GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Short Communication

Screening of basidiomycetes in submerged cultivation based on antioxidant activity

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Genet. Mol. Res. 14 (3): 9907-9914 (2015)
Received February 13, 2015
Accepted July 1, 2015
Published August 19, 2015
DOI http://dx.doi.org/10.4238/2015.August.19.25

ABSTRACT. Submerged cultivation of medicinal basidiomycetes is a reproducible and efficient method of producing mycelia and metabolites. The antioxidant activity indicates its medicinal properties and is an important tool for basidiomycete screening. In this study, we analyzed the production of mycelial biomass and exopolysaccharides and the antioxidant activity of basidiomycete strains in submerged cultivation. Twenty-five strains were used for submerged cultivation in extract malt medium, and the production of mycelial biomass and exopolysaccharides was evaluated. Antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl method. Among the 25 evaluated strains, *Lentinus crinitus* produced the highest biomass, reaching 1190 ± 52 $mg \cdot L^{-1} day^{-1}$; *Agaricus subrufescens* strains had the highest exopolysaccharide production from 18.96 ± 0.15 to 20.97 ± 2.10 mg $L^{-1} \cdot day^{-1}$. Additionally, *A. subrufescens* showed the highest total antioxidant activity, reinforcing the therapeutic potential of this basidiomycete. No significant correlation was found between mycelial biomass or exopolysaccharide production and antioxidant activity;

however, the results depended on each species and the strains of the same species. We found large variations in the production of mycelial biomass and exopolysaccharides and in antioxidant activity among different species and among strains of the same species. Thus, evaluating the total antioxidant activity is an important tool for identifying strains with biotechnological potential.

Key words: *Agaricus blazei*; *Agaricus brasiliensis*; *Lentinula edodes*; *Lentinus crinitus*; *Pleurotus ostreatus*; *Schizophyllum commune*

INTRODUCTION

The fungus kingdom includes approximately 1.5 million species; however only 14,000 are basidiomycetes (Blackwell, 2011). Few of these genera are cultivated on an industrial scale worldwide, including *Pleurotus*, *Lentinula*, *Auricularia*, *Agaricus*, *Flammulina*, *Coprinus*, *Agrocybe*, and *Volvariella* (Li, 2012). Mushrooms are used for a variety of biotechnological applications, particularly for the production of food, enzymes, dietary supplements, pharmaceutical compounds (Elisashvili, 2012), feed supplements (Santos et al., 2015), and flavoring agents (Jong and Birminghamn, 1993), among other uses.

According to Lindequist et al. (2005), 80-85% of all medicinal mushroom products is derived from fruiting bodies and only 15-20% is from mycelia in submerged cultivation. However, submerged cultivation is more efficient and under better control for producing medicinal biocompounds from basidiomycetes (Elisashvili, 2012; Bertéli et al., 2014; Almeida et al., 2015). The first step for submerged cultivation success is the screening of basidiomycetes that are well-adapted to the environment and capable of yielding large amounts of biotechnological bioproducts.

Basidiomycete medicinal properties originate from various cellular components and secondary metabolites that can be isolated and identified in the fruiting body, vegetative mycelia, or in the culture broth (Elisashvili, 2012). One important indicator of the medicinal properties of an organism is its antioxidant activity (Izawa and Inoue, 2004). Although numerous studies have investigated antioxidant production in medicinal mushrooms (Lindequist et al., 2005), few have examined antioxidant production of mycelial submerged cultivation. Basidiomycete strains with high mycelial biomass, exopolysaccharide production, and antioxidant activity, growing under submerged cultivation conditions, are of great industrial interest. Exopolysaccharides resulting from secondary metabolism of microorganisms are polymers excreted from the outside of the cell walls with potential applications in pharmaceutical, biochemical, and medical areas because of their specific biological activities such hepatoprotective, immunomodulatory, antiangiogenic, antiviral, and antioxidant activities (Matou et al., 2005; Borchers et al., 2008; Arena, et al., 2009). Additionally, these molecules show potential use in agriculture and in the industry (Barbosa et al., 2004). In this study, we analyzed mycelial biomass, exopolysaccharide production, and antioxidant activity of basidiomycete strains in submerged cultivation.

