J Ind Microbiol Biotechnol (2012) 39:1179–1188
DOI 10.1007/s10295-012-1112-2

Lasiodiplodan, an exocellular (1→6)-β-D-glucan from *Lasiodiplodia theobromae* MMPI: production on glucose, fermentation kinetics, rheology and anti-proliferative activity

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Received: 15 December 2011 / Accepted: 17 February 2012 / Published online: 8 March 2012

Abstract Lasiodiplodan, an exopolysaccharide of the (1→6)-β-D-glucan type, is produced by *Lasiodiplodia theobromae* MMPI when grown under submerged culture on glucose. The objective of this study was to evaluate lasiodiplodan production by examining the effects of carbon (glucose, fructose, maltose, sucrose) and nitrogen sources (KNO_3, $(NH_4)_2SO_4$, urea, yeast extract, peptone), its production in shake flasks compared to a stirred-tank bioreactor, and to study the rheology of lasiodiplodan, and lasiodiplodan's anti-proliferative effect on breast cancer MCF-7 cells. Although glucose (2.05 ± 0.05 g L^{-1}), maltose (2.08 ± 0.04 g L^{-1}) and yeast extract (2.46 ± 0.06 g L^{-1}) produced the highest amounts of lasiodiplodan, urea as N source resulted in more lasiodiplodan per unit biomass than yeast extract (0.74 ± 0.006 vs. 0.22 ± 0.008 g g^{-1}). A comparison of the fermentative parameters of *L. theobromae* MMPI in shake flasks and a stirred-tank bioreactor at 120 h on glucose as carbon source showed maximum lasiodiplodan production in agitated flasks (7.01 ± 0.07 g L^{-1}) with a specific yield of 0.25 ± 0.57 g g^{-1} and a volumetric productivity of 0.06 ± 0.001 g L^{-1} h^{-1}. A factorial 2^2 statistical design developed to evaluate the effect of glucose concentration (20–60 g L^{-1}) and impeller speed (100–200 rpm) on lasiodiplodan production in the bioreactor showed the highest production (6.32 g L^{-1}) at 72 h. Lasiodiplodan presented pseudoplastic behaviour, and the apparent viscosity increased at 60°C in the presence of $CaCl_2$. Anti-proliferative activity of lasiodiplodan was demonstrated in MCF-7 cells, which was time- and dose-dependent with an IC_{50} of 100 μg lasiodiplodan mL^{-1}.

Keywords *Lasiodiplodia theobromae* · Lasiodiplodan, (1→6)-β-D-glucan · Carbon and nitrogen nutrients · Anti-proliferative activity · Breast cancer cells (MCF-7)

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Introduction

Microbial exopolysaccharides (EPS) are a class of high-value biopolymers with a wide variety of commercial applications [6]. β-Glucans of fungal origin are polysaccharides consisting of D-glucose units variously linked by glucosidic bonds (usually (1→3), but also of mixed linkages (1→3;1→6)), and are principally found in the cell wall, but some can also be secreted exocellularly [20]. They belong to a group of physiologically active compounds named biological response modifiers (BRM) [3] and have been described as being effective in treating disease conditions such as cancer, microbial infections, hypercholesterolaemia, diabetes and reducing cardiovascular risks [5, 11].

The most commonly occurring fungal β-glucans described in the literature are those of the (1→3)- and (1→3;1→6)-β-D-glucan types. Those possessing biological activity appear to be branched (1→3)-β-D-glucans and have been isolated from the yeast cell wall, fungal mycelia, fungal fruiting bodies, and they can also be produced exocellularly by fungi cultured on defined nutrient media [11]. Exocellular fungal (1→6)-β-D-glucans are rather uncommon (only two have been described [14, 21]), and although they are widely known as pustulan [10] produced by lichens of Umbilicariaceae species from which they are extracted with boiling water, there is paucity of information on the microbial physiology of their production by fungi and their biological activities.

An exocellular (1→6)-β-glucan named lasiodiplodan has been described that is produced by a *Lasiodiplodia theobromae* isolate (MMPI), and exists in a triple-helix conformation [21]. (1→6)-β-D-Glucans are commonly found as water-insoluble components of the cell walls of ascomycetous and basidiomycetous fungi [9]. Their isolation is complicated, involving multiple extraction steps performed sequentially such as hexane (lipid removal), followed by cold and hot water, and alkali [7]. A polysaccharide of the (1→6)-β-glucan type was structurally characterized and found to be present in the cell wall (mycelium) of *Botryosphaeria rhodina* MAMB-05 [7]. The exocellular (1→6)-β-glucan produced by *L. theobromae* MMPI is secreted in the culture medium during submerged fermentation and is precipitated from the culture fluid simply by adding ethanol. Its isolation is considered more cost-effective than extraction of fungal cell walls.

In the present study, we report on the requirement of some essential nutrients for *L. theobromae* MMPI to produce the (1→6)-β-glucan (lasiodiplodan) and compare the production of this EPS when cultivated on glucose as carbon source by submerged fermentation in shake flasks and in a stirred-tank bioreactor. We also report for the first time on the fermentative kinetic and rheological properties of lasiodiplodan and its anti-proliferative activity in breast cancer MCF-7 cells.

