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Polysaccharide Production by Submerged and Solid-State Cultures from Several Medicinal Higher Basidiomycetes

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ABSTRACT: Polysaccharides produced by microorganisms represent an industrially unexploited market. An important number of polysaccharides have been isolated from fungi, especially mushrooms, with many interesting biological functions, such as antitumor, hypoglycemic, and immunostimulating activities. In the search of new sources of fungal polysaccharides, the main goal of this research was to test the ability of several species of basidiomycetes, among them various edible mushrooms, to produce both extracellular polysaccharides (EPSs) and intracellular polysaccharides (IPSs). Among 10 species screened for production of EPSs in submerged cultures with glucose, soy oil, and yeast extract, the best results were obtained with *Ganoderma lucidum* (0.79 g/L EPS) and *Pleurotus ostreatus* (0.75 g/L EPS). Agitation strongly improved EPS production in most of the studied strains. Eight of 10 species assayed successfully developed basidiomes during synthetic "bag-log" cultivation on a substrate consisting of oak sawdust and corn bran. This work describes for the first time the environmental factors required for fruiting of 4 species under such conditions: *Schizophyllum commune*, *Ganoderma applanatum*, *Trametes versicolor*, and *T. trogii*. IPSs were extracted from the carpophores. The IPS content of the carpophores varied from 1.4% (*G. applanatum*) up to 5.5% and 6% in *G. lucidum* and *Grifola frondosa*, respectively.

KEY WORDS: medicinal mushrooms, higher Basidiomycetes, submerged cultivation, solid-state fermentation, exopolysaccharides, fructification, endopolysaccharides

ABBREVIATIONS: EPS, exopolysaccharides; **IPS,** intracellular polysaccharides

I. INTRODUCTION

Polysaccharides are used industrially as thickeners, stabilizers, and gelling agents in food products; they also are employed as depolluting agents. There is a growing interest in their biological functions, such as antitumor, antioxidant, or prebiotic activities.[1] They are derived from a wide variety of sources: bacterial, fungi, algae, and plants. Despite the many sources of polysaccharides, the world market is dominated by polysaccharides from algae and higher plants. Production of exopolysaccharides (EPSs) from microorganisms, including bacteria, yeasts, and molds, demands shorter periods of time. EPSs produced by microorganisms represent an industrially untapped market.[2]

Production of EPSs is widely distributed among fungi.[3] They fulfill different tasks during growth on natural substrates, such as adhesion to surfaces, immobilization of secreted enzymes, prevention of hyphae from dehydration, and increased residence time of nutrients inside the mucilage.[4] Research interest has concentrated on polysaccharides produced by medicinal mushrooms because of their various biological and pharmacological activities. These include immunostimulating, antitumor, antibacterial, antiviral, hypocholesterolemic, antioxidant, and hypoglycemic activities; vitality and performance-enhancement effects; and beneficial cosmetic effects on skin.[5,6] Medicinal mushroom polysaccharides, such as lentinan and schizophyllan, among others, are mainly glucans

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with different structures. β-D-glucans are the most common polysaccharides of higher Basidiomycetes, with a branched structure with varying degrees of substitution, containing β(1→3) linkages in the main chain and additional β(1→6) branch points.[7] To obtain bioactive polysaccharides from edible and medicinal mushrooms, different authors have cultivated mushrooms on solid media (for production of fruiting bodies) or in submerged cultures. In addition to EPSs excreted into fermentation broth, mycelial cultures also produce internal polysaccharides localized within the mycelia (endo- or intracellular polysaccharides [IPSs]). Submerged cultures of higher Basidiomycetes can be used to produce polysaccharides in a shorter time when compared to the production and extraction process of mushroom fruiting bodies. This encouraged many authors to develop a variety of culture media to optimize the production of these polymers.[8] Most reports have focused on EPSs, but not on IPSs.[5] The IPSs can be extracted from fruiting bodies and their mycelia by solid–liquid extraction using heated water or mixtures of organic solvents. Because the demand for fruiting bodies, mycelium biomass, or both is constantly increasing, artificial cultivation has become essential. Solid-state cultivation is one way of meeting the rising demand for fungal mycelium and its bioactive metabolites.[9] In the search for new sources of mushroom polysaccharides, the main goal of this research was to screen the ability of several species of basidiomycetes for the production of EPSs under submerged cultivation and to evaluate the IPS content in the fruiting bodies produced by these mushrooms under solid-state cultivation.

