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A polysaccharide fraction extracted from *Pleurotus ostreatus* mycelial biomass inhibit Sarcoma 180 tumor

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ABSTRACT

Fungi of *Pleurotus* genus have attracted a great interest due to their medicinal properties such as anti-inflammatory, antimicrobial and antitumor. These properties are attributed mainly to polysaccharides synthesized by *Pleurotus*. This work aimed to study the mycelial growth of *P. ostreatus* in submerged culture, evaluating the influence of the initial concentration of substrate (20 and 40 g/L of glucose) and the pH (4 and 6) on kinetic parameters of production of biomass. The effectiveness of different doses (10, 30 and 50 mg/kg) of a mycelium polysaccharide fraction extracted from *P. ostreatus* in reducing Sarcoma 180 development in mice was also verified. In the range of this study, maximum concentration of mycelial biomass (about 12.8 g/L) was obtained using 40.0 g/L of glucose, at pH 4.0. The total biomass productivity (Px) was not significantly affected by substrate concentration and pH, reaching values of 0.034 g/L.h. Sarcoma 180 tumor weight was reduced in 74.1, 75.5 and 53.7% when 10, 30 and 50 mg/kg were administered, respectively. These results show the high antitumor potential of intracellular polysaccharide fraction of mycelial biomass of *P. ostreatus*, particularly at lower doses of 10 and 30 mg/kg.

Key words: Antitumor Activity, Mycelium, *Pleurotus ostreatus*, Polysaccharides, Sarcoma 180.

INTRODUCTION

Fungi of the class of basidiomycetes are well known for producing a great number of bioactive molecules (Wasser 2002, Lindequist et al. 2005). Among the basidiomycetes, the genus *Pleurotus* has been intensively studied due to its antitumor activity (Xu et al. 2012). The increase of the number of these studies is related to the search of new and natural molecules that can act against tumor cells without the collateral effects associated to the conventional therapies that involve chemical and physical agents producing side effects or toxic reactions (Bast et al. 2000). According to Liu (2004), epidemiological and preclinical tests have demonstrated the great potential of natural products in combating cancer and other chronic diseases that result from oxidative stress.

Among the several molecules with bioactivity produced by fungi, polysaccharides seems to be the

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main responsible by the antitumor effect (Ren et al. 2012). Regarding the genus *Pleurotus*, several polysaccharidic extracts from the fruiting bodies with antitumor activity have been reported (Liu et al. 2015, Assis et al. 2013, Patel et al. 2012), although a few reports are found describing the mycelium antitumor effect.

Depending on the fungi culture conditions (submerged or solid culture, pH, temperature, oxygen concentration, composition of the culture medium etc) and of the method of extraction, polysaccharides with different structures and, consequently, different bioactivities can be obtained from the fruiting bodies, from the fermented culture broth or from the mycelial biomass (Zhang et al. 2007, Synytsya and Novák 2013, Ruthes et al. 2015). Silveira et al. (2015) reported a mannogalactan with antinociceptive and anti-inflammatory effects extracted from the culture broth of *Pleurotus sajor-caju* by freeze-thawing and dialysis. A (1 → 3)-β-D-glucan with anti-inflammatory activity was isolated from fruiting bodies of *Pleurotus sajor-caju* via extraction with hot water followed by fractionation by freeze-thawing and finally by dimethyl sulfoxide extraction (Silveira et al. 2014). Komura et al. (2014) isolated a water-soluble mannogalactan from *Pleurotus ostreatus* var. florida mycelial biomass concluding that is possible to obtain similar and also different molecules from those found in the fruiting body and mycelial biomass of the same mushroom species.

The factorial design has been widely applied in optimization of medium composition (Gern et al. 2008, Papaspyridi et al. 2010) replacing the classical or empirical methods such as one-factor-at-a-time-method, which is time consuming process and incapable of searching the global optimal condition, especially when interaction between independent factors exists (Papaspyridi et al. 2010). This work studied the mycelial growth of *Pleurotus ostreatus* DSM 1833 in submerged culture, using a factorial design to evaluate the effect of the initial concentration of glucose and the medium pH over the kinetic parameters of the process. In addition, further experiments were conducted to verify the efficacy of different doses of a polysaccharidic fraction extracted from the mycelium biomass, on the inhibition of Sarcoma 180 induced in mice.

