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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Production of β-glucan exopolysaccharide lasiodiplodan by *Lasiodiplodia theobromae* CCT 3966 from corn bran acid hydrolysate

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Abstract

The potential utilization of corn bran acid hydrolysate (CBAH) was evaluated as an inexpensive feedstock for the production of a rich carbohydrate and protein medium for lasiodiplodan (LAS) production using the filamentous fungus *Lasiodiplodia theobromae* CCT 3966. Experiments were performed according to a 2^2 CCRD experimental design aiming to evaluate the influence of agitation speed (rpm) and temperature (°C) over the production of total cell biomass (TCB) and LAS concentration released to the medium (LAS-M), adhered to biomass (LAS-C), and total (LAS-T). Under the selected conditions (temperature of 28°C and agitation of 200 rpm), 8.73 g·L^{-1} of LAS-T and 4.47 g·L^{-1} of TCB were obtained. Recovery of LAS-C with hot water was shown as an alternative to increase the production concentration, although it might require further purification steps. CBAH potential for substitution of synthetic media was demonstrated, indicating that it is an adequate raw material containing all necessary nutrients for LAS production.

Key points

- *Corn bran acid hydrolysate is presented as a suitable substrate for β-glucan production.*
- *Lasiodiplodia theobromae CCT 3966 have the potential for the industrial β-glucan production.*
- *Simple recovering of biomass-adhered lasiodiplodan by hot water extraction.*

Keywords β-glucan · Biopolymers · Corn bran · Exopolysaccharides · Lasiodiplodan · Starchy hydrolysates

Introduction

Glucans are worldwide commercialized due to their beneficial properties to both animal and human welfare. They are produced by bacteria, fungi, and some lichens, although a few species are used by industry (Freitas et al. 2017; Synytsya and Novák 2013), and are present as a constituent in the cell wall or extracellularly as capsule or slime. Extracellular glucans, so-called exopolysaccharides (EPS), present some benefits over other glucans, such as increased production in short time span and facilitated recovery (Mahapatra and Banerjee 2013). They include alpha or β-glucans, this last with interesting applications for the development of products of human interest.

The utilization of β-glucans covers several industrial sectors, including food and feed (dietary fiber, edible films, emulsifiers, and food thickeners), cosmetics (hair and skincare), medical and pharmaceutical (anticoagulant, antimicrobial, antiviral, antitumor), chemical (nanotechnology, wastewater treatment, and heavy metal adsorption), the agricultural field, and others (Cunha et al. 2017; Gupta and Thakur 2016; Jindal et al. 2012; Kagimura et al. 2015a, b).

Applications for β-glucans may vary according to the chosen microorganism strain, physical conditions, and media components. Mechanistically, EPS are first produced intracellularly and later secreted into the medium by the microorganism, although detailed metabolic pathways for production by fungi remain unrevealed (Mahapatra and Banerjee 2013). For the fermentative production of EPS, important parameters such as medium composition (carbon and nitrogen sources,

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minerals, and specific additives such as oils and vitamins) and cultivation conditions (pH, oxygen level, temperature, and incubation time) have been studied (Mahapatra and Banerjee 2013; Philippini et al. 2019). For fungal EPS production, several carbon sources such as glucose, arabinose, cellobiose, sucrose, or maltose and polyalcohols such as xylitol and mannitol have been studied (Barbosa et al. 2004; Cunha et al. 2012). Regarding nitrogen sources, the most commonly used are organic, such as glutamate, L-asparagine, peptone, urea, and yeast extract, or inorganic, such as ammonium, potassium and sodium nitrates, and ammonium phosphate and sulfate (Chen et al. 2008; Gientka et al. 2016; Hilares et al. 2017; Lee et al. 2015; Rusinova-Videva et al. 2011).

The production of glucans by fungi has been significantly increased in the last two decades, promoting the discovery of new EPS molecular structures and study of strains, such as the filamentous fungus *Lasiodiplodia theobromae* CCT 3966, the producer of the EPS lasiodiplodan (LAS). *L. theobromae* is reported as the anamorphic form of *Botryosphaeria rhodina*, and it is reported in the literature as a producer of a variety of other industrial interest metabolites, such as indole-3-carboxylic acid (anti-inflammatory, phytohormone), jasmonic acid (cosmetic, phytohormone), lasiodiplodin (antitumoral, phytohormone), and mallein (anti-bacterial, antifungal) (Dhandhukia and Thakkar 2007; Huang et al. 2011; Qian et al. 2014; Vasconcelos et al. 2008). Particularly, LAS is a β(1→6)-D-glucan and presents important potential applications due to its anticoagulant, antitumor, antioxidant, antiproliferative, and immunomodulatory properties (Cunha et al. 2012; Kagimura et al. 2015a, b; Oliveira et al. 2015; Vasconcelos et al. 2013). Even though LAS presents important properties and promising industrial potential, its production using agroindustrial byproducts is scarcely reported in the literature with few previous reports describing the use of agricultural biomass sources as a raw material in the elaboration of media for its production (Philippini et al. 2018; Acosta et al. 2020; Abdeshahian et al. 2020a).

Starchy biomass is a group of agroindustrial products which are directly linked to human and animal in the development of staple diets (Martiniano et al. 2020). Among starchy sources, corn (*Zea mays*) is a major crop with worldwide distribution. According to the Food and Agriculture Organization (FAO 2020), more than 1 billion tons of corn were harvested worldwide in 2017. Corn kernels are composed of four major structures: endosperm (83%), germ (11%), pericarp (5%), and tip cap (1%). The endosperm is mainly composed of starch and can be milled in different processes such as stone-grinding (whole grain) or steel-roller (de-germinated) for the corn bran (CB) production. CB is presented in different commercial forms, varying from fine powders to more coarse products. These products present several applications for both human and livestock nutrition. Indeed, 50–70% of corn production is used as livestock feed (cattle and poultry). The remaining corn is applied for the generation of human consumables such as ethanol, sweeteners, and starch (Mohanty and Swain 2019).

As CB is primarily composed of starch, presenting high carbohydrate composition (~77%), it is a good source of proteins (~7%) and lipids (~4%), presenting low fiber content (~7%). CB is also a good source of amino acids and is a good source of minerals, such as potassium, phosphorus, and magnesium, which turns this feedstock an attractive material for the development of rich carbohydrates and protein hydrolysates in biotechnological processes (Gwirtz and Garcia-Casal 2013; Martiniano et al. 2020). The utilization of CB in the elaboration of hydrolysates for carbon and nitrogen supplementation in submerged fermentation for LAS production was not previously reported.

