the method described by Laemmli (1970) using 12 % polyacrylamide gel.

^{1}H and ^{13}C NMR spectrometry were performed according to the method described by Abraham and Loftus (1978). The polysaccharide sample was dissolved in one ml of concentrated dimethyl sulphoxide (DMSO) by ultrasonication for 10-15 min. The solution was introduced into a precision ground tube (5mm diameter, 2-3 cm depth) then subjected to measurement. To achieve well resolued NMR spectra, sample tubes were placed in a bath type sonicator, cooled with ice for 20 min.

Detection of immune stimulation activity of exopolysaccharides : Peripheral blood mononuclear cells (PBMC) were isolated from healthy individual by Ficoll-Hypaque (Sigma, St. Louis, MO, USA) gradient centrifugation. The purified cells were cultured at 1.0 X 10^6 cells ml^{-1} in RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 25 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma), 4mM L-glutamine (Cambrex), 100U of penicillin and 100 µg streptomycin (Cambrex) and 10% FES (GEPCO, BRL, USA). PBMC were stimulated with 200, 100, 80, 40, and 20 µg ml^{-1}, of each of *P. sajor-caju*, and *P. eryngii* mycelia exopolysaccharides. A positive control culture was included, where PBMC was stimulated with 2 µg/ml^{-1} Phytohemaglutinin-L (PHA, Sigma). Proliferation was determined after incubation for 3 days at 37°C, 5% CO_2, and 95% humidity, by addition of 20 µl of BrdU labeling reagent (Roche, Penzerg, Germany) in the last 2 hours of the culture. The labeled cultures were harvested and the bromo deoxy uridine (BrdU) uptake was determined using Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche) following the manufacturer instructions (Porstmann *et al,* 1985). All samples were assayed in triplicates. Data were presented as stimulation index (SI), where proliferation is considered positive if SI is ≥ 2.

RESULTS AND DISCUSSION

It is clear from Table 1 that all of the tested *Pleurotus* spp. successfully produced exopolysaccharides in different quantities. *P. sajor-caju* and *P. florida* gave the highest yields reached 1.4 gl^{-1} while *P. erengii* and *P. ostreatus* gave the lowest of 0.8 gl^{-1} dry EPS after 14 days of fermentation.

There was no correlation between fungal biomass and its protein content with the exopolysaccharides production with the majority of tested *Pleurotus* spp. The

highest biomass producer, however, was *P. florida* which gave 9.6 gl^{-1} with a 2.76 gl^{-1} total protein.

In this respect, Maziero *et al.* (1999) screened different basidiomycetes for the production of exopolysaccharides in submerged culture for 14 days. They mentioned that most of the different *Pleurotus* spp. tested gave the highest biomass yield after 14 days which ranged between 4.50 and 11.72 gl^{-1} for *P. ostreatus* and *P. florida*, respectively. On the other hand, the same authors have found that the concentration of EPS has either increased as the fermentation period increased as in the case of *P. flabellatus* and *P. ostreatoroseus* or decreased as in the case of *P. ostreatus*, *P. sajor caju* and *Pleurotus florida*. Exopolysaccharides produced reached 1.72 and 1.36 gl^{-1} after 14 days of fermentation for *P. sajor caju* and *Pleurotus florida*, respectively.

Also, Kim *et al.* (2002) when tested different media for the production of exobiopolymers in submerged cultures by various edible mushrooms reached the same conclusions. They reported that while fungal biomass generally reached its maximum after 15 days of fermentation, the EPS reached their maxima after 10 - 15 days according to fermentation media used.

According to obtained results and those of others (Maziero *et al.*, 1999, Kim *et al.*, 2002) it could be concluded that exopolysaccharide yield and mycelial growth of mushrooms vary widely with respect to the fungal species. Moreover, the profile of polysaccharides production from the different species was not consistent with that of the mycelial growth.

Table 1. Screening of different *Pleurotus* spp. for the production of bio-exopolysaccharides (EPS) and fungal biomass in submerged culture

Tested *Pleurotus* spp.	Biomass			Effluent	
	Mycelial D.W.	Crude protein		(EPS) D.W.	Final pH
	gl^{-1}	%	gl^{-1}	gl^{-1}	
P. sajor-caju	6.3	28.22	1.78	1.4	5.8
P. erengii	3.5	31.82	1.11	0.8	5.3
P. ostreatus	8.3	27.7	2.30	0.8	3.7
P. florida	9.6	28.7	2.76	1.4	4.8

The NMR spectra of the exopolysaccharides isolated from the different *Pleurotus* species are similar to each other although there are significant differences in the relative intensities of the peaks.

The ^{1}H NMR spectra (Fig. 1) of the exopolysaccharides exhibited signals at different resonance which represent the anomeric proton and protons of the different hydroxyl groups.