MATERIALS AND METHODS

Pleurotus ostreatus DSM 1833 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh, German, was maintained in WDA (Wheat-Dextrose-Agar) solid medium composed by 20 g of glucose, 15 g of agar and 1 L of wheat extract (Furlan et al. 1997). Inoculum was produced in Duran flask of 2 L containing 400 mL of POL medium (Cavazzoni and Adami 1992) with the following composition (g/L): glucose, 20.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 1.0; yeast extract, 2.0 and peptone, 1.0; pH 6.5 – 7.0. The flask was inoculated with the whole mycelium of a 7-day-old culture grown in a Petri dish, and incubated at 30ºC, 120 min^{-1} (reciprocal), for 6 days.

Cultivation was carried out in a Biostat B (B. BRAUN) bioreactor containing 4 L of POL medium described above added with 1.0 g/L of $CaCO_3$, inoculated with 400 ml of inoculum and incubated at 30ºC. Initial K_La of 15 h^{-1} was achieved under agitation of 300 min^{-1} and 0.25 L/min of air supply. The initial concentration of glucose and the pH varied according to the 2^2 factorial design shown in Table I. Experiments were carried out in duplicate. Pareto Analysis (Barros Neto et al. 1995) was used to estimate the effects of each variable and their interactions on the response parameters.

Mycelial biomass was measured by gravimetry. Samples were filtered in Whatman no. 1, washed, transferred to pre-weighed crucibles, and dried for 48 h, at 90ºC. Biomass concentration was reported as the dry weight of biomass per volume of sample. Determination of glucose concentration was

TABLE I
Factorial design 2^2 varying the initial glucose concentration and the medium pH. Symbols (-) and (+) represent inferior and superior levels, respectively.

Variables	Levels	
	–	+
pH	4	6
Glucose (g/L)	20	40

performed using the enzymatic method of glucose oxidase-peroxidase (Glicose-E, CELM, Brasil).

Mycelial biomass resulting from the best condition obtained from the factorial design was removed from the culture broth by vacuum filtration using Whatmann no. 1. The biomass was washed with distilled water, frozen, and fractionated according to the methodology proposed by Zhang et al. (1994), modified by Dalonso et al. (2010). Briefly, 2 volumes of ethanol were added to the frozen mycelial biomass and the suspension was maintained at 80ºC for 3 h. The mixture was centrifuged (3.400 g) and the supernatant containing low molecular weight molecules was discarded. This procedure was repeated 4 times. The solid residue "S" generated was boiled in water for 3 h and filtered. This procedure was repeated 4 times. The new residue obtained (Residue I) was mixed with 1% solution of NH_4-oxalate, boiled for 3 h and filtered. This procedure was repeated 4 times and aimed to extract polysaccharides from Residue I. Five volumes of ethanol was added to the filtrate and the mixture was maintained at 4ºC for about 24 h and centrifuged (3.400 g). The supernatant was discarded, and the solid residue containing mainly polysaccharides (Precipitate II) was lyophilized and named FII (Facchini et al. 2014).

FII fraction was administered in doses of 10, 30 and 50 mg/kg in male Swiss mice (*Mus musculus*) weighing 30±5 g, purchased from the Instituto Tecnológico do Paraná – TECPAR, Curitiba/PR/Brazil. Animals were divided into 4 groups according to Table II. The fraction was solubilized in phosphate buffer (PBS) 0.01 M, pH 7.0 at concentrations of 1 g/L (for the dose of 10 mg/kg) and 10 g/L (for the doses of 30 and 50 mg/kg). The treatment was performed intraperitoneally (ip) for 10 days (Zhang et al. 1994), starting 24 h after the induction of the tumor by the inoculation of S-180 in the ascitic form (5 x 10^6 cells/animals) subcutaneously on the back of each mouse of the positive and control groups (Nakamura et al. 2004).