MATERIAL AND METHODS

Biological material

Twenty-five strains of several basidiomycetes from the Fungus Collection of the

Laboratory of Molecular Biology of the Universidade Paranaense, including *Agaricus subrufescens* Peck (*Agaricus blazei* Murrill *ss*. Heinemann; *Agaricus brasiliensis* Wasser et al.), *Lentinula edodes* (Berk.) Pegler, *Lentinus crinitus* (L.) Fr., *Pleurotus eryngii* (DC.) Quél., *Pleurotus ostreatus* (Jacq.) P. Kumm., and *Schizophyllum commune* Fr., were used. For the recovery of cryopreserved fungi (Mantovani et al., 2012), the mycelium of each fungus was transferred to 20 g/L malt extract agar medium, which had been sterilized at 121°C for 20 min and stored in the dark at 28 ± 1°C. Mycelium showing homogenous growth without sectioning was selected as the inoculum.

Production of mycelial biomass and exopolysaccharides

Three malt extract agar discs, measuring 5 mm in diameter and containing the mycelium of each strain, were transferred to 250-mL Erlenmeyer flasks containing 30 mL 20 g/L malt extract broth. Flasks were stored at 28 ± 1°C in the dark. After 10 days, another 30 mL malt extract broth was added to each Erlenmeyer flask. Mycelial biomass was allowed to grow for 21 days, after which it was separated by centrifugation at 2900 *g* for 15 min at 4°C. The supernatant was separated (supernatant-1) and the precipitate containing mycelial biomass was washed twice with 30 mL ultra-purified water and again separated by centrifugation at 2900 *g* for 15 min at 4°C. To determine total exopolysaccharides, 10 mL supernatant-1 was mixed with 30 mL cold (5°C) ethanol and kept at 5°C for 12 h. The precipitate containing the exopolysaccharides was separated by centrifugation at 2900 *g* for 15 min at 4°C. The resulting mycelial biomass and the exopolysaccharides were frozen at -20°C and freeze-dried to determine dry mass. All experiments were conducted in triplicate.

Antioxidant activity of mycelial biomass and exopolysaccharides

First, 1.5 mL methanol was added to the freeze-dried mycelial biomass or 0.2 g exopolysaccharides and stored at 60°C for 60 min (Mourão et al., 2011); the mixture was centrifuged at 4400 *g* for 20 min at 4°C. The supernatant was considered the raw extract. Free radical scavenging activity was determined using the 1,1-diphenyl-2-picrylhydrazyl method. For this test, 0.1 mL raw extract was mixed with 2.9 mL 60 µM fresh 1,1-diphenyl-2-picrylhydrazyl methanolic solution. After incubation for 30 min at 22 ± 2°C, decreased absorbance at 515 nm was observed. All tests were repeated 3 times. The antioxidant activity was calculated relative to a standard solution of 60 µM quercetin, the absorbance of which was considered to be 100% (Molyneux, 2004) and total antioxidant capacity was calculated by multiplying the percentage of antioxidant activity by the mass of the mycelial biomass or exopolysaccharide produced. The results were analyzed by variance analyses and the differences among averages determined by Scott-Knott's test ($P \leq 0.05$). Multiple regression and correlation analyses were conducted to examine associations among mycelial biomass, exopolysaccharide production, and antioxidant activity.

