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IN VITRO CULTIVATION OF *LAETIPORUS SULPHUREUS* AND EVALUATION OF ITS ANTIMICROBIAL PROPERTIES

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Abstract

Laetiporus sulphureus (Bull. Fr.) Murill., is a wood-rotting basidiomycete mushroom well known for it nutritional value. In this study, alcoholic and aqueous extracts obtained from a Romanian isolate of L. sulphureus cultivated on various culture media were investigated for the antimicrobial properties. PDA, malt extract as solid media and PD (I), malt extract (II), YPG (III) and Hwang (2008)(IV) -as liquid media were used for in vitro cultivation of L. sulphureus, in order to evaluate the optimal medium for an efficient biomass production of L. sulphureus. Between all media tested, best results regarding the growth of mycelia, were obtained when I, II, IV media were used. Only on IV (Hwang) culture medium was observed the typically orange pigment elaborated by fungus. Alcoholic and aqueous extracts of fruit bodies and submerged mycelium developed in liquid media were analyzed against strains of Candida albicans ATCC10321, Candida parapsilopsis CBS604, Escherichia coli, Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas fluorescens and Pseudomonas aeruginosa. The results shown that the types of culture media for biomass production of L. sulphureus and their aqueous and alcoholic extracts tested against these pathogens have different effects on inhibitory activity. Between the two types of extracts tested aqueous extracts were inferior to alcohol extract in their inhibitory activity on all organisms except Candida sp. in interaction with L. sulphureus aqueous extract from biomass developed on IV medium.

Keywords: *antimicrobial properties, in vitro cultivation, Laetiporus sulphureus*

INTRODUCTION

Laetiporus sulphureus (Bull. Fr) Murril (Aphyllophorales, Polyporaceae) is a wood – rotting basidiomycete mushroom which grow on mature and old-growth trees in forests or in urban parks. It is know as a destructive pathogen of the trees that cause butt and trunk rots (Holsten et al., 2001; Sinclair and Lyon, 2005). *L. sulphureus* is characterized by an intense orange colour, fleshy basidiocarps and tubular hymenopores (Banik et al., 1998) and can be harvested as an edible fungus with reliable nutritional value. Because of its medical properties *L. sulphureus* has been used in some therapies as antitumor, antiviral, antimicrobial treatments (Wasser and Weis, 1999). Among several other mushrooms like *Trametes versicolor, Ganoderma applanatum* and *G.lucidum*, *Laetiporus sulphureus* has been found to be an excellent source of natural products with therapeutic properties. Those mushrooms provide a rich variety of secondary metabolites and polysaccharides that have been proved to posses significant antimicrobial activities (Siljegovic et al., 2011). The fruiting bodies of *L. sulphureus* contain N-methylated tyramine derivatives (Rapior et al., 2000), polysaccharides (Alquini and Carbonero, 2004), terpenoids, laetiporic acids and other compounds (Weber et al., 2004; Davoli et al., 2005). From *L. sulphureus* submerged mycelia cultures have been isolated various polysaccharides (Hwang et al., 2008; Hwang and Yun, 2010) with therapeutic evidences. For these reasons, the goals of our studies were to

find the optimal medium for an efficient biomass production of *L. sulphureus* and to investigate the antimicrobial activities of alcoholic and aqueous extracts from fruit bodies and submerged mycelium developed in liquid media, against some pathogenic agents.

MATERIALS AND METHODS

***In vitro* culture establishment.** Samples of the fruit bodies of *Laetiporus sulphureus,* collected from Sinaia woods, were surface sterilizing and cutting out a piece of trama using a sterile scalpel. The pieces were placed in Petri dishes on 2% malt extract agar and PDA (potato-dextrose-agar) media and incubated at 25^0C for a week. After the mycelium growing on the medium surface, mycelia agar discs (5 mm diameter) obtained from the active growth areas were placed in 100 ml Erlenmeyer flasks, each with 50 ml PD (potato-dextrose) (I), 2% malt extract (II) and YPG (yeast peptone glucose) (III) liquid media. We also used a medium (IV) prepared according Hwang et al (2008) (20g/l glucose, 2g/l peptone, 2g/l yeast extract, 0,46g/l KH_2PO_4, 1g/l K_2HPO_4, 0,5 g/l $MgSO_4$), in order to determine the optimal growing medium for *in vitro* culture of *L. sulphureus*. After inoculation the samples were incubated at 25^0C in a rotary shaker at 148 rpm for 10 days. The biomass obtained from each liquid medium was filtrated and weighed.

