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Production and Characterization of the Exopolysaccharides Produced by *Agaricus brasiliensis* in Submerged Fermentation

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Abstract The aim of the work was to study the production of the exopolysaccharides by *Agaricus brasiliensis* and the isolation of exopolysaccharides (EPSs) with biological effects. *A brasiliensis* LPB03 was cultured in submerged fermentation in a medium containing glucose, yeast extract, hydrolyzed soybean protein, and salts (pH 6.1) at 29 °C and 120 rpm for 144 h. The maximum biomass and EPS yield was 7.80±0.01 and 1,430.70±26.75 mg/L, respectively. To isolate the produced EPSs, two methods were compared: (1) with alcohol precipitation and (2) treatment with tricloroacetic acid (TCA), followed by alcohol precipitation. The use of TCA facilitated the purification of the EPS, reducing the amount of the contaminant soy proteins. For monosaccharide identification, the EPSs were hydrolyzed, derivatized to alditol acetates, and analyzed by gas chromatography (GC) and GC-mass spectrometry, which showed the presence (in molar percentage) of mannose (58.7), galactose (21.4), and glucose (13.1) as major sugars, with lower amounts of rhamnose (3.9) and xylose (2.8). Scanning electron microscopy was used to observe the morphological structure of the EPS. The experiments in vivo including EPS in the mice diet during 8 weeks indicated the hipocholesteremic and hypoglycemic effects.

Keywords *Agaricus brasiliensis* · Submerged fermentation · Exopolysaccharide · Monossacharides composition · Biological properties

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Introduction

The use of edible mushrooms for medical purposes is widely used in Asian countries [1]. However, they have gained interest in the Occident only in the last couple of decades. This interest is due to the observations that these mushrooms have been used for hundreds of years, without apparent collateral effects, while the effects of some modern drugs have contraindications [2, 3]. The extracts or the compounds derived from the mushrooms have been used as infusions (tea) or have been mixed together with other herbs [4, 5]. Some of the properties described for these Basidiomycetes include antibacterial, antifungal, and antiviral activity, as well as hypoglycemic, antioxidant, antiallergic, antinflamatory, immunomodulatory, antiaterogenic, and antitumor activities [6].

Agaricus brasiliensis belongs to the Basidiomycetes family. It is considered to be originated in São Paulo state, Brazil, and has been traditionally used as a health food source in Brazil [7, 8]. Studies have proved that the glucan–protein complex with β-(1→6) bonds in the main chain of the exopolysaccharide (EPS) has potential immunomodulating and antitumor activities [9]. There is evidence that β-D-glucans induce a biological response by binding to the membrane complement receptor type 3 (CR3, alpha Mb2 integrin or CD11b/CD18) on the immune effectors cells. The ligand–receptor complex can be internalized. The events that occur after glucan–receptor binding have not been fully determined until now [10]. Mizuno et al. [11] have reported another antitumor polysaccharide isolated from a liquid fermentation of the mycelium of *A. blazei* Murril, which has a β-D-mannan structure with a ramification β-D-Glc-(1→3)-β-D-Glc-1. Itoh et al. [9] described a polysaccharide produced by *A. blazei* with a β-(1→6) backbone, which differed from the β-(1→3) backbone with β-(1→6) branches shared by many other antitumor glucans. In addition, a glucomannan with the main chain of β-(1→2)-linked D-mannopyranosyl residues has been isolated from this mushroom and found to inhibit tumorgenesis [12]. Other reported activities of glucans are included in Table 1. These glucans are linear or branched molecules having a backbone composed of α- or β-linked glucose units, and some of them contain side chains that are attached at different positions. Heteroglucan side chains containing the glucuronic acid, xylose, galactose, mannose, arabinose, or ribose may be in different combinations. Another large group of the bioactive polysaccharides is called heteroglycans and classified as galactans, fucans, xylans, and mannans. The heteroglycan side chains may contain arabinose, mannose, fucose, galactose, xylose, glucuronic acid, and a glucose moiety as a main component or in other combinations [13].

Brazil is the second biggest soybean exporter and the main soybean meal exporter, with 32% of the global market. Soybean meal and other byproducts could be utilized in bioprocesses for value addition as this could be economically useful for the production of

Table 1 Structure and activity of different polysaccharides isolated from *Agaricus brasiliensis*.

