

Comparison of Antibacterial Activity of the Spent Substrate of *Pleurotus ostreatus* and *Lentinula edodes*

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Received: January 21, 2016 Accepted: February 24, 2016 Online Published: March 15, 2016

doi:10.5539/jas.v8n4p43

URL: <http://dx.doi.org/10.5539/jas.v8n4p43>

Abstract

Nowadays, the uncontrolled use of antibiotics has created the problem of bacterial resistance to them, what has motivated the search for new alternatives of drug for the treatment of bacterial diseases. Here, we compare antimicrobial activity of spent substrate of mushroom *Pleurotus ostreatus* and *Lentinula edodes*, against *Escherichia coli*, *Salmonella tiphymorium*, *Staphylococcus aureus* and *Micrococcus luteus*. We designed two mixtures, barley straw to be used as a substrate of cultivation of mushroom *Pleurotus ostreatus* and oats or cedar for the cultivation of mushroom *Lentinula edodes*; and were obtained aqueous extracts from spent substrates; extracts were tested for antibacterial activity. The protocol was a completely randomized assay with a factorial arrangement design. The data were analyzed with PROC GLM, SAS. The results showed that in the case of *Escherichia coli* the greatest inhibition zone was of 12.66 mm at a concentration of 6 mg mL⁻¹, with treatment of *Lentinula edodes*/Cedar; *Salmonella tiphymorium* showed a greatest inhibition zone of 31.10 mm to a concentration of 5.12 mg mL⁻¹, with treatment of *Pleurotus ostreatus*/Barley straw; *Staphylococcus aureus* showed a greatest inhibition zone of 9.33 mm to a concentration of 100 mg mL⁻¹, with the treatment of *Lentinula edodes*/Cedar and finally, *Micrococcus luteus* showed a greatest inhibition zone of 15.00 mm to a concentration of 50 mg mL⁻¹, with the treatment *Lentinula edodes*/Oats. In conclusion, the results suggest that it is possible to use indistinctly the spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* as source of extracts with antibacterial activity.

Keywords: mushroom, *Escherichia coli*, *Salmonella tiphymorium*, *Staphylococcus aureus* and *Micrococcus luteus*

1. Introduction

The uncontrolled use of antibiotics has caused serious problems in human and animal health, causing that bacterias develop resistance to them, so World Health Organization considered to infections caused by bacteria resistant to drugs as a public health problem; therefore, it is necessary to find new pharmacological strategies, among which we can find natural products such as plants and fungi (Roca et al., 2015).

Due to the excess production of agricultural wastes it has increased world production of edible fungi to over 7 million tons, of which 70% are Basidiomycetes as *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes* (Sánchez, 2010; Lin, Ge, & Li, 2014). Basidiomycetes produce several bioactive substances such as polysaccharides, peptides, nucleosides and phenols which act as hypoglycemic, immunomodulatory, anti-inflammatory, antitumor, antiviral, antibacterial or antiparasitic (Wasser & Wis, 1999; Wasser, 2002; Sánchez, 2010). By increasing the production of edible mushrooms, also increases accumulation of spent substrate residues after cultivation (Grodzinskaya, Infante, & Piven, 2002).

Spent mushroom substrate containing carbohydrates as cellulose and hemicellulose, lignin, remnant of edible fungi, is a byproduct of mushroom production industry. Substrate used as growth media to produce mushroom is composed of maize cobs, wheat straw, grass straw, sugarcane bagasse, field hay, corn cobs, cotton seed hulls and some other. After several mushroom harvesting cycles, the productivity of the substrate could decrease so that the substrate is declared as spent (Guoa & Choroverb, 2006; Onyango, Palapala, Arama, Wagai, & Gichimu, 2011). One of the main problems in the production of mushrooms still the treatment and disposal of spent

mushroom substrate; many studies have already been carried out for the use of such substrates, among which we mention feeding and/or antimicrobial activity (Zhu, Sheng, Yan, Qiao, & Lv, 2012).

