Antioxidant and antibacterial properties of the extracts from
Pleurotus ostreatus EVFB1 and EVFB4

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Abstract

The aim of this work was focused on the obtaining of Pleurotus ostreatus mycelium by submerged fermentation, in order to get freeze-dried biomass and extracts with high antioxidant and antimicrobial activities. The tests were performed at 25°C, in 300 ml Erlenmeyer flasks. The most effective carbon source and the optimum agitation speed were determined, in order to cultivate the two strains in a medium containing 2% malt extract and 2% peptone.

For both strains the optimum agitation speed was established at 150 rpm. The most effective carbon source for Pleurotus ostreatus EVFB1 was glucose (10%) and, for Pleurotus ostreatus EVFB4, lactose (15%). The obtained biomass was freeze-dried and was submitted to ethanol and pure methanol extraction. It resulted that Pleurotus ostreatus EVFB1 and EVFB4 strains had an antioxidant activity by 10% higher on average when ethanol was used as solvent, compared to methanol. The phenols quantities were different, notwithstanding the solvent used, for Pleurotus ostreatus EVFB1 strain being by 29% higher as compared to Pleurotus ostreatus EVFB4, namely 68.6 mg/g gallic acid. In the case of reducing power, the ethanol extracts were the most effective. The two Pleurotus species proved narrow antibacterial activity against Gram-negative and Gram-positive bacteria tested.

Key words: mushrooms, glucose, lactose, DPPH, antimicrobial capacity

Introduction

A feature of mushrooms is the fact that they can be classically cultivated in fungi farms, but also in bioreactors, by growing the mycelium. [1]

Their cultivation is relatively similar to that of other microorganisms. The most important differences are related to the fermentation time, the culture medium and the stirring speed. A fermentation meant to obtain mycelium from Pleurotus ostreatus takes between 10 – 12 days. [2] The carbon source is usually the glucose, but sucrose and starch can also be used, even lactose in more advanced studies. Stirring plays a special role in the fungi multiplication speed. If cultivating mycelium in stirred flasks or in a bioreactor, the stirring must be set up at 100 – 150 rpm. If cultivating micelyum in a bioreactor, the oxygen level and that of emitted carbon dioxide must be strictly controlled. The decrease of the fermentation time can be obtained under the condition of an exponentially growth of the inoculum and a lower rate of the agitation rate, not through the increasing of the carbon source quantity inside the culture medium. [2, 3, 4]
The mushrooms bioactive metabolites can be industrially produced by their submerged cultivation in bioreactor, but the efficiency depends on the possibility to develop a good technological procedure adapted to the used strain. [4] The development of this technology is facilitated by the increasing of the product quantity and the development of new production systems.[2] Such compounds are released at a lower rate in the culture medium, while most of them can be released through extraction with various solvents. It is supposed that the antioxidant effects of the filtered culture medium are the result of the polysaccharides action. Researches have proven that alcoholic extracts, especially ethanol ones from Pleurotus strains have a strong inhibition effect of the oxidative stress at the level of the liver and brain. [5, 6] Also, there are studies revealing that the alcoholic extracts of the Pleurotus strains have a high reducing power and a significant phenol quantity. All these are related to a strong antioxidant activity and to an antimicrobial and antifungal activity. The latest researches show that the aqueous extracts of Pleurotus sajor-caju and Pleurotus pulmonarius also have a strong antiviral effect. [7]

Materials and Methods

**Strains and cultivation conditions.** The researches were conducted within the Animal and Vegetal Biotechnologies Lab, Biotechnology Faculty – USAMV Bucharest, between August - October 2010. A Pleurotus ostreatus EVFB1 strain was used, sampled from a willow lump outside the IRASM Hall – Magurele, Ilfov and a Pleurotus ostreatus EVFB 4 sampled from the Baneasa forest. The strains were kept in Nalgene cryotubes, in 20% glycerol, at -80°C. The inoculum was obtained through cultivation at 100 rpm, during seven days, in a medium containing 2% malt extract and 2% peptone.

For each strain, the carbon source was established, selected between the glucose, the lactose and the sucrose. Also, the concentration of this source and the stirring in lab conditions were determined, at 25°C. The studies were performed in a cooling LabTech incubator. The biomass was isolated through centrifuging, with a Centurion C2041 centrifuge and it was submitted to freeze-drying in a Alpha 1-2 LD freeze-dryer, in the absence of a cryoprotecting agent. [3, 4]

**Obtaining Pleurotus ostreatus extracts.** The freeze-dried biomass was submitted to the ethanol and methanol extraction through stirring at 150 rpm, at room temperature, while the ratio was 1g freeze-dried biomass for 10 ml solvent. The extract was separated after 24 hours through filtering, with Whatman no. 1 paper. The perfectly clear solution was freeze-dried with the same Alpha 1-2 LD freeze-dryer and it was tested at a 10 mg/ml concentration. [5, 9, 10, 12]