II. MATERIALS AND METODS

A. Medicinal Mushroom Material

The medicinal mushrooms studied in this work were selected according to their commercial potential and their attributes. Thus, species like *Grifola frondosa*, *Lentinus edodes*, *Ganoderma lucidum*, and others are becoming better known and their markets are growing because of their active components and usefulness. On the other hand, species like *Schizophyllum commune*, *Ganoderma applanatum*, and *Trametes trogii* have been studied by different researchers, who have found that their components are also important for several segments of industry, although their cultures have not been disseminated. In this way, they represent both scientific and industrial interest.

Ten species of higher Basidiomycetes were screened: *L. edodes* (CICL54), *Sch. commune* (UCS004), *T. trogii* (BAFC463), *Trametes* (=*Coriolus*) *versicolor* (PSUWC430), *Pycnoporus sanguineus* (UCS003), *Ganoderma applanatum* (UCF001), *G. lucidum* (UCC002), *Grifola frondosa* (PSUMCC 922), *Pleurotus ostreatus* (UCC001), and *Auricularia delicata* (UCS002). Pure cultures are deposited at Culture Collection of Macrofungi at University of Caldas (Manizales, Colombia). The species were maintained on potato dextrose agar at 4°C, with periodic transfer.

B. Culture Medium for Production of EPSs Under Submerged Fermentation

Inocula consisted of a 50-mm^2 surface agar plug from a 10-day-old culture grown on potato dextrose agar. Cultures were performed in 100-mL Erlenmeyer flasks containing 25 mL of liquid medium. The medium consisted of 1 g ammonium sulfate, 1 g monopotassium sulfate, 0,5 g magnesium sulfate heptahydrate, 0.1 g calcium chloride, 30 g glucose, 14.15 g soy oil, 2 g yeast extract, and distilled water up to 1 L. Initial pH of the medium was 5.5. The medium was autoclaved at 121°C for 15 minutes. Incubation was carried out at 25°C. Static as well as stirring conditions (100 rpm) were assayed. Cultures were harvested after 14 and 21 days. Six replicas of all the experiments were performed.

C. EPS Measurement

Cultures were harvested over time and mycelium was separated from the culture broth by vacuum filtration using a preweighed glass microfiber filter (Whatman GF/F, Maidstone, England). To estimate growth, mycelial weight was determined after drying overnight at 80ºC the mycelial mats obtained

from filtration. For determination of both water- and alkali-soluble EPSs, the EPSs were precipitated from the resulting culture filtrate (0.5 mL mixed with 4 times the volume of 96% ethanol over 45 minutes) and then separated by centrifugation at 14000 rpm for 15 minutes. The insoluble components were resuspended in 0.5 mL sodium hydroxide 1 M at 60°C for 1 hour. After solubilization of the EPS, 5- to 50-µL samples were taken (proper dilutions in distilled water were done when necessary), the volume was completed to 0.5 mL with distilled water, then 0.5 mL of 5% (w/v) phenol were added and the mixture was shaken thoroughly. Three milliliters of 98% sulphuric acid also were added and stirred vigorously. The tubes were allowed to stand 30 minutes at room temperature. A blank test was prepared by substituting the solubilized EPSs with distilled water. Total carbohydrates were determined by measuring the absorbance at 490 nm.[10] The EPS content of supernatants was calculated by subtracting the amount of alcohol-insoluble carbohydrates from uninoculated media. EPS yields were expressed as grams of EPS per liter of culture medium and specific yields as milligrams of EPS per gram dry mycelium.