The assessment of tumor development was performed 21 days after tumor induction (Harhaji et al. 2008) by determining the weight (g) of the tumor according to Misaki et al. (1984) and the rate of tumor inhibition (I%) (Mizuno 1999), calculated according to the Equation 1.

$$I(\%) = \frac{C-T}{C} * 100 \quad (1)$$

Where:

C = weight of the tumor of the Positive Control Group (g)

T = weight of the tumor of the Test Group (g).

Data were evaluated by Dixon's "Q" test for rejection of deviant values at the 95% confidence level (Rorabacher 1991) and by Tukey test for analysis of variance of the mean values (ANOVA), with significance level of 5% ($p < 0.05$).

All the procedures were approved by the Ethics Committee on Research with Animals of the University of Joinville Region and are in

TABLE II
Distribution of the animals according to the experiment.

Group	Treatment	Dose (mg/kg)	No. of animals
Test **(TG)**	Tumor induction and administration of FII	10, 30 and 50	10
Substance Control **(SC)**	Administration of FII without tumor induction	10, 30 and 50	10
Positive Control **(PC)**	Tumor induction and administration of PBS	10	10
Negative Control **(NC)**	Administration of PBS without tumor induction	10	10

accordance with the *EU Directive 2010/63/EU* for animal experiments.

RESULTS

Figure 1 shows the profiles of mycelial biomass (X) and glucose concentrations (S) with time.

Data extracted from Figure 1 allowed the construction of Table III. Table IV shows the effects of glucose and pH on the mycelia biomass concentration (ΔX), overall yield of glucose on mycelial biomass ($Y_{X/S}$) and overall mycelial biomass productivity (Px). The effects shown on Table IV are considered significant only when their absolute values are higher than the correspondent absolute value statistically significant (AVSS). A negative and a positive effects express that the value of the variable increases towards the inferior and superior levels, respectively.

Evaluation of the antitumor effect of fraction FII was carried out through a dose x response assay, using 10, 30 and 50 mg/kg. The results of tumor weight (g) and tumor inhibition rate (I%) are shown in Figure 2. Doses of 10 and 30 mg/kg promoted the highest inhibition rate, of about 75%, without significant differences between them. The dose of 50 mg/kg promoted the lowest inhibition rate (53.7%).

DISCUSSION

Glucose uptake is faster in pH 6.0 (experiments 2 and 4) than in pH 4.0 (experiments 1 and 3) for the same initial glucose concentration (Figure 1). However, pH 4.0 led to higher values of biomass, showing

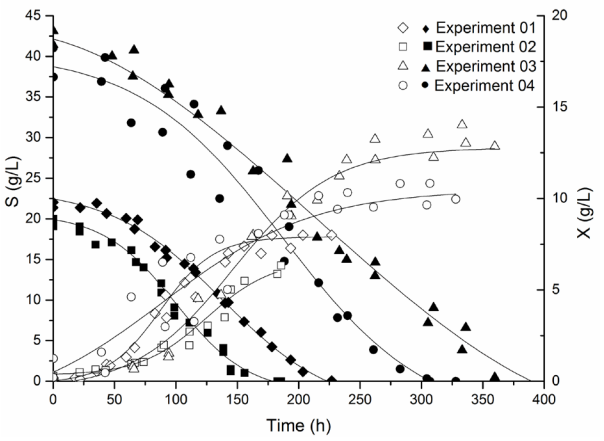

Figure 1 - Kinetic profile of mycelial biomass concentration (X) and substrate (glucose) concentration (S) obtained for the experiments 01 (◊,♦), 02 (□, ■), 03 (Δ, ▲) and 04 (○, ●). Open and closed symbols represent X and S, respectively.