The fungi have an important role in the degradation of organic matter (Chang & Miles, 1984), in addition to being a source of bioactive substances to produce antibiotics or pharmaceutical drugs, such as functional food and additives in feeding stuffs (Santoyo, Ramírez-Anguiano, Reglero, & Soler-Rivas, 2009). Crop of mushrooms (*Pleurotus ostreatus*) it is a source of products agricultural, their organic waste generated can be used as a source of food with high protein content and as an alternative pharmaceutical treatment; *Pleurotus ostreatus* is a fungus that has the ability to grow on agricultural wastes, accelerates the biodegradation and recycling them, avoiding its burning and the subsequent environmental pollution (Varnero, Quiroz, & Álvarez, 2010). *Lentinula edodes*, fungi edible, is the most studied, has been shown that fruiting body and mycelium have antimicrobial properties; likewise, the lentine inhibits mycelium growth of other fungi as *Physalospora piricola*, *Mycosphaerella arachidicola* and *Botrytis cinerea* (Rojas, 2013; Romero-Arenas, Martínez, Damian, Ramírez, & López-Olguín, 2015).

The production of mushroom has been used with a large number of substrates; one of the main is straw, used as a source of carbon to increase the nutritional characteristics and palatability of the fruiting body, getting a better nutritional quality (Sánchez, 2010). The crop of fungi and the quick growth in mushroom production worldwide has resulted in large quantities of spent substrate mushroom (about 13.6 million t year⁻¹). The massive amounts of waste can cause environmental problems; this causes, led more research to develop technologies for its treatment or use (Lin et al., 2014).

In recent years, there has been a need study antimicrobial phytochemicals with potential to generate new pharmacological options. Our group previously demonstrated that the use of spent substrate of *Pleurotus ostreatus* mixed or not with medicinal plants, has antibacterial activity (Ayala et al., 2015). Therefore, the objective of this study was to determine the antibacterial activity of spent substrate of *Pleurotus ostreatus* and/or *Lentinula edodes* against *Escherichia coli*, *Salmonella tiphymorium*, *Staphylococcus aureus* and *Micrococcus luteus* at different concentrations.

2. Method

2.1 Strain, Substrates and Cultivation Mushrooms

The blocks (1 Kg) were obtained from Centro de Investigaciones Biológicas of the Universidad Autónoma del Estado de Hidalgo, México. The blocks were formed by a mixture of barley straw; which were purchased in Central Abastos in Pachuca Hidalgo, Mexico, the taxonomic identification did it the botanist Dr. Miguel Angel Villavicencio Nieto; the specimens were deposited at the Herbarium of the Centro de Investigación de Ciencias Biológicas, of the Universidad Autónoma del Estado de Hidalgo, México. To form the substrate, barley straw was colonized with mycelium of *Pleurotus ostreatus* UAEH-004 in solid substrate fermentation; mushrooms were harvested at 23 d and was obtained spent substrate; on the other hand, oats or cedar were colonized with mycelium of *Lentinula edodes* UAEH-015 in solid substrate fermentation; mushrooms were harvested at 90 d and were obtained spent substrate of each one.

2.2 Preparation of Organic Extracts

The extracts were obtained by mixing 100 g of spent substrate mushroom of each treatments and 300 mL distilled water in case of *Pleurotus ostreatus* and 600 mL distilled water in case of *Lentinula edodes*. Then, the mix was macerated in blender during 1 min. Subsequently, mix macerated was filtered using gauze, to separate solid and liquid parts, was filtered through filter paper Whatman® #41. The extract was placed in a water bath to 70 °C for 48 h, to obtain the sample dry; for each 25 ml of liquid extract were obtained 0.472 g of *Pleurotus ostreatus* and 0.451 g of *Lentinula edodes* of dry extract.

2.3 Colony Forming Units (CFU)

For obtention of CFU, 100 µl mother sample was placed in 50 ml of nutrient broth and incubated at 37 °C for 24 h. Subsequently, through serial dilutions of this solution, to concentrations of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 200 µl were inoculated on plates; *Escherichia coli* in medium culture Mac Conkey (DIBICO, México), and *Salmonella tiphymorium*, *Staphylococcus aureus* and *Micrococcus luteus* in medium culture Estafilococos No. 110 (DIBICO, México), and incubated at 37 °C for 24 h, CFU they were counted.

2.4 Test Organisms

The test organisms were *Escherichia coli* (ATCC25922), *Salmonella tiphymorium* (ATCC14028), *Staphylococcus aureus* (ATCC25923) and *Micrococcus luteus* (ATCC9341).