D. Basidiome Production

Spawn of all the species evaluated, with the exception of *G. frondosa*, was prepared on wheat grains (40% moisture content) according to the method described by Chang and Miles.[11] *G. frondosa* was inoculated on corn grains according to the method described by Montoya et al.[12] The substrate consisted of (dry weight basis) 73% oak sawdust (25% moisture content), 23% corn bran (13% moisture content), 2% sucrose, and 2% calcium carbonate. The substrates were packed in polypropylene bags and tyndallized. The moisture content was adjusted according to the development requirements of each species *L. edodes* (55%), *Sch. commune* (60%), *T. trogii* (65%), *T. versicolor* (60%), *G. applanatum* (65%), *G. lucidum* (60%), *G. frondosa* (58%), and *P. ostreatus* (70%).[11–17] Each bag of (20-cm diameter and 30-cm height) contained 1 kg of substrate. One square hole (2.54 cm^2) was made at the top of each bag and covered with a microporous breather strip to allow for gas exchange. The bags containing the substrate were aseptically inoculated with 4% (wet basis) of the spawn.

E. IPS Measurement

Carpophores were dehydrated in a Deni dehydrator tray (Keystone Manufacturing Company, Inc., Buffalo, NY, USA), with forced convection of hot air, at a temperature between 55 and 60°C and up to 5% moisture content. Subsequently, the dried fruit bodies were milled with a Tecator knife mill (model Cyclotec 1093, Foss Tecator AB, Höganäs, Sweden) to achieve particle-size mesh 180–200. For determination of IPSs, 1 g dry powder (5% moisture content) was mixed with 50 mL of 80% (v/v) ethanol and extracted with an ultrasound-assisted extractor (Elmasonic E Elma extractor, Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) for 30 minutes, with an ulterior 20-hour rest contact between the fungus and the solvent. The extract was filtrated at reduced pressure by using a preweighed glass microfiber filter (Whatman GF/F) and then centrifuged for 15 minutes at 14000 rpm. The precipitate was suspended in 1M sodium hydroxide solution at 60°C for 1 hour. Total carbohydrates were determined in this suspension using the phenol-sulfuric method.[10]

F. Statistical Analysis

To select the best EPS-producing species among those evaluated, a multifactorial design with 3 factors—10 species, incubation day (14 or 21) and stirring conditions (100 rpm or none)—was performed. The response variable was the total carbohydrate concentration in grams of EPS per liter. The data presented are the average of the results of at least 3 replicates with a standard error of <5%. Analysis of variance and repeated measures analysis were tested by Statistica software version 7 (StatSoft, Inc., Tulsa, OK, USA). The significant differences between treatments were compared by Tukey's test at 5% level of probability.

III. RESULTS AND DISCUSSION

A. Production of EPS in Submerged Cultivation

EPS production in submerged cultivation under stirred and static conditions was evaluated after 14 and 21 days of incubation in 10 species of higher Basidiomycetes. Results are summarized in Table 1. The analysis of variance showed that the species and incubation conditions influenced EPS production, whereas the incubation time was not a significant factor in the production of EPS. Among the species assayed, best results were obtained with *G. lucidum* at 21 days of incubation under agitation (0.79 g/L of EPS) and *P. ostreatus* (0.75 g/L) under the same conditions. Tukey test indicated that *G lucidum*, produced higher EPS concentrations on average than *A. delicata*, *P. sanguineus*, *L. edodes*, *Sch. commune*, *G. frondosa*, *T. trogii*, and *G. applanatum*, but no significant differences were obtained with EPS production by *P. ostreatus* and *T. versicolor*. Agitation strongly improved EPS production in most of the studied species. For example, for *L. edodes* and *P. ostreatus*, EPS production at 21 days under static conditions was around 30% of that produced under agitation. Nevertheless, for some of the species tested (*T. trogii*, *G. applanatum*, *G. frondosa,* and *A. delicata*), agitation and incubation time were not significant factors in EPS production. Differences between EPS concentrations obtained under static and agitated conditions in both incubation periods did not exceed 15%. Although the statistical analysis of experimental data showed no significant differences in the incubation period, differences in EPS production were observed in some of the species tracked, as was the case of *L. edodes* without stirring, which decreased from 0.21 g/L EPS at 14 days of incubation to 0.08 g/L after 21 days; *T. trogii*, which rendered 0.28 g/L under agitated conditions after 14 days but only 0.16 g/L at 21 days; or *P. sanguineus*, whose EPS production increased under static conditions from 0.07 g/L at 14 days to 0.20 g/L at 21 days. Stirring conditions may affect fungal biomass production and, in turn, EPS production. Maintenance of high dissolved oxygen levels was important for both cell growth and EPS formation in *Tremella fuciformis*.[18] However, Park et al.[19] reported an inhibitory effect of high aeration rates on EPS production by *Cordyceps militaris*.

