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Article

Bioprospecting the Endophytic Fungus, *Lasiodiplodia theobromae* MMPI, for the Integrated Production of Mycoprotein and Exocellular (1→6)-β-Glucan

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Abstract: In response to the growing demand for alternative protein sources and functional biomolecules for industrial applications, this study investigated the production of mycoprotein and extracellular (1→6)-β-D-glucan (lasiodiplodan) by the fungus *Lasiodiplodia theobromae* MMPI, establishing an integrated biotechnological platform. Soybean molasses was evaluated as a low-cost fermentation substrate and was compared to a sucrose-based medium. Experimental design and response surface methodology defined conditions that maximized mycelial biomass and lasiodiplodan production. Mycelial biomass from soybean molasses was rich in essential amino acids, lipids, and polyunsaturated fatty acids like gamma-linolenic and alpha-linolenic acids, while sucrose favored higher lasiodiplodan production. Antioxidant compounds like gallic acid and catechin were also found in the biomass, showing potential for scavenging free radicals. Soybean molasses promoted lipid-rich biomass, suggesting *L. theobromae* MMPI's potential for biofuel production. The study highlights the fungus's utility in producing mycoproteins, lipids, and lasiodiplodan for food, animal feed, and industrial uses.

Keywords: exopolysaccharide; lasiodiplodan; mycoprotein; Oleaginous fungus; soybean molasses

1. Introduction

There is an increasing demand worldwide for foods due to the continuing growth of the human population globally. The need for feed production to support livestock and the animal pet market and environmental issues, such as the depletion of natural resources, create the need for research to find new alternative food and protein sources [1,2]. The United Nations has estimated that the global population will reach 9.8 billion by 2050 and 11.2 billion in 2100 [3]. The estimated increase in the world human population will require a greater food supply, including protein sources, and chemical and pharmaceutical inputs [4].

The global production of meat, particularly poultry, and pork, has increased in recent decades due to the territorial expansion of production and the increase in productivity. The United States Department of Agriculture (USDA) predicts that Brazil, the third-largest cattle producer in the world,

behind China and India, will remain the world's largest exporter of beef in 2024 [5]. Similarly, Brazil is the world's second-largest producer of chicken meat, after the United States, and the largest exporter of chicken meat globally [6]. Regarding pork production, Brazil is the fourth-largest producer worldwide, with a production of 4.45 million metric tons (2023/2024), amounting to 4% of the global market, according to the US Foreign Agricultural Service [7].

In many countries, expanding land for food production is a limiting factor for increasing agricultural output, with such expansion often linked to the destruction of native forests [8]. Other aspects debated regarding animal protein production include ruminant's release of greenhouse gases (methane, CO₂, among others), high water consumption, and the demand for alternative protein sources from vegetarian and vegan markets [9,10]. In recent years, various reports in the scientific literature have described the production and applications of proteins from alternative sources, including proteins of fungal origin (mycoproteins), as well as from bacteria and algae, plants, and insects (crickets, grasshoppers, and silkworm pupae). Mycoproteins are also known as mycelium-based or fungal proteins; they are a form of "single-cell protein" derived from fungi. Preparations of fungal-produced mycoproteins will include other nutritional components besides proteins, such as amino acids, lipids, carbohydrates, vitamins, and minerals. These are considered alternative protein sources that can be used for human or animal nutrition purposes [11].

In seeking to contribute to obtaining and the study of alternative protein sources and ingredients for animal feed production, the work reported herein evaluates the production of mycelial biomass (mycoprotein) by the ascomycetous endophytic fungus, *Lasiodiplodia theobromae* MMPI, cultivated under submerged conditions using feedstocks based on commercial sucrose and soybean molasses. In addition, our study assesses the associated production of the carbohydrate biopolymer lasiodiplodan, an extracellular (1→6)-β-D-glucan that presents various biological and technological properties [12] and is produced concomitantly with mycelial growth. Among the biological functions of this β-glucan are antioxidant activity, antitumor activity, hypocholesterolemic and hypoglycemic effects, transaminase activity, and DNA protective activity [12,13]. β-Glucans have the potential as prebiotics or as prebiotic adjuvants and are used as functional ingredients in some foods and animal feeds, especially for their immunomodulatory effects [14,15]. It is essential to highlight that the production of extracellular β-glucans is advantageous compared to their extraction from the yeast cell wall, as the process only involves precipitation with ethanol and purification by dialysis, without using solvents or alkalis [16]. Lasiodiplodan is also an unusual glucan, as it is a linear (1→6)-β-D-glucan, whereas cell wall glucans are mainly of the (1→3)-β type [17].

This study sought to contribute to developing an integrated biotechnological platform, associating the production of mycelial biomass (mycoprotein) and the biomacromolecule (β-glucan: lasiodiplodan) of high commercial value, adding greater value to the process. As a strategy for cost reduction, the industrial by-product soybean molasses was evaluated as a potential fermentation substrate for this bioprocess.

2. Materials and Methods

2.1. Inoculum Preparation, Soy Molasses Clarification, and Rice Bran Extraction

The ascomycete fungus *Lasiodiplodia theobromae* MMPI, originally isolated from the tropical fruit pinha (*Annona squamosa*), was maintained on Sabouraud-chloramphenicol agar medium at 4 °C through successive sub-cultures. To prepare the inoculum, a portion of the stock culture mycelium was transferred to plates containing Sabouraud-chloramphenicol agar medium and cultivated at 28 °C for 96 h. The colonized mycelium on the agar plates was transferred aseptically to 250 mL Erlenmeyer flasks containing 100 mL of Vogel's minimum salts medium (VMSM)[18] and 5 g L⁻¹ of glucose and incubated at 28 °C in an orbital shaker (150 rpm) for 48 h. The mycelial biomass from the pre-culture was aseptically homogenized in a blender to obtain a concentrated mycelial suspension. Under aseptic conditions, the mycelial suspension was diluted with sterilized saline solution (0.9 %

w v⁻¹) to obtain a suspension with a standardized concentration based on absorption readings at 400 nm between 0.4 and 0.5.

According to the procedure outlined by Acosta et al [19], clarifying soybean molasses was carried out to improve the fermentation conditions. Initially, the pH of the raw molasses was adjusted to 3.0 by the addition of concentrated sulfuric acid and stirred continuously for 24 h at 4 °C. The molasses was then centrifuged (1500 x g / 30 min), and the pH of the supernatant recovered was adjusted to 5.5 with 6 mol L⁻¹ sodium hydroxide solution followed by re-centrifugation. The resulting supernatant (clarified molasses) was used in the fermentation experiments.

Rice bran extract (RBE) was prepared at a concentration of 200 g L⁻¹. An aqueous suspension of rice bran (200 g rice bran L⁻¹) was autolyzed in an autoclave at 121 °C for 15 min. The mixture was cooled and centrifuged at room temperature (1500 x g) for 30 min. The resulting supernatant was transferred to sterile capped-reagent bottles for later use as a nitrogen source in cultivating *L. theobromae* MMPI [20].

2.2. Submerged Fermentation for the Co-Production of Mycelial Biomass and Lasiodiplodan

Experimental planning methodology using a Rotational Central Composite Design (RCCD 2³) was applied to study the influence of the concentrations of substrate (carbon source) and rice bran extract (nitrogen source), as well as agitation speed on mycelial growth for the production of cellular biomass and lasiodiplodan.

Submerged fermentations were carried out in 250-mL Erlenmeyer flasks containing 100 mL of synthetic sucrose-based medium or medium formulated with soybean molasses (see below). An aliquot of 10 mL of the standardized inoculum was added to the previously prepared culture media (see above), and the flasks were incubated in an orbital incubator (shaker) for 96 h at 28 °C.

The initial pH of the culture media was adjusted to 5.5 with 1 mol L⁻¹ HCl or 1 mol L⁻¹ NaOH solution. The synthetic sucrose-based medium (SBM) consisted of sucrose and rice bran extract following the concentrations described in the experimental design (Table 1) and supplemented with KH₂PO₄ (2 g L⁻¹) and MgSO₄·7H₂O (2 g L⁻¹). The molasses-based medium (SMM) was formulated with soybean molasses (total sugar concentration was adjusted by dilution with water according to the RCCD 2³ design) and rice bran extract at the concentrations described in the experimental design and supplemented with the mineral salts described above. The production of lasiodiplodan was considered a co-produced bioproduct inherent to the cultivation and cellular growth of the fungus *L. theobromae* MMPI in the two-culture media studied.

Table 1. Production and yields of mycelial biomass and lasiodiplodan at 96 h by *Lasiodiplodia theobromae* MMPI cultivated on SMM and SBM media.

Runs	Variable levels			Values obtained							
	X ₁	X ₂	X ₃	Soy molasses medium *				Sucrose-based medium **			
				P _X (g L ⁻¹)	P _{Las} (g L ⁻¹)	Y _{P/S} (g g ⁻¹)	Y _{X/S} (g g ⁻¹)	P _X (g L ⁻¹)	P _{Las} (g L ⁻¹)	Y _{P/S} (g g ⁻¹)	Y _{X/S} (g g ⁻¹)
1	-1	-1	-1	3.450	2.640	0.154	0.202	3.160	2.267	0.290	0.404
2	-1	-1	1	10.627	1.520	0.095	0.667	4.633	0.827	0.062	0.348
3	-1	1	-1	6.580	1.120	0.060	0.353	8.360	3.333	0.287	0.721
4	-1	1	1	9.783	1.840	0.099	0.527	6.073	1.627	0.104	0.387
5	1	-1	-1	10.077	3.487	0.069	0.199	6.493	5.700	0.178	0.203
6	1	-1	1	10.583	1.893	0.037	0.205	11.400	1.813	0.056	0.354
7	1	1	-1	12.637	0.667	0.013	0.241	6.207	1.653	0.036	0.136
8	1	1	1	10.837	2.267	0.044	0.211	14.860	0.400	0.009	0.334
9	-1.68	0	0	2.443	0.427	0.074	0.426	3.183	0.240	0.049	0.653
10	1.68	0	0	16.627	0.533	0.009	0.282	11.103	1.013	0.024	0.262
11	0	-1.68	0	10.223	1.120	0.030	0.272	6.497	0.720	0.047	0.426
12	0	1.68	0	8.157	0.800	0.021	0.215	8.823	1.013	0.034	0.292

13	0	0	-1.68	8.927	1.093	0.029	0.236	5.937	0.800	0.025	0.184
14	0	0	1.68	18.490	2.320	0.067	0.538	5.997	1.493	0.056	0.227
15 (C)	0	0	0	8.110	0.560	0.016	0.230	11.205	0.760	0.029	0.423
16 (C)	0	0	0	9.315	0.800	0.022	0.259	10.930	1.560	0.060	0.424
17 (C)	0	0	0	9.475	0.520	0.014	0.251	9.425	1.000	0.037	0.351
18 (C)	0	0	0	8.785	0.280	0.007	0.230	8.985	0.720	0.027	0.335
Variables			Levels (real values)								
			-1.68	-1	0	1	1.68				
X ₁ substrate (g L ⁻¹)			6.4	20	40	60	73.6				
X ₂ RBE (%)			1.6	5	10	15	18.4				
X ₃ agitation (rpm)			99.6	120	150	180	200.4				

C, center point; RBE, rice bran extract.