Materials and methods

Microorganism and inoculum preparation

A fungal strain of *L. theobromae* MMPI isolated from the tropical fruit pinha (sugar-apple, *Annona squamosa*) [13] was maintained through periodic transfer on Sabouraud agar plates containing chloramphenicol at 4°C. Pre-inoculum was prepared by growing the fungal isolate on Sabouraud agar plates at 28°C for 96 h. With the aid of an inoculating loop, a small amount of mycelium and hyphae was scraped from agar plates colonized with freshly growing fungal mycelium, transferred to 250-mL Erlenmeyer flasks containing 100 mL of nutrient medium (Vogel minimum salts medium (VMSM) [22] and 5 g L^{-1} glucose) and incubated at 28°C on a rotary shaker at 180 rpm for 48 h. Fungal mycelium was harvested by centrifugation (1,500×g for 15 min), washed twice with sterile distilled water, and sterile physiological saline solution was added followed by homogenization for 30 s in a chilled sterile blender. A standardized inoculum was prepared by diluting the homogenate with sterile physiological saline solution to an optical density between 0.4 and 0.5 at 400 nm [17].

Effect of carbon and nitrogen sources on lasiodiplodan production

Four carbon (C) sources (glucose, fructose, maltose, sucrose) were evaluated for lasiodiplodan production in submerged fermentation. Experiments were conducted in 250-mL Erlenmeyer flasks containing 100 mL of nutrient medium (VMSM and 20 g L^{-1} carbon source). The initial pH was adjusted to 6.0; the mixture was then inoculated with 5 mL of standardized inoculum and left for 48 h at 28°C. All experiments were conducted in duplicate, and the results represent mean ± SD.

Five different nitrogen (N) sources consisting of organic (yeast extract, peptone, urea) and inorganic (KNO_3, $(NH_4)_2SO_4$) nitrogen were examined for their effect on lasiodiplodan production. The nutrient medium comprised 20 g L^{-1} glucose, 2 g L^{-1} K_2HPO_4, 2 g L^{-1} $MgSO_4 \cdot 7H_2O$ and water (1 L), and one of the above N sources (2 g L^{-1}). The initial pH of medium and the conditions of cultivation were as described above.

Isolation of lasiodiplodan

Following growth, fungal biomass (mycelium) was separated by centrifugation (1,500×g, 30 min), washed twice with distilled water, and dried in an oven at 70°C to achieve constant weight. The supernatant was recovered, and EPS was precipitated from solution by adding four volumes of absolute ethanol and leaving the mixture to stand at 4°C (overnight). The precipitate was recovered by filtration and dried and its weight recorded.

Lasiodiplodan production in submerged cultivation using shake flasks (SFs)

Fungal isolates were cultivated in 250-mL Erlenmeyer flasks containing 100 mL of nutrient medium and 5 mL of standardized inoculum and were left at 28°C. The nutrient medium consisted of 40 g L^{-1} glucose, 2 g L^{-1} K_2HPO_4,

2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 g L^{-1} yeast extract and water (1 L). The initial pH of the medium was 6.0. The conditions of cultivation were as described above, and each experiment was conducted in duplicate. At each sampling time, two flasks were removed from the shaker, the mycelium was separated by centrifugation, and lasiodiplodan in the supernatant was precipitated with ethanol and its dry weight measured.

Lasiodiplodan production in a stirred-tank bioreactor (STBR)

Discontinuous fermentation was carried out on a bench-scale STBR (Biostat B, B. Braun Biotech International, Germany). The bioreactor was loaded with 1,000 mL of nutrient medium (as defined above and containing 40 g L^{-1}) and was inoculated with 50 mL of standardized inoculum. The bioreactor was run at 28°C with an airflow of 0.8 vvm and an impeller speed of 180 rpm. The pH, temperature, agitation speed of the impeller, and airflow were monitored during the course of the fermentation run (up to 120 h). The nutrient medium composition was the same as that used in the SF experiments.

Statistical factorial design for lasiodiplodan production in an STBR

A factorial 2^2 design was developed with triplicates at the central point to quantify the influence of the variables glucose concentration (X_1; 20–60 g L^{-1}) and impeller speed (X_2; 100–200 rpm) on the responses (lasiodiplodan production (P_P), yield ($Y_{P/S}$) and volumetric productivity (Q_P)) analysed. The kinetic parameters to evaluate lasiodiplodan production by *L. theobromae* MMPI followed the defined conditions established by the experimental design (Table 1) and were conducted in an STBR. The results of the factorial design were analysed using the software STATGRAPHICS Centurion XVII. In all fermentation runs the residual glucose content was determined using a glucose oxidase kit (Glicose PAP Liquiform, Labtest Diagnóstica SA, Brazil). Standard curves were calibrated with D-glucose.

Table 1 Central-composite 2^2 factorial design matrix defining conditions for lasiodiplodan production by *L. theobromae* MMPI in an STBR on glucose for 72 h and the responses obtained

Runs	Variables in coded levels		Observed responses		
	X_1	X_2	P_P (g L^{-1})	$Y_{P/S}$ (g g^{-1})	Q_P (g L^{-1} h^{-1})
1	+1	+1	6.32	0.23	0.09
2	+1	−1	5.70	0.24	0.08
3	−1	+1	2.38	0.14	0.03
4	−1	−1	4.28	0.17	0.06
5[a]	0	0	5.26	0.36	0.07
6[a]	0	0	5.27	0.28	0.07
7[a]	0	0	5.30	0.31	0.07

Factors	Real levels		
	−1	0	+1
X_1 (glucose, g L^{-1})	20	40	60
X_2 (impeller speed, rpm)	100	150	200

P_P production, $Y_{P/S}$ yield, Q_P volumetric production

[a] Central point runs

Monosaccharide analysis and FT-IR spectroscopy

For chemical analysis purposes, the EPS precipitates recovered after treating the culture supernatants with ethanol were first exhaustively dialyzed against distilled water with frequent changes of distilled water and then lyophilized. Lasiodiplodan samples (10 mg dry weight) were hydrolysed with 2 M HCl (1 mL) at 100°C in a sealed glass tube for 4 h and then neutralized with 1 mL of 2 M NaOH. The composition of the monosaccharides in the EPS hydrolysate was determined by high-performance anion-exchange chromatography using a pulsed amperometric detector (HPAEC-PAD). The analyses were performed on a Dionex ICS 3000 chromatograph system using a CarboPac PA-20 column (and PA-20 guard column), and eluted with 500 mM sodium acetate (eluent 1) and 100 mM sodium hydroxide (eluent 2) at a flow rate of 1.0 mL min^{-1}. A sodium acetate gradient concentration of 3–50% was used.