Submerged cultures showed different concentrations of EPS, varying from 0.22 to 0.79 g/L for *A. delicata* and *G. lucidum*, respectively. Nevertheless, culture medium and incubation conditions need to be optimized for each strain. Komura et al.[8] recently compared EPS production under submerged fermentation in various basidiomycetes and showed that EPS concentrations varied between 0.24 and 0.91 g/L. Kim et al.[20] examined the effect of different synthetic liquid media on EPS production using shake flask cultures of 19 mushrooms. The best EPS production was attained by *G. lucidum* and *Phellinus linteus* in potato malt peptone medium, with EPS concentrations of 1.17 and 1.52 g/L, respectively. It is remarkable that high values were obtained recently by *G. lucidum* (15 g/L EPS) in an optimized medium for EPS production under submerged fermentation.[3] The screening results shown in this work can undoubtedly be useful to address research efforts on the optimization of culture media for EPS production using different higher Basidiomycetes.

Antrodia cinnamomea and *G. frondosa* exhibited an increase in EPSs that paralleled growth response.[21,22] However, *T. versicolor* and *Gloeophyllum trabeum* showed an inverse relationship between biomass and EPS production. Thus, low biomass production was associated with high EPS production and vice versa. For this reason, specific EPS yield (milligrams of EPS per grams of dry mycelium) provides more information on how different culture conditions could influence the enhancement of EPS production.[3] Table 1 depicts specific EPS yields. The highest specific EPS yield was attained by *G. applanatum* under static conditions after 21 days (204 mg/g). Specific EPS production compares favorably to previous reports. A maximum of 139 mg EPS/g was obtained by *G. lucidum* in an optimal medium for EPS production.[3]

TABLE 1. Production of Exopolysaccharides (EPSs) Under Submerged Cultivation in Stirred and Static Conditions

	14 Days						21 Days					
	Shake Flasks			Static Conditions			Shake Flasks			Static Conditions		
Species	EPS (g/L)	Biomass (g/L)	spEPS (mg/g)	EPS (g/L)	Biomass (g/L)	spEPS (mg/g)	EPS (g/L)	Biomass (g/L)	spEPS (mg/g)	EPS (g/L)	Biomass (g/L)	spEPS (mg/g)
Lentinus edodes	0.19	1.92	96.35	0.21	1.73	118.50	0.28	3.41	82.35	0.08	2.03	41.38
Schizophyllum commune	0.16	2.88	56.25	0.13	1.93	68.91	0.33	4.01	79.30	0.16	2.54	64.31
Trametes trogii	0.28	2.34	120.51	0.25	1.88	134.57	0.16	3.93	41.73	0.18	2.10	84.29
T. versicolor	0.35	2.98	116.11	0.25	2.32	109.05	0.59	4.67	126.12	0.24	2.12	112.74
Pycnoporus sanguineus	0.24	1.87	128.34	0.07	1.23	56.91	0.19	3.05	61.31	0.20	1.98	100.51
Ganoderma lucidum	0.38	2.99	126.76	0.25	1.77	138.42	0.79	4.97	158.72	0.34	2.68	125.37
G. applanatum	0.19	1.76	107.39	0.19	1.02	185.29	0.23	3.56	60.04	0.28	1.35	204.44
Grifola frondosa	0.17	2.54	65.75	0.17	1.45	120	0.24	4.38	55.71	0.28	3.01	93.36
Pleurotus ostreatus	0.48	2.97	160.61	0.19	2.00	96.5	0.75	4.87	154.62	0.24	3.77	64.72
Auricularia delicata	0.15	1.54	98.70	0.13	0.98	131.63	0.22	2.85	78.60	0.19	1.79	107.26