2.3. Analytical Methods

The fungal mycelial biomass and lasiodiplodan content were determined by gravimetry. The fermented broth was separated from the biomass by centrifugation (1500 × g) for 15 min. The mycelial biomass recovered was washed with distilled water at 60 °C to remove the lasiodiplodan that adhered to the mycelium and then dried in an oven with air circulation at 50 °C until constant mass. The exopolysaccharide (EPS) lasiodiplodan was separated from the cell-free fermentative broth by adding three volumes of ethanol (95 %) and left overnight at 5 °C. The precipitated EPS was recovered and dried in an oven with air circulation at 50 °C until constant mass. Residual total sugars were determined by the phenol-sulfuric method [21] in the fermentation broth after removing the exopolysaccharide by ethanol precipitation. The fermentative parameters P_x (cell biomass production), P_f (lasiodiplodan production), Y_{x/s} (yield in cell biomass), Y_{p/s} (yield in lasiodiplodan), Y_e (specific yield), Y_c (percentage of substrate consumption), Q_x (volumetric productivity in cell biomass), Q_p (volumetric productivity in lasiodiplodan), and Q_s (global rate of substrate consumption) were determined.

2.4. Characterization of Mycelial Biomass and Lasiodiplodan

2.4.1. Proximal Composition

The proximal composition of the fungal biomass was determined in terms of moisture, fixed mineral residue (ash), crude protein, total lipid content, and dietary fiber. The moisture content of the samples was determined in triplicate using the official AOAC method (Nº. 925.10), in which 5 g of each fungal biomass was dried in an air-dried oven at 105 °C until of constant mass. The fixed mineral residue was determined gravimetrically as ash after incineration in a muffle furnace at 550 °C, according to the AOAC method (Nº. 923.03). Total lipids were determined gravimetrically following Soxhlet reflux extraction; AOAC method (Nº. 920.39c), where the lipid fraction was extracted with diethyl ether solvent. The protein content was determined by the Kjeldahl method (AOAC method Nº. 2001.11), which consists of determining the total nitrogen concentration and subsequent conversion to protein using a correction factor (6.25). The dietary fiber content was determined by the enzymatic-gravimetric method (AOAC method No. 991.43) [22].

2.4.2. Assessment of Total Phenolics, Antioxidant Activity, and Bioactive Compounds

Mycelial biomass was extracted using a hydroalcoholic solution. The extractions were carried out in 250 mL Erlenmeyer flasks containing 1 g d.w. of freeze-dried biomass in a proportion of 10 mL of hydroalcoholic solution (80 % v v⁻¹), and were heated at 60 °C in an ultrasound bath for 1 h. The biomass extracts obtained were then filtered (Whatman filter paper Nº. 1) and used to analyze total phenolics, bioactive compounds, and antioxidant activity [11].

The total flavonoid content was determined on aliquot samples of 0.5 mL following the procedure outlined by Park et al [23], and employed 4.3 mL of ethanol, 0.1 mL of 1 M potassium

acetate, and 0.1 mL of aluminum nitrate (10 % w v⁻¹). As a control, 0.1 mL of distilled water replaced the aluminum nitrate solution. The absorbance of the samples was read at 415 nm after 40 min. A quercetin standard curve was prepared in the 5 to 100 µg mL⁻¹ range. The absorbance obtained was related to the total flavonoid content based on the quercetin standard.

Total phenolic compounds were determined by the Folin-Ciocalteu method [24]. A 0.5 mL aliquot of ethanolic extract was transferred to test tubes containing 2.5 mL of Folin-Ciocalteu aqueous solution (1:10, v v⁻¹). The samples were left in the dark for 5 min, and then 2 mL of 4% (v v⁻¹) sodium carbonate solution was added. The tubes were then kept for 2 h (in the dark) at room temperature, and the absorbance read at 740 nm in a UV/VIS Digilab-Hitachi U-2800 Spectrophotometer (Lambda Advanced Technology, Wembley, UK).

The antioxidant potential was evaluated by the classical methods: ABTS [25], DPPH [26], Ferric Reducing Antioxidant Power – FRAP,[27] Hydroxyl Radical Removal [28], and total antioxidant capacity (TAC) by the phosphomolybdenum complex reduction method [29].

A Varian 920 LC HPLC system (Varian Inc., Walnut Creek, CA, USA) coupled to a photodiode array detector (PAD) and using a reverse-phase C-18 RP (250 × 4.6 mm × 5 µm) column (Eclipse Plus, Agilent Technologies, Wilmington, DE, USA) maintained at 30 °C was used to analyze the profile of phenolic compounds. Volumes of 10 µL of extracts were injected at a concentration of 80 g L⁻¹. The mobile phase consisted of a gradient mixture of solvent A (2% aqueous acetic acid solution) and solvent B (40% acetonitrile acidified with 2% acetic acid solution), eluting at a flow rate of 1 mL min⁻¹.

In the chromatographic analyses, 16 standards were used (gallic acid, chlorogenic acid, vanillic acid, caffeic acid, coumaric acid, ferulic acid, salicylic acid, cinnamic acid, catechin, epicatechin, rutin, isoquercetin, astragalin, myricetin, quercetin and kaempferol) to identify the main compounds present in the mycelium extracts. Phenolic compounds were identified by comparing their retention times with authentic chromatographic standards and quantified by integrating the respective chromatographic peaks. The wavelengths used in the detection of compounds were 280 nm for gallic and vanillic acids, as well as for catechin and epicatechin; 300 nm for *p*-coumaric and salicylic acids; 320 nm for caffeic, cinnamic, chlorogenic, and ferulic acids, and 360 nm for astragalin, isoquercetin, quercetin, kaempferol, myricetin and rutin.

2.4.3. Characterization of Mycelial Biomass and Lasiodiplodan by Scanning Electron Microscopy, Thermal Analysis, X-ray Diffraction, and FT-IR Spectroscopy

The morphological aspects, thermal behavior, diffractometric profile, and FT-IR spectra of mycelial biomass and lasiodiplodan obtained from the cultivation of *L. theobromae* MMPI on culture media based on soybean molasses (SMM) and sucrose (SBM) were investigated. The samples were placed on carbon tape, and SEM images were obtained using a benchtop electron microscope model TM3000 (Hitachi, Irving, TX, USA) at amplitudes of 200 X, 600 X, and 1000 X, using 5 to 15 k. The thermogravimetric profile was analyzed on a SDT Q600 thermal analyzer (TA Instruments, New Castle, DE, USA). Thermal analyses of the mycelial biomass samples were monitored in a temperature range ranging from 25 to 800 °C with a heating rate of 10 °C min⁻¹ and synthetic air flow of 50 mL min⁻¹. The diffraction profile of the samples was obtained using an X-ray diffractometer model XDR-6000 (Shimadzu, USA) with a copper lamp radiation source (CuKα= 1.5418 Å), current of 30 mA and voltage of 40 kV, angle of sweep (2θ) from 10° to 90°, speed of 0.5 ° min⁻¹ and a step of 0.02 degrees. Infra-red spectra (IR-ATR; Attenuated total reflection) were obtained on a Frontier spectrophotometer (Perkin Elmer, USA) in the region of 4000-400 cm⁻¹, with 32 accumulations and a resolution of 0.5 cm⁻¹, using the ATR method.

3. Results and Discussions

3.1. Co-Production of Mycelial Biomass and Exocellular Lasiodiplodan by *Lasiodiplodia theobromae* MMPI

As shown in Table 1, *L. theobromae* MMPI grew in both media formulations (SBM and SMM) under the cultivation conditions stipulated. Higher production of mycelial biomass was obtained in media formulated with soybean molasses, as was evident in experimental run 10 ($P_x = 16.63 \text{ g L}^{-1}$) and 14 ($P_x = 18.49 \text{ g L}^{-1}$). Appreciable mycelial biomass contents were also found in sucrose-based media; runs 6 ($P_x = 11.40 \text{ g L}^{-1}$) and 8 ($P_x = 14.86 \text{ g L}^{-1}$), although such values were lower than those found in cultivations on SMM media.

Another aspect that must be highlighted is the effect of substrate concentration on mycelial growth (P_x). A punctual evaluation of runs 1 and 9 suggests lower concentrations of soybean molasses and sucrose-based media promote less mycelial growth. In fact, runs 1 and 9, where the lowest levels of substrate concentration were used (-1 and -1.68, respectively), resulted in the lowest production values (P_x).

In relation to the mycelial biomass yield ($Y_{x/s}$), the cultivation conditions used in runs 2 ($Y_{x/s} = 0.67 \text{ g g}^{-1}$, SMM medium) and 3 ($Y_{x/s} = 0.72 \text{ g g}^{-1}$, SBM medium) promoted the highest yield values. In contrast to what was observed with production (P_x), the maximum yield values were similar for both media.