In this context, the present study aimed to evaluate lasiodiplodan production from *L. theobromae* CCT 3966 under submerged fermentation using corn bran acid hydrolysate (CBAH) as a nutrient source. CB starch was hydrolyzed by dilute acid under mild conditions, and the obtained hydrolysate was used to prepare the cultivation medium. Process conditions were evaluated and optimized. The performance of experiments carried out using CBAH was compared with the use of the synthetic medium.

Materials and methods

Microorganism and storage conditions

The filamentous fungus *L. theobromae* CCT 3966 (WDCM 885) was kindly gifted by the Fundação André Tosello Pesquisa e Tecnologia (Campinas, São Paulo, Brazil). The strain was originally isolated from Atlantic forest soil at the Juréia-Itatins Ecological Station (Peruíbe, São Paulo, Brazil). The fungal mycelium was cultivated on yeast malt agar (YMA), containing yeast extract 0.3 (w/vol%), malt extract 0.3 (w/vol%), glucose 1 (w/vol%), and agar 2 (w/vol%), pH: 5.5. Plates were incubated for 120 h at 28 °C and stored at 4 °C.

Dilute acid hydrolysis of corn bran

CB was obtained from Capivariana (Pavan Indústria e Comércio de Produtos Alimentícios LTDA, Capivari, São Paulo, Brazil). The material was stored in plastic bags in the cold room at 4 °C. CB moisture content was determined as 10 %. Dilute acid hydrolysis was performed in 250 mL Erlenmeyer flasks. For this, CB (20 g dry weight) was added to each flask followed by the addition of 100 mL of aqueous H_2SO_4 (1 w/vol%) solution. The material was properly sealed up with aluminum foil and autoclaved for 15 min at 121 °C. After autoclaving, the flasks were cooled down, filtered in qualitative filter paper, and centrifuged at 2500×*g* for 10 min. The resultant CBAH was properly diluted for use in subsequent fermentation steps as a carbon and nitrogen source.

Optimization of lasiodiplodan production

For fermentation of CBAH and glucose synthetic media, 4 discs of agar (0.7 mm of diameter) from fully grown culture containing the mycelium of *L. theobromae* CCT 3966 were inoculated in CBAH media (100 mL, pH 5.5) in 250 mL Erlenmeyer flasks and incubated at 28 °C for 96 h. Samples were taken periodically every 24 h. Product (*P*p) and biomass concentration (*P*x) were used for the calculation of fermentative parameters, such as lasiodiplodan yield ($Y_{P/S}$), biomass yield per sugar unit ($Y_{X/S}$), rate of substrate consumption (Q_S), volumetric productivity of fungal biomass (Q_X), volumetric productivity of lasiodiplodan (Q_P), specific yield (Y_e), and substrate consumption (Y_c).

Lasiodiplodan production was optimized by 2^2 Central Composite Rotational Design (CCRD), evaluating two process variables, i.e., shaker agitation speed (X_1) and temperature (X_2). These variables were chosen considering their influence on mycelial growth and oxygen availability for the microorganism during cultivation (Cunha et al. 2012; Khanzada et al. 2006). The studied ranges were 0 to 282.8 rpm for agitation and 18 to 35 °C for temperature. Design-Expert 8.0 (Stat-Ease, Inc., USA) and Statistica 8.0 (StatSoft Inc. 2004) software were used to compose and analyze the empirical models and describe response variables as a function of agitation and temperature. The response variables were total cell biomass (TCB) and LAS concentration, which last was divided into two groups: LAS-M is the extracellular lasiodiplodan freely released in fermentative broth, while LAS-C is the lasiodiplodan that is adhered to cell biomass. The sum of the concentrations of LAS-M and LAS-C was named LAS-T.

Process optimization was performed aided by the numerical analysis tool of the Design-Expert 8.0 software, based on function desirability. The model prediction was confirmed by experimental runs carried out in triplicate. The intention was to achieve maximum lasiodiplodan production utilizing the CBAH as an inexpensive fermentation media in substitution of synthetic media. In this way, to compare results obtained with CBAH-based medium, an experiment with synthetic media containing 40 $g{\cdot}L^{-1}$ of glucose and 20 $mL{\cdot}L^{-1}$ of Vogel Minimum Salts Medium (VMSM) (Vogel 1956) was also performed. The selected operational parameters for synthetic media were the same as for CBAH for LAS and TCB production.

Product recovery and analytical methods

Lasiodiplodan recovery

For LAS-M recovery, the fermented broth was centrifuged at 2500×*g* for 10 min to separate biomass (which was used for LAS-C recovery, as following described). The supernatant was precipitated with 3 volumes of absolute ethanol and stored frozen at −20 °C for 24 h. The precipitate was recovered, dissolved in deionized water (60 °C), and dialyzed at room temperature (24 °C) for 48 h to remove small molecules and residual sugars. The obtained material was precipitated one more time with 3 volumes of absolute ethanol for subsequent centrifugation and drying (60 °C) until constant weight.

LAS-C was obtained from cell biomass, which was suspended in deionized water to original media volume, mixed in the vortex at 2500 rpm. The cell suspension was then heated at 60 °C for 1 h, and the cell slurry was centrifuged at 2500×*g* for 10 min to obtain TCB. For LAS-C recovery, the supernatant was then submitted to the same process above described to obtain LAS-M.

Sugars, potential inhibitors, and protein content

Total sugars (TS), total reducing sugars (TRS), and total protein (TP) in the hydrolysate and fermentation medium supernatant (after biomass removal) were evaluated using spectrophotometric methods, as described by Dubois et al. (1956), Miller (1959), and Lowry et al. (1951), respectively. Glucose, xylose, arabinose, acetic acid, and glycerol were analyzed by high-performance liquid chromatography (HPLC) Agilent 1200 series (Agilent Technologies Inc., USA) equipped with a refractive index detector RID-6A and an HPX-87H (300 × 7.8 mm) column (Bio-Rad, USA). The following conditions were used: 45 °C column temperature, 0.01 N H_2SO_4 as the mobile phase, 0.6 mL min^{-1} flow rate, and 20 μL injection volume. Maltose was analyzed using an Aminex HPX-87P (300 × 7.8 mm), at 60 °C, utilizing distilled water as eluent at a flow rate of 0.4 mL min^{-1}. All samples were previously diluted and filtered in Sep-Pak C18 cartridges (Millipore). For analysis of furfural and hydroxymethylfurfural (HMF) content, the same HPLC equipment was used but equipped with RP 18 column (200 × 4.6 mm) at 25 °C; injection volume 20 μL, UV detector SPD-10a UV-VIS (276 nm); water/acetonitrile 1:8 with 1 % acetic acid; and flow rate of 0.8 mL min^{-1}.