Fourier transform infrared spectroscopy (FT-IR) of lasiodiplodan was conducted on a Bomem MB 100 FT-IR spectrometer (ABB Bomem Inc., Quebec, Canada). Lasiodiplodan was pressed into KBr pellets (lasiodiplodan/KBr ratio of 1:100) and the FT-IR spectrum recorded in the region of 4,000–500 cm^{-1} at a resolution of 4 cm^{-1}.

Determination of fermentation kinetic parameters in the production of lasiodiplodan

Kinetic parameters used to evaluate the fermentative profile of *L. theobromae* MMPI conducted in SFs and STBR on nutrient medium defined above containing 40 g L^{-1} glucose, including the yield of lasiodiplodan, volumetric productivity of lasiodiplodan, volumetric productivity of fungal biomass, rate of substrate (glucose) consumption and specific yield, were calculated from Eqs. 1–6. The conversion yield of glucose to lasiodiplodan ($Y_{P/S}$) was calculated as the amount of lasiodiplodan produced from the substrate (glucose) consumed (Eq. 1). The volumetric productivity of lasiodiplodan (Q_P) was calculated as the

ratio of the maximum lasiodiplodan concentration to the fermentation time (Eq. 2). The volumetric productivity of biomass (Q_X) was calculated as the ratio of the maximum biomass concentration to the fermentation time (Eq. 3). The total rate of substrate consumption (Q_S) was calculated as the ratio of the glucose consumed to the fermentation time (Eq. 4). Biomass production per unit of glucose consumed ($Y_{X/S}$) was calculated as the ratio of biomass yield to the glucose consumed (Eq. 5). The specific yield (Y_e) was calculated as the amount of lasiodiplodan produced on the biomass formed (Eq. 6).

$$Y_{P/S} = \frac{\text{EPS (g)}}{\text{Consumed glucose (g)}} \quad (1)$$

$$Q_P = \frac{\text{Maximum EPS concentration } (gL^{-1})}{\text{Fermentation time (h)}} \quad (2)$$

$$Q_X = \frac{\text{Maximum biomass concentration } (gL^{-1})}{\text{Fermentation time (h)}} \quad (3)$$

$$Q_S = \frac{\text{Consumed glucose } (gL^{-1})}{\text{Fermentation time (h)}} \quad (4)$$

$$Y_{X/S} = \frac{\text{Biomass (g)}}{\text{Consumed glucose (g)}} \quad (5)$$

$$Y_e = \frac{\text{Maximum EPS concentration (g)}}{\text{Produced biomass (g)}} \quad (6)$$

Rheological analysis of lasiodiplodan

Viscosity measurements were performed at 25° and 60°C on aqueous solutions of lasiodiplodan (2 g L^{-1}) and aqueous solutions of lasiodiplodan (2 g L^{-1}) containing $CaCl_2$ (1 g L^{-1}) using a Brookfield digital rheometer (model LVDV III) equipped with a spindle CP 18 operating at shear rates within the range of 4–264 s^{-1} and a thermostatic programmable bath (Brookfield model TC-502P). The measurements were recorded at 5-s intervals increasing the shear rate from 4 to 264 s^{-1}.

In vitro cell proliferation (MTT) assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay for measuring the activity of dehydrogenases, which in living cells reduce the yellow tetrazolium salt to produce a purple formazan dye that can be measured spectrophotometrically. This assay is used to measure cell viability, cytotoxicity and cell proliferation [2].

MCF-7 cells (150 µL; 2.5×10^4 cells/well) were cultured in 96-well microplates and allowed to adhere to the well walls overnight at 37°C under an atmosphere containing 5% CO_2. After incubation for 24 h, the culture medium was aspirated, an equal volume (150 µL) of lasiodiplodan solution (25–1,500 µg mL^{-1}) was added to each well, and the cells were incubated for another 24 and 48 h at 37°C and 5% CO_2. Following this procedure, the cells were treated with MTT solution (15 µL; 5 mg mL^{-1} in PBS Sigma-Aldrich, Canada), and the plate was incubated for an additional 4 h. Thereafter, the incubation medium was aspirated and 50 µL of dimethyl sulfoxide was added per well. Following 10 min of agitation on a Belly Dancer shaker the reduction of MTT was determined spectrophotometrically by measuring the absorbance (A) at 490 and 620 nm, and the difference (ΔA) calculated from the formula: $\Delta A = A_{490} - A_{620}$ was used to analyse the results. The treatment groups were compared with control groups in the absence of lasiodiplodan. Results were expressed as percentage of inhibition of growth compared to a control. The value of IC_{50} represents the concentration of lasiodiplodan required to inhibit the MCF-7 cells by 50% of the control level.

