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Comparative Highly Efficient Production of β-glucan by *Lasiodiplodia theobromae CCT 3966* and Its Multiscale Characterization

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Abstract: Lasiodiplodan, a (1→6)-β-d-glucan, is an exopolysaccharide with high commercial value and many applications in food, pharmaceuticals, and cosmetics. Current industrial production of β-glucans from crops is mostly by chemical routes generating hazardous and toxic waste. Therefore, alternative sustainable and eco-friendly pathways are highly desirable. Here, we have studied the lasiodiplodan production from sugarcane bagasse (SCB), a major lignocellulosic agricultural residue, by *Lasiodiplodia theobromae* CCT 3966. Lasiodiplodan accumulated on SCB hydrolysate (carbon source) supplemented with soybean bran or rice bran (nitrogen source) was 16.2 [6.8×10^3 Da] and 22.0 [7.6×10^3 Da] g/L, respectively. Lasiodiplodan showed high purity, low solubility, pseudoplastic behavior and was composed of glucose units. Moreover, the exopolysaccharides were substantially amorphous with moderate thermal stability and similar degradation temperatures. To our knowledge, this is the first report on the highest production of SCB-based lasiodiplodan to date. *L. theobromae*, as a microbial cell factory, demonstrated the commercial potential for the sustainable production of lasiodiplodan from renewable biomass feedstock.

Keywords: sugarcane bagasse; rice bran; soybean bran; optimization; *Lasiodiplodia theobromae*; lasiodiplodan

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1. Introduction

The excessive production and use of certain hazardous wastes within the industrial-chemical sectors have generated a growing public concern over their notorious impact on health and environmental well-being. Therefore, the market for sustainable natural products through a greener route has drawn tremendous attention from producers and consumers in the last decade [1]. β-glucans are natural polysaccharides produced from bacteria, fungi, algae, and cereals, which have significantly grown in industrial demand in recent years due to their extensive physical and biological properties [2]. The biological functions of β-glucans are directly related to their source and structural characteristics, such as branching frequency, solubility, molecular weight, polymer charge, and conformation in solution [3].

Currently, the global β-glucan market is dominated by traditional cereal grains like oats, barley, wheat, and rye [4,5]. However, the exploitation of edible crops for this process challenges global food security. Furthermore, industrial β-glucan extraction is based predominantly on chemical methods (bases, acids, and solvents) and consequently, it could

generate toxic and harmful residues [6–8]. Likewise, the extraction method selected can interfere with long extraction time, low yields, reduced purity, high process costs, and lower environmental sustainability [5].

Microbial β-glucan production is a potential alternative, with the main advantages including sustainability, a high degree of purity, prominent production efficiency, rapid microbial growth, and independence from seasonal factors [8]. Lasiodiplodan is a β-glucan-type exopolysaccharide with great commercial potential considering its versatile biological functions such as antimicrobial, antioxidant, anticoagulant, protective activity against induced DNA damage by doxorubicin, hypoglycemic and transaminase activities, and other properties [9]. It is a homopolysaccharide composed of glucose monomers with β-(1→6) linkages in an unbranched linear chain [10]. Lasiodiplodan synthesized extracellularly in the culture medium, mainly glucose and sucrose, by the filamentous fungus *L. theobromae,* is essential for its easy extraction and recovery [11]. *L. theobromae* exhibit asexual reproduction through mycelial growth in submerged fermentation or sporulation induced to stimulate conidia formation by specific nutritional media like potato dextrose agar (PDA) [12].

Like any other fermentative bioproduct, the production of lasiodiplodan also depends on the type of microorganism used and the culture conditions, among others [13]. Biotechnological lasiodiplodan production can cater to its growing demand in society. Nonetheless, the low production levels of lasiodiplodan through a microbial route is one of the bottlenecks for its commercial manufacturing [9,10,14–16]. Sugarcane bagasse (SCB) is a low-cost renewable carbon source primarily used for energy and steam generation in sugar mills and distillery [17]. However, there is still a sizeable amount of SCB available for biofuels and biochemical production. Due to the presence of a high amount of carbohydrates, SCB represents a sustainable carbon source for the microbial production of lasiodiplodan.

Hence, the current study was undertaken to enhance the production levels of lasiodiplodan and, at the same time, to make the bioprocess economical. To this end, the bioproduction of lasiodiplodan was attempted from crude renewable carbon (glucose-rich hydrolysate from SCB) and nitrogen sources (rice bran extract (RBE); soybean bran extract (SBE)), followed by the optimization of culture conditions and the characterization of the accumulated biopolymer.

To the best of our knowledge, this is not only the highest production of lasiodiplodan reported to date but also the first demonstration of C/N ratio optimization from sustainable sources for microbial β-glucan production. Moreover, the lasiodiplodan obtained from the optimized culture media were recovered, quantified, and characterized by various methods. Our findings suggest that *L. theobromae* CCT 3966 possesses the potential as an integrated platform for the green production of microbial β-glucan and represents a good target for biotechnological applications.

2. Materials and Methods

2.1. Raw Materials and Microorganism

The raw materials were previously air-dried until reaching 7% moisture. They were stored in plastic bags and kept at 4 °C. SCB supplied by the Ipiranga Agroindustrial plant (Descalvado, Brazil) was milled and sieved through a 20 mesh (0.841 mm). Rice and soybean bran were purchased from the Cooperativa de Laticínios Serramar-Lorena/SP and sieved through a 45 mesh (0.35 mm).

Lasiodiplodia theobromae CCT 3966 was kindly provided by the Fundação André Tosello Pesquisa e Tecnologia (Campinas, Brazil). The strain was sub-cultured in Petri dishes containing YMG agar (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1.0% glucose, and 2.0% agar) pH 5.5 at 28 °C for 6 days and stored at 4 °C [16]. In this study, the red pigment accumulation in the mycelia determined the fungal cell viability as the indicator for lasiodiplodan production.

2.2. Carbon Source: Sugarcane Bagasse Cellulosic Hydrolysate

Sugarcane bagasse cellulosic hydrolysate (SCBCH) was obtained from the saccharification of SCB pretreated sequentially with dilute acid-alkali according to Ascencio et al. [18]. Sequential diluted nitric acid-sodium hydroxide pretreatment was conducted in an autoclave under the following conditions: 1% (*w/v*), HNO_3 1% (*w/v*), NaOH, and 10% of dry biomass at 121 °C for 30 min. Enzymatic hydrolysis of the cellulosic pulp was carried out in a wet state to eliminate the hornification effect. Moisture was determined to consider the final water content. The experimental conditions were: 10% of cellulosic pulp, sodium citrate buffer (0.05 M, pH 5.5), 15 Filter Paper Units (FPU)/g dried cellulosic pulp using cellulase enzyme Cellic® CTec2 at 50 °C, and 200× rpm for 5 days. The SCBCH sugars were collected in the liquid phase and analyzed by HPLC.