Lasiodiplodan characterization

Fourier transform infrared spectroscopy and XRD analysis

Fourier transform infrared spectroscopy (FT-IR) of lasiodiplodan samples was measured by Perkin Elmer® Spectrum™ GX (Shelton, USA). Samples were mixed with KBR (1.5 mg of LAS and 250 mg of KBR), dried at 50 °C, and then pressed in pellets. FT-IR spectrum was recorded in the region of 4000–600 CB^{-1}. X-ray diffractogram was obtained by PANalytical (Malvern Panalytical, UK), with copper tubes. Samples were pressed over a lamellar container with 20-mm diameter and scanned over a diffraction angle (2θ) of 10–80 ° and 0.02 step by Cu K radiation (k = 1.54 Å) with 20-s counting.

Lasiodiplodan purity

The purity of lasiodiplodan was determined by a hydrolysis step using 20 mg of dried lasiodiplodan added to 1 mL of 72 % H_2SO_4 followed by incubation in the thermostatic bath at 30 °C for 1 h. After that, the volume was completed to 10 mL of distilled water and the mixture autoclaved at 121 °C for 1 h. The hydrolysis procedure was also performed using a pure glucose solution for the measurement of sugar degradation products. Samples were neutralized to pH 5–6 using calcium carbonate and centrifuged at 2500×*g*.

Results

Effects of agitation and temperature on lasiodiplodan production using CBAH

The CBAH composition was characterized for TS, RS, and TP, besides some specific sugars (glucose, xylose, maltose, and arabinose) and potential inhibitors (acetic acid, furfural, glycerol, and HMF). Results are presented in Table 1. As shown, RS concentration (70 g·L^{-1}) corresponded to about 40 % of TS (170 g·L^{-1}) in the hydrolysate. Among the present RS, glucose was presented in higher concentration. Besides about 15 g/L of TP was obtained in the hydrolysate, without a significant quantity of known fermentation inhibitors as acetic acid, furfural, and HMF.

The lasiodiplodan production using CBAH as raw material was influenced by agitation and temperature, as shown in Table 2. All fermentation graphics corresponding to the CCRD 2^2 experiments are presented in the supplementary material (Figure S1). As can be observed in Table 2, LAS-M production varied from zero (runs 1, 5, and 7) to 6.92 g·L^{-1} (run 9), at 96 h of fermentation, and higher concentrations were obtained at conditions of higher agitation. Similar behavior about the influence of agitation speed was observed for LAS-C and LAS-T production. Besides, the recovery of LAS-C in runs with higher biomass production using the hot water process for the extraction of glucans from cells improved the total lasiodiplodan recovery up to 1.5-fold. As also shown in Table 2, during the fermentation, pH changed only slightly from its initial value, 5.5, with the final value varying from 5.17 to 5.69. TS and RS consumptions varied from 17 to 86 % and from 23 to 81 %, respectively.

Table 1 Sugar, protein, and inhibitor content in corn bran acid hydrolysate (CBAH)

Component	Concentration (g/L)
Total sugars	171 ± 3.88
Reducing sugars	70.3 ± 3.25
Maltose	1.19 ± 0.18
Glucose	43.6 ± 0.61
Xylose	3.69 ± 0.25
Arabinose	3.35 ± 0.38
Total proteins	14.9 ± 1.89
Acetic acid	0.33 ± 0.05
5-HMF	n.p.
Furfural	n.p.
Glycerol	0.67 ± 0.13

n.p. not produced

Empiric quadratic models were composed for the answer variables LAS-M, LAS-C, LAS-T, and TCB (Eq. 1, Eq. 2, Eq. 3, and Eq. 4). Models were reduced by excluding non-significant terms (95 % confidence level), except when they were necessary for model hierarchy (Table 3). As shown, all models were significant ($p<0.05$) and without a significant lack of fit ($p>0.05$). For TCB, the R^2 value was 0.85, and, for the other answer variables, it was ≥0.95. Thus, models were considered to explain the experimental variability of the results in the evaluated range. Correspondent contour plots and response answers are shown in Fig. 1.

$$LAS{-}M\ \left(\mathrm{g}.L^{-1}\right) = -53.3 + 0.0624\,x_1 + 3.87x_2{-}1.52 \times 10^{-4}\,x_1^2{-}0.0692\,x_2^2 \quad (1)$$

where x_1 and x_2 correspond to the actual values of agitation speed (rpm) and temperature (° C), respectively.

$$LAS{-}C\ \left(\mathrm{g}.L^{-1}\right) = -14.6 + 8.44 \times 10^{-4}x_1 + 1.25\,x_2 + 1.13 \times 10^{-3}\,x_1\,x_2{-}7.29 \times 10^{-5}\,x_1^2{-}0.0254\,x_2^2 \quad (2)$$

where x_1 and x_2 correspond to the actual values of agitation speed (rpm) and temperature (° C), respectively.

$$LAS{-}T\left(\mathrm{g}.L^{-1}\right) = -63.7 + 0.0338\,x_1 + 4.96\,x_2 + 2.24^{-3}x_1\,x_2{-}2.25^{-4}x_1^2{-}0.0947\,x_2^2 \quad (3)$$

where x_1 and x_2 correspond to the actual values of agitation speed (rpm) and temperature (° C), respectively.

$$TCB\ \left(\mathrm{g}.L^{-1}\right) = -37.1 + 0.0197\,x_1 + 3.06\,x_2{-}0.0571\,x_2^2 \quad (4)$$

where x_1 and x_2 correspond to the actual values of agitation speed (rpm) and temperature (°C), respectively.

Figure 2 shows that intermediary to higher levels of agitation speed resulted in high values of LAS and TCB production. Regarding the temperature effect on the process, lower levels were not favorable for TCB or LAS production (Fig. 1).