A. brsailiensis	Chain principal	Characteristics	Activity	Reference
Fruiting body	β-d-Glc*p*-(1→6)	Lineal	Antitumor	[12]
Fruiting body	β-d-Glc*p*-(1→6)(1→4) β-d-Glc*p*-(1→3)(1→6) β-d-Glic*p*-(1→6)(1→3)	5.2% glucuronic acid 4.5% glucuronic acid	Antitumor	[48]
Fruiting body	β-d-Glc*p*-(1→6)	Substitution in O-3 each tree chain for 3-O- β-D-Glc- β-D-Glc*p*	–	[38]
Mycelium	β-d-Man*p*-(1→2)	Substitution in O-6 for β-d-Glc*p*-(1→3).	Antitumor	[49]

enzymes, organic acids, mushrooms, flavor and aroma compounds, pigments, polysaccharides, hormones, human food, and animal feed. However, much remains to be done in these areas to develop commercial processes with techno-economical feasibility [14]. One system consists to produce first microbial growth to guarantee a maximum accumulation of biomass, followed by the production phase, where the biosynthesis of the metabolite of interest is maximized [15]. The use of agro-industrial products as the substrates is common in the bioprocesses and could be useful in biopolymers synthesis also using soy industry residues.

The production and the extraction of EPSs depend on the cultivation media used in the fermentation and the methods for isolation. The simplest method for the extraction and isolation of EPS involve broth dialysis, followed by freeze drying, ethanol precipitation after the dialysis, or ethanol/acetone precipitation. Other techniques include the use of filter membranes, microfiltration, ultrafiltration, and diafiltration [16, 17]. When the cultivation medium is complex, an additional purification step has been proposed, to reduce the additional protein of the medium. For the EPS production from a medium with a high concentration of proteins, the use of trichloroacetic acid (TCA), the digestion with proteases, or the combination of both is recommended, followed by ethanol precipitation [16].

The objective of the present study was to improve the production of EPS produced by *A. brasiliensis* using the hydrolyzed soybean protein (HSP) as a low-cost component of the culture medium. To optimize the EPS isolation, the usual alcohol precipitation was compared with the other method that incorporated a precipitation with TCA, followed by alcohol precipitation. Attempts were also made to determine the monosaccharide composition by gas chromatography (GC) analysis. The biological properties of the partially purified EPS were studied in vivo supplementing chow diet for the mice with *A. brasiliensis* EPS.

Materials and Methods

Microorganism

The LPB03 strain of *A. brasiliensis* was obtained from the standard stock of the Laboratory of Biotechnological Processes at the Federal University of Paraná, Brazil. It was maintained on potato dextrose agar [18] and incubated at 29±2 °C for 7 days, followed by refrigeration [19] or stocked in sterile distilled water at 4 °C [20, 21].

Hydrolyzed Soybean Protein

The soybean meal containing 70% of protein was weighed (150 g/L) and diluted in distilled water. The pH was adjusted to 2.0 with 1 M HCl. The sample was autoclaved at 121 °C for 1 h. The solid fraction was separated by centrifugation at 10,000×*g* for 7 min. The supernatant was neutralized with 1 M NaOH and again centrifuged at 4,700×*g* for 20 min. The precipitate was removed, and the supernatant was stored at −20 °C. The supernatant had about 0.48 g of protein per milliliter.

Culture Media

The mycelia growing on the surface on the potato dextrose agar in a Petri dish was softly taken out, measuring 90 mm of diameter, and was transferred to an Erlenmeyer

flask of 250 mL, containing 100 mL of the basal medium containing (g/L) [22]: glucose 20, yeast extract 3.95, $MgSO_4 \cdot 7H_2O$ 0.3, and $K_2HPO_4 \cdot 3H_2O$ 0.6, pH 6.1. The flask was incubated at 29±0.2 °C and 120 rpm for 6 days. The mycelium was filtered under aseptic conditions. The biomass was triturated with 50 mL sterile distilled water using a slice and a mesh (0.5 mm^2) [23]. After this, the mycelium was reinoculated in 1-L Erlenmeyer flasks, with 500 mL of basal medium containing 5% of HSP and incubated under the same conditions of temperature and agitation for 6 days. The control was the basal medium described by Fan [22] and did not contain HSP. The mycelial biomass was isolated from the medium by filtration using a low-pressure pump. The supernatant was concentrated to one fourth of the original volume by the rotary evaporator at reduced pressure and below 45 °C. The mycelial mass retained was dried at 47 °C.