2.3. Nitrogen Sources: Rice and Soybean Bran Extracts

Rice bran and soybean bran were auto-hydrolyzed separately in 1 L Erlenmeyer flasks using 200 g/L of dry biomass at 121 °C for 15 min [19]. Protein extracts were collected in the liquid phase and transferred to sterilize flasks. Total protein [20] and reducing sugar [21] were determined by colorimetric methods.

2.4. Media and Cultivation Conditions for Lasiodiplodan Production

Fermentation experiments evaluated RBE and SBE in high concentrations (10 g/L) as alternative nitrogen sources for lasiodiplodan production in 125 mL Erlenmeyer flasks [13]. Firstly, 50 mL of synthetic glucose medium (40 g/L glucose; 10 g/L RBE or SBE) and Vogel's Minimal Salts Medium (VMSM) (40 g/L glucose; 20 mL/L VMSM) were used as a comparison control previously optimized [16,22]. Secondly, 50 mL of alternative medium (40 g/L SCBCH; 10 g/L RBE or SBE) was used. Two agar discs (0.7 cm diameter) of *L. theobromae* CCT 3966 with red pigment secretion by the mycelia were used as inoculum. All flasks were kept in an orbital shaker at 28 °C, 200× rpm for 96 h. Samples were collected at periodic intervals to monitor sugar and protein consumption, fungal biomass, and lasiodiplodan production.

2.4.1. Lasiodiplodan and Fungal Biomass Quantification

Lasiodiplodan and fungal biomass were quantified using gravimetric analysis by centrifugation and washing two-fold at 60 °C for 24 h in an air circulation oven until a constant weight was achieved [16]. Fermented broth supernatant (5 mL) was precipitated with 15 mL of chilled absolute ethanol (−20 °C). The lasiodiplodan precipitated was dissolved in 5 mL of deionized water preheated at 60 °C to remove the fungal biomass and/or precipitated protein from the nitrogen supplementation. Then, it was re-precipitated with 15 mL of chilled absolute ethanol. Finally, to eliminate the culture medium and lasiodiplodan traces, the fungal biomass pellet was washed twice with 50 mL of deionized water (60 °C).

2.4.2. Sugars Quantification

Monomeric sugars were analyzed by HPLC (Agilent 1200 series, Santa Clara, CA, USA) equipped with an Aminex HPX-87H Column by Bio-Rad (Hercules, CA, USA) (300 × 7.8 mm) [18]. Prior to carrying out the HPLC analyses, samples were filtered through a Sep Pak C18 filter (Waters Corporation, Milford, MA, USA), and 20 µL of a sample was injected into the column at 45 °C. The column was eluted with 0.01 N sulfuric acid at 0.6 mL/min. The eluents monitored by a RID-6A refractive index detector allowed the calculated concentrations by peak area to be compared to known standards.

2.5. Central Composite Rotational Design (CCRD): Interplay of Carbon and Nitrogen Sources

2^2 CCRD investigated the interactive impact of carbon (X_1) and nitrogen (X_2) sources on improving lasiodiplodan production and was developed by Design Expert v. 7.0.0 [16]. The chosen variables are the main nutritional components of the culture media and improve mycelial growth and exopolysaccharide production [13]. In this study, the selected

independent variables X_1 (40–80 g/L) and X_2 (0.5–4 g/L) were evaluated at five different levels (Table S1 in the Supplementary Materials). The center points contain three repetitions, while the axial points were unperformed. The response variables were the production and yield of lasiodiplodan. Equations (S1) and (S2) dimensionalized the carbon and nitrogen concentrations, and Equations (S3) and (S4) estimated lasiodiplodan yield at 96 and 120 h, respectively (Supplementary Materials).

A symbolic regression was performed by the genetic programming using Eureqa Formulize v. 1.2.4.0 software (Nutonian, Inc., Boston, MA, USA) [23]. Four fit models were selected according to the maximum coefficient of determination ($R^2 \geq 0.99$). Statistical analysis of the fit models was carried out by the *p*-value, *F*-test, lack of fit, and analysis of variance (ANOVA) to demonstrate the overall model significance. Response surface graphs were plotted by Wolfram Mathematica v. 12.0.0.0 software to understand the improved production behavior of lasiodiplodan. Prediction of the models was confirmed by experimental fermentation. In addition, the kinetic parameters were calculated at 120 h using Equations (S5)–(S11) (Supplementary Materials).

2.6. Multiscale Characterization of Lasiodiplodan

2.6.1. Purity and Solubility Properties

Crude lasiodiplodan was dissolved in deionized water and dialyzed using a Spectrum Spectra/Por™ (Fisher Scientific, Gardena, CA, USA) dialysis membrane tubing (12,000 to 14,000 Da) [24]. Later, purity and chemical composition were analyzed using 20 mg of lasiodiplodan as described by Philippini et al. [16]. Pure glucose was used as a control to assess possible sugar degradation. The identification of Monosaccharides was carried out by HPLC. Total sugar [25] and protein [20] were analyzed.

Lasiodiplodan solubility in water used 10 mg of sample in 10 mL of deionized water, stirred for 24 h at 25 °C [14]. After centrifuging, total sugars in the supernatant expressed a percentage (%) of soluble mass in 100 mL of deionized water (mg of sample/100 mL).

2.6.2. Viscosity and Molecular Weight Analysis

Lasiodiplodan apparent viscosity (2 g/L) was determined using a Rheocalc T1.2.19 viscometer (Brookfield Engineering Labs Inc., Middleboro, MA, USA) [11]. This equipment was equipped with an SC4-18 spindle that operates at cutting speeds within the range from 1 to 264/s at 25 °C. Measurements were recorded at 5 s intervals, increasing the shear speed from 1 to 264/s.

The molecular weight of lasiodiplodan was determined by Gel Permeation Chromatography (GPC) using a Superose 12 10/300 GL column (GE Healthcare, Chicago, IL, USA) with NaOH (pH 12) as an eluent at 0.6 mL/min [26]. Lasiodiplodan molecular weight was determined using a standard curve based on known molecular weights. A group of standards, xylose, raffinose, xylohexose, dextran T1, dextran, dextran T6, dextran T10, cytochrome C, carbonic anhydrase, albumin, alcohol dehydrogenase, and blue dextran (150.13; 504.42; 810.7; 1000; 3500; 6000; 10,000; 12,384; 29,000; 66,000; 1,500,000; and 2,000,000 Da) were applied to calibrate the column and eluted at the same conditions.