RESULTS

Table 1 shows the production of mycelial biomass and the antioxidant activity for the basidiomycete mycelial biomass. The mycelial biomass production was higher ($P \leq 0.05$) for *Lentinus crinitus* U9-1 at 1190 ± 52 $mg \cdot L^{-1} \cdot day^{-1}$ and lower ($P \leq 0.05$) for strains of *Agaricus*

subrufescens U2-6 and U2-4 with 98 ± 8 and 87 ± 9 $mg \cdot L^{-1} \cdot day^{-1}$, respectively (Table 1). The latter showed some of the lowest results, while other strains of *A. subrufescens* showed intermediate results for mycelial biomass production, such as *A. subrufescens* U2-7, the second most productive strain with 993 ± 45 $mg \cdot L^{-1} \cdot day^{-1}$ (Table 1).

Table 1. Mycelial biomass (MB), antioxidant activity (AA), and total AA of basidiomycete strains.

Strain	Code	MB ($mg \cdot L^{-1} \cdot day^{-1}$ of cultivation medium)	AA (% Quercetin/mg mycelium)*	Total AA (% quercetin $\cdot L^{-1} \cdot day^{-1}$ cultivation medium)
Agaricus subrufescens	U4-2	842 ± 91^{c}	11.17 ± 1.50^{b}	9384 ± 317^{a}
Agaricus subrufescens	U2-7	993 ± 45^{b}	7.16 ± 1.47^{e}	7109 ± 335^{b}
Lentinus crinitus	U9-1	1190 ± 52^{a}	5.84 ± 0.22^{f}	6930 ± 156^{b}
Schizophyllum commune	U6-7	868 ± 24^{c}	7.02 ± 0.22^{e}	6078 ± 330^{e}
Agaricus subrufescens	U2-3	475 ± 12^{e}	11.74 ± 1.71^{b}	5576 ± 279^{d}
Agaricus subrufescens	U11-2	487 ± 16^{e}	9.85 ± 0.28^{c}	4796 ± 170^{e}
Agaricus subrufescens	U7-3	629 ± 28^{d}	7.24 ± 0.10^{e}	4554 ± 120^{e}
Agaricus subrufescens	U4-1	368 ± 27^{f}	11.35 ± 1.80^{b}	4176 ± 98^{f}
Pleurotus ostreatus	U2-11	615 ± 38^{d}	6.61 ± 0.18^{f}	4065 ± 102^{f}
Pleurotus ostreatus	U2-9	573 ± 21^{d}	7.09 ± 0.34^{e}	4062 ± 80^{f}
Pleurotus ostreatus	U6-9	652 ± 38^{d}	6.20 ± 0.10^{f}	4042 ± 69^{f}
Agaricus subrufescens	U2-1	654 ± 74^{d}	5.94 ± 0.49^{f}	3878 ± 145^{f}
Agaricus subrufescens	U2-2	615 ± 78^{d}	6.40 ± 0.35^{f}	3936 ± 77^{f}
Pleurotus ostreatus	U6-8	651 ± 14^{d}	6.01 ± 0.15^{f}	3912 ± 95^{f}
Pleurotus ostreatus	U6-10	639 ± 51^{d}	5.96 ± 0.26^{f}	3808 ± 133^{f}
Agaricus subrufescens	U7-1	479 ± 51^{f}	4.64 ± 0.62^{g}	2224 ± 177^{g}
Pleurotus eryngii	U8-11	548 ± 14^{e}	4.02 ± 1.11^{g}	2203 ± 168^{g}
Lentinula edodes	U6-11	319 ± 16^{g}	6.16 ± 0.10^{f}	1965 ± 190^{g}
Agaricus subrufescens	U6-16	105 ± 9^{i}	17.63 ± 0.10^{a}	1851 ± 255^{g}
Lentinula edodes	U6-12	369 ± 45^{f}	5.05 ± 0.21^{g}	1863 ± 184^{g}
Agaricus subrufescens	U2-6	98 ± 8^{i}	11.62 ± 0.48^{b}	1138 ± 64^{h}
Lentinula edodes	U8-1	259 ± 12^{h}	4.02 ± 0.11^{g}	1041 ± 122^{h}
Agaricus subrufescens	U4-3	115 ± 16^{i}	8.43 ± 0.34^{d}	969 ± 163^{h}
Agaricus subrufescens	U4-4	87 ± 9^{i}	8.68 ± 0.14^{d}	755 ± 105^{i}
Agaricus subrufescens	U2-4	329 ± 22^{g}	2.29 ± 0.21^{h}	753 ± 94^{i}

*Percentage in relation to 60 µM quercetin solution. Averages indicated by the same letter do not differ statistically according to the Scott-Knott test ($P \leq 0.05$).