Isolation of the Exopolysaccharide

Precipitation with Ethanol For one part of the sample, four parts of ethanol (96°GL) was added and maintained overnight at low temperature (4 °C) [13, 24]. The precipitated material was recovered by the centrifugation, redissolved in distilled water, dialyzed in the membrane with cutoff of 8,000 Da, and freeze dried [13, 24].

TCA Treatment Combined with Ethanol Precipitation The TCA (80%) was added to the supernatant (1:6). The sample was quickly homogenized using a vortex, followed by centrifugation (10,000×*g* for 10 min). The supernatant was recovered, and alcohol precipitation was carried out with two parts of ethanol (96°GL; 4 °C for 18 h) centrifuged, dialyzed, and freeze dried as above.

Analytical Methods

Sugars Determination Total sugars quantification was done by the method of Dubois et al. [25] and modified by Cuesta et al. [24], and the quantification of the reducing sugar (glucose) was done by Somogyi-Nelson [26]. Both methods were adapted to microplates of 96 wells (flat bottom).

Quantification of Proteins The proteins were determined by the method of Lowry et al. [27].

Total Acid Hydrolysis of EPS Approximately 2 mg of the sample was treated with 0.5 mL trifluoracetic acid (2 M) for 1 h at 121 °C [17]. The acid was removed by evaporation under reduced pressure, followed by $NaBH_4$ reduction and acetylation [28] and GC and GC-mass spectrometry (MS) analysis for the corresponding alditol acetates.

Gas Chromatography and Gas Chromatography–Mass Spectrometry Analysis GC-MS analysis was performed using a Varian 3.300 GC equipped with a DB-225 (30 m× 0.25 mm) column interfaced to Finnigan Mat ITD 800 MS. A fused silica capillary column (30 m×0.25 mm) coated with DB-225 was used to analyze the alditol acetates. The injector and flame ionization detector temperatures were at 250 °C. Helium was used as the carrier gas (1.0 mL/min).

Microscopic Morpho-structural Observation

Scanning electronic microscopy was used to observe the microstructure of the isolated EPS using an EVE Jeol JSM-6360 LV scanning electron microscope.

Biological Studies in Vivo

Swiss female mice (*Mus musculus*) aged 30–35 days weighing 24–30 g were divided into two groups: The control group received normal chow (C) during 8 weeks; another set of mice (Ag) was fed normal chow supplemented with 30 mg/kg per day EPS of *A. brasiliensis*. The water and chow were supplied ad libitum, and the body weight was monitored weekly. The University Federal of Paraná Committee of Animal Welfare approved all the procedures involving the animals.

Serum Glucose and Cholesterol Concentrations

At the end of the eighth week, mice blood was taken for the analysis. The samples were centrifuged (3,500×*g* for 5 min), and the plasma concentrations of glucose and total cholesterol were measured in ADVIA 1650 (Bayer) automated equipment.

Statistical Analysis

The Student's *t* test was used for the statistical analysis [29].

Results and Discussion

Production of Biomass and EPS with and without HSP

The control basal medium showed the highest production of the EPS at the tenth day of fermentation, with a biomass of 8.30±0.02 g/L; however, the production of the EPS in the medium with HPS had the maximum at the fifth day with a mycelial biomass yield of 7.80±0.01 g/L (Fig. 1). It was evident that HSP stimulated the growth of the culture. Table 2 shows the kinetic parameters of the biomass production of *A. brasiliensis* and EPS in submerged fermentations with and without HPS.

These results could be of commercial interest, since the mycelium of *A. brasiliensis* is used as a component in the nutraceutical and functional foods, giving the products a light sweetish taste due to the presence of amino acids such as alanine and sugars, which mask the presence of aspartic and glutamic acid, responsible for the bitter taste [30]. The chemical composition and the compounds that determine the flavor present in the mycelium explain the good acceptance of the food using this mushroom [31]. Besides the ingestion of the mycelial mass of the fungus, it also showed the antitumor activity against Sarcoma 180 in the rats [12, 32–34].