2.6.3. Scanning Electron Microscopy (SEM)

SEM was used to examine the surface morphology of lyophilized lasiodiplodan. Micrographs were taken using an electron detector at 50× and 1200× (Hitachi TM3000, Chiyoda, TYO, Japan).

2.6.4. Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR characterization of lasiodiplodan was recorded using a Perkin Elmer® Spectrum™ GX (Shelton, CT, USA) with a spectral range between 4000 and 400 at 4 cm^{-1}. The sample pellet was prepared using 1.5 mg of lasiodiplodan mixed with 250 mg of KBr powder and dried at 50 °C under reduced pressure before analyzing.

2.6.5. Differential Scanning Calorimetry (DSC)

The analysis was recorded using a DSC Q20 V24.11 (New Castle, DE, USA). Lasiodiplodan was scanned from 30 to 500 °C at 10 °C/min under synthetic air atmosphere as eluent using a flow of 50 mL/min.

2.6.6. X-ray Diffraction (XRD)

An Empyrean diffractometer (Malvern Panalytical, Malvern, Worcestershire, UK) with MoK–α radiation [λ = 0.7107 Å] was used to determine the XRD lasiodiplodan diffractogram. The measurement conditions used the angular range [20] from 8 to 60°, 0.02° step size, and 70 s/step counting.

2.7. Statistical Analysis

Data related to fermentation and lasiodiplodan characterization are represented in terms of average ± standard deviation less than 10%. The experiments were run in triplicate and the values were averaged. The different letters in the graphs and tables denote difference significance ($p \leq 0.05$). In addition, the ANOVA and Tukey's test were used jointly at the 95% confidence level.

3. Results and Discussion

3.1. Importance of Alternative Nitrogen Sources on Lasiodiplodan Production

Figure 1 shows the profile of lasiodiplodan production and *L. theobromae* growth during solid-state and submerged fermentation using glucose. Notably, the reducing sugar content was low having 0.9 g/L in the SBE and 4.0 g/L in RBE, for the culture media preparation.

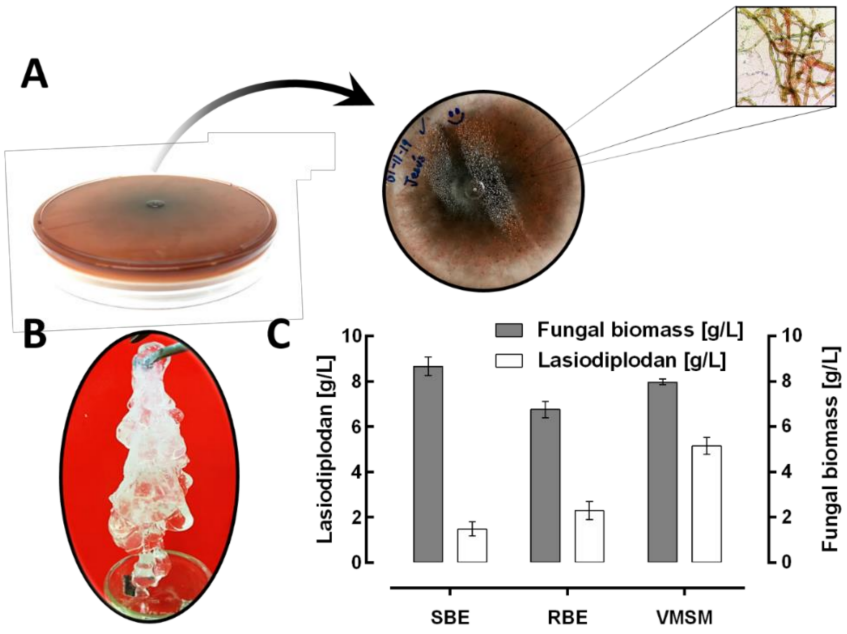

Figure 1. Lasiodiplodan production by *L. theobromae* CCT 3966: (**A**) Fungal growth on YMG agar exhibiting red pigmentation; (**B**) Lasiodiplodan recovered after fermentation; (**C**) Impact of crude nitrogen sources on fungal biomass and lasiodiplodan accumulation using synthetic glucose medium.

The fungal mycelia produce red pigmentation in solid-state fermentation (Figure 1A). This red pigmentation could be due to low moisture content stress, and consequently, a lower solubility of nutrients and increased gas exchange [27]. Likewise, the red pigment in the strain used as a crucial strategy to increase lasiodiplodan production (Figure 1B) stopped during submerged fermentation. Throughout the submerged fermentation, SBE

and RBE, as nitrogen sources, were feasible for lasiodiplodan production (Figure 1C). Their use showed 1.5 g/L and 2.2 g/L lasiodiplodan, respectively. In addition, the synthetic glucose medium supplemented with SBE allowed higher fungal biomass of 8.7 g/L versus RBE supplementation of 6.8 g/L. In most cases, a high nitrogen concentration, more than 10 g/L, can drastically reduce exopolysaccharide production [13]. However, RBE and SBE proved to be suitable for lasiodiplodan accumulation, even at high nitrogen concentrations.

Cunha et al. [11] determined yeast extract (2 g/L) as the best nitrogen source, between organic and inorganic, and reported 2.46 g/L of lasiodiplodan. Naturally, RBE and SBE are complex nitrogen sources, similar to yeast extract, i.e., carbon, sulfur, protein, amino acids, minerals, vitamins, and other essential growth factors for various microorganisms [28,29]. Alternatively, the VMSM control experiment resulted in 5.21 g/L of lasiodiplodan and 7.97 g/L of fungal biomass. Similar results, 5.25 g/L lasiodiplodan, 8.40 g/L fungal biomass, were reported using the same fungal strain under optimized conditions [16].

The use of synthetic glucose medium and RBE/SBE led to higher fungal biomass production similar to the control experiment, although the accumulation of lasiodiplodan was lower (Figure 1C). Hence, lasiodiplodan production is not highly dependent on the fungi amount but rather on the nutritional conditions. The chemical nature and concentration of carbon and nitrogen sources can induce catabolic repression during the secondary metabolism of filamentous fungi [30]. Catabolic repression regulated by nutrients, metals, growth rate, and enzymatic control could produce fungal mycelia, necessary for secondary metabolites, such as exopolysaccharides [31].