Table 1 (Suppl. Mat.) shows an analysis of variance (ANOVA) of the experimental design results related to mycelial production in the SMM medium. The experimental data were analyzed by multiple linear regression, and the mathematical model obtained was significant at a 95% confidence interval ($p < 0.05$). The F test indicated that the generated model is predictive since the calculated F value was higher than the tabulated F. The model's coefficient of determination was 0.8147, which indicates that the model can explain 81.47% of the data variability. Coefficients of determination above 75% can be considered adequate in mathematical models that describe biological experiments with living cells. The mathematical model (Eq. 1) that represents mycelial growth (P_x) in cultures in SMM medium described by the response surface (Figure 1) considered only the significant variables (X_1 : substrate and X_3 : agitation):

$$P_x = 3.98198 + 0.522156 X_1 - 0.237733 X_3 \quad (1)$$

The analysis of variance (ANOVA) of the results of the experimental design of fermentations conducted in SBM medium for the production of mycelial biomass is shown in Table 2 (Suppl. Mat.). The mathematical model obtained by multiple linear regression (Eq. 2) was significant at a 95% confidence interval ($p < 0.05$), with an F value of 5.69 and a tabulated F of 3.39. Although the model's coefficient of determination was relatively high ($R^2 = 0.8649$), the model was not predictive.

$$P_x = -22.8466 - 0.1240 X_1 + 0.9167 X_2 - 0.0014 X_3^2 + 0.0030 X_1 X_3 \quad (2)$$

The analysis of variance (ANOVA) of the results of the experimental designs of fermentations in SMM and SBM media on the yield in mycelial biomass ($Y_{x/s}$) are described in Tables 3 and 4 (Suppl. Mat.). The mathematical model of the $Y_{x/s}$ yield obtained in the SMM medium was significant within a 95% confidence interval ($p < 0.05$) and is described in Eq. 3. The calculated F value was 9.82, and the tabulated F was 3.39, a predictive model with 91.69% of the data variability explained by the model ($R^2 = 0.9169$).

$$Y_{x/s} = 0.161018 + 0.000055 X_3^2 + 0.008544 X_1 - 0.005422 X_3 - 0.000138 X_1 X_3 \quad (3)$$

Likewise, the mathematical model of yield ($Y_{x/s}$) obtained in the SBM medium (Eq. 4) was predictive, finding a calculated F of 7.23, a tabulated F of 3.39, and a coefficient of determination R^2 of 0.8905.

$$Y_{x/s} = -0.342087 - 0.000064 X_3^2 - 0.29297 X_1 + 0.000154 X_1 X_3 \quad (4)$$

The variables of soybean molasses concentration and agitation (Figure 1a and Figure 2a) demonstrated a statistically positive influence at the 95% confidence level ($p < 0.05$) on mycelial growth (P_x) (positive linear effect) when the fungus was cultivated on SMM medium. The positive

effect of such variables on the response indicates that the increase in the value of these variables within the range studied contributes to the improvement in the response.

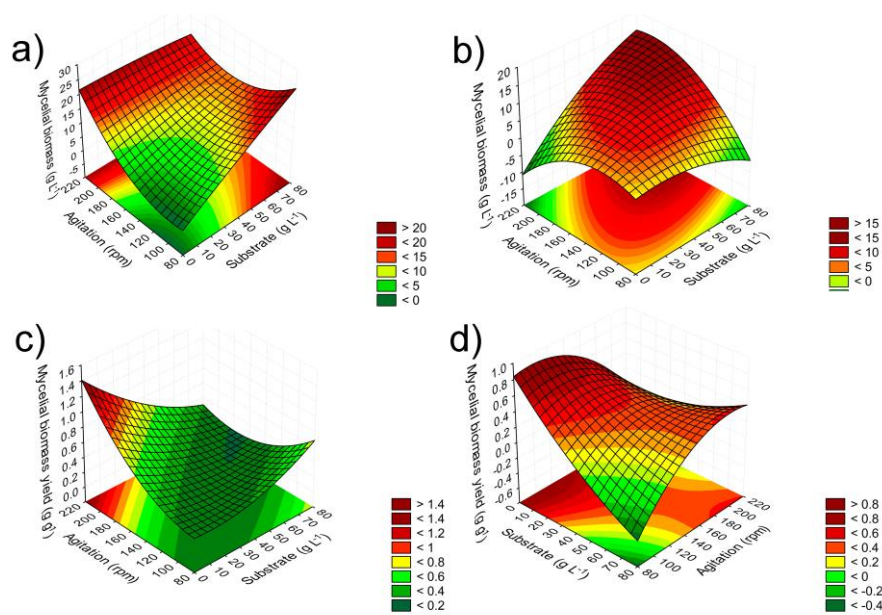


Figure 1. Response surface 3-D and contour plots for the production of biomass (Px) and biomass yield (Y_{x/s}) in culture media formulated with soybean molasses (a and c), and sucrose (b and d). The response surface was obtained for substrate concentration and agitation, using RBE (10%) as the nitrogen source.

Table 2. Critical and experimental values for the production and yield of mycelial biomass by *Lasiodiplodia theobromae* MMPI cultivated for 96 h on media based on soybean molasses (SMM) and sucrose (SBM).

Variables	Soy molasses medium (SMM)			Sucrose (SBM)		
	Critical value	Experimental value	Recovery (%)	Critical value	Experimental value	Recovery (%)
Substrate (g L ⁻¹)	64.43	64.43	-	40.68	40.68	
RBE (%)	8.81	8.81	-	3.44	3.44	
Agitation (rpm)	152.83	152.83	-	161.22	161.22	
P _x (g L ⁻¹)	12.13	12.44	102.6	7.90	10.09	127.7
Y _{x/s} (g g ⁻¹)	0.196	0.214	109.2	0.378	0.328	86.8

RBE: Rice Brain Extract; P_x: mycelial biomass production; Y_{x/s}: yield in mycelial biomass.

Table 3. Fermentation parameters of *Lasiodiplodia theobromae* MMPI cultivated for 96 h on media based on soybean molasses and sucrose.

Fermentation parameters	Cultivation Medium	
	SMM *	SBM **
P _x (g L ⁻¹)	12.440 ^a	10.087 ^a
P _F (g L ⁻¹)	0.573 ^a	0.547 ^a
TRS (g L ⁻¹)	6.275 ^b	9.964 ^a
Y _{P/S} (g g ⁻¹)	0.010 ^a	0.018 ^a
Y _{X/S} (g g ⁻¹)	0.214 ^a	0.328 ^a
Y _e (g g ⁻¹)	0.046 ^a	0.054 ^a
Y _c (%)	90.261 ^a	75.507 ^b
Q _x (g L ⁻¹ h ⁻¹)	0.130 ^a	0.105 ^a
Q _P (g L ⁻¹ h ⁻¹)	0.006 ^a	0.006 ^a

Q_s (g L ⁻¹ h ⁻¹)	0.606 ^a	0.320 ^b
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* Soybean Molasses Medium; ** Sucrose-Based Medium. Values followed by the same letter in the lines do not vary significantly at the 95 % confidence level ($p \leq 0.05$), P_x: mycelial biomass production, P_F: lasiodiplodan production, TRS: Total Residual Sugars, Y_{P/S}: yield in lasiodiplodan, Y_{X/S}: yield in mycelial biomass, Y_e: specific yield, Y_c: percentage of substrate consumption, Q_P: volumetric productivity in lasiodiplodan, Q_X: volumetric productivity in mycelial biomass, Q_s: overall substrate consumption rate.

Table 4. Proximal composition and profiles of amino acids and fatty acids of *Lasiodiplodia theobromae* MMPI mycelial biomasses cultivated on SMM and SBM media.

Proximal Composition [#]					
	SMM*	SBM**		SMM*	SBM**
Moisture (% at 105 °C)	9.5	9.7	Dietary fiber	7.5	17.0
Crude Protein	16.27	19.88	Total carbohydrates	24.67	50.96
Total fat	43.77	7,56	Mineral residue (ash)	5.79	12.57
Caloric value (Kcal 100 g ⁻¹)	557.69	351,4			
Essential Amino Acids ^{##}					
	SMM*	SBM**		SMM*	SBM**
Histidine	20.28	0.00	Phenylalanine	38.11	7.04
Isoleucine	49.17	16.10	Threonine	43.64	7.04
Leucine	97.11	39.24	Tryptophan	8.60	6.04
Lysine	91.58	19.11	Valine	61.46	18.61
Methionine	25.20	7.04			
Non-essential amino acids ^{##}					
	SMM*	SBM**		SMM*	SBM**
Aspartic Acid	119.85	30.18	Tyrosine	29.50	2.01
Glutamic Acid	159.19	37.22	Glycine	51.63	8.05
Alanine	76.83	15.59	Proline	1.23	0.00
Arginine	86.05	11.07	Serine	71.30	11.57
Cystine	15.98	0			
Monounsaturated fatty acids (MUFA) [#]					
	SMM*	SBM**		SMM*	SBM**
Elaidic Acid (C18:1n9t)	0.01	-	Palmitoleic Acid (C16:1n7) (ω -7)	0.08	0.05
Oleic Acid (C18:1n9c) (ω-9)	5.12	1.85	cis-11-Eicosenoic acid (C20:1n9)	0.04	0.01
Polyunsaturated fatty acids (PUFA) [#]					
	SMM*	SBM**		SMM*	SBM**
Linoleic Acid (C18:2n6c) (ω-6)	24.38	2.24	Linoleic Acid (C18:2n6t) cis-11,14-	0.02	-
α-Linolenic acid (C18:3n3) (ω-3)	2.99	0.16	Eicosadienoic acid (C20:2)	0.04	-
Saturated fatty acids (SFA) [#]					
	SMM*	SBM**		SMM*	SBM**
Mystic acid (C14:0)	0.04	0.03	Caprylic acid (C8:0)	0.12	0.00
Pentadecanoic acid (C15:0)	0.04	0.01	Arachidic acid (C20:0)	0.06	0.05
Palmitic acid (C16:0)	8.81	2.05	Heneicosanoic acid (C21:0)	0.02	0.00
Margaric acid (C17:0)	0.07	0.01	Behenic acid (C22:0)	0.14	0.03

Stearic acid (C18:0)	1.60	1.03	Tricosanoic acid (C23:0)	0.05	0.01
Lignoceric acid (C24:0)	0.14	0.03			
Total lipids[#]					
	SMM*	SBM**		SMM*	SBM**
Monounsaturated	5.25	1,91	Saturated	11.09	3.25
Polyunsaturated	27.43	2.40	<i>trans</i> lipids	0.03	0.00
Unsaturated	32.68	4,31	Total Lipids	43.77	7,56

Media based on: *soybean molasses and **sucrose, as carbon sources, [#]grams per 100 grams of dry mycelial biomass (g 100 g⁻¹), **milligrams per gram of protein (mg g⁻¹).