Table 2 Matrix of the 2^2 central composite rotational design used for the evaluation of the influence of shaker agitation speed and temperature in the results of the fermentation of corn bran acid hydrolysate (CBAH) by *L. theobromae* cultured in Erlenmeyer flasks

Run	Experimental variables*		Lasiodiplodan (LAS-M), $g \cdot L^{-1}$	Lasiodiplodan (LAS-C), $g \cdot L^{-1}$	Total lasiodiplodan (LAS-T), $g \cdot L^{-1}$	Total cell biomass (TCB), $g \cdot L^{-1}$	Total sugar (TS) consumption**, %	Reducing sugar (RS) consumption**, %	Total protein (TP) consumption**, %	Final pH
	Agitation speed, rpm	Temperature, °C								
1	41.4 (−1)	20.5 (−1)	0	1.32	1.32	1.33	17	23	56	5.41
2	241.4 (+1)	20.5 (−1)	1.87	1.51	3.38	5.07	49	47	20	5.69
3	41.4 (−1)	32.5 (+1)	1.63	0.61	2.24	2.47	39	32	48	5.15
4	241.4 (+1)	32.5 (+1)	6.17	3.51	9.68	5.88	85	81	26	5.17
5	0 (−1.41)	26.5 (0)	0	0.35	0.35	3.58	43	33	30	5.27
6	282.8 (+1.41)	26.5 (0)	6.39	3.87	10.26	9.62	86	79	78	5.44
7	141.4 (0)	18 (−1.41)	0	1.20	1.20	3.69	39	52	45	5.45
8	141.4 (0)	35 (+1.41)	2.47	2.26	4.73	3.66	66	71	44	5.31
9	141.4 (0)	26.5 (0)	6.92	3.41	10.33	6.97	77	74	50	5.42
10	141.4 (0)	26.5 (0)	5.98	3.81	9.79	8.42	86	76	43	5.29
11	141.4 (0)	26.5 (0)	6.12	3.21	9.33	7.34	81	72	43	5.31

*Coded levels in parenthesis

**Calculated with basis on initial content in the medium

Table 3 ANOVA for a second-order model composed for LAS-M, LAS-C, LAS-T, and TCB after 96 h of fermentation of corn bran acid hydrolysate (CBAH) varying agitation and temperature

Source	LAS-M					LAS-C					LAS-T					TCB				
	SQ	Df	Ms	*F*-value	*p*-Value	SQ	Df	Ms	F-value	p-Value	SQ	Df	Ms	F-value	p-Value	SQ	Df	Ms	F-value	p-Value
Model	80.07	4	20.02	30.22	0.0004*	17.01	5	3.40	24.17	0.0016*	170.61	5	34.12	46.56	0.0003*	57.55	3	19.18	13.21	0.0029*
Agitation (X_1)	29.80	1	29.80	44.99	0.0005*	8.14	1	8.14	57.80	0.0006*	69.09	1	69.09	94.28	0.0002*	30.81	1	30.81	21.22	0.0025*
Temperature (X_2)	11.09	1	11.09	16.75	0.0064*	0.97	1	0.97	6.91	0.0466*	18.63	1	18.63	25.43	0.0040*	0.45	1	0.45	0.31	0.5957
X_1X_2	**					1.83	1	1.83	13.03	0.0154*	7.22	1	7.22	9.86	0.0257*	**				
X_1^2	13.08	1	13.08	19.75	0.0044*	3.00	1	3.00	21.31	0.0058*	28.62	1	28.62	39.05	0.0015*	**				
X_2^2	35.35	1	35.35	53.37	0.0003*	17.01	5	3.40	24.17	0.0016*	66.08	1	66.08	90.18	0.0002*	26.29	1	26.29	18.10	0.0038*
Residual	3.97	6	0.66			0.70	5	0.14			3.66	5	0.73			10.17	7	1.45		
Lack of fit	3.47	4	0.87	3.41	0.2396	0.52	3	0.17	1.85	0.3701	3.17	3	1.06	4.29	0.1947	9.03	5	1.81	3.18	0.2566
Pure Error	0.51	2	0.25			0.19	2	0.093			0.49	2	0.25			1.14	2	0.57		
Total	84.04	10				17.72	10				174.27	10				67.72	10			
R^2 adjusted	0.95					0.96					0.98					0.85				

SQ sum of squares, *Df* degrees of freedom, *Ms* mean square

*Significant at confidence level of 95%

**Coefficient excluded from the model due to low significance level

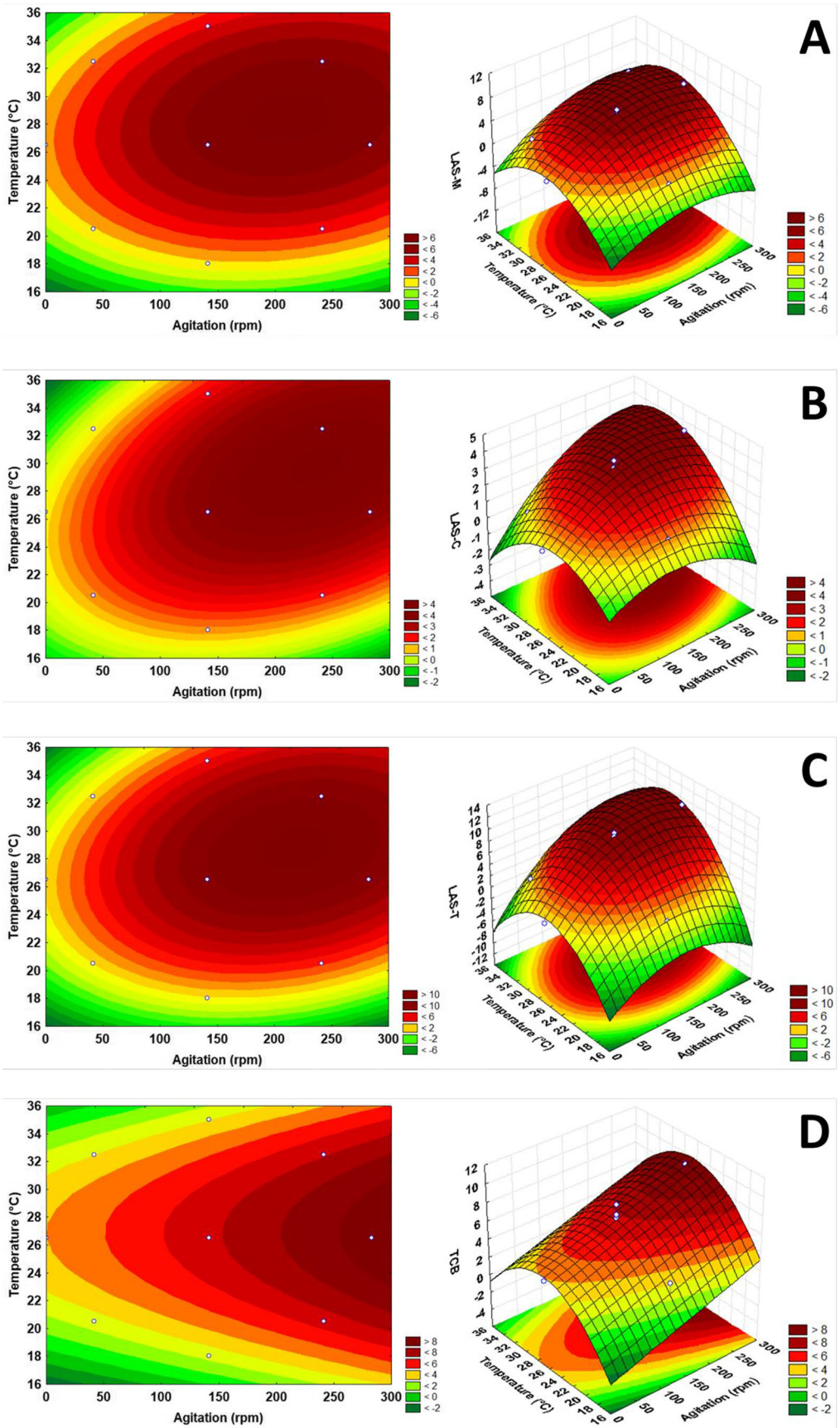

Fig. 1 Contour plots and response surfaces for LAS-M (**a**), LAS-C (**b**), LAS-T (**c**), and TCB (**d**) produced from corn bran acid hydrolysate (CBAH)