Hitherto our attention has been given to RBE and SBE as alternative nitrogen for lasiodiplodan production. However, RBE and SBE also represent a good source of trace elements and vitamins for microorganisms. Therefore, it is also imperative to properly evaluate lignocellulosic hydrolysates utilization and establish the optimal carbon and nitrogen concentrations for the sustainable production of lasiodiplodan.

3.2. Impact of Lignocellulosic Carbon Source on Lasiodiplodan Production

Lignocellulosic biomass is the abundant and renewable carbon source in second-generation biorefineries for renewable chemicals, materials, and fuels production [17]. In this experiment, glucose was replaced with SCBCH (sole carbon source) using RBE and SBE, and the potential of *L. theobromae* to produce lasiodiplodan was investigated (Figure 2). Cellulose constitutes the maximum fraction of SCB, which upon depolymerization yields glucose only, which is highly attractive for obtaining various value-added compounds through microbial routes [18]. HPLC analysis of SCBCH overwhelmingly showed 100 g/L glucose, while other sugars and inhibitors such as acetate, HMF, furfural, etc., were absent. Acid-alkali pretreatment removed the whole hemicellulose and a significant lignin fraction [18]. Consequently, the clean sugar hydrolysate is due to the sequential pretreatment applied to the SCB.

Figure 2 shows a 24 h lag phase was witnessed, followed by a rapid glucose assimilation concomitant with biomass growth. More than 90% glucose was exhausted within 72 h, and there was a marginal improvement in fungal biomass beyond this time. Lasiodiplodan accumulation began at 48 h when fungal biomass reached the stationary phase, and after 72 h, it became almost constant beyond this period.

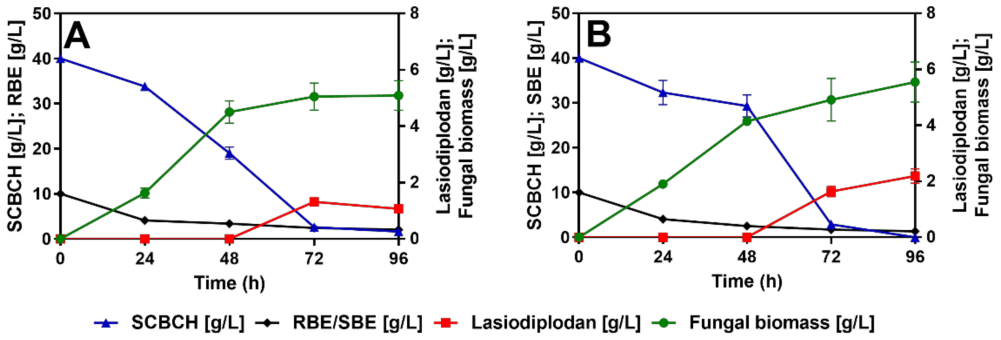

Figure 2. Impact of the lignocellulosic carbon source on the bioproduction of lasiodiplodan and fungal biomass by *L. theobromae*: (**A**) SCBCH/RBE medium; (**B**) SCBCH/SBE medium.

With all things considered, SCBCH/RBE contributed positively to obtain the maximum lasiodiplodan production of 1.31 g/L and 5.05 g/L fungal biomass at 72 h (Figure 2A). Nonetheless, lasiodiplodan production suffered a sudden decrease of 18.98% at 96 h, while the fungal biomass remained constant at 5.08 g/L. This finding suggests that lasiodiplodan, composed of glucose units, served as a carbon reservoir for fungal growth after the exhaustion of supplied glucose. Felix et al. [32] showed that *L. theobromae* cultivated at 25 and 30 °C has a significantly endoglucanolytic activity. Therefore, enzymes could be responsible for hydrolyzing the β-glycosidic bonds on the polymer backbone to release glucose for fungal growth. Unlike SCBCH/RBE, the SCBCH/SBE utilization produced 2.19 g/L of lasiodiplodan and 5.54 g/L of fungal biomass at 96 h (Figure 2B). Although the fermentation concluded at 96 h, Figure 2B shows that the concomitant fungal biomass and lasiodiplodan production was yet uncompleted. Thereby, we examined the following optimization experiments up to 120 h. There is no metabolic pathway described in the literature for lasiodiplodan production by *L. theobromae*. However, more than once, the established relationship between fungal growth and lasiodiplodan synthesis indicated lasiodiplodan as a non-growth-associated product.

3.3. Carbon and Nitrogen Optimization Through CCRD and Genetic Programming

The efficient use of low-cost LCB in a bioprocess ensures its success and increases economic viability. Further, the optimization of culture media can enhance product yield, minimize by-products, and reduce other production costs [33]. In this study, 2^2 CCRD was used to determine the optimal concentration of SCBCH (carbon abundance) and RBE/SBE (nitrogen-limiting) to increase lasiodiplodan production by *L. theobromae*. Tables S2 and S3 in the Supplementary Materials summarize the experimental results using SCBCH and RBE/SBE. Symbolic regressions by genetic programming were used to generate mathematical expressions that fit the entire dataset. As shown in Eq. A12–A15, four mathematical models were meticulously constructed for the successful production of lasiodiplodan (Supplementary Materials). Response surfaces were plotted for each model, considering the production and yield of lasiodiplodan at 96 and 120 h (Figures 3 and 4).

Figure 3 depicts the production and yields of lasiodiplodan varying the concentrations of SCBCH and RBE at 96 and 120 h of fermentation. Lasiodiplodan production was 21.54 g/L ($Y_{P/S}$: 0.26 g/g) at 96 h, but the most efficient production was 25 g/L ($Y_{P/S}$: 0.32 g/g) at 120 h. The titers of lasiodiplodan reported corresponding to the middle SCBCH (60 g/L) and high RBE (4 g/L) concentrations. In contrast, Figure 4 presents the same information as Figure 3 but using SCBCH and SBE. As follows, this culture medium led to the maximal lasiodiplodan production of 13.92 g/L ($Y_{P/S}$: 0.14 g/g) at 96 h and 10.94 g/L ($Y_{P/S}$: 0.11 g/g) at 120 h. For this purpose, the response surfaces showed a low SCBCH [45.86 g/L] and low SBE [1.01 g/L] concentrations to reach these titers of lasiodiplodan.