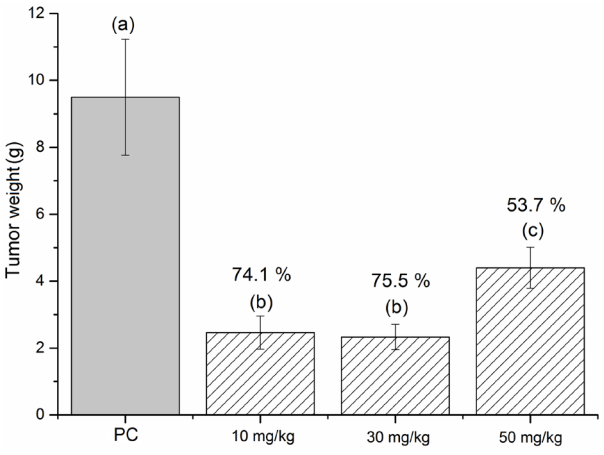

Figure 2 - Tumor weight and tumor inhibition rate (%) of Sarcoma 180 after 21 days of mice treatment with 10, 30 and 50 mg/kg of FII fraction extracted from mycelial biomass of *Pleurotus ostreatus*. Bars indicate mean values ± standard error. Equal letters indicate values without significant differences according to the Tukey test, at a confidence level of 95%. PC = Positive Control group.

TABLE III

Mean values± standard deviation of ΔX (mycelial biomass concentration), $Y_{X/S}$ (overall yield of glucose on mycelial biomass) and Px (overall mycelial biomass productivity).

Experiment	Glucose (g/L)	pH	ΔX (g/L)	$Y_{X/S}$ (g/g)	Px (g/L.h)
01	20	4	7.53 ± 0.41	0.37 ± 0.03	0.035 ± 0.002
02	20	6	5.81 ± 0.29	0.30 ± 0.02	0.031 ± 0.001
03	40	4	12.86 ± 0.83	0.32 ± 0.03	0.037 ± 0.004
04	40	6	9.65 ± 1.29	0.25 ± 0.03	0.030 ± 0.005

TABLE IV
Effects of the variables glucose (g/L) and pH on the mycelia biomass concentration (ΔX), overall yield of glucose on mycelial biomass ($Y_{X/S}$) and overall mycelial biomass productivity (Px).

Variables	Estimated effects ± standard error		
	ΔX (g/L)	$Y_{X/S}$ (g/g)	Px (g/L.h)
glucose	4.589 ± 0.57*	-0.0049 ± 0.02	0.00025 ± 0.0024
pH	-2.462 ± 0.57*	-0.0715 ± 0.02*	-0.00525 ± 0.0024
Interaction glucose and pH	-0.747 ± 0.57	0.0035 ± 0.02	-0.00125 ± 0.0024
AVSS**	|1.58|	|0.056|	|0.0068|

*Statistically significant values at confidence limits of 95%. ** Absolute value statistically significant.

the negative effect of the pH over the mycelial biomass concentration (X) and on the overall yield of glucose on mycelial biomass ($Y_{X/S}$) (Table IV). The overall mycelial biomass productivity (Px) was not significantly affected by the pH or by the initial glucose concentration (Table IV). Despite the increase of 70% in the cultivation time when initial concentration of glucose increases from 20.0 to 40.0 g/L, the increase in mycelial biomass is proportional (Figure 1, Table III). Experiment 3 (40.0 g/L of glucose and pH 4.0) led to an overall mycelial biomass productivity of 0.037 g/L.h. This result is higher than those reported by Borges et al. (2013) (0.022 g/L.h), cultivating *P. djamor* using the same medium, at pH 4.0 and 40 g/L of initial glucose concentration. Gern et al. (2008), cultivating *P. ostreatus* in a medium containing corn steep liquor as nitrogen source, 40.0 g/L of initial glucose concentration and pH 4.0, reach lower values of maximum productivity, achieved in 6 days of cultivation (0.032 g/L.h).

The effect of the initial concentration of glucose was only significant (positive effect) for the concentration of mycelial biomass (X) (Table IV). The same behavior was observed by Burns et al. (1994) for *Pleurotus* sp. florida. In this case, the increase in the initial glucose concentration led to the increase of biomass (9.7 g/L of biomass after 17 days, using 20 g/L of initial glucose and pH 4.0). Confortin et al. (2008) evaluated glucose and sucrose as carbon sources for *P. sajor-caju*, achieving 8.18 and 5.94 g/L of mycelial biomass using 10 g/L of glucose and 10 g/L of sucrose, respectively. Assis et al. (2013), working with 20 g/L of glucose and pH 4.0 reached 5.7 g/L of mycelial biomass of *P. sajor-caju*. Borges et al. (2013), cultivating *P. djamor* using 40 g/L of glucose and pH 4.0 reached 6.28 g/L of biomass in 13 days. Higher value of biomass (12.9 g/L) was observed in our work (experiment 3, Table III), achieved in 14 days of cultivation. Thus, this condition (40.0 g/L of initial glucose concentration and pH 4.0) was chosen to produce biomass aiming the extraction of FII fraction. Biomass was separated from the culture broth by filtration, washed with distilled water, and frozen.