The control medium without the addition of the HSP produced 993.5±0.2 mg/mL in 6 days, followed by a small reduction and an increase on the seventh and eighth days, while the highest production of EPS in the broth with HSP occurred in the ninth day with 1,430.70±26.75 mg/L, which is 30% higher than that from the medium without

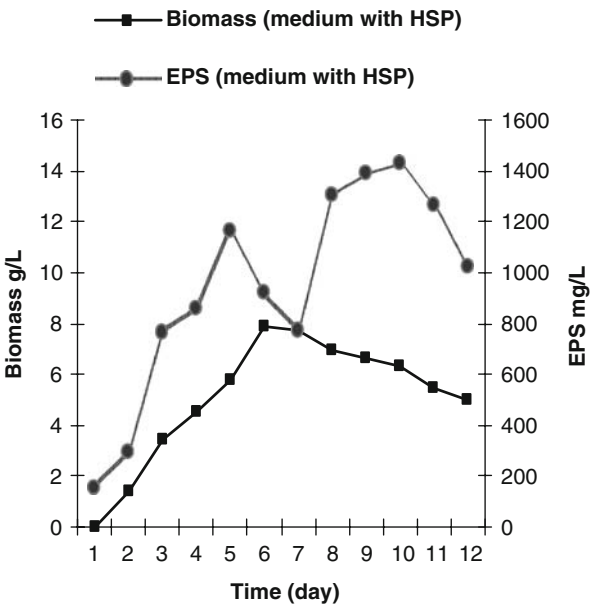

Fig. 1 Dosage of biomass and EPS yield in the medium with HSP

HSP (Fig. 2). Fan et al. [35] using a similar culture broth with controlled aeration and with 1% glucose obtained a maximum EPS yield of 321.20 mg/L. Gern [36] achieved an EPS yield of 1,235.96 mg/L in the bioreactor with an aeration rate of 2 vvm (vol air vol broth^{-1} min^{-1}) and pH controlled at 7.0. In the present study, an inoculum ratio of 3% was used, which could explain the higher EPS production. In all the cases, the glucose was rapidly consumed and disappeared after 1 or 2 days of cultivation; the reduction in the carbon source in the medium was followed by an increase in the pH (Figs. 3 and 4).

Determination of Protein in Isolated EPS

It was observed that the precipitation with prior treatment with the TCA gave a carbohydrate/protein ratio of 92:8, while the precipitation with ethanol gave a ratio of

Table 2 Kinetic parameters and EPS production by *A. brasiliensis* under optimal medium submerged fermentation.

Kinetic parameters (shake-flask)	Basal medium	Basal medium + HSP
Maximum specific growth rate (μ_m), h^{-1}	0.0112	0.0105
Maximum biomass concentration, g/L	8.30±0.02	7.80±0.01
Maximum EPS concentration, g/L	1,430.70±26.75	993.50±0.20
Biomass yield coefficient ($Y_{x/s}$)[a]	0.55	0.56
Conversion of substrate to product ($Y_{p/s}$)[b]	0.10	0.07
Maximum productivity (R_m)[c]	0.0112	0.0229