Extracts preparation. For extracts preparation the biomass developed on each liquid medium tested was used. A mixture of mycelium and medium from the *in vitro* culture was separated by filtration. The filtrated mycelia mass was grounded and used for the extracts preparation. For aqueous extract, 1 ml of distilled water per 1 g of mushroom material was added. In the case of alcohol extract, 1 ml 70% ethyl alcohol was added to sample (1 g wet weight). Also, ethyl alcohol (70%) was used as negative control. The aqueous and alcoholic solutions were then centrifuged at 5000 rpm for 10 minutes. The supernatant was kept at 4 ^{0}C and used to determine the antimicrobial activity of *L. sulphureus* extracts.

Antimicrobial activity. Alcoholic and aqueous extracts of submerged mycelium developed in liquid media were analyzed against strains of *Candida albicans* ATCC10321*, Candida parapsilopsis* CBS604 (from the collection of MICROGEN, Bucharest)*, Escherichia coli, Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas fluorescens* and *P. aeruginosa* (from the collection of Faculty of Biotechnologies Bucharest). To determine the antimicrobial activities of tested extracts, 1 ml from each bacterial and yeasts suspensions was inoculated on Luria Broth or YPG media, respectively, in Petri plates. After removing the excess suspension using a micropipette, sterile filter paper discs soaked in fungal extracts were placed on the surface of the inoculated medium. 24 hours after incubation at 37^0C and 27^0C respectively, occurrence of inhibition halos was observed.

RESULTS AND DISCUSSIONS

The submerged cultivation of mushrooms is a promising method for obtaining pharmaceutical compounds and was applied for various edible and medicinal species (Petre et al., 2012). For *L. sulphureus* there are only few reports regarding the submerged cultivation and fungal pellet obtaining in order to obtain various metabolites (Sivulski et al., 2009; Hwang et al., 2008).

For this reason, one of the aims of our experiments was to evaluate the effects of different media compositions on the submerged mycelium growth of *L. sulphureus* and to achieve maximum biomass production. For this purpose *L. sulphureus* fresh mushroom was firstly cultivated on two agar media (malt extract and PDA). The mycelium grown on the surface of these media was used for inoculation of four different liquid media: I-PD (potato-dextrose), II- malt extract, III-YPG (yeast peptone glucose) and IV- Hwang (2008). After ten days of culture on these tested media the results shown that the best growth of the mycelia biomass (as pellets) was observed on Hwang medium followed by PD and malt extract media. The poorest growth was encountered on YPG medium. The wet weight of filtrated mycelia biomass harvested from Hwang medium (8.26 g/100ml) was significantly superior to that obtained on PDA

(2.4 g/100 ml) and malt extract (1.30 g/100ml) media, respectively. The mycelia growth as pellets is of great interest from practical point of view, facilitating the extraction procedures of biological active compounds and which can be used to prepare functional food (Petre et al., 2012).

I-PD II-Malt extract III- YPG IV- Hwang

Figure 1. Aspect of submerged mycelia of *Laetiporus sulphureus* in different liquid media

An interesting aspect of our results was the accumulation of orange pigment in mycelium in submerged conditions (in Hwang medium) (figure 2); in the other media the mycelium was uncoloured.

Figure 2. *Laetiporus sulphureus* orange pigment elaborated in Hwang medium

Davoli et al (2005), investigating the orange pigment produced by fruit-bodies of *L. sulphureus* using modern spectroscopic techniques, found that the pigment is a polyene of non-isoprenoid biosynthetic origin named laetiporic acid. The practical significance of this compound is not very clear but it could be used as food colorant.