The ^{13}C NMR spectra (Fig. 2) showed the presence of 6 carbon atoms. The proton magnetic resonance is the most accurate spectroscopic method used to determine the structure of new compounds. It gives information about the number of each type of hydrogen. ^{1}H NMR spectra for the isolated polysaccharides are comparable to those obtained by Zhuang *et al.* (1994). These authors reported the presence of β-anomeric proton at 4.8-5.1 ppm in the extracted polysaccharides from different mushroom species. ^{13}C NMR spectra revealed signals that are characteristic for glucan.

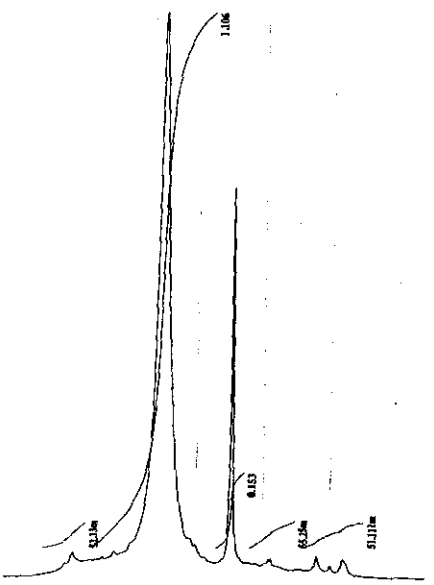

Fig. 1. ^{1}H NMR of polysaccharides isolated from *P. erengii*

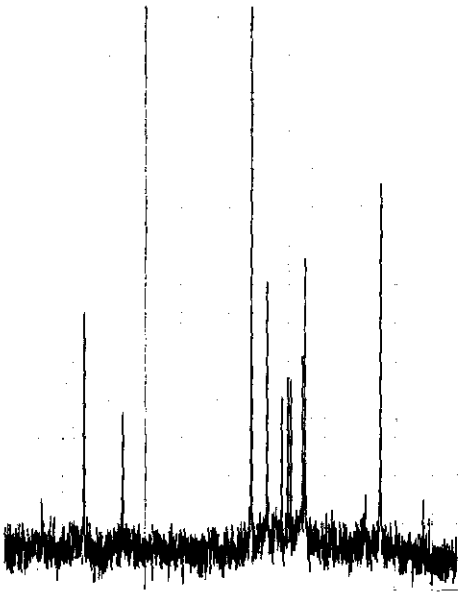

Fig. 2. ^{13}C NMR of polysaccharides isolated from *P. erengii*

The obtained NMR spectral data could be compared with those reported by Gorin (1981) for Lentinan polysaccharide, a potent antitumour agent, extracted from Shiitake. The immunostimulant activity of the exopolysaccharides studied by Gorin (1981) indicates that mushroom polysaccharides act as a host defense potentiator

through maturation, differentiation and proliferation of the important cells in host defense mechanisms (Hamuro and Chihara, 1985 and Chihara *et al.*, 1987).

The electrophoresis pattern (Fig. 3) indicates that each isolated polysaccharide was actually a mixture of several polysaccharides, some of them with high molecular weights and the others with low molecular weights.

Data presented in Fig. 4 showed that the exopolysaccharides might stimulate proliferative response of normal peripheral blood mononuclear cells (PBMC) in an independent dose manner.

The electrophoretic analysis indicates that the isolated polysaccharides are bound to a protein portion. Hobbs (1995) reported that fungal polysaccharides are often molecularly bound to various proteins. Proteins are molecules that closely represent the unique identity of organisms, and thus often activate the immune system as they enter the body. Sometimes, they might initiate an immune response of the body.