Results are the mean of 6 replicas of each experiment.
spEPS, specific yield of EPS.

TABLE 2. Development of Basidiome on Synthetic Bag-Log Cultivation by Several White Rot Mushrooms and Environmental Conditions Applied

Species	Spawn Run (Mycelial Growth)	Primordial Formation	Fruiting Development	Basidiomes' Morphological Properties[a]	Cultivation Methodology
Lentinus edodes	25–28°C, darkness, 14.5% O_2, 5.5% CO_2, 60% RH, 50 days	Thermal shock at 8°C for 24 hr, 14.5% O_2, 5.5% CO_2, darkness, 7 days at 18–22°C, 800 lux, 80% RH	18% O_2, 2.5% CO_2, 18–20°C, 800 lux, 80–85% RH, 7 days	Pileus 8–10 cm diameter; scaly, brown, with well-formed lamellae beige; stipe 5 cm long, 1 cm in diameter	Adapted from Chang & Miles[11]
Schizophyllum commune	25–30°C, darkness, 60% RH, 20 days	18–20°C, 18% O_2, 2.5% CO_2, 700–1000 lux, 80–85% RH, 5 days	18–20°C, 18% O_2, 2.5% CO_2, 700–1000 lux, 80–85% RH, 7 days	Fan-shaped pileus; grayish-white with white lamellae; no stem, in clusters	Method developed in this work
Trametes trogii	25–30°C, darkness, 11% CO_2, 9.5% O_2, 60% RH, 30 days	18–20°C, 18% O_2, 2.5% CO_2, 400–500 lux, 70–80% RH, 25 days	18–20°C, 18% O_2, 2.5% CO_2, 400–500 lux, 70–80% RH, 7 days	Shelf-type pileus; whitish-yellow to honey; corrugated and continues as vertical blinds all around the substrate block.	Method developed in this work
Trametes versicolor	25–30°C, 17.2% O_2, 7.3% CO_2, darkness, 60% RH, 30 days	18–20°C, 18% O_2, 2.5% CO_2, 500–800 lux, 70–80% RH, 20 days	18–20°C, 18% O_2, 2.5% CO_2, 500–800 lux, 70–80% RH, 14 days	Shelf-type pileus; 4–6 cm wide, showing typical concentric zones of different colors (light tan, yellow to brown); individuals around the side of the substrate block	Method developed in this work
Ganoderma lucidum	27–30°C, darkness, 11% CO_2, 9.5% O_2, 60% RH, 35 days	18–20°C, 18% O_2, 2.5% CO_2, 500–700 lux, 70–75% RH, 40–50 days	18–20°C, 18% O_2, 2.5% CO_2, 500–700 lux, 70–80% RH, 15 days	Pileus 8–12 cm in diameter, shelf-type; light yellow to red; stipe 1–3 cm long; clustered, especially at the top of the substrate block	Adapted from Chang & Miles[11]
Ganoderma applanatum	27-30°C, darkness, 8% CO_2, 12.5% O_2, 60% RH, 25 days	18–20°C, 18% O_2, 2.5% CO_2, 300–500 lux, 70% RH, 45–60 days	18–20°C, 18% O_2, 2.5% CO_2,300–500 luxes, 70–80% RH, 15 days	Pileus 4–7 cm de diameter, shelf-type, beige to dark brown, significant stipe, formed at the top and around the substrate block	Method developed in this work
Grifola frondosa	25°C, shadow, 14.5% O_2, 5.5% CO_2, 60% RH, 75 days	Thermal shock at 10°C for 24 hr, 16–18°C, 19.5% O_2, 0.7% CO_2, 100–500 lux, 70–80% RH, 7 days	16–18°C, 19.5% O_2, 0.7% CO_2, 100–500 lux, 70–80% RH, 7 days	Pileus elongated, brown-dark gray to light beige, mature fruiting clusters with overlapping petals (caps and lateral stems) extending outward, resembling a cluster flower	Adapted from Montoya et al.[23]
Pleurotus ostreatus	27–30°C, darkness, 9% CO_2, 11.5% O_2, 60% RH, 15 days	18–20°C, 18% O_2, 2.5% CO_2, 700–1000 lux, 90% RH, 5 days	18–20°C, 18% O_2, 2.5% CO_2, 700–1000 lux, 90–95% RH, 7 days	Pileus 7–10 cm in diameter, oyster-shaped, purplish to light beige, stipe 1–3 cm, gregarious	Adapted from Rodríguez & Jaramillo[24] and Montoya et al.[25]