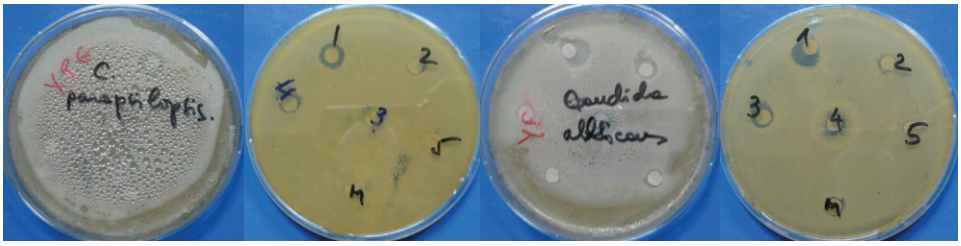
In the second investigation, alcoholic and aqueous extracts obtained from biomass developed on each liquid medium tested (I, II, III, IV) were analyzed for their ability to inhibit six strains of Gram positive and Gram negative bacteria: *E. coli, B. cereus, S.aureus, E. faecalis, P. fluorescens* and *P. aeruginosa* and two pathogenic yeasts: *Candida albicans* ATCC 10231 and *Candida parapsilopsis* CBS 604. The results obtained shown that the culture media used for submerged cultivation of *L. sulphureus* mycelium influenced not only the growth rate but also the biological activities of aqueous and alcoholic extracts against pathogens (Table 1).

Table 1. Pathogen interactions with *L. sulphureus* extracts obtained from biomass developed on different liquid culture media (I, II, III, IV)

Variant	Liquid media	Extracts	Pathogens							
			E. coli	*B. cereus*	*S. aureus*	*E. faecalis*	*P. fluorescens*	*P. aeruginosa*	*C. albicans*	*C. parapsilopsis*
I	PD	aqueous	-	-	-	-	-	-	-	-
		alcoholic	+	-	++	+	+	-	++	++
II	Malt extract	aqueous	-	-	-	-	-	-	-	-
		alcoholic	-	-	-	-	-	-	-	-
III	YPG	aqueous	-	-	-	-	-	-	-	-
		alcoholic	++	-	-	-	-	-	+	-
IV	Hwang	aqueous	-	-	-	-	-	-	+	+
		alcoholic	-	-	++	-	-	-	+	-

It was shown that the inhibitory action of **aqueous extracts** were inferior to alcohol extracts against all the pathogenic organisms tested. However, the aqueous extract from biomass developed on IV (Hwang) medium presented clear inhibition area against both *Candida* species (Figure 3). **Alcoholic extracts** of biomass provided from IV (Hwang) medium were optimal for inhibition of *S. aureus* and *C. albicans.* Clearly inhibition halo was observed on *S. aureus, E. coli E. faecalis, P. fluorescens* and against both *Candida* species when the alcoholic extract from biomass provided to I (PD) medium was used (Figure 3).

Alcoholic extract from biomass growth on III (YPG) medium had the best inhibitory activity against *C. albicans* and *E. coli*, but the alcoholic extracts from mycelium growth in malt extract medium inhibited only the growth of *E. coli* strain.



a b

Figure 3. Inhibitory activity of *L. sulphureus* alcoholic extracts from biomass developed on I (PD) medium against *Candida* sp. (a, b -1). M=control (70% alcohol)

CONCLUSIONS

The goals of our studies were to find the optimal medium for an efficient biomass production of *L. sulphureus* and to investigate the antimicrobial activities of alcoholic and aqueous extracts from submerged mycelium developed in liquid media, against some pathogenic agents. The data from *in vitro* growth tests provides significantly evidences on biological differences between mycelia biomass developed on various media used. Between all media tested for maximum biomass production, the best growth of the fungal biomass, including as pellets, was observed on Hwang medium follow by PD and malt extract media. Only in Hwang culture medium was observed the typically orange pigment elaborated by fungus. Alcoholic and aqueous extracts from submerged mycelium cultivated in different liquid media tested against several microbial strains demonstrate the influence of culture conditions on inhibitory activity. It was shown that aqueous extract from submerged mycelia developed on Hwang media was inferior to alcohol extract in their inhibitory activity on all pathogenic organisms tested except *Candida* sp. Alcoholic extracts of biomass provided from IV (Hwang) medium had the best inhibitory action for inhibition of *S. aureus* and *C. albicans*. Moreover, alcoholic extract from *L. sulphureus* biomass developed in PD medium inhibited *Candida* sp., *S. aureus, E. coli E. faecalis* and *P.fluorescens*.

The results obtained are promising but optimisations of the growth conditions as well as the extraction procedures are necessary in order to recover larger quantities of biological active compounds.

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