Results and discussion

Chemical analysis of lasiodiplodan

Isolation of an EPS (lasiodiplodan) from the fermentation broth through precipitation with ethanol resulted in a precipitate, which upon acid hydrolysis revealed only a single peak by HPAEC-PAD analysis. The peak was identified as glucose (retention time of an authentic glucose standard, T_R 2.2067 min), indicating that the polymeric material was composed of glucose, i.e. it was a glucan. An FT-IR spectrum of the EPS sample showed bands at 891 and 1,371 cm^{-1} indicating that all the glucosidic linkages were of the β-configuration. The results demonstrated that the sample produced by *L. theobromae* MMPI was a β-glucan and confirmed evidence of the polysaccharide previously described as lasiodiplodan produced by this fungal isolate when cultivated on sucrose [21].

Effect of different C and N sources on the production of lasiodiplodan and fungal growth by *L. theobromae* MMPI

Four C sources (glucose, fructose, maltose, sucrose) were evaluated to find a suitable C source for growth of *L. theobromae* MMPI to produce lasiodiplodan. Glucose and maltose resulted in the highest amounts of lasiodiplodan (2.05 ± 0.05 and 2.08 ± 0.04 g L^{-1}, respectively) (Fig. 1a). Fructose and sucrose, by contrast, resulted in lower amounts of lasiodiplodan (1.55 ± 0.08 and 1.36 ± 0.03 g L^{-1}, respectively). Fructose promoted the highest fungal growth (8.32 ± 0.18 g biomass L^{-1}), and sucrose the least (6.85 ± 0.23 g L^{-1}). The ratio of

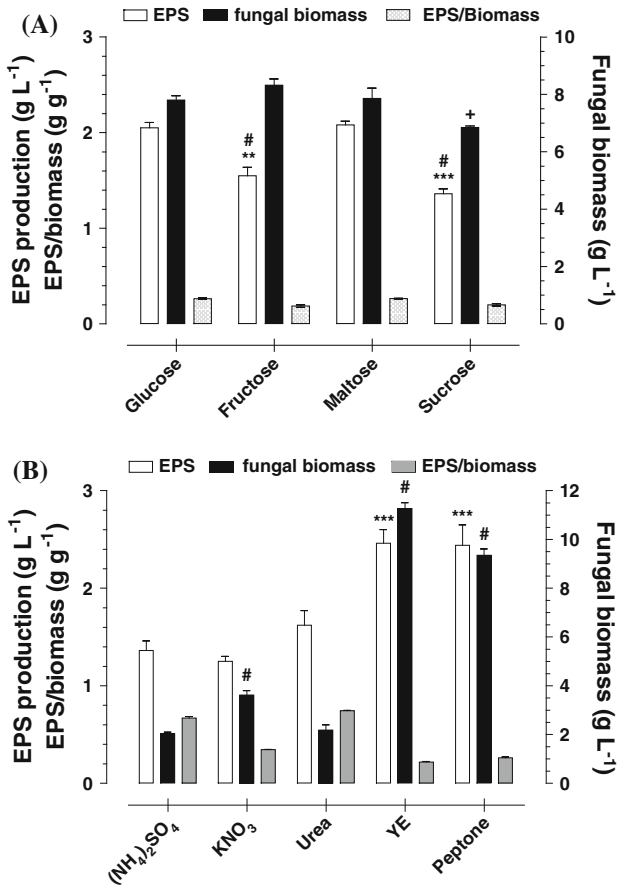

Fig. 1 Effects of different carbon (**a**) and nitrogen (**b**) sources on fungal growth and production of lasiodiplodan by *L. theobromae* MMPI. ($^{\#}p < 0.001$ vs. glucose; $^{**}p < 0.001$ vs. maltose; $^{***}p < 0.0001$ vs. maltose; $^{+}p < 0.001$ vs. glucose, fructose and maltose; *YE* yeast extract; $^{***}p < 0.0001$ vs. $(NH_4)_2SO_4$, KNO_3 and urea; $^{\#}p < 0.0001$ vs. others)

lasiodiplodan-to-mycelial biomass (lasiodiplodan specific productivity) for the four sugars evaluated ranged from 0.186 to 0.265 g g^{-1} (mean 0.228 ± 0.009 g g^{-1}), with the highest specific productivity for glucose and maltose, but there was no great variation between all of the sugars examined. The results are important, as there is no information in the literature on the types of C sources for the production of lasiodiplodan by *Lasiodiplodia* spp.

Each microbial species shows a preference for a specific source of C for growth and production of EPS. For example, Steluti et al. [17] showed the influence of glucose and other monosaccharides and some disaccharides on the production of botryosphaeran, an EPS from *B. rhodina* MAMB-05, and the highest yields occurred on sucrose. Fan et al. [8] also observed that sucrose resulted in the highest EPS production by *Agaricus brasiliensis*. Similarly, Park et al. [12] described sucrose as the best C source for exobiopolymer and mycelial biomass production by *Cordyceps militaris*. By contrast, Shih et al. [16] reported that maltose and glucose resulted in higher mycelial biomass in *Antrodia cinnamomea*, while EPS production was higher with lactose and sucrose.