[a] Grams biomass per gram glucose

[b] Grams crude EPS per gram glucose

[c] Grams crude EPS per liter per hour

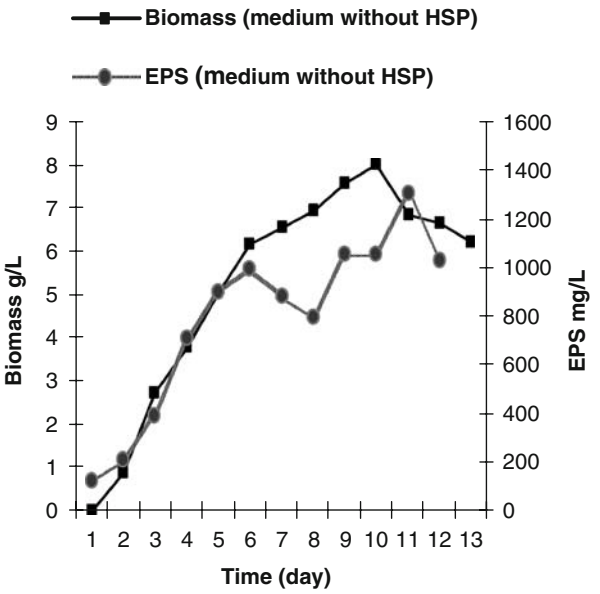

Fig. 2 Dosage of biomass and EPS yield in the medium without HSP

77.5:22.5 (Figs. 5 and 6). The results obtained by the extraction with TCA agreed with those of Lin and Yang [37], who precipitated the proteins from the broth with 5-sulfosalicilic acid, obtaining a EPS with a protein content between 0 and 1.63±0.05% and the total carbohydrates of 82.27±0.99 to 92.14±0.74%. The extraction with precipitations with ethanol gave similar results to the ones reported by Dong et al. [38], who obtained 21.6% of protein and 57.5% of carbohydrates in the EPS of *A. brasiliensis*.

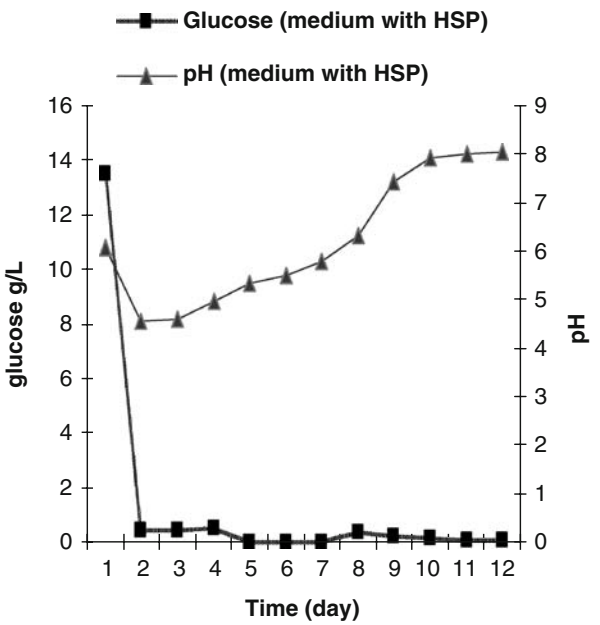

Fig. 3 Dosage of glucose and pH during culture in the medium with HSP

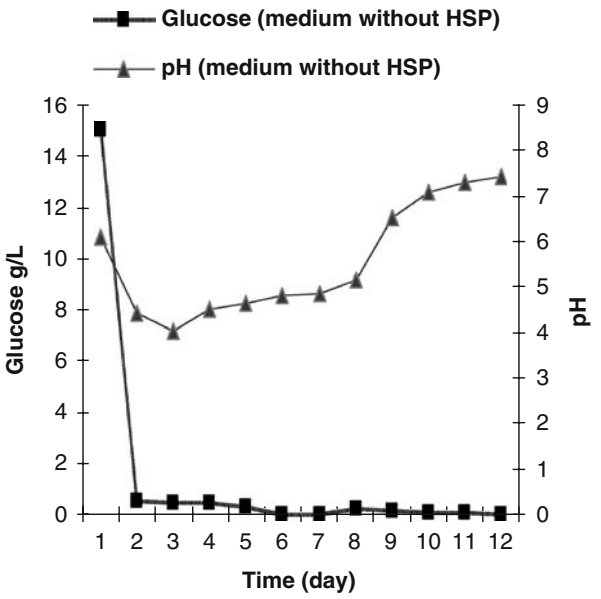

Fig. 4 Dosage of glucose and pH during culture in the medium without HSP

Monosaccharide Composition

The monosaccharide composition of the EPS was obtained after total hydrolysis [17], reduction and acetylation [39], and analysis of the corresponding alditol acetates by GC and confirmed by GC-MS. These analyses revealed in the molar percentage that the principal sugar present in the EPS was mannose (58.7%), followed by galactose (21.4%) and glucose (13.1%) and lower amounts of rhamnose (3.9%) and xylose (2.8%), a typical composition of the galactomannas. Chen and Lu [40] analyzed the polysaccharides from the medicinal fungi, including *A blazei* Murril (Brazilian mushroom), and found that myo-inositol, sorbitol, fucose, galactosamine, glucosamine, galactose, glucose, and mannose were the neutral sugars in these polysaccharides and fructose, glucose, and mannose were the