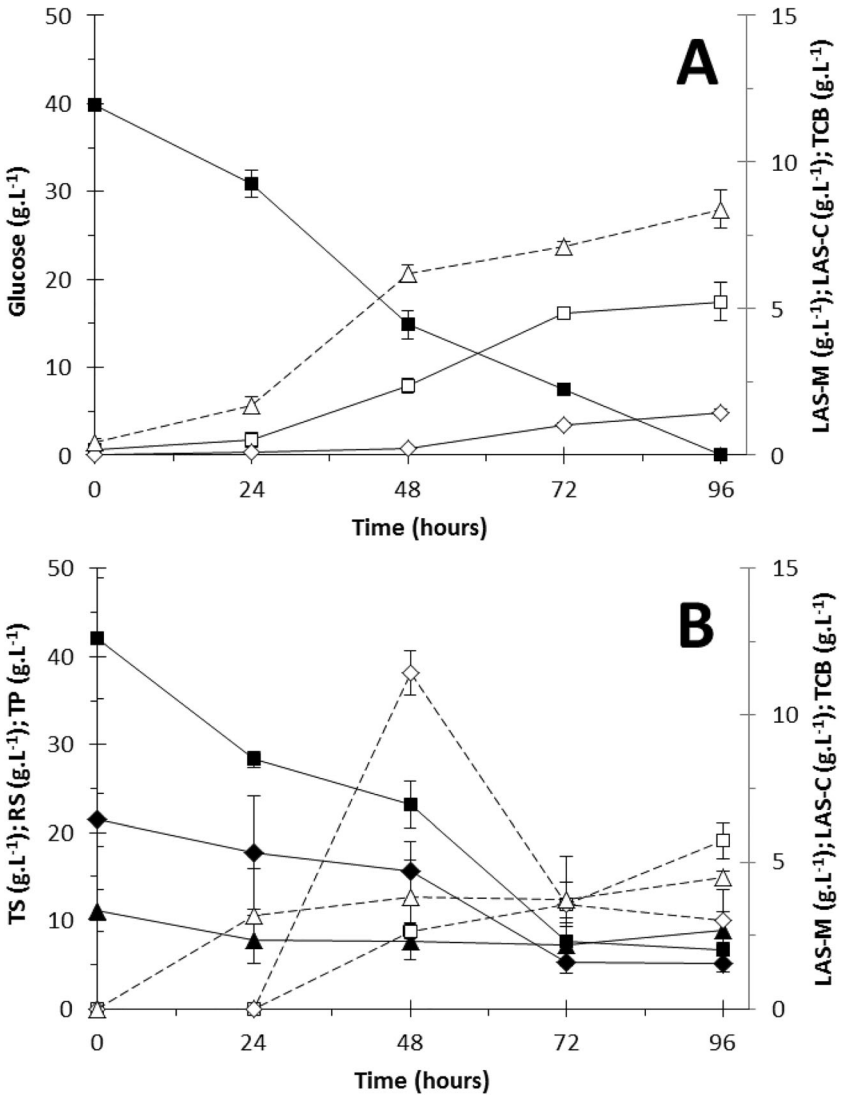

Fig. 2 Comparative production of lasiodiplodan (LAS-M and LAS-C), total cell biomass (TCB), and the nutritional consumption for glucose synthetic media (**a**) and corn bran acid hydrolysate (CBAH) (**b**). Filled square: TS, total sugars/ glucose; filled diamond: RS, reducing sugars; filled triangle: TP, total proteins; unfilled square: LAS-M; unfilled diamond: LAS-C; unfilled triangle: TCB

Fermentation under optimal conditions

Assessing the surface response profiles (Fig. 1), 200 rpm of agitation speed and 28 °C were selected as the best conditions for β-glucan production. Under the optimal conditions, values predicted by the models (average ± 95 % confidence interval) were 7.18 $g{\cdot}L^{-1}$ ± 0.90 $g{\cdot}L^{-1}$ for LAS-M, 3.95 $g{\cdot}L^{-1}$ ± 0.51$g{\cdot}L^{-1}$ for LAS-C, 11.1 $g{\cdot}L^{-1}$ ± 1.16 $g{\cdot}L^{-1}$ for LAS-T, and 7.86 $g{\cdot}L^{-1}$ ± 1.32 $g{\cdot}L^{-1}$ for TCB. The values obtained in an experiment carried out in triplicate under these conditions were (average ± standard deviation) 5.73 $g{\cdot}L^{-1}$ ± 0.61 $g{\cdot}L^{-1}$ for LAS-M, 3.00 $g{\cdot}L^{-1}$ ± 1.04 $g{\cdot}L^{-1}$ for LAS-C, 8.73 $g{\cdot}L^{-1}$ ± 1.65 $g{\cdot}L^{-1}$ for LAS-T, and 4.47 $g{\cdot}L^{-1}$ ± 0.22 $g{\cdot}L^{-1}$ for TCB. As observed, considering the range of one standard deviation for experimental values, the obtained results were in the 95 % confidence interval calculated by using the models for LAS-M, LAS-C, and LAS-T.

The kinetic profile of fermentation performed under optimal conditions was compared with results obtained in a glucose-based synthetic medium (Fig. 2). As can be observed in Fig. 2a, glucose was completely consumed in 96 h of process, and, in this fermentation, about 5.25 $g{\cdot}L^{-1}$ of LAS-M and 1.55 $g{\cdot}L^{-1}$ of LAS-C were produced, with a TCB of 8.40 $g{\cdot}L^{-1}$. Regarding the fermentation in CBAH-based medium, comparatively faster biomass growth was observed in the first 24 h (Fig. 2b). As shown in Fig. 2b, the TS, RS, and TP consumption achieved 84 %, 74 %, and 19 %, respectively, after 96 h.