Figure 1b shows that the organic N sources yeast extract and peptone resulted in the highest fungal growth and lasiodiplodan production. Yeast extract produced 2.46 ± 0.06 g lasiodiplodan L^{-1} and 11.26 ± 0.48 g L^{-1} of mycelial biomass. Urea, by contrast, produced lower amounts of lasiodiplodan, and this was probably due to the low amount of biomass produced. The inorganic N sources (KNO_3 and $(NH_4)_2SO_4$) also resulted in poor growth, and consequently low amounts of lasiodiplodan were produced. However, on considering the ratio of lasiodiplodan-to-biomass (Fig. 1b), urea and ammonium sulfate produced more EPS per g of biomass (0.744 ± 0.006 and 0.668 ± 0.026 g g^{-1}, respectively) than yeast extract (0.22 ± 0.008 g g^{-1}) and peptone (0.26 ± 0.015 g g^{-1}). Yeast extract and peptone are complex organic sources of N containing amino acids, vitamins and minerals, factors that contribute to cellular growth, but did not have a significant influence on lasiodiplodan production. Each microorganism responds differently in relation to the source of N used in the cultivation medium. Selbmann et al. [15] demonstrated that $(NH_4)_2SO_4$ was a poor source of N for EPS production by *Botryosphaeria rhodina* DABAC-P82 BTS, but urea contributed to high levels of the EPS produced. On the other hand, Fan et al. [8] reported that complex organic N sources were better for EPS production by *A. brasiliensis*.

In the present study, glucose and yeast extract were chosen as the best sources of C and N, respectively, to produce lasiodiplodan by *L. theobromae* MMPI and were used in the nutrient media in subsequent studies to evaluate lasiodiplodan production in submerged fermentation in SFs and in an STBR.

Fermentation profile of *L. theobromae* MMPI in SFs and in an STBR

The profile of fungal growth and lasiodiplodan production by *L. theobromae* MMPI on 40 g L^{-1} glucose in SF cultivation is shown in Fig. 2. Lasiodiplodan concentration reached a maximum level of 7.01 ± 0.07 g L^{-1} at 120 h of cultivation, whereas the maximum mycelial concentration (17.3 ± 0.57 g L^{-1}) was reached after 72 h. Fungal growth significantly increased over the first 72 h (log phase) and levelled off thereafter (stationary phase). Glucose was consumed during the whole 120-h course of growth, and at this stage approximately 30% glucose remained in the culture medium. The cessation of fungal growth at 72 h may be associated with a reduction in the dissolved oxygen content in the medium as sufficient levels of glucose remained to sustain growth. A condition of low

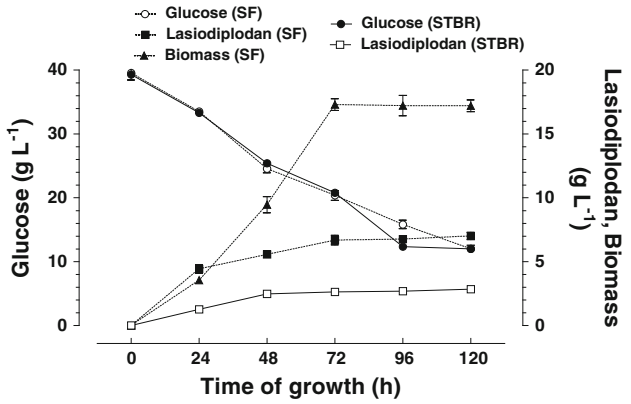

Fig. 2 Comparison of lasiodiplodan production by *L. theobromae* MMPI in SFs and an STBR

oxygen availability to the fungal cells might be attributed to the high viscosity of the culture medium due to lasiodiplodan, as well as the high biomass content. Both conditions would affect oxygen transfer to the fungal cells [1, 6]. The possible condition of low concentration of dissolved oxygen (DO) present in the system might have been responsible for the stagnation in the production of lasiodiplodan beyond 72 h.

Biopolymer synthesis generally occurs only when the microorganism is grown aerobically, and biopolymers of a higher molecular weight are usually produced under non-oxygen-limited conditions [18]. The increased viscosity of the fermentation broth as during biopolymer production may allow the formation of a layer on the fungal cell surface that can act as a diffusion barrier to the transfer of oxygen to the cells. The DO concentration becomes a limiting factor in processes of high oxygen demand or when the rheological properties of the fermentation broth offer a high resistance to mass transfer [1].

Glucose was consumed during the fermentation process at a rate of 0.23 ± 0.002 g L^{-1} h^{-1} and was not completely exhausted from the medium after 120 h. In the culture flasks, nearly 70% (Y_C) of the carbon source was consumed, leaving a residual glucose level of 12.02 ± 0.03 g L^{-1}.

As shown in Table 2 and Fig. 2, the fermentative performance of the fungal isolate in the STBR was similar to that conducted in SFs, but production of lasiodiplodan and fungal biomass was somewhat higher in agitated flasks (7.01 ± 0.07 and 17.3 ± 0.80 g L^{-1}, respectively) than in the STBR (5.7 ± 0.16 and 11.9 ± 0.57 g L^{-1}, respectively). Fermentation in the STBR produced 18.7% less lasiodiplodan than that in SFs.

The volumetric productivity of lasiodiplodan and fungal biomass was 0.06 ± 0.001 and 0.14 ± 0.01 g L^{-1} h^{-1}, respectively, in agitated flasks and was comparable to data obtained for *L. theobromae* MMPI cultivated in the STBR

Table 2 Kinetic parameters for the production of lasiodiplodan by *L. theobromae* MMPI cultivated in submerged fermentation for 120 h

Fermentation parameters	Agitated flasks	STBR
P_P (g L^{-1})	7.01 ± 0.07	5.70 ± 0.16
P_X (g L^{-1})	17.2 ± 1.13	11.9 ± 0.57
$Y_{P/S}$ (g g^{-1})	0.25 ± 0.57	0.21 ± 0.003
$Y_{X/S}$ (g g^{-1})	0.62 ± 0.01	0.43 ± 0.002
Y_c (%)	69.6 ± 0.25	69.5 ± 0.06
Y_e (g g^{-1})	0.41 ± 0.02	0.48 ± 0.01
Q_P (g L^{-1} h^{-1})	0.06 ± 0.001	0.05 ± 0.001
Q_X (g L^{-1} h^{-1})	0.14 ± 0.01	0.10 ± 0.005
Q_S (g L^{-1} h^{-1})	0.23 ± 0.002	0.23 ± 0.004