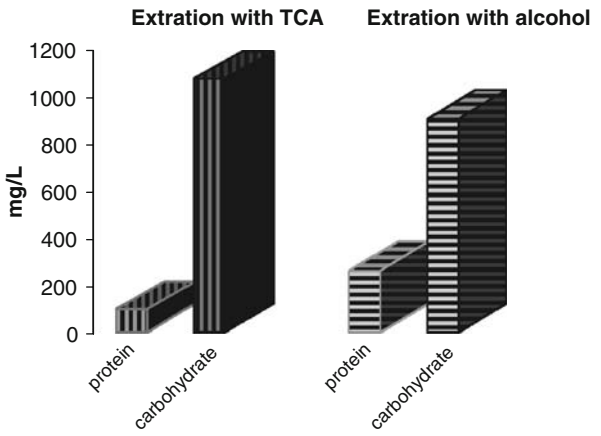

Fig. 5 Dosage of protein and carbohydrate in medium with HSP

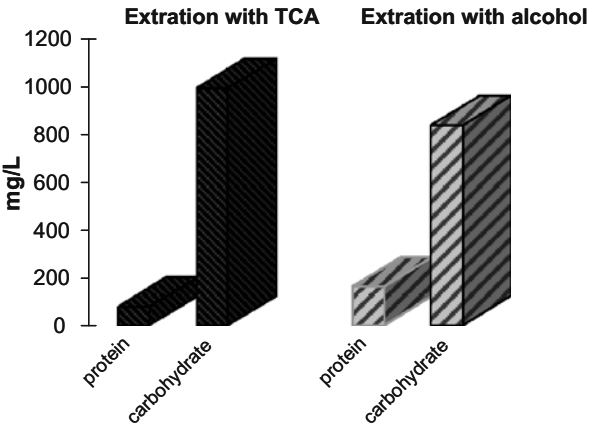

Fig. 6 Dosage of protein and carbohydrate in medium without HSP

predominant monosaccharides from this fungus [41]. These results agreed with Mizuno [42] for the mannogalactoglucan, β-(1→6); α-(1→3)-glucan, α-(1→4); β-(1→6) glucan, α-(1→6); and α-(1→4)-glucan. Gern [36] using the same methodology for the analysis of *A. brasiliensis* EPS, found mannose, 57.68%, galactose, 28.17%, and glucose, 8.35%, which was similar to the present results.

Microscopic Morphological Structure of EPS

Figures 7a and b shows the morphological structure of the EPS produced by *A. brasiliensis*. It appeared as constituted by the heterogeneous mucoid rod type components, excluding the possibility of a globular structure for the protein–galactomannan complex.

Biological Properties

At the end of 8 weeks of daily feeding with chow with and without supplementation with the EPS, it was observed that the mice fed with the normal diet increased their weight about 30%. The mice fed with normal chow supplemented with *A. brasiliensis* EPS increased about 6.4% with respect to the weight in the control (Fig. 8).

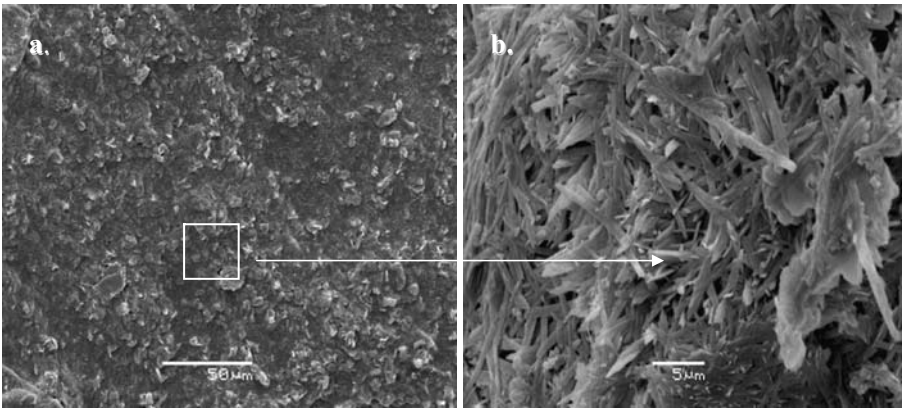

Fig. 7 **a** Morphological structure of the EPS produced by *A. brasiliensis* (50 μm). **b** Electron micrograph of EPS producing the both with HSP (5 μm)