2.5 Antimicrobial Assay

The antibacterial activity of the spent substrate of *Pleurotus ostreatus* and/or *Lentinula edodes* extracts were studied by the method of paper disc diffusion assay with slight modification (Kil et al., 2009). The bacterial pathogens strain were grown in liquid medium for 24 h to yield a final concentration of *Escherichia coli* 7.7×10^6 CFU/200 μ L, *Salmonella tiphymorium* 1.11×10^7 CFU/200 μ L, *Staphylococcus aureus* 1.0×10^7 CFU/200 μ L and *Micrococcus luteus* 1.04×10^7 CFU/200 μ L. Next, aliquots of 0.1 ml of the test microorganisms were spread over the surface of agar plates. Sterilised filter paper discs of 6 mm diameter (paper Whatman® #41) were saturated with 50 μ l of different concentrations (0, 6, 12.5, 25, 50 and 100 mg mL⁻¹) spent substrate of *Pleurotus ostreatus* and/or *Lentinula edodes* extracts. The soaked discs were then placed in the middle of the plates and incubated for 24 h at 37 °C (Forma Series II Water Jacket CO₂, Incubator, Model 3100, Thermo Scientific, USA), after which the diameter (in mm) of each inhibitory zone was measured (scalimeter). Negative control was prepared with distilled water; as positive control was used commercial antibiotic (Penicillin G sodium salt, Sigma-Aldrich, St. Louis, MO, USA) to a concentration 100 mg mL⁻¹ on medium culture Mac Conkey and Estafilococos No. 110.

2.6 Statistical Analysis

Data were analyzed using factorial design 3 \times 3 and blocked by extract type and extract concentration and bacterial strain as factors. A PROC GLM procedure and LSMEANS option were used (SAS, 2002).

3. Results

Treatment with spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* extracts were effective against four bacterial strains tested; antibacterial activity at different concentrations is showed in Table 1; the tested bacteria were quantitatively assessed by measuring the diameter of inhibition generated for each sample; each result is the mean of three replicates. The results showed that the spent substrate *Pleurotus ostreatus*/Barley straw extracts presented highest inhibitory effect against *Escherichia coli* (7.7×10^6 CFU/200 μ L) at a concentration of 12.5 mg mL⁻¹ with 9.86 mm inhibition halo; *Staphylococcus aureus* (1.0×10^7 CFU/200 μ L) at a concentration of 25 mg mL⁻¹ with 9 mm inhibition halo and *Micrococcus luteus* (1.04×10^7 CFU/200 μ L) at a concentration of 50 mg mL⁻¹ with 9.66 mm inhibition halo. *Salmonella tiphymorium* (1.11×10^7 CFU/200 μ L) at a concentration of 12.5 mg mL⁻¹ with 31.10 mm inhibition halo, showing significant differences with the concentrations 6, 25, 50 and 100 mg mL⁻¹ (P < .05).

In case of spent substrate *Lentinula edodes*/Oats extracts, results showed that the highest inhibitory effect against *Escheriquia coli* (7.7×10^6 CFU/200 μ L) to 12.5 mg mL⁻¹ with 9.20 mm inhibition halo; *Salmonella tiphymorium* (1.11×10^7 CFU/200 μ L) to 6 mg mL⁻¹ with 30.30 mm inhibition halo; *Staphylococcus aureus* (1.0×10^7 CFU/200 μ L) to 50 mg mL⁻¹ with 8.66 mm inhibition halo and *Micrococcus luteus* (1.04×10^7 CFU/200 μ L) to 50 mg mL⁻¹ with 15 mm inhibition halo; in this treatment, there was no significatives differences between each of the concentrations tested for each bacteria.

Spent substrate *Lentinula edodes*/Cedar extracts, results showed the highest inhibitory effect against *Escheriquia coli* (7.7×10^6 CFU/200 μ L) to 6 mg mL⁻¹ with 12.66 mm inhibition halo, showing significant differences with the concentrations 12.5, 25, 50 and 100 mg mL⁻¹ (P < .05) and *Salmonella tiphymorium* (1.11×10^7 CFU/200 μ L) to 50 mg mL⁻¹ with 29.80 mm inhibition halo, showing significant differences with the concentrations 6, 12.5 and 25 mg mL⁻¹ (P < .05). The highest inhibitory effect against *Staphylococcus aureus* (1.0×10^7 CFU/200 μ L) was to 100 mg mL⁻¹ with 9.33 mm inhibition halo and *Micrococcus luteus* (1.04×10^7 CFU/200 μ L) to 100 mg mL⁻¹ with 10 mm inhibition halo. Positive control (penicilin) showed larger diameter of inhibition (45 and 30.6 mm), than all spent substrate extracts tested.