Higher antioxidant activity ($P \leq 0.05$) was observed for *A. subrufescens* U6-16 with 17.63 ± 0.10%$_{Quercetin}$/mg, but it produced only 105 ± 9 $mg \cdot L^{-1} \cdot day^{-1}$ of mycelial biomass (Table 1). In contrast, *Lentinus crinitus* U9-1 showed the highest production of mycelial biomass, with antioxidant activity of just 5.84 ± 0.22%$_{Quercetin}$/mg (Table 1).

The total antioxidant activity was higher ($P \leq 0.05$) for *A. subrufescens* U4-2 with 9384 ± 317%$_{Quercetin}$ L/day. This strain was the third-highest producer of mycelial biomass (842 ± 91 mg/L) and had the second-highest antioxidant activity (11.17 ± 1.5%$_{Quercetin}$/mg); thus, when combined biomass production and antioxidant activity the *A. subrufescens* U4-2 becomes the highest antioxidant producer (Table 1).

Table 2 shows the production of the exopolysaccharides and its antioxidant activity as well as total antioxidant activity for the basidiomycete exopolysaccharides. Exopolysaccharide production was higher ($P \leq 0.05$) for the *A. subrufescens* U11-2, U7-3, U2-4, U2-2, and U2-1 strains, ranging from 18.96 ± 0.15 to 20.97 ± 2.10 $mg \cdot L^{-1} \cdot day^{-1}$ (Table 2). The highest ($P \leq 0.05$) exopolysaccharide production with relatively higher ($P \leq 0.05$) antioxidant activity was observed for *A. subrufescens*, emphasizing the therapeutic potential of this basidiomycete. The total antioxidant activity of exopolysaccharides was the highest ($P \leq 0.05$) for *A. subrufescens* U11-2 with 347 ± 28%$_{Quercetin}$ L/day (Table 2).

Table 2. Exopolysaccharides (EPS), antioxidant activity (AA), and total AA of basidiomycete strains.