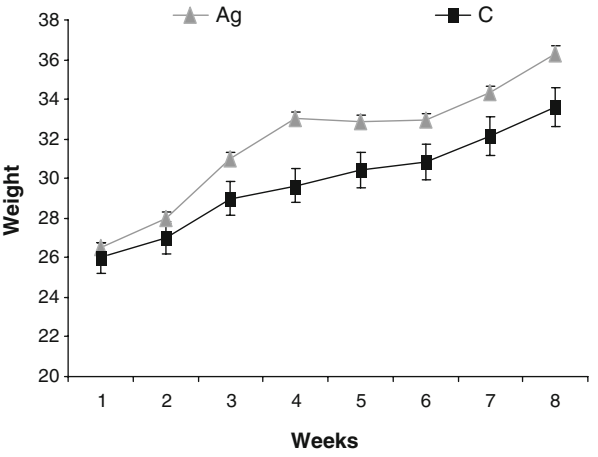

Fig. 8 Effect of diet enriched with EPS from *A. brasiliensis* on body weigh gain during 8 weeks. *Ag A. brasiliensis* group, *C* control group

Hypoglicemic and Antichoresterolemic Activity in Mice

The diet supplemented with *A. brasiliensis* EPS fed during 8 weeks to the mice produced a reduction in glucose plasma concentration around 22%. It has been also demonstrated in another study [43] that the supplementation with β-glucans and oligosaccharides obtained from the fruiting body of *A. brasiliensis* caused reduction in the glucose serum concentration in rats. These authors suggested that the mushroom could have an antidiabetic activity by promoting the insulin release by the Langerhans cells in the pancreas.

The total cholesterol ratio in the mice was reduced around 27% with *A. brasiliensis* EPS supplementation. Other authors demonstrated that the fruiting body biomass and the mushroom polysaccharides have significant antihyperglycemic activity and the abilities to increase glucose metabolism and insulin secretion in type 2 diabetes mellitus [44] (Fig. 9).

However, the mechanisms of action for the EPS of *A. brasiliensis* on cholesterol and glucose metabolism are still unknown. The effect of *A. brasiliensis* on the enzyme activities was considered to be sustained for at least 2 to 3 months after the supplementation in the calves. Although the absorption of *A. brasiliensis* was considered to be good, the amount and form of *A. brasiliensis* supplementation in combination with different kinds of the feed should be further studied [45–47].

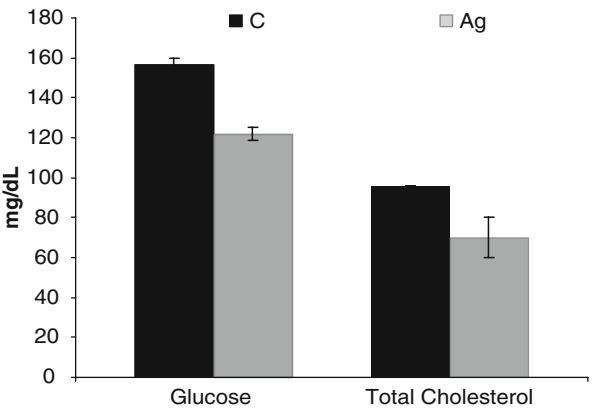

Fig. 9 Effect of diet enriched with EPS from *A. brasiliensis* on glucose and total cholesterol plasmatic concentration. *Ag A. brasiliensis* group, *C* control group

Conclusions

The hydrolyzed soy protein present in the culture medium for *A. brasiliensis* improved the yield of the EPS. The free amino acids, proteins, and peptides present in the HSP after HCl treatment were good sources of nutrients promoting the growth and higher polymer production, reducing the time of the fermentation for the EPS and biomass production. The prior treatment with TCA was useful when compared to the alcohol precipitation commonly used. The studies in vivo showed that the supplementation of mice chow with EPS extracted from *A. brasiliensis* could have a potential preventive and therapeutic action against the diseases related to diabetes and hypercholesterolemia. Further studies involving the HSP in the submerged cultivation of fungal mycelia in the bioreactors at a larger scale with controlled aeration, pH, and agitation could improve the EPS production for its application to metabolic diseases and eventually as the imunomodulating agent. It could also be interesting to elucidate the structure of EPS.

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