LAS-M production started after 24 h of fermentation (Fig. 2b). In 48 h, a maximum was observed for LAS-C production that reached 11.5 g/L, decreasing to 3.53 g/L in the correspondent sample at 72 h of process. At 96 h of fermentation, LAS-M concentration corresponded to 5.73 g/L. At this time, aiming to increase the lasiodiplodan production, recovering

of LAS-C would correspond to an increase of 52 % on the total produced lasiodiplodan. The fermentative parameters of glucose synthetic media and CBAH were properly presented in Table 4 and were evaluated according to Tukey's range test.

Product purity, FTIR, and XRD analysis

Figure 3 depicts LAS-M recovery on ethanol (a), after dialysis (b), and drying (c). The results for the lasiodiplodan purity are presented in Table 5. The recovered LAS-M and LAS-C showed high reducing sugar content (88 % and 76 % w·w^{-1}, respectively) and low protein (7.73 and 11.7 % w·w^{-1}, respectively).

No extra proteins were detected in LAS obtained in fermentation carried out in synthetic glucose medium.

Lasiodiplodan obtained from the synthetic medium and CBAH were submitted to the analysis of FTIR spectroscopy. Figure 4 shows typical peaks and bands of polysaccharides in FTIR spectra and the structural similarity of these compounds. The X-ray diffraction analysis showed that the two samples of lasiodiplodan produced by *L. theobromae* CCT 3966 in culture media containing different carbon sources presented some similarities, as shown in Fig. 5.

Discussion

The composition of CBAH presented a high concentration of RS, and glucose was the main component. This monomer was obtained from the starch after the use of mild acid hydrolysis conditions. Besides the analyzed reducing sugars (maltose, glucose, xylose, and arabinose), maltotriose and other oligosaccharides might be responsible for the remaining RS presented in CBAH, once they are easily obtainable from starch hydrolysis. Considering the sugar concentration used in previous reports about LAS production (Cunha et al. 2012), the native hydrolysate was diluted to have TS, RS, and TP approximately of 40 g·L^{-1}, 20 g·L^{-1}, and 2.5 g·L^{-1} respectively, to compose the fermentation medium.

The lasiodiplodan production using CBAH as substrate was influenced by the evaluated parameters (agitation and temperature). Higher LAS-M concentrations were generally observed in runs with higher biomass, indicating that the extracellular β-glucan generation is related to cell production. Thus, the extraction of fungal glucans by hot water solution is well known and commonly used for polysaccharides recovery from mushrooms in the development of nutraceutical products (Camelini et al. 2005; Synytsya and Novák 2013; Veverka et al. 2014).

Intermediary to higher levels of temperature and agitation speed favored higher consumption of sugars and consequently the cell biomass production. In most of the runs of the experimental design, TS consumption was higher than RS consumption (both calculated based on initial TS and RS content in the medium), indicating that the fungus has performed additional carbohydrate hydrolysis during the process. Regarding TP, the consumption varied from 20 to 78 %, demonstrating that the protein concentration presented in the hydrolysate was well utilized by this strain for growing and LAS production. There are in literature examples of the use of other starchy biomasses such as rice bran as a cheap source of nitrogen and other nutrients for the production of industrial interest biomolecules, such as second-generation ethanol (Martiniano et al. 2014; Milessi et al. 2013).

Intermediary to higher levels of agitation speed led to higher LAS and TCB production. This influence is related to the biomass and exopolysaccharide production dependence on aeration, considering that the lack of oxygen is not favorable for EPS production (Mahapatra and Banerjee 2013). Thus, an adequate amount of agitation can provide the

Table 4 Fermentative parameters for LAS-M production on glucose synthetic media and corn bran acid hydrolysate (CBAH)

Parameter	Glucose synthetic media	Corn bran hydrolysate (CBAH)
Pp (g.L^{-1})	5.25 (± 0.16)^{a}	5.73 (± 0.61)^{a}
Px (g.L^{-1})	8.40 (± 0.66)^{b}	4.25 (± 0.22)^{c}
$Y_{P/S}$ (g.g^{-1})	0.13 (± <0.01)^{d}	0.14 (± 0.04)^{d}
$Y_{X/S}$ (g.g^{-1})	0.21 (± 0.02)^{e}	0.10 (± 0.02)^{f}
Q_S (g.L^{-1}.h^{-1})	0.41 (± <0.01)^{g}	0.44 (± 0.08)^{g}
Q_X (g.L^{-1}.h^{-1})	0.09 (± 0.01)^{h}	0.04 (± <0.01)^{i}
Q_P (g.L^{-1}.h^{-1})	0.05 (± <0.01)^{j}	0.06 (± 0.01)^{j}
Ye (g.g^{-1})	0.63 (± 0.07)^{k}	1.36 (± 0.02)^{k}
Yc (%)	99.8 (± 0.18)^{i}	84.8 (± 4.35)^{j}

Results were shown as average of triplicates ± standard deviation. Same letter in lines indicates no difference according to Tukey's range test (95% of confidence level)

Yp/s LAS-M yield in substrate consumption, Yx/s cell biomass yield in substrate consumption, Qs volumetric consumption of sugars, Qx volumetric productivity of biomass, Qp LAS-M volumetric productivity, Ye specific yield, Yc specific yield of LAS-M on biomass production

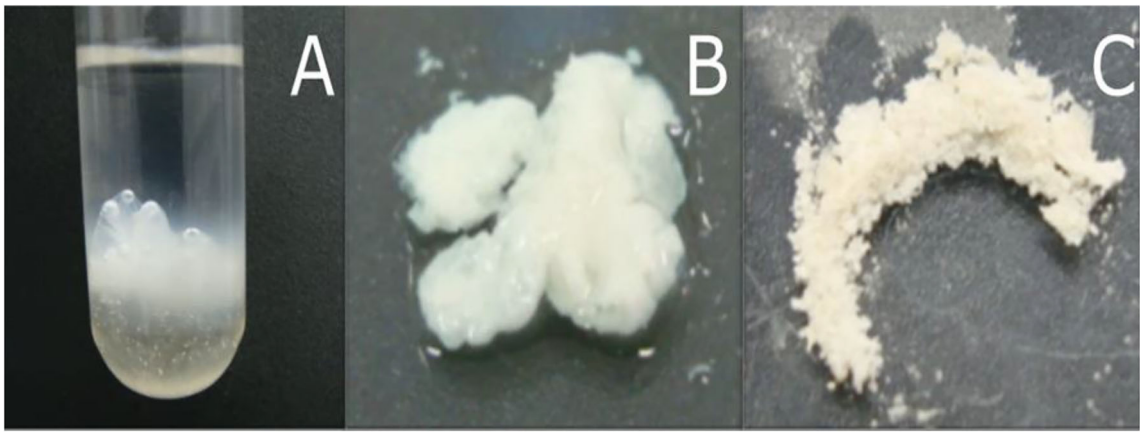

Fig. 3 Lasiodiplodan (LAS-M) recovery from corn bran acid hydrolysate (CBAH). **a** Ethanol precipitated LAS-M, **b** LAS-M dialyzed and re-precipitated on ethanol, and **c** dried at 60°C and milled LAS-M