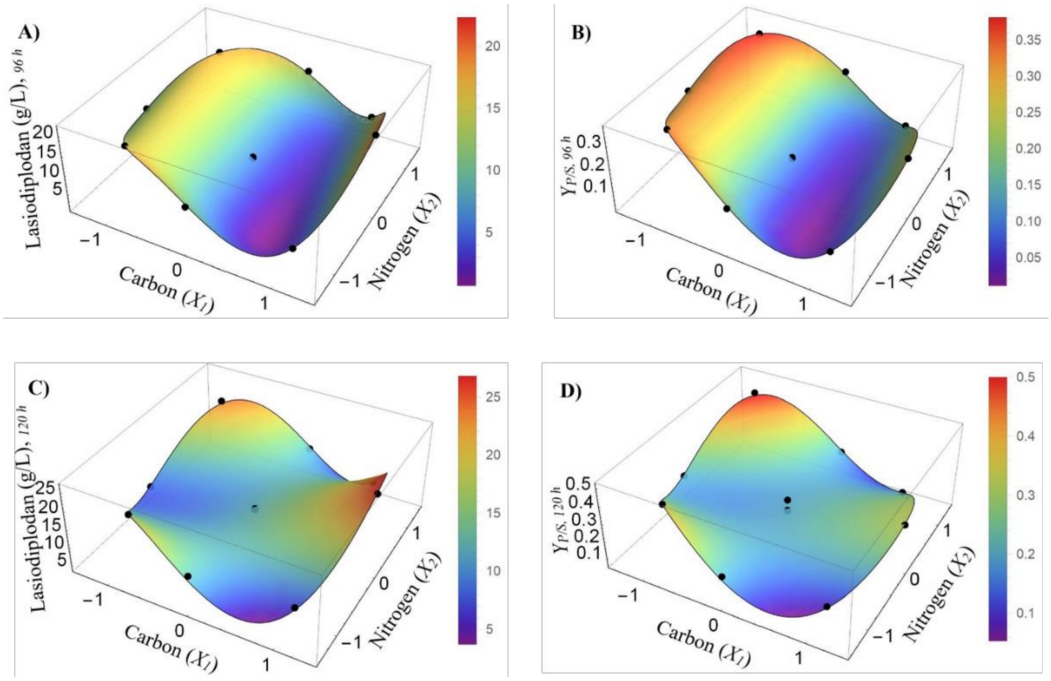

Figure 3. SCBCH and RBE interaction for lasiodiplodan production: (**A**) Production at 96 h; (**B**) Yield at 96 h; (**C**) Production at 120 h; (**D**) Yield at 120 h.

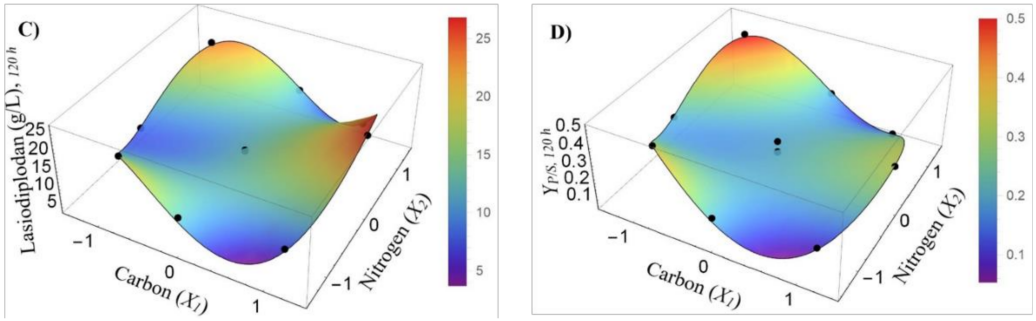

Figure 4. SCBCH and SBE interaction for lasiodiplodan production: (**A**) Production at 96 h; (**B**) Yield at 96 h; (**C**) Production at 120 h; (**D**) Yield at 120 h.

All individual terms in the models appropriately represent the interactions between carbon and nitrogen, showing a high significance with a 95% confidence level ($p < 0.05$), as shown in Table S4. In this way, Table S5 shows the statistical ANOVA of the mathematical models. Regressions of the models have a high statistical significance with a 95% confidence level according to the *F*-test ($p < 0.05$) and the lack of fit insignificant ($p$lf > 0.05). Likewise, the models represent more than 99.8% of the variation ($R^2 = 0.998$), indicating an excellent fit quality to experimental data. Similarly, Gomes et al. [23] built optimization models that exhibited minimal relative error using genetic programming by Eureqa. The optimization of the models considered the yields through the *NMinimize* function of Wolfram Mathematica because of the exceeding cost of commercial cellulase used in enzymatic hydrolysis [17]. Carbon and nitrogen optimized (46.2 g/L SCBCH, 3.5 g/L RBE; 43.2 g/L SCBCH, 1.3 g/L SBE) for lasiodiplodan accumulation were better at 120 h for lasiodiplodan accumulation. The predicted production and yield of lasiodiplodan were 23.4 g/L ($Y_{P/S}$: 0.50 g/g) for SCBCH/RBE and 12.3 g/L ($Y_{P/S}$: 0.28 g/g) for SCBCH/RBE.

3.4. Lasiodiplodan Production Using the Optimal Carbon and Nitrogen Concentration

This experiment validated the optimized carbon and nitrogen for lasiodiplodan accumulation by CCT 3966 strain and its kinetic parameter profile up to 120 h. Figure 5 demonstrated that lasiodiplodan production started quickly after 24 h for both conditions (SCBCH and RBE/SBE). Moreover, the considerable carbon and protein uptake were recorded at 96 h, while a high production of lasiodiplodan and fungal biomass was achieved at 120 h.

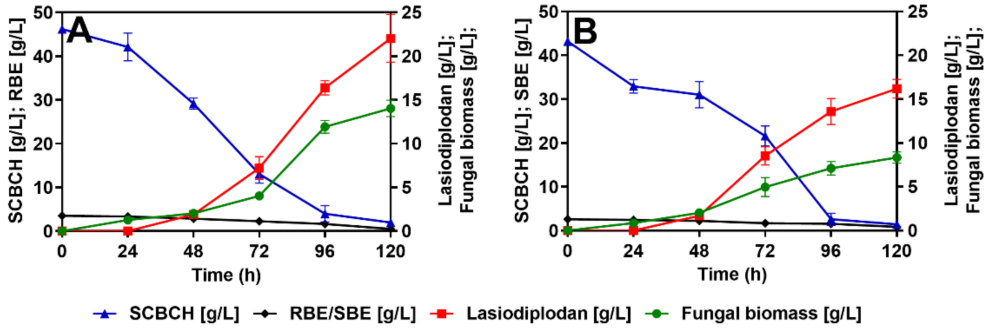

Figure 5. Production of lasiodiplodan and fungal biomass by *L. theobromae* under optimized culture media: (**A**) SCBCH/RBE culture medium; (**B**) SCBCH/SBE culture medium.

