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Antitumour Activity of *Grifola frondosa* Exopolysaccharides Produced by Submerged Fermentation Using Sugar Cane and Soy Molasses as Carbon Sources

Jacqueline Sugitani Chimilovski[1], *Sascha Habu*[1], *Richard Fhillipy Bosqui Teixeira*[1], *Vanete Thomaz-Soccol*[1], *Miguel Daniel Noseda*[2], *Adriane Bianchi Pedroni Medeiros*[1], *Ashok Pandey*[3] *and Carlos Ricardo Soccol*[1]*

[1]Bioprocess Engineering and Biotechnology Division, Centro Politécnico, Federal University of Paraná, 81531-990 Curitiba, Paraná, Brazil

[2]Department of Biochemistry and Molecular Biology, Centro Politécnico, Federal University of Paraná, 81531-990 Curitiba, Paraná, Brazil

[3]Biotechnology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, 695 019 Trivandrum, India

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Summary

Grifola frondosa is an edible Basidiomycete and produces exopolysaccharides (EPS) known for their antitumour activity. The objectives of this study are to produce exopolysaccharides in submerged fermentation using alternative carbon sources (sugar cane and soy molasses), and to evaluate their anti-proliferative activity against tumour cells. Exopolysaccharides were extracted by ethanol and tested against mice tumour cells, then characterized by gas chromatography. Carbon sources represent the major cost of the bioprocess, so a search for new alternatives such as agro-industrial residues is important to establish the viability on an industrial scale. Moreover, the data about the kinetics of the EPS production allow studying the optimization of the process.

Key words: *Grifola frondosa,* kinetics, molasses, exopolysaccharides, antitumour activity, submerged culture

Introduction

Mushrooms have become an attractive option for functional food or as a source for the development of drugs and nutraceuticals (*1,2*). *Grifola frondosa* is a Basidiomycete belonging to the order Aphyllopherales and family Polyporaceae. It is a traditional edible mushroom in the Southeast Asia, and is commonly used in the treatment of various diseases such as hepatitis, hepatopathy, hypertension, nephritis, bronchitis, and cancers due to its considerable biological activities (*3–5*).

Anticancer drugs have side effects on the haematopoietic tissues, the gastrointestinal mucosa, gonads and skin. The haematopoietic tissue injuries cause immunosuppression, which negatively affects therapy (*2,4,5*). On the other hand, *G. frondosa* contains polysaccharides comprising β-glucans, such as grifolan, known for their immunostimulatory and antitumour activities (*4,6,7*). These polysaccharides are obtained either from fruiting bodies or by fungal culture in liquid fermentation. The submerged culture in liquid medium increases the potential production of biocompounds in a compact space, in less time and with greater security (*3,8–10*). The submerged

*Corresponding author; Phone/Fax: ++55 41 3361 3695; E-mail: soccol@ufpr.br

fermentation is a practical alternative, feasible and it enables adaptability to large scale production in industry.

Carbon sources in the culture medium represent a major cost of the bioprocess. The use of cheap alternative carbon sources makes it feasible on an industrial scale. Apart from that, the reuse of industrial residues such as molasses is beneficial to the environment (*8,10, 11*). Production of polysaccharides by mushrooms has wide application in food industries, pharmaceuticals, cosmetics and other industries (*3,10–12*).

Materials and Methods

Mushrooms

The strain of *G. frondosa* was obtained from the standard stock of Bioprocesses and Biotechnology Laboratory (LPB) at the Federal University of Paraná (UFPR), Curitiba, Brazil. It was maintained on potato dextrose agar (PDA), incubated at (30±2) °C for seven days followed by refrigeration at 4 °C.

Pre-inoculum preparation

Mushrooms were initially grown on PDA medium in a Petri dish 90 mm in diameter, and then the mycelium was transferred to a 500-mL flask containing 250 mL of basal modified medium composed of (in g/L): glucose 20, yeast extract 3.0, $MgSO_4·7H_2O$ 0.3 and $K_2HPO_4·3H_2O$ 0.6, pH=6.1, according to Fan *et al*. (*13*). The flask was incubated under agitation at 120 rpm and (28±2) °C for 10 days. After fermentation, the mushroom was homogenized under aseptic conditions.

Submerged fermentation

The submerged fermentation was done in 1-litre flask containing 500 mL of basal medium according to Fan *et al*. (*13*), with carbon source substituted with soy and sugar cane molasses.

Pre-inoculum was added to the fask in a fraction of 4 % (by volume). The fermentation was carried out at 28 °C, agitation of 120 rpm and initial pH=6.1 for 5 days. Aliquots were taken daily for kinetic analyses. The culture was filtered using Whatman no. 1 filter paper, under low pressure. The basal medium with glucose as the carbon source was also fermented under the same conditions as the control.