[a]Obtained in this work.
RH, relative humidity.

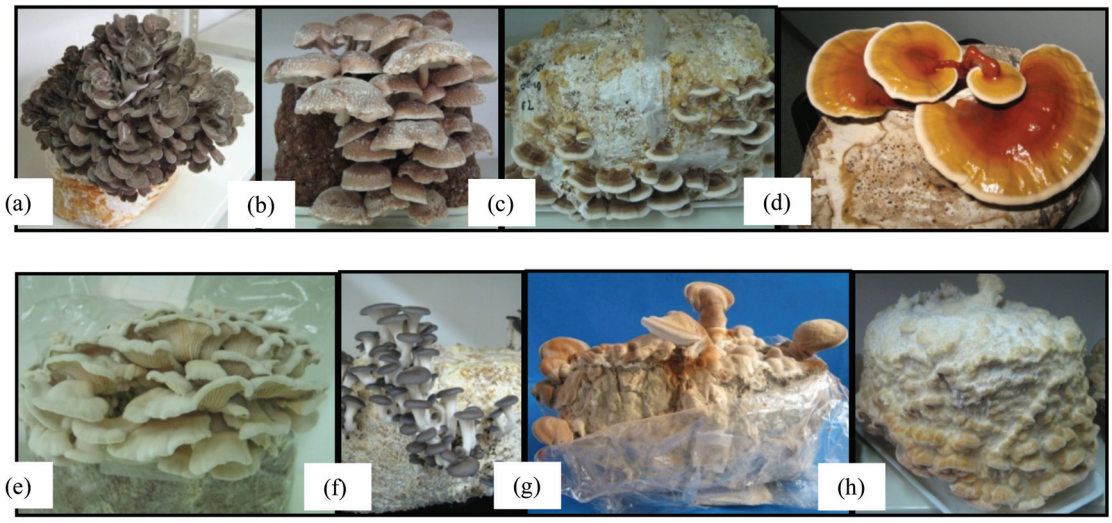

FIGURE 1. Development of fruiting bodies under the conditions assayed in this work. (**a**) *Grifola frondosa*, (**b**) *Lentinula edodes*, (**c**) *Trametes versicolor*, (**d**) *Ganoderma lucidum*, (**e**) *Schizophyllum commune*, (**f**) *Pleurotus ostreatus*, (**g**) *Ganoderma applanatum*, and (**h**) *Trametes trogii*.