Thus, the optimized conditions of SCBCH/RBE led to the maximal production of 22.0 g/L lasiodiplodan ($Y_{P/S}$: 0.49 g/g) along with 14.03 g/L of fungal biomass ($Y_{X/S}$: 0.31 g/g) (Figure 5A). This lasiodiplodan titer is the highest reported, equivalent to 2.3-fold compared to the best from a synthetic glucose medium [14]. Fermentation ended using 95.6% glucose contained in SCBCH and 85.6% protein contained in RBE. Alternatively, the SCBCH/SBE medium showed similar behavior to Figure 5A but with less production of lasiodiplodan (Figure 5B). The maximum lasiodiplodan accumulation reached was 16.20 g/L ($Y_{P/S}$: 0.38 g/g) with 8.34 g/L fungal biomass ($Y_{X/S}$: 0.19 g/g). For this, uptake was 96.5% glucose (SCBCH) and 66.7% protein (RBE).

Unquestionably, the low nitrogen concentration significantly favors lasiodiplodan. Several studies have shown that nitrogen-limiting is a critical factor for exopolysaccharide production [13]. The experimental results correctly validated the models built through genetic programming. Furthermore, fermentation parameters demonstrated a significant enhancement in profitable lasiodiplodan production compared to other alternative carbon sources, as shown in Table 1.

Table 1. Comparison of fermentative parameters for lasiodiplodan production on various carbon sources.

Kinetic Parameter	SCBCH/RBE Medium [1]	SCBCH/SBE Medium [1]	Soybean Molasses [2]	Corn Bran Hydrolysate [3]	Glucose Medium [3]
P (g/L)	22.03	16.20	1.06	5.73	5.25
X (g/L)	14.03	8.34	8.40	4.25	8.40
$Y_{P/S}$ (g/g)	0.49	0.38	0.13	0.14	0.13
$Y_{X/S}$ (g/g)	0.31	0.19	0.93	0.10	0.21
$Y_{X/P}$ (g/g)	0.63	0.51	0.13	0.13	0.63
Q_P (g/L.h)	0.18	0.13	0.01	0.06	0.05
Q_X (g/L.h)	0.11	0.06	0.11	0.04	0.09
Q_S (g/L.h)	0.36	0.34	0.12	0.44	0.41
Y_C (%)	95.6	96.6	87.4	84.8	99.8

P, lasiodiplodan; X, fungal biomass; $Y_{P/S}$, lasiodiplodan yield; $Y_{X/S}$, fungal biomass yield; $Y_{X/P}$, specific yield; Q_P, volumetric productivity of lasiodiplodan formation; Q_X, volumetric productivity of fungal biomass growth; Q_S, volumetric productivity of substrate consumption; Y_C, percentage consumption of total substrate. [1] Lasiodiplodan from this study. There is no statistical difference according to the Tukey test (95% confidence level); [2] Lasiodiplodan from soybean molasses [9]; [3] Lasiodiplodan from corn bran hydrolysate and synthetic glucose medium [16].

3.5. Multiscale Characterization of Lasiodiplodan from Sustainable Process

3.5.1. Physicochemical Properties Analysis

The biopolymer purity is essential, to know the total sugar content, and the presence of impurities, i.e., proteins, lipids, aromatics, nucleic acids, minerals, etc., [29]. Table 2 summarizes the physicochemical properties found for lasiodiplodan samples.

Table 2. Physicochemical characterization of lasiodiplodan from SCBCH using RBE/SBE.

Parameter	Lasiodiplodan [1]	Lasiodiplodan [2]	Glucose [3]
Total sugar (%, *w/w*)	93.75 ± 0.01	80.11 ± 0.01	100.09 ± 0.05
Glucose (%, *w/w*)	94.20 ± 0.05	80.40 ± 0.04	100.12 ± 0.04
Total protein (%, *w/w*)	4.56 ± 0.01	15.25 ± 0.01	ND
Solubility (%)	16.81 ± 0.01	4.53 ± 0.03	ND
Monosaccharide composition	Glucose	Glucose	Glucose

[1] Lasiodiplodan from SCBCH/RBE; [2] Lasiodiplodan from SCBCH/SBE; [3] Pure glucose as an experimental control of sugar degradation. ND–not determined.

In general, both (1→6)-β-d-glucan (lasiodiplodan) contained more than 80% carbohydrate-derived sugars and less than 15% protein. Pure β-glucans are closely related to a single type of monosaccharide [34]. Accordingly, the identification of glucose as the only monomer type in lasiodiplodan indicates high purity and linear homopolysaccharide nature. Lasiodiplodan showed 94.2% (SCBCH/RBE) and 80.4% (SCBCH/SBE) of glucose derived from the structural carbohydrate present in the polymer (values equivalent to the purity of lasiodiplodan). The lasiodiplodan purities reported here were similar to lasiodiplodan [87.6%] from corn bran hydrolysate [16]. However, oat bran β-glucan, composed of (1→3/1→4)-β-d-glucan–linked glucose units, showed 95% purity following a rigorous extraction process (fat removal using hot isopropanol and petroleum ether, water solubilization at 95 °C, starch hydrolysis applying α-amylase enzyme, and β-glucan precipitation using $(NH_4)_2SO_4$ [35]. Therefore, the solubility properties and extraction process represent a specific role in the purity of β-glucans.

Lasiodiplodan is soluble in water due to the hydroxyl making hydrogen bonds. Nonetheless, its solubility is affected when the polymer is lyophilized, removing, and crosslinking the hydroxyl. In this sense, lasiodiplodan solubility (16.81%) from SCBCH/RBE was higher than lasiodiplodan (4.53%) from SCBCH/SBE. Likewise, lasiodiplodan from synthetic glucose had a similar solubility (4.02%) to lasiodiplodan from SCBCH/SBE [14]. Moreover, lasiodiplodan from SCBCH/RBE showed a 3.7-fold increase in solubility compared to other native lasiodiplodan.

3.5.2. GPC, SEM, and Viscosity Analysis

Figure 6 presents details on molecular weight, rheological properties, and morphological changes on lasiodiplodan. A GPC standard curve estimated the lasiodiplodan molecular weight of 7.6×10^3 Da (SCBCH/RBE) and 6.8×10^3 Da (SCBCH/SBR) (Figure 6A). In this way, Oliveira et al. [36] identified two (1→6)-β-d-glucans, the first with similar molecular weight (7.0×10^3 Da) to report here, and the second with a larger size (1.8×10^6 Da) from VMSM and sucrose by *L. theobromae*. Many factors can be responsible for the molecular weight of lasiodiplodan, i.e., the fermentation condition, culture medium, strain used, and extraction method.