The production of exopolysaccharides (EPS), mycelia, residual sugar, and pH changes were analyzed daily. The submerged fermentation values are the mean of triplicate independent experiments with standard deviation.

Culture medium with sugar cane and soy molasses

Molasses contain different types of sugar; however, they also contain inhibitory substances. Four different concentrations were tested (5, 10, 30 and 35 g/L) to define which quantities of molasses would be ideal to substitute the glucose of the standard medium. The yield of EPS is directly related to the yield of biomass. Therefore, the best concentrations of sugar cane and soy molasses were chosen depending on the production of biomass in dry mass. The concentration of sugar was defined by soluble solid percentage measured by a refractometer (RT-30ATC, Instrutherm, São Paulo, Brazil). The sugar cane molasses medium had 3 °Brix (10 g/L) and the soy molasses medium had 4 °Brix (30 g/L). The molasses were donated by the Bioprocesses and Biotechnology Laboratory (LPB) at the Federal University of Paraná (UFPR), Brazil.

Exopolysaccharide extraction

The filtrate was concentrated to ¼ of the original volume by rotary evaporator under reduced pressure below 50 °C. Four parts of 95 % ethanol were added to the concentrated sample at low temperature (4 °C) overnight for EPS precipitation (*14*). The precipitate was recovered by centrifugation at 4000 rpm for 20 min and washed twice with ethanol.

Analytical methods

Exopolysaccharides (EPS) were determined by phenol-sulphuric acid method (*15*). Residual glucose was measured according to the method of Somogyi-Nelson (*16,17*). The biomass was measured as dry mass (g/L) and pH was verified with pH meter.

Methods of EPS treatment

The EPS recovered from fermentation were lyophilized and then they were treated to improve their purity by two different treatments: (*i*) the EPS were dialyzed using membrane of 8–12 kDa for 24 h; (*ii*) the EPS were reprecipitated with ethanol. After the treatments, they were lyophilized again.

Determination of antiproliferative activity on Ehrlich tumour in vitro

Treated and lyophilized EPS were weighed, added to distilled water and only the soluble fraction was used for tests. The samples were filtered in 0.22-μm filter for antiproliferative tumour cell tests.

Ehrlich tumour cells were grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % heat-inactivated fetal bovine serum, 1 % glutamine and 1 % antibiotics, and incubated at 37 °C in humidified incubator containing 5 % CO_2.

The cell concentration was adjusted to 10^5 cells/well and distributed in a 96 well-plate. The microplate was incubated for 24 h with different concentrations (10, 30, 60 and 100 μg/mL) of EPS from mushrooms. Cultures were kept for 48 h at 37 °C in an incubator, maintaining a constant atmosphere of 5 % CO_2. PBS buffer was used as a negative control. Tumour cell proliferation was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method. All tests were carried out in triplicate. The results were compared to the control groups. The inhibitory rate (in %) of Ehrlich tumour cells and sarcoma 180 cells were calculated as follows:

$$\text{Inhibitory rate}=[(B-A)/B]\cdot 100 \qquad /1/$$

where A is the average absorbance at 550 nm of the treated groups and B is the average absorbance at 550 nm of the untreated group (control group).

Monosaccharide composition

Approximately 1 mg of EPS was treated with 0.5 mL of 1 M trifluoracetic acid (TFA), for 1 h at 120 °C. The acid was evaporated until desiccation, followed by $NaBH_4$ reduction, acetylation and analyzed by gas chromatography (*18*).

Results and Discussion

Kinetics of EPS production from Grifola frondosa

Alternative carbon sources showed significantly better results than the fermentation with the standard medium containing glucose. After 5 days, there were (19.11± 2.22) g/L of dry biomass and (3.86±0.02) g/L of EPS using the soy molasses medium. Sugar cane molasses medium showed the best production, with (22.93±3.37) g/L of dry biomass and (5.14±0.26) g/L of EPS. The yields of EPS production achieved are presented in Table 1.

Molasses from sugar cane and soybeans have complex composition and can stimulate the metabolism of enzymes. The mean composition of soy molasses is (in %): protein 10.44, ashes 9.37, lipids 10.05, carbohydrates 40.77, with (in g/L): glucose 8.43, fructose 8.26, sucrose 77.12, raffinose 23.12, stachyose 84.53 and other sugars (*19*). According to the United States Sugar Corporation (*20*), the typical composition of sugar cane molasses is (in %): protein 6.3, ashes 16.0, carbohydrates 48.3, with glucose 2.6, fructose 5.6, sucrose 35.9 and other sugars.