Table 1. Antibacterial activity of spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* extracts at different concentrations *in vitro*

Treatment	Concentration (mg mL ⁻¹)	Diameter of inhibition (mm)			
		<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i>	<i>Micrococcus luteus</i>
		Means±SD			
<i>Pleurotus ostreatus</i> /Barley straw	6	9.53±0.40	22.33±2.51 ^c	0.00	0.00
	12.5	9.86±0.51	31.10±8.61 ^a	0.00	0.00
	25	9.46±0.68	26.96±10.01 ^b	9.00±1.00	0.00
	50	9.10±1.05	24.63±5.22 ^b	8.86±1.69	9.66±1.52
	100	9.00±1.17	20.50±3.43 ^c	8.36±0.71	8.83±1.25
<i>Lentinula edodes</i> /Oats	6	8.83±1.04	30.30±2.30	0.00	0.00
	12.5	9.20±0.72	29.60±4.35	0.00	0.00
	25	8.50±0.50	29.40±2.02	0.00	0.00
	50	8.06±1.32	28.76±3.99	8.66±1.15	15.00±2.29
	100	8.83±0.28	27.66±3.78	8.33±1.52	12.00±1.80
<i>Lentinula edodes</i> /Cedar	6	12.66±2.88 ^a	27.83±1.44 ^b	0.00	0.00
	12.5	8.66±0.35 ^b	26.00±1.00 ^b	0.00	9.33±0.57
	25	10.10±1.92 ^b	28.46±5.68 ^b	8.66±0.57	9.66±1.15
	50	7.83±1.61 ^b	29.80±1.55 ^a	8.43±1.40	9.66±1.52
	100	8.80±1.85 ^b	29.46±3.99 ^a	9.33±1.15	10.00±1.00
Penicilin	100	30.6±3.05	45.00±0.00	45.00±0.00	45.00±0.00

Note. ^{abc} Literal different ranks indicate significant difference between the concentrations of each treatment ($P < 0.05$) with the Tukey test.

When comparing the largest zone of inhibition of the three treatments tested (*Pleurotus ostreatus*/Barley straw, *Lentinula edodes*/Oats y *Lentinula edodes*/Cedar) against each bacteria (Table 2), the results showed in the case of *Escherichia coli* the largest zone of inhibition was at a concentration of 6 mg mL⁻¹ (12.66 mm) with treatment of *Lentinula edodes*/Cedar; *Salmonella typhimurium* showed the largest zone of inhibition at a concentration of 5.12 mg mL⁻¹ (31.10 mm) with treatment of *Pleurotus ostreatus*/Barley straw; *Staphylococcus aureus* showed the largest zone of inhibition at a concentration of 100 mg mL⁻¹ (9.33 mm) with the treatment of *Lentinula edodes*/Cedar and *Micrococcus luteus* showed the largest zone of inhibition at a concentration of 50 mg mL⁻¹ (15.00 mm) with treatment of *Lentinula edodes*/Oats. *Salmonella typhimurium* and *Staphylococcus aureus* no showed significant differences between each treatments; *Escherichia coli* treated with *Lentinula edodes*/Oats is significantly different to the treatment *Lentinula edodes*/Cedar ($P < 0.05$), but without showing difference significant with treatment *Pleurotus ostreatus*/Barley straw, while *Micrococcus luteus* treated with *Lentinula edodes*/Oats is significantly different with treatment of *Lentinula edodes*/Cedar and *Pleurotus ostreatus*/Barley straw ($P < 0.05$).

Table 2. Comparison of antibacterial activity of spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* extracts against *Escherichia coli*, *Salmonella typhimorium*, *Staphylococcus aureus* and *Micrococcus luteus* *in vitro*.