P_P production of lasiodiplodan, P_X production of mycelial biomass, $Y_{P/S}$ conversion yield of glucose to lasiodiplodan, $Y_{X/S}$ yield of biomass on consumed glucose, Y_c percentage of consumed substrate, Y_e specific yield, Q_P volumetric productivity of lasiodiplodan, Q_X volumetric productivity of biomass, Q_S global rate of glucose consumption

(0.05 ± 0.001 and 0.10 ± 0.005 g L^{-1} h^{-1}, respectively, Table 2). Similar behaviour was observed regarding the yield of biomass on glucose consumed, which was higher in agitated flasks (0.62 ± 0.01 g g^{-1}) than in the STBR (0.43 ± 0.002 g g^{-1}). The consumption of glucose in the STBR also was incomplete; residual glucose of 12.01 ± 0.20 g L^{-1}, which was similar to that observed in SFs (Fig. 2).

Generally, higher EPS production and fungal growth are expected in an STBR because of the fine control of fermentation parameters. However, this did not appear to occur in this case. Although a relatively high airflow was used in the STBR (0.8 vvm), low oxygen transfer might have occurred in relation to the high viscosity of the medium associated with intense mycelial growth inside the STBR. Furthermore, the impeller speed might have been insufficient to promote adequate dissolution of oxygen in the medium under these conditions. A higher impeller speed would normally have contributed to more effective transfer of oxygen, which might have increased substrate consumption, and consequently the production of more EPS. In the present study, two Rushton turbines (flat disk with six vertical flat blades vertically mounted used primarily for very high intensity mixing) were used as a double impeller device operating up to 200 rpm, allowing for a high capacity of mass transfer. Under these conditions, however, no increases in lasiodiplodan production resulted. The problem may have been caused because of the type of mycelial growth inside the STBR; tight clumps of mycelial biomass adhered to the baffles, pH and temperature probes and the impeller, and this may have impeded mass transfer. An examination of agitation speed of the impeller system under conditions where the diffusion

of oxygen is hindered by the inherent rheological properties of the medium (viscosity imparted by EPS) and increased fungal biomass will be the subject of further study. Moreover, the impeller itself when operated at high rpm can also contribute to cellular stress with consequent lower performance. However, at 200 rpm, the speed is relatively low, although the Rushton-type impeller has a high shear capacity.

Mass transfer was hampered by the increased viscosity of the fermentation broth due to the presence of lasiodiplodan concomitantly with fungal biomass built up with time of growth, which increased the consistency of the fermentation medium during cultivation of the fungus. Viscosity and biomass impose restrictions on oxygen transfer to the growing fungal cells, and these would limit glucose consumption. Other investigators [1, 6] have described that the rheological properties of the broth can interfere in the mass transfer and influence the performance of EPS production and cellular growth.

Effects of glucose concentration and impeller speed on lasiodiplodan production in an STBR

Lasiodiplodan production in the STBR may be improved through studies of process optimization. To enhance lasiodiplodan production, the initial concentration of substrate and aeration of the system are key variables that should be taken into account. The Pareto graph (Fig. 3a) showed that the concentration of glucose had a significant effect on and positive interaction with the production of lasiodiplodan (P_P; $p < 0.05$) by *L. theobromae* MMPI. At low impeller speeds, the P_P increased less than at higher speeds (1.42 vs. 3.94 g L^{-1}) with increasing concentrations of glucose. These effects are best visualized in geometric form (Fig. 4a). However, at high impeller speeds, P_P increased less prominently at low glucose concentrations than at higher glucose concentrations (−1.90 vs. +0.62 g L^{-1}). Impeller speed as a factor, on the other hand, had no significant effect on lasiodiplodan production, and there was no interaction with the concentration of glucose.

In relation to the volumetric productivity (Q_P), a positive and significant effect of the glucose concentration was observed, and significant interaction existed between glucose concentration and impeller speed (Fig. 3b). An increase in glucose concentration from 20 to 60 g L^{-1} at increasing impeller speeds (100–200 rpm) increased lasiodiplodan productivity compared to low agitation rates (0.09 vs. 0.06 g L^{-1} h^{-1}, Fig. 4b). At low concentrations of glucose (20 g L^{-1}) and high impeller speed (200 rpm), Q_P decreased. On the other hand, factors such as impeller speed and glucose concentration showed no significant