Previous studies had lower yields of EPS and biomass. *Grifola frondosa* cell growth yielded relatively high mycelial dry biomass (11.22±1.14) g/L and the maximum EPS production was (2.248±0.107) g/L, which was achieved in 4 % glucose medium with 0.5 % soybean oil. *Grifola frondosa* showed production yield of exopolymer of 1.326 g/L, according to Cui *et al*. (*21*).

The initial pH of the culture medium was adjusted to 6.1. The pH of the sugar cane molasses medium decreases more than standard and soy molasses media as it is shown in Fig. 1.

Acidification is related to the fungus metabolism. It produces organic acids as by-products. This can also be observed in the sugar cane molasses medium (Fig. 1).

Antiproliferative activity on Ehrlich tumour

The dialyzed EPS from *Grifola frondosa* showed higher antiproliferative activity on Ehrlich tumour cells of (65.05± 1.06) % at 100 μg/well with soy molasses medium, while the reprecipitated EPS showed the activity of (67.06±1.13) % at 100 μg/well with sugar cane molasses medium. Differences in the inhibitory activity of different EPS concentrations on tumour cells suggest that they are dose--dependent.

Exopolysaccharides from mushrooms are a well-known biological response modifier (BRM). EPS were extracted from the fermentation broth and then received two different treatments to test the improvement in the purity. The two treatments were tested by the proliferation of tumour cell tests. EPS produced with alternative carbon sources showed more inhibitory activity than the EPS from standard medium with glucose (control; Fig. 2).

Antitumour and immunomodulating activities of the sulphated derivative of EPS from *G. frondosa* were estimated *in vitro* and *in vivo* by Nie *et al*. (*22*). EPS inhibited the proliferation of SGC-7901 cells and induced apoptosis, in a dose-dependent manner. The results from *in vivo* experiments demonstrated that EPS significantly inhibited the tumour growth and enhanced the peritoneal macrophage phagocytosis in sarcoma 180-bearing mice.

Characterization of EPS

Polysaccharides offer a high capacity for carrying biological information because of their increased potential for structural variability. Therefore, this variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanisms of various cell-cell interactions in higher organisms.

The major sugar component of *Grifola frondosa* fruiting body is glucose, while fucose, xylose, mannose and galactose are minor components (*22*). Polysaccharide struc-

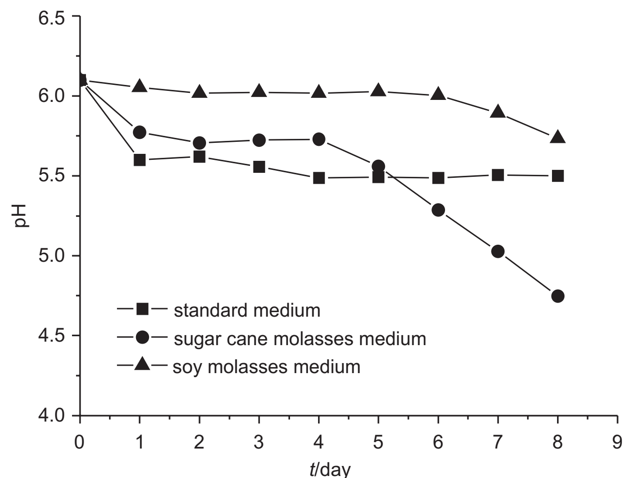

Fig. 1. pH of fermentation broth with three different carbon sources

Table 1. Best yields of fermentation with three different carbon sources

Carbon source	Production peak/day	γ(biomass) g/L	γ(EPS) g/L	$Y_{P/X}$ γ(EPS)/γ(biomass)	Productivity (EPS) g/(L·day)
Soy molasses	5	19.11±2.22	3.86±0.02	0.202±0.01	0.040
Sugar cane molasses	6	22.93±3.37	5.14±0.26	0.195±0.07	0.033
Glucose	8	13.33±1.33	2.03±0.02	0.152±0.02	0.019