Strain	Code	EPS ($mg \cdot L^{-1} \cdot day^{-1}$ cultivation medium)	AA (% Quercetin/mg mycelium)*	Total AA (% Quercetin/L cultivation medium)
Agaricus subrufescens	U11-2	19.21 ± 1.30^{a}	18.08 ± 1.23^{a}	347 ± 28^{a}
Agaricus subrufescens	U7-3	18.96 ± 0.15^{a}	14.80 ± 1.57^{b}	273 ± 35^{b}
Agaricus subrufescens	U2-4	19.06 ± 0.94^{a}	11.23 ± 0.17^{d}	217 ± 86^{b}
Agaricus subrufescens	U2-2	20.97 ± 2.10^{a}	13.11 ± 0.36^{c}	275 ± 20^{b}
Agaricus subrufescens	U4-2	18.16 ± 0.36^{b}	12.86 ± 0.23^{c}	236 ± 58^{b}
Agaricus subrufescens	U2-3	15.13 ± 0.87^{c}	17.47 ± 1.66^{a}	264 ± 28^{b}
Agaricus subrufescens	U4-1	17.96 ± 0.39^{b}	12.74 ± 0.04^{c}	228 ± 52^{b}
Agaricus subrufescens	U2-1	19.63 ± 1.93^{a}	11.33 ± 0.32^{d}	222 ± 25^{b}
Agaricus subrufescens	U7-1	12.60 ± 1.06^{d}	15.35 ± 1.76^{b}	193 ± 17^{c}
Agaricus subrufescens	U2-6	18.13 ± 0.66^{b}	9.35 ± 0.04^{e}	175 ± 29^{c}
Agaricus subrufescens	U4-3	10.08 ± 0.99^{d}	15.85 ± 0.99^{b}	159 ± 38^{c}
Agaricus subrufescens	U2-7	10.12 ± 1.99^{d}	11.07 ± 0.52^{d}	112 ± 10^{d}
Agaricus subrufescens	U4-4	3.49 ± 0.08^{g}	15.08 ± 0.45^{b}	53 ± 2^{e}
Agaricus subrufescens	U6-16	3.41 ± 0.07^{g}	14.93 ± 0.41^{b}	51 ± 1^{e}
Pleurotus eryngii	U8-11	7.01 ± 0.76^{e}	7.32 ± 0.06^{f}	51 ± 4^{e}
Pleurotus ostreatus	U2-9	6.56 ± 0.33^{e}	7.14 ± 0.05^{f}	47 ± 6^{e}
Pleurotus ostreatus	U6-9	5.51 ± 0.28^{f}	7.41 ± 0.06^{f}	41 ± 3^{f}
Pleurotus ostreatus	U6-8	5.47 ± 0.16^{f}	7.44 ± 0.08^{f}	41 ± 2^{f}
Schizophyllum commune	U6-7	3.77 ± 1.70^{g}	11.09 ± 0.19^{d}	42 ± 1^{f}
Lentinula edodes	U8-1	3.10 ± 0.34^{g}	7.17 ± 0.17^{f}	22 ± 3^{g}
Lentinus crinitus	U9-1	3.20 ± 0.04^{g}	7.25 ± 0.28^{f}	23 ± 1^{g}
Pleurotus ostreatus	U6-10	3.05 ± 0.19^{g}	7.28 ± 0.15^{f}	22 ± 1^{g}
Pleurotus ostreatus	U2-11	2.98 ± 0.25^{g}	6.90 ± 0.68^{f}	20 ± 1^{g}
Lentinula edodes	U6-12	2.51 ± 0.30^{g}	6.95 ± 0.29^{f}	17 ± 2^{g}
Lentinula edodes	U6-11	1.95 ± 0.22^{h}	7.41 ± 0.01^{f}	14 ± 1^{h}

*Percentage in relation to 60 µM quercetin solution. Averages indicated by the same letter do not differ statistically according to the Scott-Knott test ($P \leq 0.05$).

The mycelial biomass, exopolysaccharide production, and the respective antioxidant activity showed high variability among species and even among different strains of the same species. Considering only the strains of *A. subrufescens*, the mycelial biomass production varied by approximately 11-fold, the antioxidant activity of the mycelial biomass varied by approximately 8-fold, the exopolysaccharide production varied by approximately 6-fold, and the antioxidant activity varied by approximately 2-fold. In addition, no significant correlation was found between mycelial biomass or exopolysaccharide production and its antioxidant activity for the strains, but values depended on each species and among strains of the same species.

DISCUSSION

Kirsch et al. (2011) cultivated *L. citrinus* in medium containing 0.5% soluble starch and 0.2% gelatin and obtained 1150 $mg \cdot L^{-1} \cdot day^{-1}$ mycelial biomass. In our study, a similar result was found for *L. crinitus* (1190 $mg \cdot L^{-1} \cdot day^{-1}$; Table 1). Elisashvili (2012) reported mycelial biomass production in submerged cultivation in the range of 5300-12,700 mg/L at different cultivation times for different basidiomycetes. Although numerous variables affect mycelial biomass production, strain type appeared to be the most important factor in cultivation (Mshandete and Mgonja, 2009). Therefore, *L. crinitus* U9-1 is an excellent choice for biotechnological processes involving mycelial biomass production.