Bacteria	<i>Pleurotus ostreatus</i> /Barley straw		<i>Lentinula edodes</i> /Oats		<i>Lentinula edodes</i> /Cedar	
	Concentration (mg mL ⁻¹)	Diameter of inhibition (mm)	Concentration (mg mL ⁻¹)	Diameter of inhibition (mm)	Concentration (mg mL ⁻¹)	Diameter of inhibition (mm)
		Means ± SD		Means ± SD		Means ± SD
<i>E. coli</i>	12.5	9.86 ± 0.51 ^a	12.5	9.20 ± 0.72 ^b	6	12.66 ± 2.88 ^a
<i>S.typhimorium</i>	12.5	31.10 ± 8.61	6	30.30 ± 2.30	50	29.80 ± 1.55
<i>S. aureus</i>	25	9.00 ± 1.00	50	8.66 ± 1.15	100	9.33 ± 1.15
<i>M.luteus</i>	50	9.66 ± 1.52 ^b	50	15.00 ± 2.29 ^a	100	10.00 ± 1.00 ^b

Note. ^{ab} Literal different ranks indicate significant difference between treatments of each bacteria ($P < 0.05$) with the Tukey test.

4. Discussion

In recent years the bacteria have acquired the ability of multi-resistance to antibiotics (Nehra, Meenakshi, & Yadav, 2012) which has generated that recent research are focus in the search for alternative treatments, such as fungi. Edible fungi such as *Pleurotus ostreatus*, have shown a high nutritional value as food (Patel, Naraian, & Singh, 2012) and anti-inflammatory, antidiabetic, antiviral, antioxidant, anticancer, antitumor, immunomodulatory and antibacterial activity; however, most of the research has been based on the study of the fruiting body and not in spent substrate (Hearst et al., 2009; Deepalakshmi & Mirunalini, 2014).

A water-soluble polysaccharide named PL was isolated and purified from spent mushroom substrate, the polysaccharide contained two fractions (PL1 and PL2), composed of glucose, rhamnose and mannose; the antibacterial activity of polysaccharide against *E. coli* was the strongest, while the weakest against *Sarcina lutea*, the minimal inhibition concentrations of PL2 were 12.5, 25 and 100 µg/mL, respectively (Zhu et al., 2012). We show that aqueous extract of spent substrate of *Pleurotus ostreatus*/Barley straw has antibacterial activity against *Escherichia coli* (9.86 mm), *Staphylococcus aureus* (9.00 mm), *Micrococcus luteus* (9.66 mm) and *Salmonella typhimorium* (31.10 mm), similar to that obtained using extracts of mushroom *Pleurotus ostreatus* obtained with different organic solvents (24.56 and 14 mm) for Gram positive and Gram negative bacteria (Nehra et al., 2012).

For the cultivation of *Lentinula edodes* for many years have used various agricultural and industrial wastes, among which we mention sorghum, sugar cane, sawdust, oak, cedar (Grodzinskaya et al., 2002) as carbon source; shiitake mushrooms (*Lentinus edodes*) is of great importance, due to its attributed not only to its nutritional value, but also potential applications in industrial food and medicine as antibacterial (Hearst et al., 2009) and/or antitumor, among other features; this activity is due to the lentina (one polysaccharide isolated from fruiting body), which acts as an enhancer of host defense; has shown action against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (Hatvani, 2001). Chowdhury, Kubrai, and Ahmed (2015) mentioned antimicrobial activity of 3 edible mushrooms (*Pleurotus ostreatus*, *Lentinula edodes*, *Hypsizigus tessulatus*) methanolic extracts, indicated considerable activity against bacteria and fungi, revealing zone of inhibition ranged from 7 ± 0.2 to 20 ± 0.1 mm; Kazue, Megumi, and Dantas (2001) found that the mycelium of 35 different strains of *Lentinus edodes*, has antibacterial activity against *B. subtilis*, with inhibition halos 5-20 mm in diameter similar to our findings.

This work is novel because for the first time is studied the use of spent substrate *Lentinula edodes* as antibacterial, since only been shown this activity in the fruiting body; they have been used different extraction techniques: high-pressure operations and low-pressure methods. The high-pressure technique was applied to obtain *Lentinus edodes* extracts using pure CO₂ and CO₂ with co-solvent or organic solvents such as n-hexane, ethyl acetate and dichloromethane (Kitzberger, Smânia Jr., Pedrosa, & Ferreira, 2007); here it is included barley straw as a substrate for the cultivation of *Pleurotus ostreatus* and oats or cedar for the cultivation of *Lentinula edodes*, in order to obtain aqueous extracts and determine its antibacterial activity; the findings suggest that it is feasible to use these substrates in the future for obtain antibacterial pharmaceutical compounds and at the same time reduce the pollution by their accumulation.

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