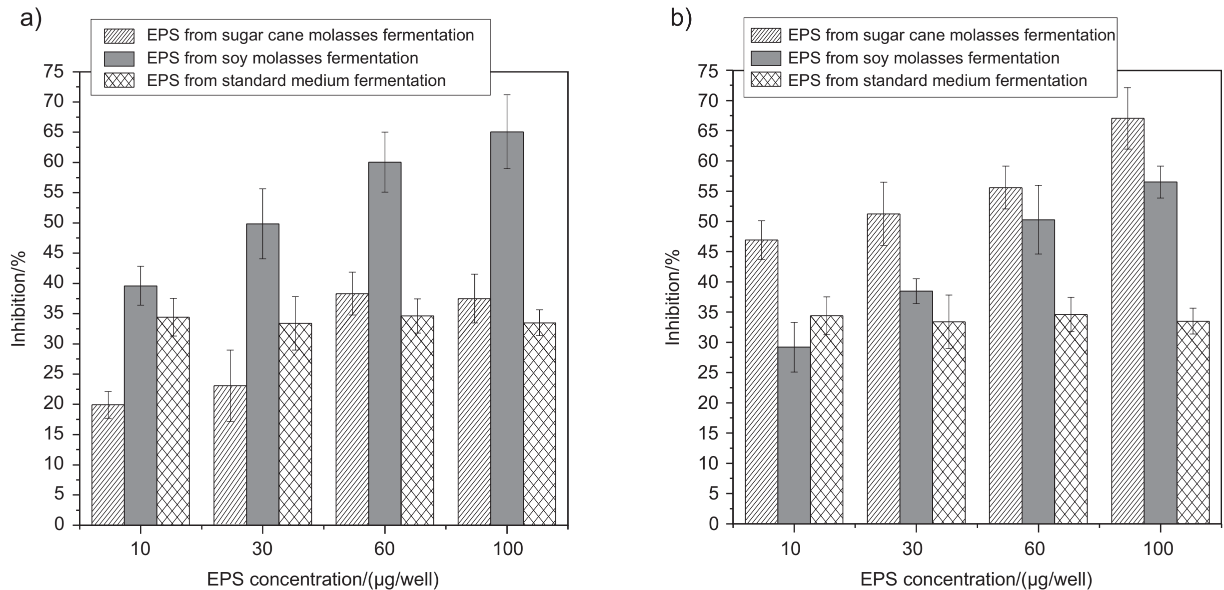

Fig. 2. Inhibitory activity against tumour cells of EPS from three different culture media: sugar cane molasses, soy molasses and standard medium (glucose): a) inhibitory activity from dialyzed EPS; b) inhibitory activity of reprecipitated EPS

Table 2. Monosaccharide composition of EPS produced by submerged fermentation of *Grifola frondosa* with different carbon sources

Carbon source	Treatment	*w*/%							
		Rha	Fuc	Rib	Ara	Xyl	Man	Gal	Glc
Sugar cane molasses	reprecipitation	–	22.94	–	36.03	13.98	11.73	2.86	12.76
	dialysis	2.10	5.48	1.31	45.54	5.86	19.30	7.12	13.29
Soy molasses	reprecipitation	23.16	5.64	13.21	7.78	–	23.99	3.49	22.73
	dialysis	0.69	3.11	1.70	6.41	1.49	72.44	6.96	7.20
Glucose	–	0.40	0.18	0.37	0.51	0.12	4.26	0.52	93.65

Rha=rhamnose, Fuc=fucose, Rib=ribose, Ara=arabinose, Xyl=xylose, Man=manose, Gal=galactose, Glc=glucose

Table 3. Monosaccharide composition of sugar cane and soy molasses without fermentation

Molasses	Treatment	*w*/%							
		Rha	Fuc	Rib	Ara	Xyl	Man	Gal	Glc
Sugar cane	reprecipitation	0.98	3.19	2.64	32.16	8.80	14.66	2.68	34.89
	dialysis	1.36	6.36	1.93	39.37	10.76	13.53	4.10	22.59
Soy	reprecipitation	–	–	5.69	7.25	–	64.75	4.71	17.60
	dialysis	0.56	0.65	3.20	5.44	0.35	70.91	5.25	13.64

Rha=rhamnose, Fuc=fucose, Rib=ribose, Ara=arabinose, Xyl=xylose, Man=manose, Gal=galactose, Glc=glucose

ture in submerged culture may depend on the composition of a nutrient medium. The conformational structure of polysaccharides and the molecular mass have played a dominant role in the antitumour activity (*23*,*24*).

Monosaccharide composition of EPS varies according to the carbon source and the treatment after fermentation (Table 2). The molasses media were also analyzed by gas chromatography and the results are shown in Table 3. Tables 2 and 3 show that fermentation changes the monosaccharide composition of EPS. The EPS from standard medium is different from the EPS from medium with alternative carbon sources, which explains the different inhibitory responses during the biological tests against tumour cells.

Conclusion

Kinetics data are essential for fermentation in a bioreactor. The alternative carbon sources showed a high production of EPS and biomass. EPS produced by alternative carbon sources showed interesting antiproliferative properties on cancer cells. Polysaccharides from *G. frondosa* resulted in distinct monosaccharide compositions when fermented with different carbon sources.

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