In relation to mycelial growth (P_x) observed in the SBM medium (Figure 2b), the variables substrate (sucrose) concentration and rice bran extract (RBE) concentration showed a positive influence (linear effect). A positive interaction effect (Figure 2b) was also verified between the variable's substrate concentration and agitation, which can also be observed in the response surface presented in Figure 1b. The positive effect of rice bran extract on mycelial growth occurred only when sucrose was used as substrate. This phenomenon was expected as soybean molasses is rich in nitrogen and minerals and does not require supplementation [19]. On the other hand, agitation was found not to contribute to greater mycelial growth, with a negative (quadratic effect) effect of this variable on growth in the SBM medium (Figure 2b). The negative effect of agitation on mycelial growth observed in the sucrose-based medium can be explained by the higher production of β -glucan (Table 1). The presence of β -glucan in the growth medium promotes an intense increase in viscosity, which can hinder the transfer of oxygen and a consequent reduction in the rate of cellular respiration, leading to lower energy production and mycelial growth.

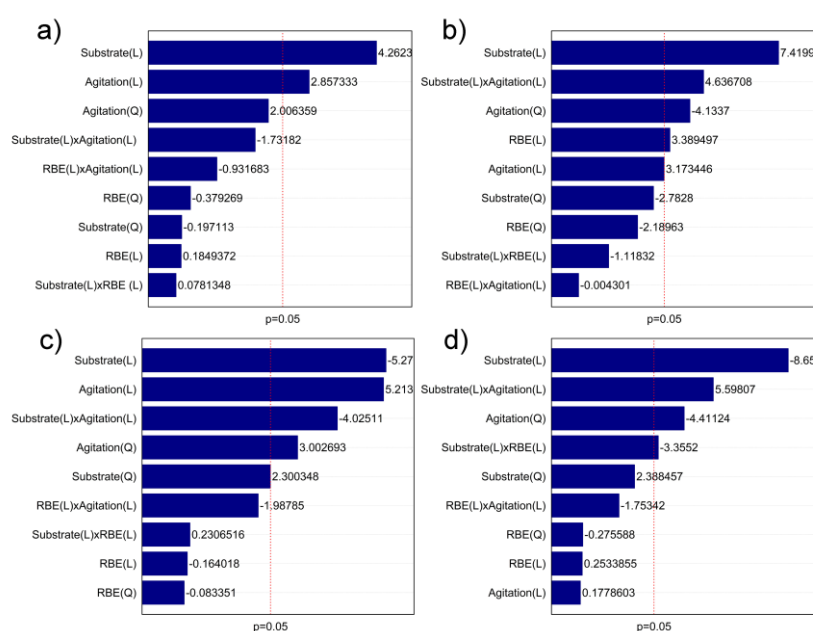


Figure 2. Pareto diagrams of standardized effects to produce biomass (PX) in media based on soybean molasses - SMM (a), and sucrose - SBM (b), and biomass yields (YX/S) in media based on soybean molasses - SMM (c) and sucrose - SBM (d).

In the fermentation runs with soybean molasses, there was a significant influence of the variable's substrate concentration (negative linear effect) and agitation (positive linear and quadratic effects), as well as a negative effect of interaction between such variables on the yield in mycelial biomass (Y_{X/S}, Figure 2c) at the 95% confidence level ($p < 0.05$). This behavior indicates that to obtain

better yields of mycelial biomass ($Y_{X/S}$) within the study range, it is recommended to use a lower concentration of soybean molasses associated with a higher agitation rate.

Similar to what was observed in the SMM medium, the Pareto chart shows a negative influence (linear effect) of substrate concentration on mycelial biomass yield ($Y_{X/S}$) in the sucrose-based medium (SBM) (Figure 2d). This result indicates that increasing the concentration of sugars in the cultivation medium does not stimulate the direct conversion of substrate into mycelial biomass, although it can promote greater mycelial growth (P_X). In the medium with sucrose (Figure 2d), both substrate concentration and agitation showed negative effects on the yield of mycelial biomass, possibly due to the production of associated β -glucan, which was much higher in the medium with sucrose.

The fungus produced lasiodiplodan in both formulated SBM and SMM media and under all cultivation conditions (Tables 1 and 2). In experimental run 5, the highest lasiodiplodan production was obtained in SMM (P_F : 3.49 g L⁻¹) and SBM (P_F : 5.70 g L⁻¹) media. Similarly, better yields in lasiodiplodan ($Y_{P/S}$) were obtained in both media when using the cultivation conditions of run 1 ($Y_{P/S}$ SMM: 0.154 g g⁻¹ and $Y_{P/S}$ SBM: 0.290 g g⁻¹). A specific evaluation of experimental runs 5 and 1 indicates that higher production (P_F) and yields ($Y_{P/S}$) in lasiodiplodan should be obtained when the lowest levels of the variables agitation and concentration of rice bran extract are used. In the case of the lasiodiplodan yield, lower concentrations of rice bran extract and low agitation must be associated with using lower substrate concentrations to obtain better results.

Analysis of variance (ANOVA) of the experimental design data regarding lasiodiplodan production and yield in SMM medium are shown in Tables 5 and 6 (Suppl. Mat.), respectively. The regression model obtained for lasiodiplodan production was statistically non-significant at the 95% confidence interval ($p < 0.05$); the data did not fit within the scientific experimental range. The mathematical model obtained was significant regarding the performance yield of lasiodiplodan (F_{calc} : 3.65; F_{tab} : 3.39). The coefficient of determination (R^2) relative to yield in lasiodiplodan was 0.8041, indicating an acceptable adjustment considering a biological assay using microorganisms.

In fermentations of the fungal isolate in the SBM medium, the regression model obtained for producing lasiodiplodan did not show statistical significance in a 95% confidence interval ($p < 0.05$), nor did the data present a good fit (R^2 : 0.5279) (Table 7, Suppl. Mat.). Similarly, for the lasiodiplodan yield, the model generated by linear regression of the data also did not show statistical significance at the 95% confidence level ($p < 0.05$) (Table 8, Suppl. Mat.).

Considering the lack of significance or adjustment of the models generated for producing lasiodiplodan in both culture media environments, evaluations of the effects of the variables on the responses were not carried out on data from the Pareto diagrams and response surfaces.

3.2. Validation of Predictive Models and Kinetic Study of the Cultivation of *Lasiodiplodia theobromae* MMPI in Media Based on Soybean Molasses and Sucrose

The predictive models for mycelial biomass production (P_X) and yield ($Y_{X/S}$) were validated by correlating the experimental values obtained for mycelial biomass production and its yield in a cultivation kinetics study. The experimental fermentation runs employed critical values (values optimized by the model) of the variables substrate and rice bran extract concentrations and agitation described in the predictive model for maximum results. The validation experiment was conducted to verify whether the predictive models were adequate and how close the predicted responses were to the experimentally verified responses.

As can be seen in Table 2, there was a good correlation between the values estimated by the models and the data obtained experimentally, both for the yield and production of mycelial biomass on both substrate feedstocks (soybean molasses or sucrose). When soybean molasses was used as a substrate, the experimental data showed a recovery of 109% for the predicted values for mycelial biomass yield. Similarly, a recovery of 102.6% was observed in the production of mycelial biomass. When sucrose was the limiting substrate, the recovery values were 86.8% (yield) and 127.7% (mycelial biomass production).

The profiles for substrate consumption, mycelial biomass, and lasiodiplodan production were similar for both culture media studied when the optimized conditions were applied. However, under these fermentation conditions, soybean molasses demonstrated slightly better performance as a substrate for mycelial growth and lasiodiplodan production. For lasiodiplodan, maximum production was verified in 72 h of cultivation in the medium based on soybean molasses (0.813 g L^{-1}). Similarly, the maximum production of mycelial biomass occurred in the soybean molasses-based medium in 96 h (12.44 g L^{-1}), as displayed in Figure 3a and Table 3. In relation to the sugar assimilation capacity, the fungus was efficient in its use. In the first 24 hours, 63% of the total sugars in the soybean molasses medium and 52% of the sucrose-based medium had already been consumed. After this period, the substrate was consumed gradually until the end of the experiment (96 h), where maximum consumption was observed in soybean molasses (Y_C : 90.2 %, Table 3). Soybean molasses contributed to a more effective rate of overall substrate consumption (Q_S : $0.606 \text{ g L}^{-1} \text{ h}^{-1}$). The effective consumption of the substrate in soybean molasses indicates that the fungus produced enzymes that act on the oligosaccharides present in the molasses preparation, whose composition includes a large amount of these sugars, mainly stachyose (tetrasaccharide), raffinose (trisaccharide), and sucrose (disaccharide) [19].