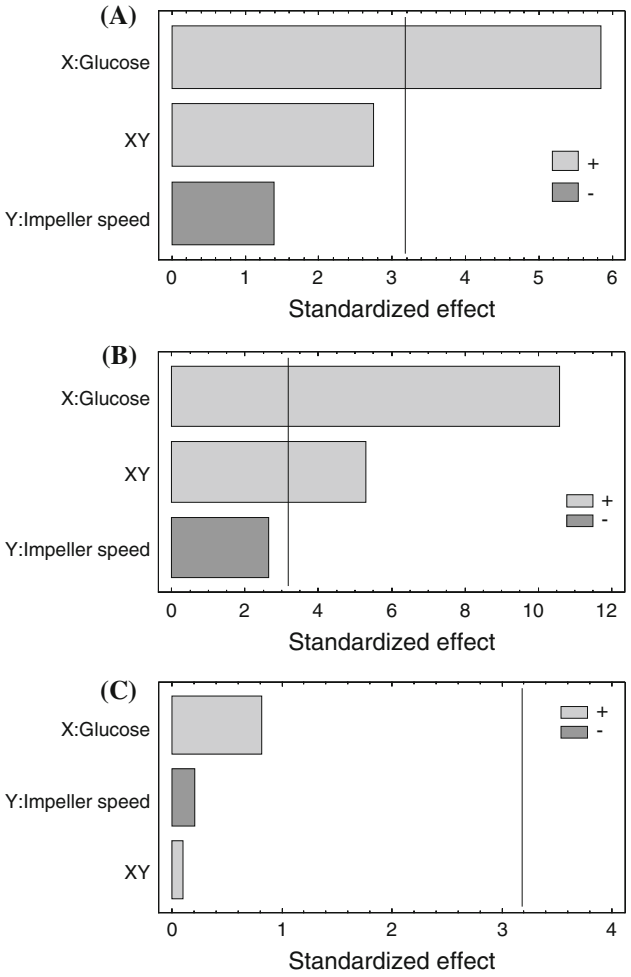

Fig. 3 Pareto diagram showing effects and interactions of glucose concentration and agitation speed on **a** lasiodiplodan production (P_P), **b** volumetric productivity of lasiodiplodan (Q_P), and **c** conversion yield ($Y_{P/S}$) of glucose to lasiodiplodan by *L. theobromae* MMPI. *X*, *Y* main effects, *XY* two-factor interactions. The *vertical line* defines 95% of the reliable interval ($p = 0.05$). *X* glucose concentration, *Y* agitation speed

effect on or interaction with the conversion yield ($Y_{P/S}$) of glucose into lasiodiplodan (Fig. 3c).

The fungus grew well during the time of growth, and the highest lasiodiplodan production observed was 6.32 g L^{-1} at 72 h. Glucose was assimilated over 96 h during the process, but glucose concentration was not reduced beyond this time.

Rheological properties of lasiodiplodan

The relationship between viscosity and shear rate of an aqueous solution of lasiodiplodan in the absence and presence of calcium chloride at 25° and 60°C are shown in Fig. 5. An increase of shear rate contributed to a steep decline in viscosity of lasiodiplodan solutions suggesting a pseudoplastic behaviour at both of the temperatures

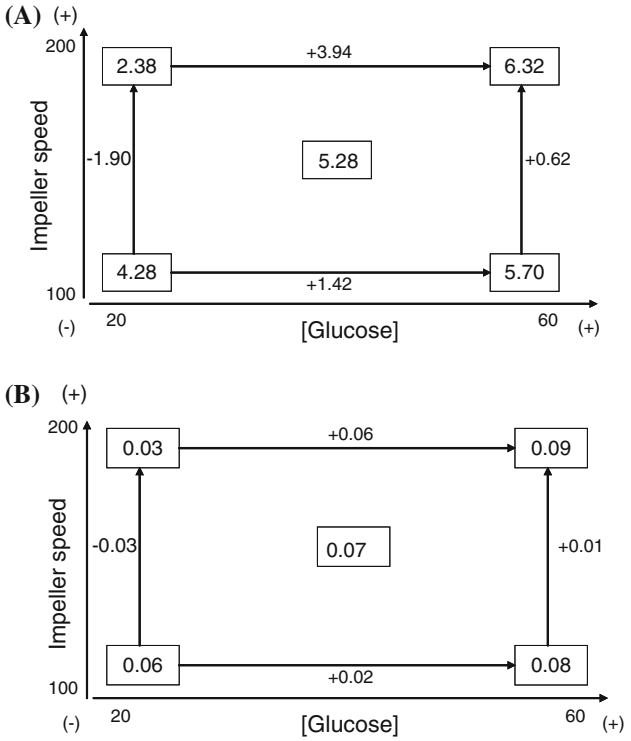

Fig. 4 Geometric representation of interaction plots for **a** lasiodiplodan production (P_P) and **b** volumetric productivity (Q_P) by *L. theobromae* MMPI as a function of the variables glucose concentration (g L^{-1}) and agitation speed (rpm)

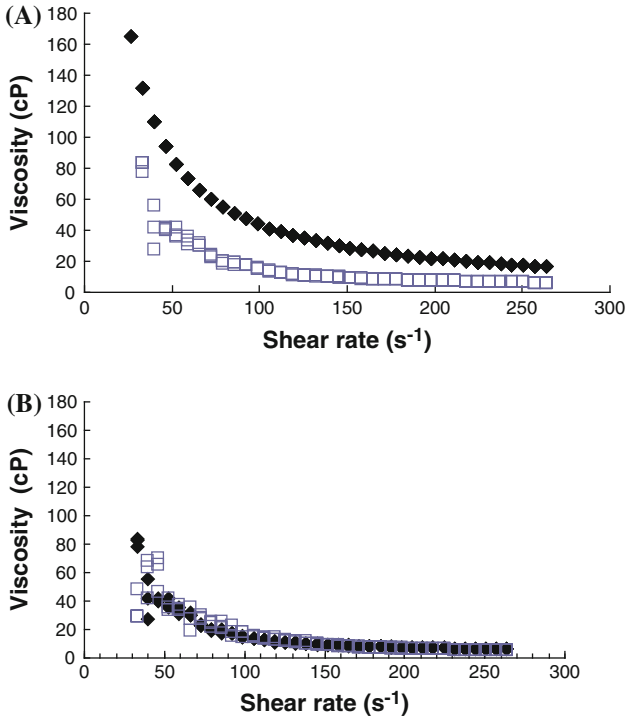

Fig. 5 Shear rate dependent apparent viscosity. **a** Lasiodiplodan solution at 25°C (*squares*) and 60°C (*filled diamonds*) with addition of calcium chloride. **b** Lasiodiplodan solution at 25°C with (*filled diamonds*) and without (*squares*) added calcium chloride. The concentration of lasiodiplodan was 2 g L^{-1}, and that of calcium chloride was 1 g L^{-1}

evaluated. This behaviour can be explained by the alignment of the polymeric chains when lasiodiplodan is submitted to a continuous increase in shear stress resulting in a decrease of the resistance to flow. An EPS (β-(1→3;1→6)-D-glucan) produced by *B. rhodina* MAMB-05 also showed pseudoplastic behaviour at 60°C [4].