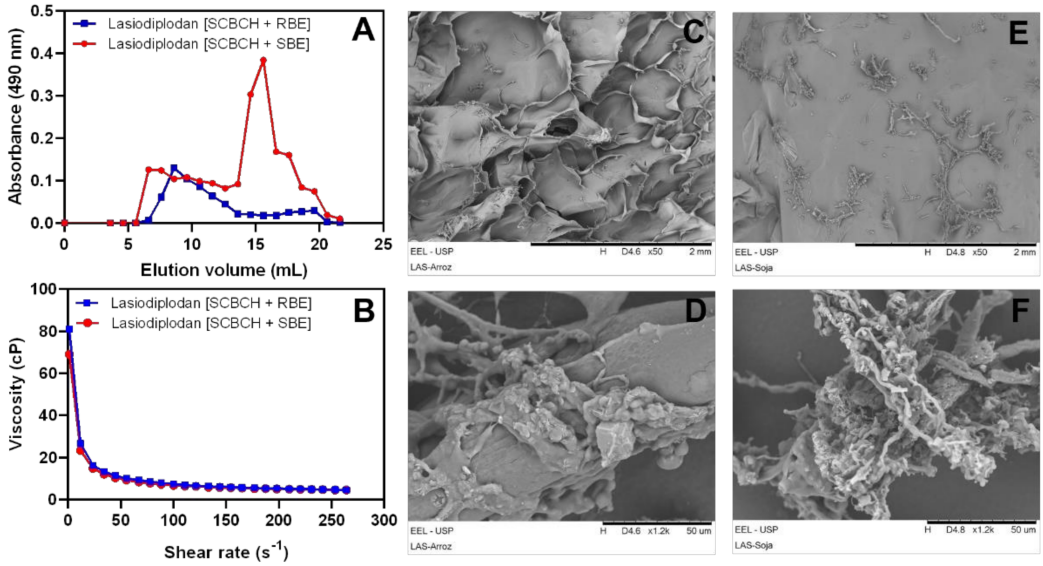

Figure 6. Physical characterization of lasiodiplodan: (**A**) Molecular weight by GPC; (**B**) Apparent viscosity; (**C,D**) 50× and 100× micrographs (SCBCH/RBE); (**E,F**) 50× and 100× micrographs (SCBCH/SBE).

Figure 6B shows that the lasiodiplodan viscosity decreases with an increasing shear rate, suggesting a pseudoplastic behavior at 25 °C for both samples. Similar results explained this behavior as an alignment of the lasiodiplodan polymer chain when exposed to the continuous increase in shear stress and decreased resistance to flow [11].

SEM revealed an irregular and fragmented surface with impurities on lasiodiplodan (SCBCH/RBE) in Figure 6C. This observation confirmed the higher solubility and purity with low protein content. By contrast, lasiodiplodan from SCBCH/SBE have a homogeneous surface with thin films, torsion formations, and impurities (Figure 6E), similar to lasiodiplodan from a synthetic glucose medium [14]. In sequence, Figure 6D,F show external structures adhered to the lasiodiplodan surface and could be related to detected protein and perhaps other types of impurities.

3.5.3. FTIR, DSC, and XRD Analysis

Lasiodiplodan samples revealed a similar chemical structure, as shown in Figure 7A. The broad bands indicated a deep intensity in the 3274 cm^{-1} regions attributed to an R-OH stretching vibration [15]. In the spectral regions, close to 2900 and 1400 cm^{-1}, the deformation vibrations are typical on lasiodiplodan [14,37]. Then, 1650 cm^{-1} bands correspond to a glucose ring, while symmetrical vibrations of C-O-C characteristic bonds on carbohydrates typically have absorption in 1075 cm^{-1} [16]. Finally, the 890 cm^{-1} bands with low intensity are typical of glycosidic bonds, attributed to the β-configuration of lasiodiplodan have been described in studies on lasiodiplodan production [14–16,38].

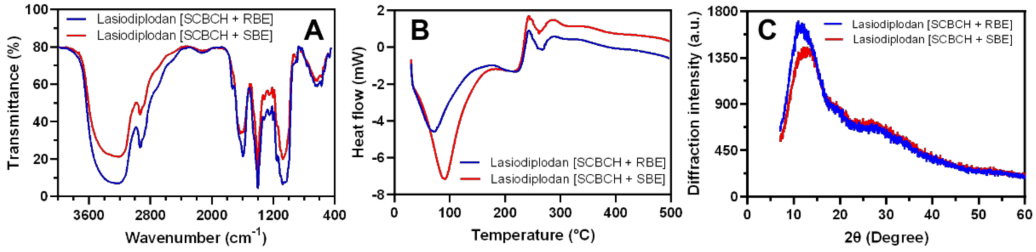

Figure 7. Spectra of lasiodiplodan samples using (**A**) FTIR; (**B**) DSC; (**C**) XRD.

Figure 7B shows three heat stages on lasiodiplodan samples. The first indicates water loss, followed by degradation and carbonization. Thus, lasiodiplodan from SCBCH/SBE show 90, 220, and 264 °C, while lasiodiplodan from SCBCH/RBE show 74, 224, and 268 °C. In contrast, lasiodiplodan from synthetic glucose presented high thermal stability (63, 310, and 475 °C) [37]. In the present work, the lasiodiplodan samples showed moderate thermal stability support by the lower molecular weight found.

The XDR showed similar lasiodiplodan profiles, as can be found in Figure 7C. Lasiodiplodan samples have an amorphous matrix demonstrated previously [14,16,37,38]. Lasiodiplodan, a non-cellulosic molecule, showed an intense and broad peak in the shoulder shape between 7.15 and 17.5°. Similarly, other β-glucans have been identified with broad peaks and nullified crystallinity [39].