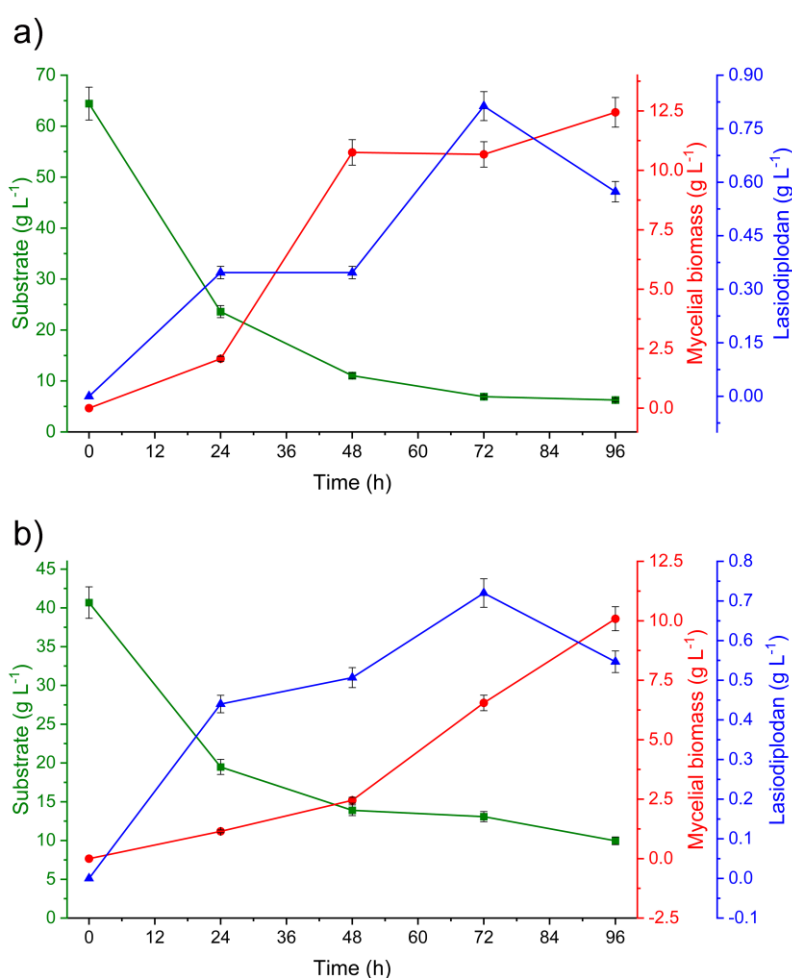


Figure 3. Fermentation profiles of *Lasiodiplodia theobromae* MMPI cultivated on media based on (a) soybean molasses and (b) sucrose.

Regarding the volumetric productivity of lasiodiplodan (Q_P) in 96 h fermentation, no differences were found between the values obtained for both the SMM and SBM media ($0.006 \text{ g L}^{-1} \text{ h}^{-1}$). On the other hand, the SMM medium promoted more effective production of mycelial biomass compared to

the SBM medium, which was observed by much higher volumetric productivity at 96 h (Q_x : 0.130 g L⁻¹ h⁻¹).

3.3. Proximal Composition and Profiles of Amino and Fatty Acids of the Mycelial Biomass from *Lasiodiplodia theobromae* MMPI.

The proximal composition and profile of amino acids and fatty acids found in freeze-dried samples of *L. theobromae* MMPI mycelial biomass are described in Table 4.

The mycelial biomass produced on the SMM and SBM media both highlight the nutritional quality, especially their crude protein content (16.27 g 100 g⁻¹, and 19.88 g 100 g⁻¹, respectively), dietary fiber (7.5 g 100 g⁻¹, and 17.0 g 100 g⁻¹, respectively) and mineral residues (5.79 g 100 g⁻¹, and 12.57 g 100 g⁻¹, respectively). Different protein contents have been reported in fungal mycelial biomass, commonly called mycoprotein. For example, Singh et al. [30] reported a crude protein of 24.52 g 100 g⁻¹ in the freeze-dried mycelium of *Pleurotus eryngii* cultivated on potato-dextrose broth under submerged conditions.

High protein contents in fungal biomass have recently been described in the scientific literature. This is driven by the growing industrial interest in alternative protein sources for human consumption and animal feeds [31]. Interest in producing unconventional proteins has increased due to global population growth, which demands foods and supplements with high nutritional quality [32].

Depending on the microbial species and the cultivation conditions used in the process, cellular biomass obtained by fermentation can have varying protein contents ranging widely from 30 to 50% [31]. In this context, Karimi et al. [33] reported concentrations of 44.7%, 57.6%, and 50.9% in the mycelial biomass from *Aspergillus oryzae* CBS 819.72, *Neurospora intermedia* CBS 131.92 and *Rhizopus oryzae* CCUG61.147, respectively, in fermentation using the vinasse-based medium.

It is important to highlight that the proteins in the mycelial biomass obtained from *L. theobromae* MMPI cultivated on both media in the present work presented all of the essential amino acids, but in the case of the mycelial biomass originating from the sucrose-based medium, histidine was not present (Table 4). Another aspect that deserves highlighting is that the biomass from the medium formulated with soybean molasses presented higher concentrations of all the essential amino acids than compared to the biomass produced in SBM. Likewise, the mycelial biomass produced in SMM presented higher amounts of each non-essential amino acid identified. Leucine and lysine were the essential amino acids in the highest concentrations in both mycelial biomass types produced (Table 4). Leucine in the diet is necessary to stimulate muscle protein synthesis and protect muscles from proteolysis (Ref required citing of this fact). Lysine has many key bodily functions, such as maintaining acid-base balance, modifying lipid metabolism through carnitine synthesis, and being important in osmoregulation [33].

The higher dietary fiber content (17 g 100 g⁻¹) observed in the biomass of *L. theobromae* MMPI cultivated in the sucrose-based medium in relation to the medium with soybean molasses (7.5 g 100 g⁻¹), can be justified due to the increased production of lasiodiplodan obtained in the medium with sucrose. Lasiodiplodan is considered a dietary fiber (prebiotic) as it is not digested in passage through the alimentary tract. In fact, even after thoroughly washing the recovered mycelial biomass with hot water to remove any β -glucan adsorbed to the mycelium, a large amount of β -glucan remained adsorbed to the fungal mycelium and, therefore, contributed in part to the overall mycelium composition, thereby increasing its dietary fiber content (data not shown). The presence of high dietary fiber content in fungal biomass, associated with the other nutritional components present, can make them attractive as ingredients in the formulation of feedstuffs and even food products.

As observed with dietary fibers, the mycelial biomass obtained from the sucrose medium also presented a higher content of mineral elements in its composition (17.0 g 100 g⁻¹) compared to the biomass from the SMM medium (7.5 g 100 g⁻¹). Such minerals arise from the culture medium and are absorbed by the mycelium during cultivation. In the case of the biomass produced on SBM, in addition to cellular absorption of minerals from the medium, chemical interactions may have

occurred between mineral ions and other constituents (e.g. proteins) in the mycelial cell wall. The mineral content in fungal biomass has been reported to be quite variable. Karimi et al [33], for example, reported values between 5.07 g 100 g⁻¹ and 9.01 g 100 g⁻¹ in mycelial biomass of *A. oryzae*, *N. intermedia*, and *R. oryzae*.

Regarding the lipid content, the composition of the culture medium led to the production of mycelial biomass, which has different concentrations of fatty acids. A high content of total lipids (43.77 g 100 g⁻¹) was found to be present in the biomass from the soybean molasses formulated medium. Lipid contents of 7.0 %, 5.5 %, and 3.5 % were reported by Karimi et al. [33] in biomass from *A. oryzae*, *R. oryzae*, and *N. intermedia*, respectively. They mentioned that the fungal biomass constituents and nutritional properties were significantly related to the medium composition and cultivation conditions. Furthermore, they observed that vinasse (an industrial by-product from the fermentation of sugars) could resist the deposition of lipids in the fungal biomass. High lipid contents in the biomass of oleaginous fungi, such as *A. terreus* IBB M1 (53.98%), *Mucor plumbeus* FRR 2412 (37.0%), *Mucor Fragilis* AFT7-4 (46.8%), *Penicillium brevicompactum* NRC 829 (57.6%), *Mortierella isabellina* NRRL 1757 (47.6%), and *Penicillium citrinum* isolate PKB20 (62.08%), were reported by Bardhan et al. [34] It is essential to mention that most oleaginous microorganisms belong to the yeast genera, with only a few exceptions that belong to the fungal genera (for example, *Aspergillus*, *Humicola*, *Fusarium*, *Mortierella*, *Cunninghamella*, and *Mucor*) that are capable of high lipid synthesis [35].

The high lipid content in the mycelial biomass of *L. theobromae* MMPI cultivated in soybean molasses (43.77 g 100 g⁻¹) suggests that this fungal biomass could be an alternative raw material for biodiesel production. In this sense, in recent years, there has been a significant increase in the number of publications related to the production of lipids by microbial sources (yeasts, algae, and filamentous fungi). There is much interest lately in the so-called oleaginous microorganisms that produce single-cell oils (SCOs - defined as microorganisms that accumulate lipids above 20% of their dry weight) [36].

The use of microbial biomass as a renewable fuel source is a technological alternative capable of reducing environmental problems. The growing demand and use of first-generation lipid-based biofuels (biodiesel) has displaced their use in foods as conventional vegetable oils (e.g., soybean, canola) and increased their costs. In this context, in recent years, there has been a growing need to discover non-conventional sources of fats and oils, which can be converted into biodiesel fuels or renewable diesel [36].

Another aspect that deserves evaluation is the possibility of using fungal biomass produced by the fermentation of soybean molasses to extract fatty acids, considering their total content and fatty acid composition. SCOs can be used as substitutes for expensive fatty acids rarely found in the plant or animal kingdoms, such as oils containing significant amounts of the medically important gamma-linolenic acid or other nutritionally important polyunsaturated fatty acids [36].

The mycelial biomass produced on soybean molasses presented a composition (Table 4) rich in unsaturated fatty acids (32.68 g 100 g⁻¹) with emphasis on the content of polyunsaturated acids (27.43 g 100 g⁻¹), especially gamma-linolenic (24.38 g 100 g⁻¹) and α -linolenic (2.99 g 100 g⁻¹) acids. Linoleic acid (23.67%) was also the most abundant fatty acid in the mycelial biomass of *Galactomyces geotrichum* TS61 produced in a sugarcane molasses-based medium reported by Altun et al. [35] Gamma-linolenic acid is considered an essential fatty acid in humans and is an important intermediate in the biosynthesis of prostaglandin derivatives. Linolenic acid is reported to be effective in preventing or curing various diseases, including rheumatoid arthritis, cardiovascular disease, hypercholesterolemia, atopic eczema, and asthma [37].

When evaluating the balance of saturated fatty acids, we observed that saturated fatty acids constitute 25.3% of the total lipids in the mycelial biomass produced with soybean molasses. On the other hand, in the mycelial biomass originating from the sucrose-based medium, this score corresponded to 47.2%. In fact, this culture medium's composition greatly influenced the amino acid profile of the *L. theobromae* MMPI mycelial biomass.