With regard to the influence of an ionic force on the viscosity of lasiodiplodan, the addition of calcium chloride to the EPS solution did not significantly alter the apparent viscosity of the material at 25°C (Fig. 5b). An increase of the temperature from 25 to 60°C, however, led to an increase in the apparent viscosity at low shear rates (<50 s^{-1}; Fig. 5a). Such behaviour might have happened as a consequence of possible gelation of the sample in the presence of $CaCl_2$, which could justify the viscosity increase at 60°C, and when subjected to increasing shear rates, the gel dissipated with subsequent viscosity reduction.

In vitro anti-proliferative activity of lasiodiplodan

EPSs produced by bacteria and fungi have been described to possess medicinal properties effective in treating disease conditions such as microbial infections, Alzheimer's disease, hypercholesterolemia, diabetes and cancer [5, 25].

The EPS lasiodiplodan produced by *L. theobromae* MMPI showed anti-proliferative activity in MCF-7 cells with an IC_{50} at a concentration of 100 μg lasiodiplodan mL^{-1}. The anti-proliferative activity of lasiodiplodan was both time- and concentration-dependent. At a concentration greater than 100 μg lasiodiplodan mL^{-1}, the effect was similar between the different doses administered, suggesting that a plateau effect was achieved with increased concentration (Fig. 6).

The structures of fungal β-glucans are reported to be important in manifesting various biological activities [24]. Lasiodiplodan is a (1→6)-β-D-glucan [21], and our results suggest that the anti-proliferative activity could be directly related to the polysaccharide structure.

Yang et al. [23] reported that *Antrodia camphorata* (fermented-broth) induced apoptosis in a dose- and time-dependent manner in MCF-7 cells. Their broth extract contained a mixture of polysaccharides, as well as other compounds (ergostan-type triterpenoids, sesquiterpenes, and phenyl and biphenyl derivatives), but the active components were not identified. Thetsrimuang et al. [19] reported that the crude polysaccharides of mature–fresh and dried-fruiting bodies of *Lentinus polychrous* Lév., as well as dried mycelia, displayed cytotoxicity against lung

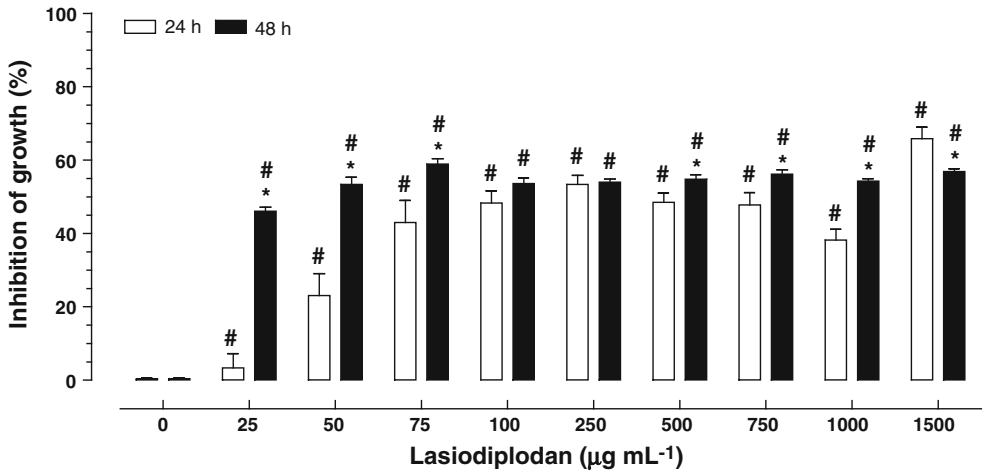

Fig. 6 Anti-proliferative effect of lasiodiplodan in MCF-7 breast cancer cells. Data are expressed as percent of inhibition of growth compared to the control. ($^{\#}p < 0.0001$ vs. control; $^{*}p < 0.05$ vs. 24 h treatment)

(A549) and breast (MCF-7) cancer cells in a dose-dependent manner. This was similar to what was observed in our present study.

Conclusion

This study showed that *L. theobromae* MMPI was able to produce the EPS lasiodiplodan, and a significant increase in viscosity of the cultivation broth appeared during the fermentation process. Glucose and maltose were the C sources that produced the highest yields of lasiodiplodan, but did not produce the highest fungal growth. The organic N sources yeast extract and peptone, although contributing to the highest mycelial growth, resulted in a lower lasiodiplodan-to-biomass ratio compared to urea and ammonium sulfate, which produced less growth. *L. theobromae* MMPI afforded good yields of lasiodiplodan when produced by fermentation in either agitated flasks or an STBR. Maximum lasiodiplodan production was obtained in agitated flasks after 120 h of cultivation. The lasiodiplodan showed anti-proliferative activity in breast cancer (MCF-7) cells.

Acknowledgments The investigators gratefully acknowledge the financial support of CNPq (Brazil), FAPESP (Brazil), NSERC-RCD (Dekker) and Lakehead University (Canada).

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