Tao et al. (2006) evaluated the antitumor action of several polysaccharides fractions of *Pleurotus tuber-regium* fruiting bodies against Sarcoma 180 in mice. Animals were treated with daily doses of 20 and 60 mg/kg, during 8 days. After 8 days, the highest tumor inhibition rate was obtained with 60 mg/kg of FII fraction (72.1%). The same fraction, at a dose of 20 mg/kg, has led to only 21.6% of the inhibition of tumor development. These results are not in agreement with those shown in Figure 2, in which the increase of the dose from 30 for 50 mg/kg promoted a decrease of 22% in the tumor inhibition rate. Moreover, results obtained with the doses of 10 and 30 mg/kg shown in our work are higher than those reported by Tao et al. (2006) with a higher dose (60 mg/kg). The difference is probably due to the species of *Pleurotus*. Other authors also observed that highest doses promoted the decrease in tumor inhibition. Jeong et al.

(2010) evaluated a polysaccharide fraction from mycelial biomass of *P. eryngii* against Sarcoma 180. Extraction was carried out in hot water and the supernatant free of biomass was treated with four volumes of ethanol for precipitation of polysaccharides. Animals were treated with 10 to 80 mg/kg of mycelial extract, during 28 days. 40 mg/kg promoted the highest tumor inhibition rate (53.1%) while 80 mg/kg inhibited tumor weight in only 25.7%. In our work, the dose of 50 mg/kg of FII fraction promoted 53.7% of inhibition, similar to the results obtained by Jeong et al. (2010) using 40 mg/kg. De Barba et al. (2011) evaluated the antitumor effect of different doses (10, 30 and 100 mg/kg) of an ethanolic precipitated from the culture broth of *Pleurotus djamor,* against Sarcoma 180 in mice. The authors observed that 30 mg/kg promoted the highest tumor inhibition (94%) and that the increase of the dose to 100 mg/kg led to a reduction of 30% in the inhibition rate. Assis et al. (2013) found an inhibition rate of Sarcoma 180 of about 84% using doses of 10, 30 and 100 mg/kg of an ethanolic precipitated from the culture broth of *P. sajor-caju* indicating that the tumor effect was not dose dependent.

According to Wasser (2002), bioactivity of polysaccharides can be associated to several factors such as water solubility, molecular weight, structure and linkage, among others, making difficult the correlation between structure and biological activity of the complex polysaccharides. Zhang et al. (1994) performed the characterization of the monosaccharide composition of extractive fractions obtained from dried fruiting bodies of *P. citrinopileatus*. The fraction FII, similar to the FII fraction studied in our work, showed 83.5% of monosaccharide as glucose, fucose, mannose and galactose, and 16.5% of proteins. Zhang et al. (2007) reported that in some mushrooms species, the polysaccharides are linked to proteins or peptides, conferring high antitumor potential to the molecule. The authors also reported that the discovery of human macrophages receptors with high specificity for glucose and mannose could confer high antitumor effect to polysaccharides that contain these monomers. Structural features such as β- (1 → 3) bonds in the main chain of the polymer, and β- (1 → 6) branches are also important factors for antitumor activity.

In the range of our study for the production of *P. ostreatus* mycelial biomass (20 and 40 g/L of initial glucose; pH 4.0 and 6.0), pH 4.0 and 40 g/L of glucose promoted the highest biomass concentration (12.8 g/L). The FII fraction obtained from frozen mycelium of *P. ostreatus* reduced about 75% Sarcoma 180 tumor weight after 10 days of administration at the doses of 10 and 30 mg/kg in mice. This suggests that low doses of FII fraction can act as antitumor, being promising as antineoplastic.

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