Palmitic acid stands out among the saturated fatty acids; amounts of 8.81 g 100 g⁻¹ and 2.05 g 100 g⁻¹ in the respective mycelial biomass cultivated on SMM and SBM media. Palmitic acid (hexadecanoic acid) has long been negatively described for its harmful effects on human health, overshadowing its multiple important physiological activities [38]. Palmitic acid accounts for 20-30% of the total fatty acids in the human body, being supplied in the diet or synthesized endogenously via *de novo* lipogenesis. This fatty acid has a wide range of pharmacological activities, including antiviral, anti-inflammatory, analgesic, and lipid metabolism regulatory activities, as well as promoting the apoptosis of neuroblastoma cells and breast cancer cells, and has the potential to inhibit the proliferation of breast cancer cells, hepatoma, and inhibit the proliferation and metastasis of prostate cancer cells [39]. Another interesting aspect regarding lipids rich in palmitic acid is that they can be interesterified to increase their applicability in the production of certain foods [40].

3.4. Profile of Phenolic Compounds in Extracts of Mycelial Biomass from *Lasiodiplodia theobromae* MMPI

Phenolic compounds were detected in *L. theobromae* MMPI biomass extracts produced on both SMM and SBM media. Both types of media presented similar amounts of total phenolics, expressed as gallic acid equivalents (GAE) per gram of dry mycelial biomass, and were determined by the Folin-Ciocalteu method. The concentrations of total phenolics found were relatively small, especially when compared to those found in plant extracts that are commonly rich in phenolic compounds [41]. The amounts of phenolic compounds found in the present work varied from 4.22 mg GAE g⁻¹ (SMM) to 4.14 mg GAE g⁻¹ (SBM). Higher amounts were reported in the mycelial biomass of *Pleurotus ostreatus* PBS281009 (35.4 mg GAE g⁻¹) and *P. ostreatus* PSI101109 (98.6 mg GAE g⁻¹) grown in a glucose-based medium [42]. It is important to highlight that the scientific literature has reported very different phenolic contents in fungal mycelia extracts. For example, Valu et al. [43] found contents between 11.1 and 23.1 mg GAE g⁻¹ of dry fruiting body extract of the basidiomycete *Hericium erinaceus* (lion's mane mushroom).

Regarding total flavonoid content, amounts of 3.4 mg QE g⁻¹ (quercetin equivalent per gram) and 3.69 mg QE g⁻¹ were found in mycelial biomass produced on the SMM and SBM media, respectively. These results are consistent with those reported by Valu et al [43], who reported values of 3.26 mg QE g⁻¹ in the lion's mane mushroom. Flavonoid concentrations between 0.011 and 1.04 mg QE g⁻¹ were reported by González-Palma et al [44], in extracts of *Pleurotus ostreatus*.

The HPLC-PAD chromatograms shown in Figure 4 revealed the presence of some compounds in the mycelial biomass extracts. In the extract obtained from soybean molasses medium (Figure 4a), it was possible to identify the phenolic acids: gallic acid (152.2 µg g⁻¹), coumaric acid (91.8 µg g⁻¹), and cinnamic acid (12.8 µg g⁻¹), and the flavonoid, catechin (533.9 µg g⁻¹). Gallic (263.8 µg g⁻¹) and cinnamic acids (34.1 µg g⁻¹) were also found in the mycelial biomass produced in the sucrose-based medium (Figure 4b), as well as the flavonoid catechin (323.8 µg g⁻¹). Conversely, coumaric acid was not found in the mycelial biomass produced in sucrose-based media. Catechin and gallic acid were the major phenolic compounds among the compounds identified in *L. theobromae* MMPI biomass extracts. Fijałkowska et al. [45] also verified the presence of catechin (58.37 mg 100 g⁻¹ of extract) and gallic acid (0.09 mg 100 g⁻¹ of extract) in mycelium extracts of the medicinal fungus *Fomitopsis officinalis*.

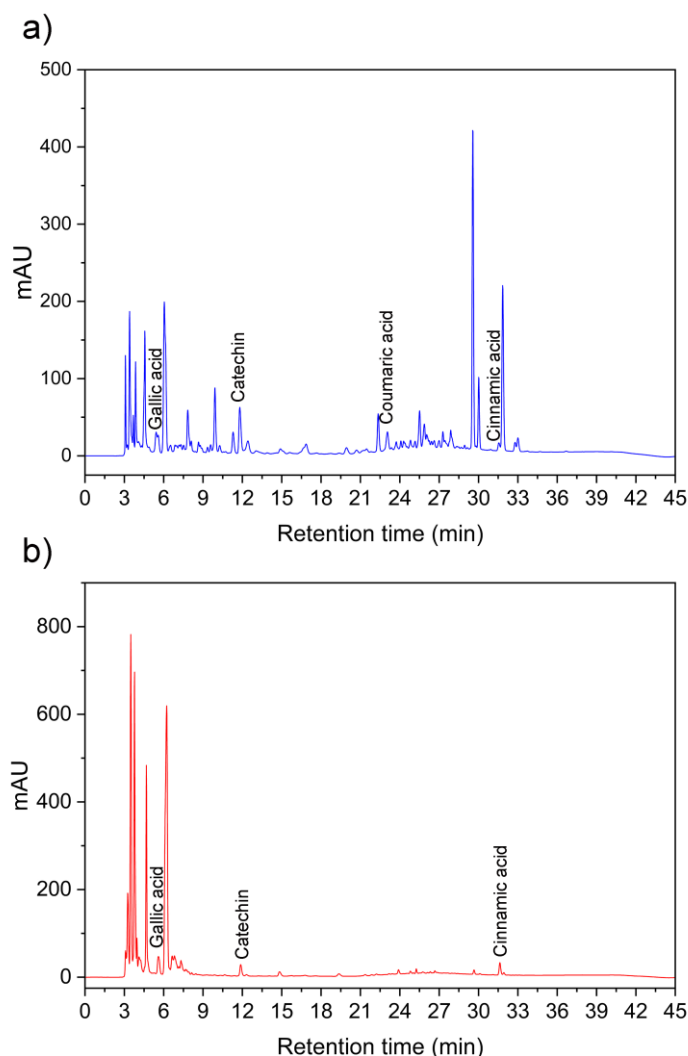


Figure 4. HPLC-PAD chromatograms identifying phenolic compounds obtained from the mycelial biomass extracts of *Lasiodiplodia theobromae* MMPI cultivated on media based on (a) soybean molasses and (b) sucrose.

Studies have demonstrated multiple benefits of catechins, such as antioxidant, anticarcinogenic, antiapoptotic, and anti-inflammatory effects, reducing inflammatory reactions, cellular damage, and lipid peroxidation [46]. Gallic acid (3,4,5-trihydroxybenzoic acid) has been reported as a health-promoting compound with antioxidant, anticarcinogenic, cardioprotective, anti-inflammatory, and antibacterial properties, gastroprotective effects, as neuroprotectors, and can also inhibit the oxidation and rancidity of oils and fats [47,48].

3.5. Antioxidant Potential of *Lasiodiplodia theobromae* MMPI Biomass Extracts

Although the extracts obtained from mycelial biomass produced on SMM and SBM media presented relatively low concentrations of total phenolic compounds, an appreciable antioxidant capacity was verified by assays that evaluated the capture potential of ABTS, DPPH, and OH radicals, and the ability to reduce ferric ions and reduction of molybdenum (VI) ions with the formation of phosphomolybdenum complex.

All of the mycelial extracts tested, effectively eliminated free radicals generated from ABTS, DPPH, and OH, and reduced ferric and molybdenum (VI) ions (Table 5).

The mycelial extracts produced in SMM medium (713.9 mmol TEq g⁻¹, Trolox equivalent per gram of mycelial biomass) and SBM medium (741.89 mmol TEq g⁻¹) showed high potential for scavenging the ABTS cation radical, with no statistically significant differences, between the SMM and SBM media extracts. Regarding the potential for scavenging the OH radical and reducing the

ferric ion, the cultivation medium (SMM, SBM) did not appear to promote mycelium production with different antioxidant abilities. On the other hand, the mycelial biomass extract from the medium formulated with sucrose (187.95 mmol TEq g⁻¹) demonstrated an ability to scavenge the DPPH radical slightly higher than that observed in the mycelial biomass extract from the medium with soybean molasses (180.72 mmol TEq g⁻¹).

The total antioxidant capacity of the mycelial extracts can show variations in different extracts due to extraction processes, culture media, fermentation times, and the types of fungal strains [49]. In this sense, regarding antiradical activity against the ABTS cation radical, values between 25.4 μmol TEq mg⁻¹ of extract and 89 μmol TEq mg⁻¹ were found in mycelial biomass extracts from *Cladosporium cladospoides* strains by Couttolenc et al. [50]. These authors also reported scavenging DPPH radicals activities of 23.5 and 43.4 μmol TEq mg⁻¹ in the mycelium extracts of *Fusarium sp.* and *Cladosporium cladospoides*, respectively. Furthermore, the respective work showed variations in the concentrations of phenolic compounds and antiradical activity against ABTS, DPPH, and galvinoxyl (2,6-di-tert-butyl-α-(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadiene-1-ylidene)-p-tolyloxy) radicals depending on the extraction processes and the culture medium used.

The total antioxidant activity, evaluated by the capacity to reduce molybdenum (VI) ions and form the phosphomolybdenum complex, was much higher in the extract obtained from the mycelium produced in SMM medium (147.72 mmol AAE g⁻¹, ascorbic acid equivalent per gram of mycelial biomass) compared to the mycelium extract of the SBM medium (84.81 mmol AAE g⁻¹). A higher content of lipophilic compounds may possibly be present in the biomass extract produced in the SMM medium, which contained a much higher lipid content (Table 4) than the biomass produced in the SBM medium. This condition may justify this greater reduction activity of the molybdenum (VI) ions. The phosphomolybdenum complex method can evaluate the antioxidant potential of both hydrophilic and lipophilic compounds.

The scavenging behavior of the OH radical (SMM: 84.56 % and SBM: 92.1 %) as well as the reducing power of the ferric ion (SMM: 244.74 mmol ferrous sulfate g⁻¹ and SBM: 230.81 mmol ferrous sulfate g⁻¹) were similar between the mycelium extracts. Another point to be noted is that the hydroxyl radical elimination capacity was quite high, considering a 100% elimination of such radicals by the ascorbic acid standard. Activities of 165.5 and 113.9 mmol ferrous sulfate g⁻¹ were reported in methanolic extracts of fresh *P. ostreatus* primordium and an aqueous extract of the fungus' fruiting body, respectively [44].