B. Basidiome Development

For basidiome production of each of 8 of the 10 species assayed (*L. edodes*, *Sch. commune*, *T. trogii*, *T. versicolor*, *G. applanatum*, *G. lucidum*, *G. frondosa*, and *P. ostreatus*) on synthetic bag-log cultivation, several methods were modified and applied in this work (Table 2). The morphological properties of the carpophores obtained are depicted in Fig. 1 and Table 2. Figure 1 shows the fruit bodies obtained from 8 of 10 isolates of study, and Table 2 describes the details of how the carpophores were obtained. Under the conditions tested, neither *A. delicata* nor *P. sanguineus* developed basidiomes. Although the environmental factors required for fruiting in synthetic bag-log cultivation of some of the species screened in this work—such as *L.* edodes,[11] *G. lucidum*,[11] *G. frondosa*,[12] and *P. ostreatus*,[16]—already have been described, this work portrays for the first time the conditions required for synthetic bag-log basidiome development for 4 species: *Sch. commune*, *T. trogii*, *T. versicolor*, and *G. applanatum* (Table 2). As far as we know, only Badcock[26] described a method for obtaining *G. applanatum*, *Polystictus versicolor* (syn. of *T. versicolor*), and *Sch. commune* fructifications (after 11, 7, and 5.5 weeks, respectively) in large tubes filled with a medium consisting of sawdust and various nutrients; Williams et al.[27] obtained morphologically typical fruit bodies of *T. versicolor* within 8–12 weeks on cut logs of birch or hazel wood, placed with their bases submerged in water in closed horticultural propagators.

C. Quantification of IPSs in the Basidiomes

The basidiomes obtained subsequently were subjected to drying and grinding for IPS quantification. Figure 2 shows IPS content (percentage of the basidiome in dry basis). The content of IPS in the carpophores varied from 1.4% (*G. applanatum*) to 5.5% and 6% in *G. lucidum* and *G. frondosa*, respectively. Up to 4.5% of IPSs were extracted by Lee et al.[5] from the mycelium of *G. applanatum* cultivated under submerged fermentation, 1.3% were obtained from mycelium of *G. frondosa* grown in liquid cultures,[28] and 1.4% from its mycelium grown under solid-state fermentation.[9] Thus, the results obtained show the potential of using some of the higher Basidiomycetes evaluated in this work to obtain significant amounts of IPSs from fruiting bodies.

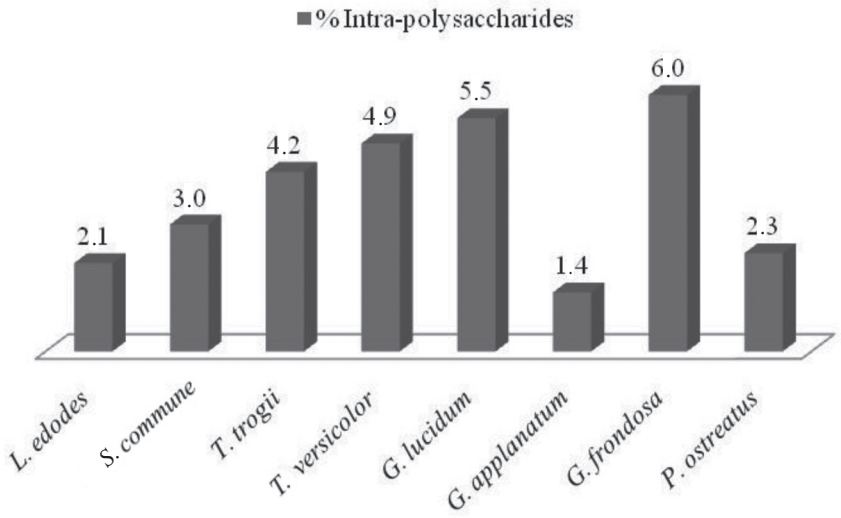

FIGURE 2. Content of intracellular polysaccharides (IPSs) in the carpophores (percentage in dry basis).

IV. CONCLUSIONS

This study contributes to expanding the knowledge of EPSs production by higher Basidiomycetes, including scantily studied species. It also demonstrates that solid-state basidiome development under synthetic bag-log cultivation could be an alternative biotechnological process for production and isolation of fungal intracellular polysaccharides.

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