required amount of oxygen during the growth of microorganisms on the medium. Also, during the fermentation, the increase in cell biomass and the exopolysaccharide production may interfere in the oxygen transfer, as both increases the viscosity of the medium (Cunha et al. 2012). TCB was higher in temperatures ranging from 25 to 30 °C, which corresponds to the value utilized in previous works (Cunha et al. 2012; Oliveira et al. 2015; Vasconcelos et al. 2013). Increased *L. theobromae* mycelial growth was reported by Khanzada et al. (2006), where the temperature ranges varying from 30 to 35 °C promoting pycnidia production. LAS production was favored in slightly higher temperatures, mainly considering LAS-C. Exopolysaccharide producing strains present optimal temperature from 22 to 30 °C, and only a few microorganisms are favored by low temperatures for biopolymer production (Kim and Yun 2005).

According to surface response profiles, the results of experimental values of LAS-M, LAS-C, and LAS-T were according to the predicted models, confirming their prediction capacity for these answers. However, the experimental TCB value was lower than the predicted by the model, indicating that the composed model was not able to explain the variability of this answer in the selected condition.

The kinetic profile of fermentation demonstrated that the TS consumption was high under optimal conditions, indicating that the *L. theobromae* CCT 3966 is a producer of enzymes that cleaved the oligosaccharides presented in the hydrolysate for fermentable sugar generation. *Lasiodiplodia* species are good enzyme producers and able to synthesize amylases, caseinases, cellulases, gelatinases, laccases, lipases, pectinases, and xylanases, demonstrating the capacity of adaptation of this microorganism to different environments and indicating its applications to produce enzymes of interest to detergent, food, starch, and biomedical industries (Félix et al. 2018). The maximum LAS-C occurred at 48 h and decreased after that. A hypothesis to explain the peak of LAS-C obtained at 48 h is an initial production of lasiodiplodan adhered to the cell wall that can be metabolized by the fungus or be liberated to the medium in later fermentation times when other carbon sources are depleting. The recovery of LAS-C after 96-h fermentation increased the total produced lasiodiplodan, demonstrating that the advantage of recovering the biopolymer adhered to the biomass, thus increasing overall fermentation yields. This increase could be higher in 48 h of fermentation when LAS-C was higher; however, its recovery could add costs to the process, requiring extra steps in the downstream

Table 5 Reducing sugars and total protein released after hydrolysis of lasiodiplodan produced on glucose and corn bran acid hydrolysate (CBAH)

Sample	Reducing sugar content per gram of lasiodiplodan (%)*	Total protein content per gram of lasiodiplodan (%)
D-Glucose standard	n.p	n.p
LAS-M (glucose)	89.9	n.p
LAS-M (CBAH)	87.6	7.73
LAS-C (CBAH)	76.1	11.7

LAS-M-glucose lasiodiplodan produced on glucose and released in the medium, *LAS-M* lasiodiplodan recovered from fermentation media, *LAS-C* lasiodiplodan recovered from cellular biomass, *n.p* not performed

*Calculated taking into account the sugar degradation of 2% (considering D-glucose standard results) and that 1 mg of pure lasiodiplodan releases 1.11 mg of reducing sugars

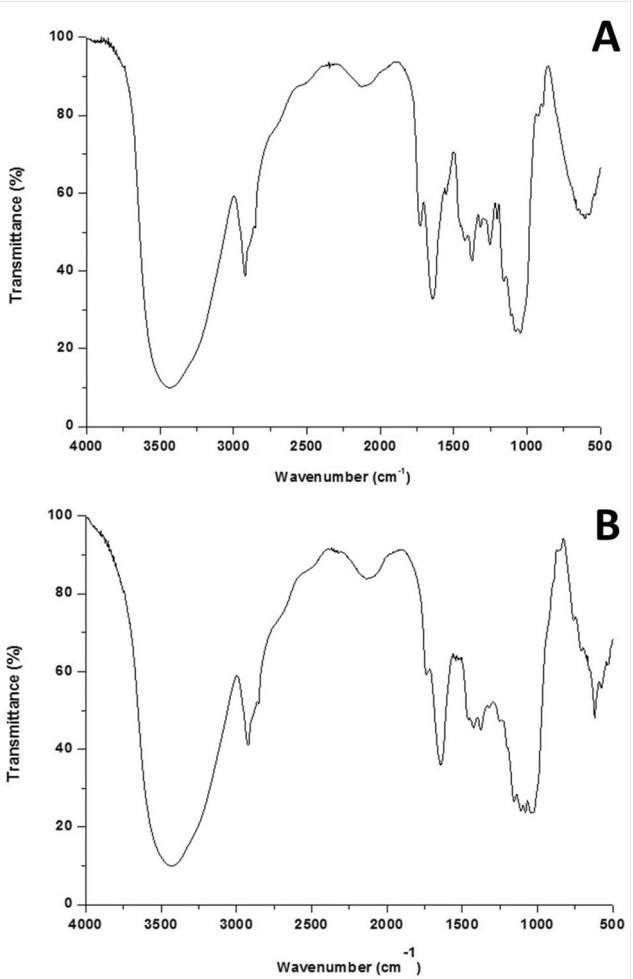

Fig. 4 FT-IR spectra of LAS-M produced on glucose synthetic media (**a**) and corn bran acid hydrolysate (CBAH) samples

process. Besides, as discussed in the following section, LAS-C has lower purity when compared to LAS-M.

Our findings were similar to other previous studies. Lasiodiplodan concentrations varied from 2.2 to 7.01 $g{\cdot}L^{-1}$, using sugar concentrations which varied from 20 to 50 $g{\cdot}L^{-1}$ and supplemented with Vogel Minimum Salts Media (VMSM) (Cunha et al. 2012; Kagimura et al. 2015a, b; Oliveira et al. 2015), producing the exopolysaccharide both in Erlenmeyer flasks and stirred tank reactors with fermentation times varying from 72 to 120 h.