Table 5. Antioxidant potential of extracts obtained from the mycelial biomass of *Lasiodiplodia theobromae* MMPI cultivated on SMM and SBM.

Antioxidant assay	Antioxidant capacity	
	SMM	SBM
ABTS (mmol TEq g ⁻¹)	713.90 ^a	741.89 ^a
DPPH (mmol TEq g ⁻¹)	180.72 ^b	187.95 ^a
OH (% reduction)	84.56 ^a	92.10 ^a
FRAP (mmol ferrous sulfate g ⁻¹)	244.74 ^a	230.81 ^a
TAC (mmol AAE g ⁻¹)	147.72 ^a	84.81 ^b

SMM, Soybean Molasses Medium; SBM, Sucrose-Based Medium. Values followed by the same letter in the lines do not differ significantly at the 95% confidence level ($p \leq 0.05$).

3.6. Morphological Aspects of Mycelial Biomass and *Lasiodiplodan*

The SEM micrographs of mycelial biomass produced on SMM and SBM media (Figure 5) show interconnected hyphae forming a three-dimensional fungal mycelium structure. The SEM images suggest that the mycelium grown in the SMM medium has a more continuous and tangled hyphae network than the mycelium produced in the SBM medium. There appears to be a disruption of the fibrillar structure of the hyphae in some portions of the mycelium grown in the SBM medium.

SEM micrographs of lasiodiplodan samples produced in media containing soybean molasses and sucrose indicate that the media's composition interferes with the biopolymer's morphological structure. Images of the lasiodiplodan sample produced in SMM medium showed an irregular surface containing folds along the surface area. Another aspect that drew our attention was the presence of “white spots” along the surface area of the biopolymer obtained in the SMM medium. The “white spots” may be due to mineral ions (Ca^{++} , Mg^{++}) binding to amphiphilic proteins carrying both positive and negative charged groups, present in the lasiodiplodan sample, as proteins coprecipitate out of solution along with lasiodiplodan on ethanol treatment of the cell-free fermentation broth. The sample of lasiodiplodan from SBM had a slightly more irregular surface than the sample from SMM, with more uneven areas and a brittle appearance, but “white spots” were not present.

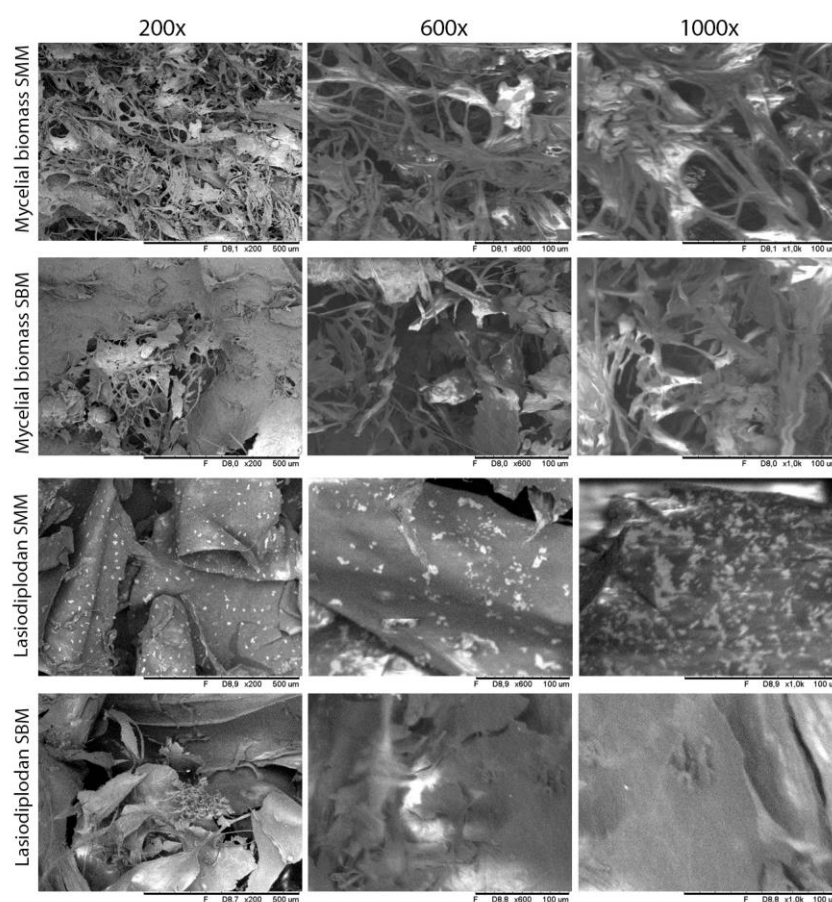


Figure 5. Scanning Electron Microscopy images of mycelial biomass and lasiodiplodan samples.

3.7. Thermal Profiles of Mycelial Biomass and Lasiodiplodan Samples

The thermal profiles of the mycelial biomass obtained from cultivation on SMM medium (Figure 6a) showed a stage of mass loss (loss of 11%) between 25 °C and 170 °C (TGA curve). The second mass loss event occurred between 170 °C and 390 °C (TGA curve) with a peak at 316 °C (DTA curve) and 54% mass loss. Subsequently, between 390 °C and 650 °C, the mass loss (32%) presented two consecutive events, with exothermic peaks at 427 °C and 549 °C on the DTA curve.

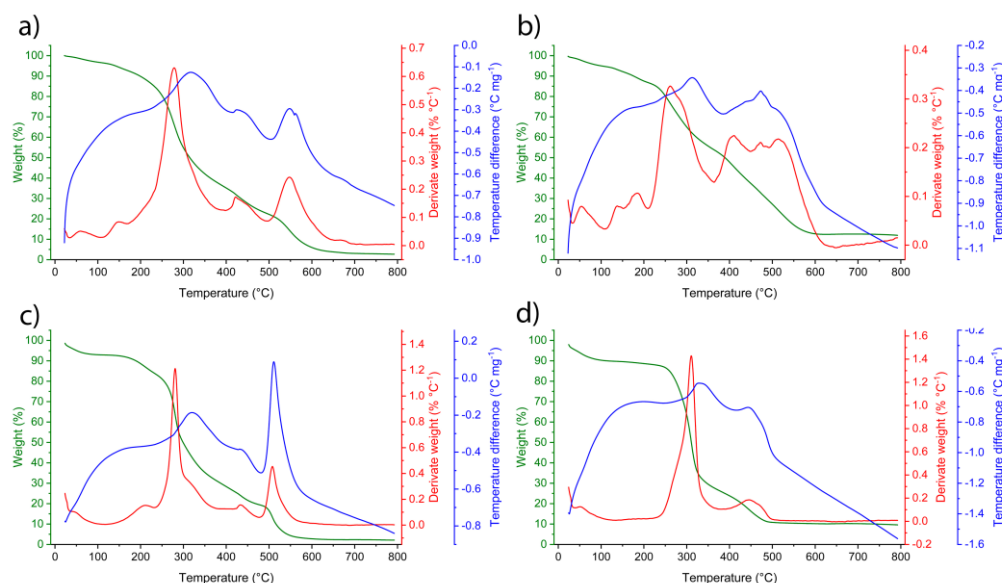


Figure 6. Thermal event profiles of the mycelial biomass produced in SMM (a) and SBM (b) and lasiodiplodan produced in SMM (c) and SBM (d). TGA (—), DTG (—), and DTA (—) curves.

The mycelial biomass obtained from cultivation in the SBM medium (Figure 6b) presented a first mass loss stage with three consecutive events (loss of 14%) between 25 °C and 210 °C (TGA curve). The second stage occurred between 210 °C and 362 °C (TGA curve) with a mass loss of 32% and an exothermic peak at 314 °C on the DTA curve. The third stage of mass loss (42%) presented three consecutive events between 362 °C and 649 °C, presenting an exothermic peak at 471 °C in DTA.

Lasiodiplodan produced in SMM medium (Figure 6c) showed four stages of mass loss. The first stage occurred up to 133 °C with a mass loss of 8% (TGA curve). This loss of mass corresponds to the elimination of water of hydration. A second mass loss occurred between 133 °C and 377 °C (TGA curve), resulting in an approximately 60% reduction in sample mass. This event is accompanied by an exothermic peak at 281 °C in the DTA curve and corresponds to the initial decomposition of the macromolecule. At higher temperatures, two consecutive mass loss events were observed between 424 °C and 600 °C, indicated by two exothermic peaks at 435 °C and 508 °C in the DTA curves. Mass loss with an exothermic peak at 435 °C can be attributed to oxidative degradation, and the last mass loss event (exothermic peak at 508 °C) corresponds to the sample's final decomposition (carbonization).

The lasiodiplodan sample produced in the SBM medium (Figure 6d) demonstrated 3 mass loss events. The first was indicated by an endothermic peak at 45 °C in the DTG curve and occurred up to around 120 °C (TGA curve), with a mass loss of 11 % (loss of hydration water). The second event corresponded to the initial decomposition of the molecule occurring from 150 °C to 373 °C (TGA curve), with an exothermic peak at 332 °C (DTA curve). At this stage, there was a loss of 63% of mass. The last event, corresponding to the final degradation of the molecule, occurred between 373 °C and 510 °C (TGA curve), with an exothermic peak in the DTA curve at 440 °C and a loss of 16% of the mass.

3.8. Infra-Red Spectroscopy

Strong intensity bands are seen in the regions of 3270 cm^{-1} (Figure 7a and Figure 7b) and 3300 cm^{-1} (Figure 7c and Figure 7d) and are attributable to -OH vibrational stretching [51]. The bands in the region of 2930 cm^{-1} are attributed to the sp^3 stretching of the methylene group, commonly present in polysaccharides (Figure 7c and Figure 7d). The appearance of this band in the mycelial biomass may indicate the presence of polysaccharides adhering to the fungal mycelium [52], or these carbohydrate biomacromolecules may exist in the fungal cell wall.