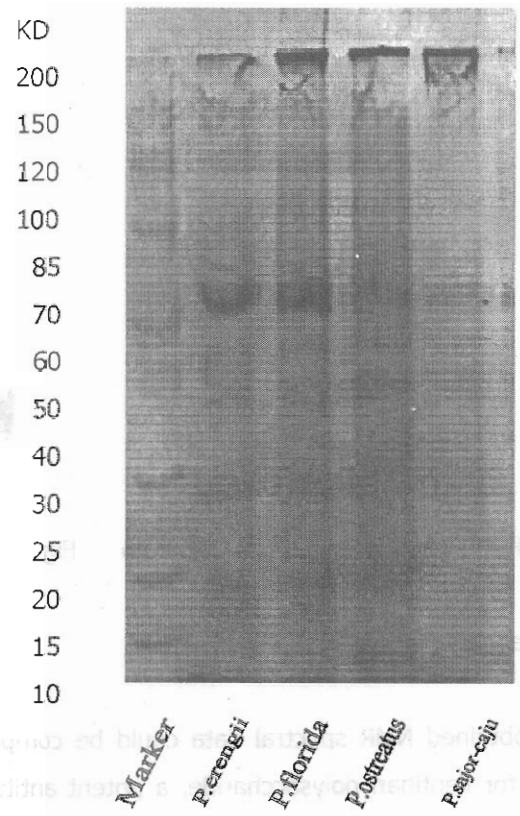

Fig. 3. SDS PAGE of protein-bound exopolysaccharides

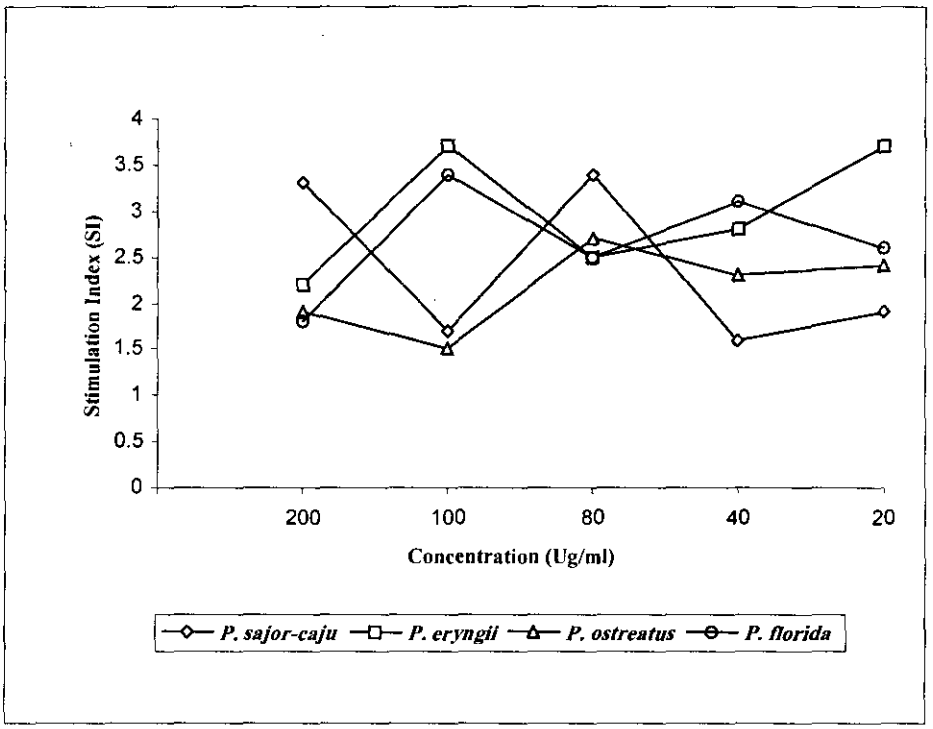

Fig. 4. Stimulatory effect of EPS produced by *Pleurotus* spp. on normal PBMC.

The fundamental information obtained in this study may be the first steps for a large-scale production of this valuable bioactive product by submerged cultures. However, there are many questions need to be answered on the production of polysaccharides in liquid culture. Future studies will, also, provide guidelines of incorporation of mushroom mycelium and /or their polysaccharides into the diet for their nutritional and pharmaceutical properties.

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إنتاج الميسليوم الفطرى والسكريات العديدة المنتجة خارجياً بواسطة المزارع السائلة لأنواع من فطر عيش الغراب من جنس الـ ***Pleurotus***

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استخدم فطر عيش الغراب المحارى *Plerotus* spp. فى إنتاج النموات الفطرية والسكريات العديدة باستخدام المزارع السائلة. ولقد أعطى فطر *P. florida* أعلى نمو للميسليوم الفطرى حيث بلغ ٩,٦ جم/لتر وكانت كمية البروتين به ٢,٧ جم/لتر بعد ١٤ يوم من فترة التحضين على ٢٨° م فى المزارع المهتزة. ووجد أن أعلى كمية من السكريات العديدة المنتجة خارج جدر الخلايا كانت لفطر *P. florida* و *P. sajor-caju* حيث وصلت إلى ١,٤ جم/لتر بينما فطر *P. ostreatus* و *P. erengii* أنتجا ٠,٨ جم/لتر فقط من السكريات العديدة.

ولقد أمكن معرفة التركيب الرئيسى لهذه السكريات والتى هى عبارة عن جلوكان عن طريق استخدام جهاز الرنين المغناطيسى (NMR). وباستخدام جهاز الفصل الكهربائى وجد أن السكريات المعزولة ترتبط بجزيئات بروتينية وأمكن معرفة الوزن الجزيئى للمركبات المفصولة والذى يتراوح ما بين ١٠ - ٢٠٠ كيلو دالتون. ولقد أثبتت الدراسة أيضاً أن هذه السكريات العديدة تعمل على تحفيز وتنشيط نمو الخلايا الطبيعية بالجهاز المناعى.

هذا البحث يلقى الضوء على أهمية التوسع فى إنتاج الميسليوم الفطرى والسكريات العديدة المنتجة خارج جدر الخلايا باستخدام المزارع السائلة لإمكان استخدامهم فى المجالات الغذائية والطبية.