**Determining the total antioxidant activity.** The antioxidant activity was measured by determining the bonding ability of the free radical 1, 1 – diphenyl – 2 – picrylhydrazyl (DPPH), based on the method used by Chirinang [5]. 0.05 ml of extract was mixed with 1.95 ml DPPH 0.01% alcohol solution. After 45 minutes, the absorbance at 515 nm was read. [8, 12]

**Determining the total content of phenols.** The phenol content was determined by the Folin – Ciocalteu method. The extract (10 mg/ml) was mixed with 5 ml Folin – Ciocalteu reagent (diluted with 1/10 distilled water). The mixture also included 4 ml 7.5% Na carbonate. The mixture was stirred for 5 seconds and afterwards it was incubated for 30 minutes at 40°C. The absorbance at 765 nm was read at Helios spectrophotometer. The total phenol content was expressed as the mg/g equivalent of the gallic acid. [7, 12]

**Determining the reduction power.** The reduction power was determined through the method described by Jeng-Leun [12]. In a test tube, 2.5 ml extract (10 mg/ml) were mixed with 2.5 ml phosphate buffer, pH 6.6 and 2.5 ml K3[Fe(CN)]6 (10 mg/ml), then the mixture was inserted in the incubator for 20 minutes at 50°C. Then, 2.5 ml trichloroacetic acid 100
mg/ml were added, while the mixture was centrifuged at 2500 rpm, for 10 minutes. The upper layer was mixed with 5 ml ultrapure water and 1 ml FeCl₃ 1 mg/ml, while the absorbance was measured at 700 nm with a Helios spectrophotometer. According to the method, a high absorbance indicated a higher reduction power. [8, 12]

Determining the antimicrobial capacity. For tests, Escherichia coli CBAB 2, Bacillus cereus CMGB 215, Listeria innocua CMGB 218 strains were used. Each strain was inoculated separately (10⁶ UFC/ml) in a Petri dish on which LB agarised medium was poured. 20 μl of extract were added and the dish was kept 30 minutes to absorb the extract. Then it was inserted in the thermostat, at 28 – 30°C, for 24 hours. The generated inhibiting areas of the microorganisms were analyzed with the specialized software of Colony Quant. [11, 13].

Results and Discussions

The first phase of the cultivation experiments in liquid medium was carried out by using the two strains: Pleurotus ostreatus EVFB1 and EVFB4. The effects of the carbon source and of stirring speed on the biomass production were highlighted. In order to find the appropriate carbon source for the production of the biomass, Pleurotus ostreatus EVFB1 and EVFB4 pure cultures were used. The inocula were obtained by cultivating each strain in the culture medium with 2% malt extract and 2% peptone, without a carbon source. The tests were made by using a 2% concentration of glucose, sucrose and lactose, at stirring speeds between 0 - 250 rpm.

![Figure 1. The fresh cell biomass quantity after cultivating Pleurotus ostreatus EVFB1 with 2% glucose, sucrose, lactose](image1.jpg)

![Figure 2. The fresh cell biomass quantity after cultivating Pleurotus ostreatus EVFB4 with 2% glucose, sucrose, lactose](image2.jpg)
The studies revealed the fact that, for *Pleurotus ostreatus* EVFB1 strain, the most productive carbon source was glucose, at a stirring speed of 150 rpm (Figure 1). For the other stirring values, the results were significantly lower, even for lactose and sucrose. To increase stirring with 50 up to 100 rpm, the decreasing of the biomass quantity ranged between 60 - 70%, if glucose was used. By decreasing the stirring speed by 50 rpm lead to a reduction of the biomass quantity of about 63%. If the other two carbon sources were added, the biomass quantity was collected also at 150 rpm. The quantity was lower by 10% for sucrose and 21% for lactose, compared to glucose.

The *Pleurotus ostreatus* EVFB4 strain indicated a maximum biomass accumulation also at 150 rpm, but only if lactose was used as carbon source (Figure 2). For the other stirring speed values, the biomass quantity was very low, except for the presence of glucose, which induced the accumulation of biomass if stirring was increased by 50 – 100 rpm. The obtained values did not exceed a maximum of 6.16 g/l, obtained at 150 rpm, when glucose 2% was used. This was only 30% of the maximum accumulated quantity, of 16.1 g/l, if 2% glucose was used. The decrease of the stirring speed determined an average accumulation of 12%, which was very low for such a strain.

The influence of the carbon source concentration, namely of glucose in the case of *Pleurotus ostreatus* EVFB1 strain is indicated in Figure 3. The maximum biomass quantity, of 41.88 g/l, was obtained if an average 10% concentration of glucose was used. An increasing of the glucose concentration determined a strong inhibition of the mushrooms growth inside the culture medium. By doubling the glucose concentration from the basic culture medium, from 5 - 10 g/100 ml, an increase of the accumulated biomass quantity of 40% in the submerged culture was generated. In the case of *Pleurotus ostreatus* EVFB4 (Figure 4), the result was a maximum of 62 g/l, if a lactose concentration of 15% was used. The 5% decreasing of the lactose concentration determined a biomass accumulation by 11.61% lower. Instead, an increasing by 5% of the lactose concentration in the culture medium determined the decreasing of the biomass accumulation quantity. In this case, the quantity decreased by 3.87%.
The second phase of the study consisted in the obtaining of the extracts from the freeze-dried biomass of the two fungi strains. Once the carbon source and its concentration were established, the two strains were cultivated, in order to obtain biomass, in 1 l Erlenmeyer flask, with 500 ml of culture medium. After 10 days of cultivation, the biomass was isolated through centrifugation (about 200 g of wet biomass for each strain) and it was freeze-dried without the cryoprotector agent in order to increase the extraction capacity. The freeze-dried product was submitted to ethanol and methanol extraction and each used strain and solvent resulted in about 40 ml of extract.