All strains evaluated produced exopolysaccharides. Fungal exopolysaccharides function in adhesion to the substrate, immobilization of extracellular enzymes, prevention of hyphal dehydration, and storage of excess nutrients, as well as participate in lignin degradation, a widespread process among basidiomycetes (Elisashvili, 2012). The highest exopolysaccharide

production was obtained for strains of *A. subrufescens*, which reached 20.97 $mg \cdot L^{-1} \cdot day^{-1}$. Lin and Yang (2006) reported an exopolysaccharide yield of 35.85 $mg \cdot L^{-1} \cdot day^{-1}$ for *A. subrufescens* grown in an optimized cultivation medium with malt extract, yeast extract, and peptone at 105 rpm for 7 days at 27°C. Fan et al. (2007) cultivated *A. subrufescens* under optimized conditions in medium containing glucose, yeast extract, K_2HPO_4, and $MgSO_4$ for 7 days, without agitation, at 30°C and obtained 32.45 $mg \cdot L^{-1} \cdot day^{-1}$. Shu and Xu (2007) produced *A. subrufescens* in medium containing glucose, yeast extract, malt extract, polypeptone, K_2HPO_4, $MgSO_4$, and vitamin B_1 for 24 days at 28°C, with aeration of 0.15 volume per volume per minute, and obtained 98.33 $mg \cdot L^{-1} \cdot day^{-1}$ exopolysaccharides. In our study, the exopolysaccharide production from strains of *A. subrufescens* varied from 3.41-20.97 $mg \cdot L^{-1} \cdot day^{-1}$, with a possible greater increase in exopolysaccharide production after the optimization of the cultivation conditions.

Lin and Chen (2007) studied the factors affecting the production of mycelial biomass and exopolysaccharides in submerged cultivation of *Antrodia cinnamomea*. They observed that high mycelial biomass production was not a determining factor for a high exopolysaccharide yield. In our study, no significant correlation was found between mycelial biomass and exopolysaccharide production. This appears to be a consensus in the literature and is in accordance with the findings of Maziero et al. (1999) who evaluated 48 native Brazilian basidiomycete strains and 8 commercial ones but found no relationship between mycelial biomass and exopolysaccharide production in submerged cultivation.

Agaricus subrufescens showed the highest antioxidant activity and the highest production of exopolysaccharides among the other strains evaluated (Table 2). Exopolysaccharides of *A. subrufescens* included glucoproteins, mainly β-D-glucans, which are primarily responsible for the biological activities of this fungus (Chen and Seviour, 2007). Ker et al. (2005) studied the antioxidant activity of 5 polysaccharide fractions extracted from *A. subrufescens* mycelium in submerged cultivation and associated the potent antioxidant effect with the high number of hydroxyl groups in the polysaccharide and the low content of polysaccharide-protein complexes. Izawa and Inoue (2004) suggested that *A. subrufescens* extracts contain a complex mixture of antioxidants and other substances that may act synergistically to reduce free radicals. The high free radical scavenging capacity of *A. subrufescens* reported in our study is in accordance with the results of previous studies. In addition, it is possible to increase the production of antioxidant molecules for *A. subrufescens* by studying several culture conditions such as temperature, carbon, and nitrogen sources, among others.

Mycelial submerged cultivation can provide a faster route for antioxidant and exopolysaccharide production in basidiomycetes. No significant correlation was found between mycelial biomass or exopolysaccharide production and antioxidant activity for the basidiomycete strains studied. The strains recommended for the production of mycelial biomass and for high total antioxidant activity include *L. crinitus* U9-1 and *A. subrufescens* U4-2 and U2-7. This is the first study to describe the antioxidant activity of *L. crinitus*. For antioxidants from exopolysaccharides, *A. subrufescens*, mainly the strain U11-2, is recommended. We observed high variability for the production of mycelial biomass, exopolysaccharides, and antioxidant activity among different species and even among strains of the same species, making it essential to evaluate the total antioxidant activity to identify strains with biotechnological potential.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Paranaense University, and the Postgraduate Program in Biotechnology Applied to Agriculture.

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