The broad band of medium intensity in the region of 2500 cm^{-1} is related to the symmetric and asymmetric stretching vibrations of skeletal CH and CH_2 in polysaccharides [53]. The band at 2160 cm^{-1} was attributed to N–H stretching vibrations of amine groups from amino acid units constituting the proteins of the mycelial biomass. The presence of this band in the lasiodiplodan samples may suggest residual protein material in the sample [54].

The bands in the regions 2029 cm^{-1} and 1967 cm^{-1} can be associated with carbon dioxide bonds, as reported in essential oil formulations microencapsulated in fructose and maltodextrin [55]. The band at 1638 cm^{-1} can be attributed to O–H bending. Bands at 1398 cm^{-1} are attributed to the symmetric and asymmetric stretching vibrations of the CH_3 groups [56], and at 1270 cm^{-1} , the stretching vibration of the C–O–C bond [57]. The band at 1029 cm^{-1} can be attributed to the C–O–C stretching vibration, which occurs in the pyranose ring [58] present in the monomers that make up lasiodiplodan (Figure 7c and Figure 7d). This band also appears in the spectra of mycelial biomass (Figure 7a and Figure 7b), and can be associated with lasiodiplodan that adhered to the mycelial biomass, as well as the presence of glucans from the fungal cell wall. The low-intensity band in the 880 cm^{-1} region was attributed to the β -configuration present in the glycosidic bond of lasiodiplodan [59].

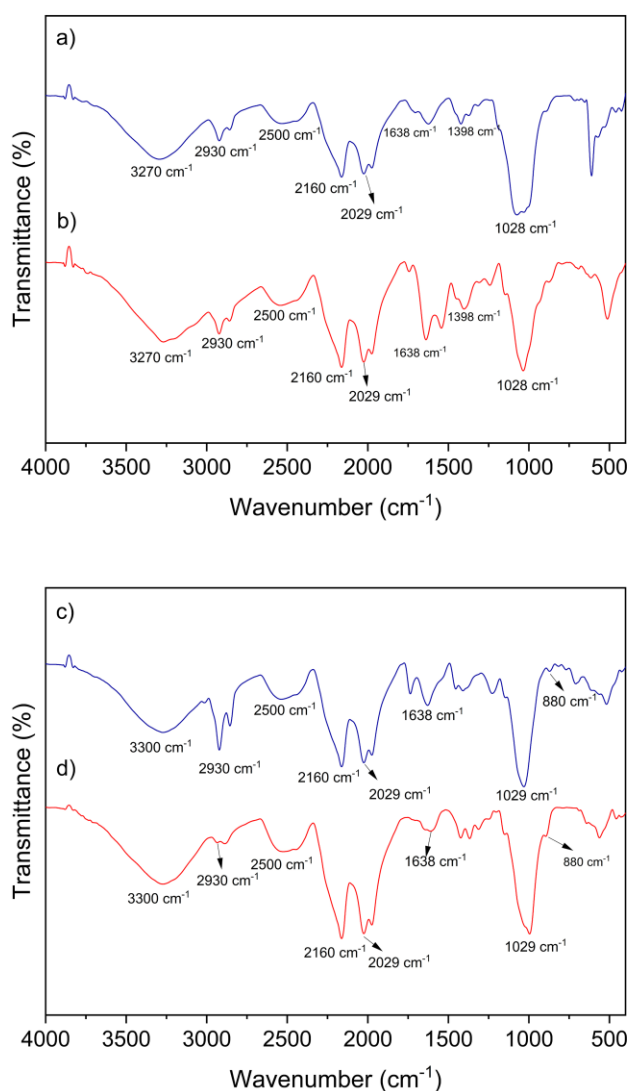


Figure 7. Infrared Attenuated total reflection spectra of mycelial biomass produced on (a) SMM and (b) SBM, and lasiodiplodan produced on (c) SMM and (d) SBM.

3.9. Diffractometric Profile of Mycelial Biomass and Lasiodiplodan Samples

The mycelial biomass samples produced in SMM and SBM media (Figure 8a and Figure 8b), as well as the lasiodiplodan produced in the soybean molasses-based medium (Figure 8c), demonstrated an essentially amorphous profile, indicated by a broad peak at 19° (2θ). Arumprasath et al. [60] described a similar profile for the mycelium of a strain of the fungus *Lasiodiplodia* sp. The lasiodiplodan sample produced in the sucrose-based medium showed regions with a certain degree of molecular organization (crystallinity), indicated by the peaks at 31° , 44° , and 56° (2θ). This characteristic suggests that the culture medium influenced the molecular organization of the (1 $\rightarrow$ 6)- β -glucan produced. Luna et al. [59] found peaks at 20.9° , 23.4° and 39.4° at 2θ in a lasiodiplodan sample produced in a synthetic medium based on glucose.

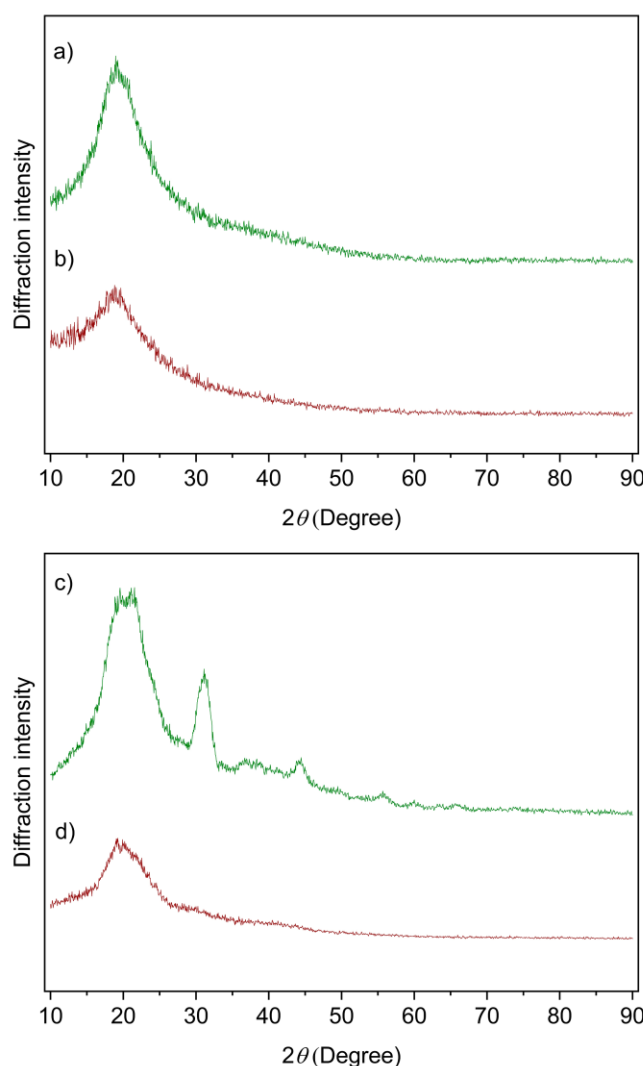


Figure 8. X-ray diffraction profiles of mycelial biomass produced on (a) SMM and (b) SBM, and lasiodiplodan produced on (c) SMM and (d) SBM.

5. Conclusions

Bioprospecting of the filamentous fungus *Lasiodiplodia theobromae* MMPI demonstrated good prospects for industrial applications, considering the high market value of β -glucans and the potential use of fungal biomass as an alternative source of protein. Media formulated with sucrose and the agro-industrial by-product, soybean molasses, showed potential for the integrated production of mycelial biomass (mycoprotein) and extracellular β -glucan (lasiodiplodan). Soybean molasses demonstrated greater potential for mycelial biomass production, whereas sucrose

contributed to higher lasiodiplodan production. The conditions that promoted the highest mycelial growth were 64.43 g L⁻¹ of total sugars, 8.81 % rice bran extract, and 152.83 rpm agitation in a soybean molasses-based medium. Under these cultivation conditions, an estimated production of 12.44 g L⁻¹ of mycelial biomass and 0.573 g L⁻¹ of lasiodiplodan was achieved. The culture medium's composition greatly influenced the biomass's nutritional quality. Mycelial biomass showed an appreciable protein content, containing all essential amino acids, but with the exception of histidine in the biomass produced in the sucrose medium. Soybean molasses promoted the production of fungal biomass rich in lipids, mainly unsaturated fatty acids, with notable contents of polyunsaturated fatty acids such as gamma-linolenic and alpha-linolenic acids. Low total phenolic contents were found in the biomass, yet their ethanolic extractives showed considerable antioxidant potential against ABTS, DPPH and hydroxyl radicals, and ferric and molybdenum VI ion-reducing power. Gallic acid and catechin were the major compounds identified in the extracts. Both types of mycelial biomass demonstrated their nutritional composition suggesting they can be used as ingredients in animal feed formulations. The mycelial biomass showed good thermal stability. Furthermore, this work described the fungus *L. theobromae* MMPI for the first time as an oleaginous microorganism cultivated on soybean molasses, opening perspectives for bioprospecting in biofuels and fatty acids of high market value.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: ANOVA and estimated main effects for mycelial biomass production in SMM medium, Table S2: ANOVA and estimated main effects for mycelial biomass production in SBM medium, Table S3: ANOVA and estimated main effects for yield in mycelial biomass in SMM medium, Table S4: ANOVA and estimated main effects for yield in mycelial biomass in SBM medium, Table S5: ANOVA and estimated main effects for lasiodiplodan production in SMM medium, Table S6: ANOVA and estimated main effects for yield in lasiodiplodan SMM medium, Table S7: ANOVA and estimated main effects for lasiodiplodan production in SBM medium, Table S8: ANOVA and estimated main effects for yield in lasiodiplodan in SBM medium.

Author Contributions: **Marcelo Luis K. Marchioro:** Writing - review & editing, Writing - original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gabrielli Aline Pietro Bom Candeia:** Investigation, Formal analysis. **Luana Malaquias Bertoleti:** Investigation, Formal analysis. **Aneli de Mello Barbosa-Dekker:** Data curation, Conceptualization. **Robert F. H. Dekker:** Data curation, Writing - review & editing, Writing - original draft. **Mário A. A. Cunha:** Writing - review & editing, Writing - original draft, Funding acquisition, Conceptualization, Supervision, Resources.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

The following abbreviations are used in this manuscript:

SMM	Soybean Molasses Medium
SBM	Sucrose Based Medium
RBE	Rice Bran Extract

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