The use of a 10 mg/ml extract concentration revealed the fact that ethanol was the most appropriate solvent. This feature was common to the two strains, even if, through the use of methanol, the *Pleurotus ostreatus* EVFB1 strain indicated a 89% antioxidant activity. It was 6% higher than the one obtained from the *Pleurotus ostreatus* EVFB4 biomass extract with the same solvent (Figure 5). If ethanol was used, the *Pleurotus ostreatus* EVFB4 strain had an antioxidant activity of 94.54%, which exceeded by only 2% the one resulting from the *Pleurotus ostreatus* EVFB1 freeze-dried biomass. In general, it is known that the methanol extracts have a lower antioxidant activity [12] of no more than 20%, but freeze-drying without a biomass protection agent determines a larger release of the active biological compounds, which increases about 4 times. Also, the value of the antioxidant activity of the ethanol...
extracts was by 2% lower than that of the ascorbic acid and by 4.5% lower than that of vitamin E, both used in a 0.5 mg/ml concentration.

According to the data presented by Chirinang [5], the phenols compounds are predominant in fungi extracts from *Pleurotus* strains. The average value of the phenols quantity in the ethanol extracts was about 38% lower than the one in the alcoholic extracts from the *Pleurotus ostreatus* EVFB1 strain, of 69 mg/ml gallic acid equivalent to the methanol extraction (Figure 6). For the *Pleurotus ostreatus* EVFB4 strain, the obtained value was about 15% higher, of 46.8 mg/ml gallic acid equivalent, also for methanol. As compared to the values obtained in the case of the methanol extracts, the ethanol ones were lower by 0.1%, for both strains, a value which can be included in the margin of error.

An important observation was that, in this research, the reduction power depended more on the strains and the solvent and less on the extract concentration. In the case of *Pleurotus ostreatus* EVFB4, the reduction power exceeded that of *Pleurotus ostreatus* EVFB1 by 9% for the ethanol extract and by 6% for the methanol one (Figure 7). For both strains, the ethanol extract indicated a reduction power exceeding that of the methanol one by about 25%. According to the literature data, the two strains have maximum values of the reduction power, which is confirmed by the high antioxidant activity, of over 90%, of the ethanol extracts.

### Table 1. The antibacterial effect of the extracts from *Pleurotus ostreatus* EVFB1 and *Pleurotus ostreatus* EVFB4 freeze-dried biomass (inhibition area diameter – cm)

<table>
<thead>
<tr>
<th>Sensitive strain</th>
<th><em>Pleurotus ostreatus</em> EVFB1</th>
<th><em>Pleurotus ostreatus</em> EVFB4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol</td>
<td>Methanol</td>
</tr>
<tr>
<td><em>Escherichia coli</em> CBAB 2</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> CMGB 215</td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td><em>Listeria innocua</em> CMGB 218</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The antimicrobial effect of the ethanol and methanol extracts of the two *Pleurotus ostreatus* strains was tested against the three Gram-positive and Gram-negative bacterial strains. Table 1 reveals the fact that the effect of the ethanol extract was a lot better than that of the methanol one. The ethanol extracts from the biomass of the two strains presented relatively similar inhibition areas. The exception to the rule was the ethanol extract from *Pleurotus ostreatus* EVFB4 which had an inhibition area diameter of 1 cm, smaller by a third than the one of *Pleurotus ostreatus* EVFB1. Instead, the methanol extract from the *Pleurotus ostreatus* EVFB4 freeze-dried biomass had a stronger antimicrobial effect than that of *Pleurotus ostreatus* EVFB1. The diameter obtained in the case of *Listeria innocua* CMGB 218 was identical, of 1.5 cm. For *Escherichia coli* CBAB 2, the diameter was by 61.5% smaller, while for *Bacillus cereus* CMGB 215 only by 38.4% smaller, namely 0.8 cm.

### Conclusions

The alcoholic extracts from *Pleurotus ostreatus* EVFB1 and *Pleurotus ostreatus* EVFB4 contain, in conclusion, a high quantity of antioxidant compounds. It is remarkable that the filtered mass resulting from the separation of the biomass had no antioxidant activity and did not contain polysaccharides. The lack of these two features can be determined by the composition of the culture medium. Instead, the freeze-dried extracts have a high phenols content, which also determined the high antioxidant activity. These observations were also doubled by the antimicrobial effect of the two types of extracts, as resulting from the research of Loganathan [7], with regard to the ethanol extract. Thus, the use of such extracts may generate a protection for the human body against the side effects of free radicals.
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