Reports on lasiodiplodan production from agroindustrial products are scarce in the literature, turning difficult to compare our study with the existing reports. In previous results, Philippini et al. (2018) obtained 7.47 g/L of LAS from *L. theobromae* CCT 3966 using CBAH as both carbohydrate and protein medium, while Abdeshahian et al. (2020a) produced 1.03 g/L of LAS, using sugarcane bagasse cellulosic hydrolysate. A different strain, *L. theobromae* MMPI, was investigated by Acosta et al. (2020), which produced 1.06 g/L of biopolymer using soybean molasses. Here, the CBAH has been proven as a potential medium for the fermentation of glucans and can be used as an inexpensive source of both carbohydrates and proteins. Thus, the obtaining of lasiodiplodan and other exopolysaccharides using agroindustrial hydrolysates proposes new production, recovery, and purification strategies against the conventional methods (Philippini et al. 2019). The utilization of other agroindustrial residues can also be employed as viable feedstocks for the cost-effective production of glucans, as these biomolecules can be incorporated in biorefineries, due to their great application potential in several industrial sectors (Abdeshahian et al. 2020b).

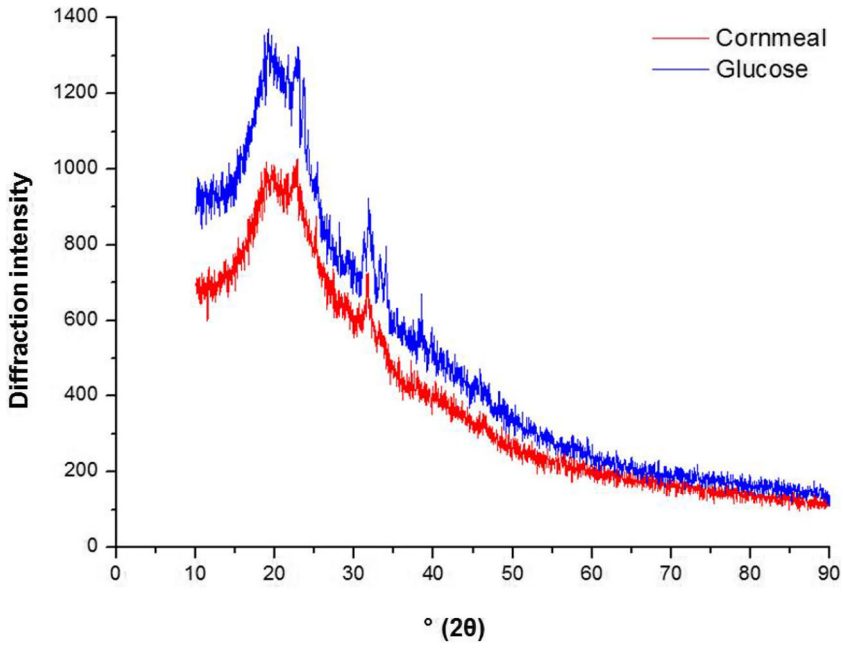

Fig. 5 Standard X-ray diffraction profiles of LAS-M produced on glucose synthetic media (blue) and corn bran acid hydrolysate (CBAH) (red)

The utilization of β-glucans for feed or food applications may require high-purity β-glucan. Purified β-glucans are much better absorbed by the digestive tract and show improved activity when compared with food-grade products containing only 65 % of glucans (Kwiatkowski and Kwiatkowski 2012). The slightly higher protein content in LAS-C, when compared to LAS-M, can be due to the removal of fractions of mannoproteins from the cell wall during the extraction process. Besides, LAS-C can include other components that were adhered to the cell wall, explaining its reduced purity.

The nitrogen supplementation in synthetic glucose medium was from VMSM addition, and no extra proteins were detected in the LAS obtained after the fermentation. CBAH may have extra macro- and micromolecules, which can get attached to the biopolymer and cause lower purity in β-glucan. Further purification and dialysis steps might be incorporated into the purification step, although it may increase the production costs.

In FTIR spectra was observed typical peaks and bands of polysaccharides. The absorption band in the region between approximately 3000 and 3600 CB^{-1} showed vibration of hydroxyl groups (R-OH) stretching. Around 2900 and 1400 CB^{-1} were observed peaks correspondent to axial deformations R-C-H linkage typical of alkyl groups such as methyls ($R-CH_3$) and methylenes ($R-CH_2-R$). The presence of a peak near 1700 CB^{-1} of carbonyl (R-C=O) corresponds to a group of the carboxylic acids present in some organic acid residues, such as uronic and pyruvic, which are commonly reported to microbial EPS structures. Peaks and bands between 1000 and 1200 CB^{-1} showed axial deformations on C-C, C-H, and R-C-O-C-R (ether) linkages. Besides, the fingerprint regions of the analyzed samples were observed between 765 and 910 CB^{-1} interval, showing the presence of bands of glycosidic linkages with β-type conformation as commonly reported in some microbial glucans (Sandula et al. 1999). The chemical structure of LAS β-glucan obtained in the present work is similar to the glucans found in *Botryosphaeria* sp. (Steluti et al. 2004; Vasconcelos et al. 2008). This fact is of fundamental importance since the fungus *L. theobromae* is an anamorphic form of *Botryosphaeria* sp., presenting some differentiation in the reproduction structural form.

The X-ray diffraction analysis showed the angular diffraction region between approximately 15 and 30 ° (2θ) in the EPS produced in CBAH, and synthetic glucose showed an intense and broad peak in the form of shoulder, and from 30 ° (2θ), with no further peak reported. The broad peaks occurred due to the polymer structure of the exopolysaccharides. These results are similar to those reported by Verveka and collaborators (Veverka et al. 2014), indicating that biopolymers presented crystalline regions although the majority described in the literature is amorphous. Also, Kagimura et al. (2015a, b) presented diffraction profiles in lasiodiplodan produced by *L. theobromae* MMPI in a synthetic medium supplemented with glucose identical to those depicted in this work.

To conclude, CBAH was proved as an adequate nutrient source for lasiodiplodan production by *L. theobromae* CCT 3966 without the necessity of medium supplementation. Determining optimal conditions of β-glucan production corresponded to 200 rpm of agitation speed and temperature of 28 °C. It was also possible to increase the recovery of lasiodiplodan adhered to cell biomass by extraction with hot water, although it might require further purification steps. CBAH can be an excellent substitution of synthetic media, promoting a considerable production yield, which may favor the feasibility of the commercial use of lasiodiplodan.

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Authors' contribution RRP conceived the project, conducted the experiments, analyzed the data, and wrote the manuscript. SEM conducted the experiments and wrote the manuscript. PRFM analyzed the data. AKC reviewed and edited the manuscript. JCS designed the research, analyzed the data, and edited the manuscript. SSS conceived the project, edited the manuscript, and supervised. All authors read and approved the manuscript.

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Data Availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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