4. Conclusions

This is the first report demonstrating the microbial production of lasiodiplodan, a β-glucan, through the valorization of sugarcane bagasse and rice/soybean bran. For this, an innovative platform of biotechnological relevance was developed through a comparative study. Genetic programming by Eureqa Formulize was a powerful tool in obtaining accurate and sensitive models for the efficient production of lasiodiplodan. Therefore, the batch system fermentation achieved high lasiodiplodan titers of 22.0 g/L using SCBCH/RBE and 16.2 g/L using SCBCH/RBE. Moreover, the lasiodiplodan multiscale characterization demonstrated the typical β-glucan configuration and exhibited new properties, which increases their potential for further applications. Sustainable production of lasiodiplodan as a bio-based product from the reuse of agro-industrial waste fits well within a lignocellulosic biorefinery. Glucose-rich enzymatic hydrolysate from SCB was an excellent substrate for lasiodiplodan production. In this work, our findings emphasize the potential of *L. theobromae* CCT 3966 as a microbial cell factory for the profitable production of lasiodiplodan on a large scale.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7030108/s1, Table S1: CCD matrix used to optimize the C/N ratio for lasiodiplodan production; Table S2: CCD responses summary of lasiodiplodan, fungal biomass, and yield using SCBCH/RBE medium; Table S3: CCD responses summary of lasiodiplodan, fungal biomass, and yield using SCBCH/SBE medium; Table S4: Statistical significance of the individual terms in the models; Table S5: Statistical ANOVA to evaluate the significance of the models.

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References

1. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *J. Nat. Prod.* **2020**, *83*, 770–803. [CrossRef]
2. Fang, X.-H.; Zou, M.-Y.; Chen, F.-Q.; Ni, H.; Nie, S.-P.; Yin, J.-Y. An overview on interactions between natural product-derived β-glucan and small-molecule compounds. *Carbohydr. Polym.* **2021**, *261*, 117850. [CrossRef]
3. Han, B.; Baruah, K.; Cox, E.; Vanrompay, D.; Bossier, P. Structure-functional activity relationship of β-glucans from the per-spective of immunomodulation: A mini-review. *Front. Immunol.* **2020**, *11*, 658. [CrossRef]
4. Zhu, F.; Du, B.; Xu, B. A critical review on production and industrial applications of beta-glucans. *Food Hydrocoll.* **2016**, *52*, 275–288. [CrossRef]
5. Kaur, R.; Sharma, M.; Ji, D.; Xu, M.; Agyei, D. Structural Features, Modification, and Functionalities of Beta-Glucan. *Fibers* **2019**, *8*, 1. [CrossRef]
6. Maheshwari, G.; Sowrirajan, S.; Joseph, B. Extraction and Isolation of β-Glucan from Grain Sources-A Review. *J. Food Sci.* **2017**, *82*, 1535–1545. [CrossRef]
7. Bzducha-Wróbel, A.; Koczoń, P.; Błażejak, S.; Kozera, J.; Kieliszek, M. Valorization of Deproteinated Potato Juice Water into β-Glucan Preparation of C. utilis Origin: Comparative Study of Preparations Obtained by Two Isolation Methods. *Waste Biomass Valoriz.* **2020**, *11*, 3257–3271. [CrossRef]
8. Żbikowska, A.; Kupiec, M.; Szymanska, I.; Osytek, K.; Kowalska, M.; Marciniak-Lukasiak, K.; Rutkowska, J. Microbial β-glucan Incorporated into Muffins: Impact on Quality of the Batter and Baked Products. *Agriculture.* **2020**, *10*, 126. [CrossRef]
9. Acosta, S.B.P.; Marchioro, M.L.K.; Santos, V.A.Q.; Calegari, G.C.; Lafay, C.B.B.; Barbosa-Dekker, A.M.; Dekker, R.F.H.; Da Cunha, M.A.A. Valorization of Soybean Molasses as Fermentation Substrate for the Production of Microbial Exocellular β-Glucan. *J. Polym. Environ.* **2020**, *28*, 2149–2160. [CrossRef]
10. Vasconcelos, A.F.D.; Dekker, R.F.; Barbosa, A.M.; Carbonero, E.R.; Silveira, J.L.; Glauser, B.; Pereira, M.S.; Silva, M.L.C. Sulfonation and anticoagulant activity of fungal exocellular β-(1→6)-D-glucan (lasiodiplodan). *Carbohydr. Polym.* **2013**, *92*, 1908–1914. [CrossRef]
11. da Cunha, M.A.A.; Turmina, J.A.; Ivanov, R.C.; Barroso, R.R.; Marques, P.T.; Fonseca, E.A.I.; Fortes, Z.B.; Dekker, R.F.H.; Khaper, N.; Barbosa, A.M. Lasiodiplodan, an exocellular (1→6)-β-d-glucan from Lasiodiplodia theobromae MMPI: Production on glucose, fermentation kinetics, rheology and anti-proliferative activity. *J. Ind. Microbiol. Biotechnol.* **2012**, *39*, 1179–1188. [CrossRef] [PubMed]
12. Alves, A.; Crous, P.W.; Correia, A.; Phillips, A.J.L. Morphological and molecular data reveal cryptic speciation in Lasiodiplodia theobromae. *Fungal Divers.* **2008**, *28*, 1–13.
13. Mahapatra, S.; Banerjee, D. Fungal Exopolysaccharide: Production, Composition and Applications. *Microbiol. Insights* **2013**, *6*, MBI S10957. [CrossRef]
14. Theis, T.V.; Santos, V.A.Q.; Appelt, P.; Barbosa-Dekker, A.M.; Vetvicka, V.; Dekker, R.F.H.; Cunha, M.A.A. Fungal Exocellular (1-6)-β-d-glucan: Carboxymethylation, Characterization, and Antioxidant Activity. *Int. J. Mol. Sci.* **2019**, *20*, 2337. [CrossRef] [PubMed]
15. Abdeshahian, P.; Ascencio, J.J.; Philippini, R.R.; Antunes, F.A.F.; Dos Santos, J.C.; da Silva, S.S. Utilization of sugarcane straw for production of β-glucan biopolymer by Lasiodiplodia theobromae CCT 3966 in batch fermentation process. *Bioresour. Technol.* **2020**, *314*, 123716. [CrossRef] [PubMed]
16. Philippini, R.R.; Martiniano, S.E.; Marcelino, P.R.F.; Chandel, A.K.; dos Santos, J.C.; Da Silva, S.S. Production of β-glucan exopolysaccharide lasiodiplodan by Lasiodiplodia theobromae CCT 3966 from corn bran acid hydrolysate. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 2319–2332. [CrossRef] [PubMed]
17. Chandel, A.K.; Garlapati, V.K.; Kumar, S.P.J.; Hans, M.; Singh, A.K. The role of renewable chemicals and biofuels in building a bioeconomy. *Biofuels, Bioprod. Biorefining* **2020**, *14*, 830–844. [CrossRef]
18. Ascencio, J.J.; Chandel, A.K.; Philippini, R.R.; Da Silva, S.S. Comparative study of cellulosic sugars production from sugarcane bagasse after dilute nitric acid, dilute sodium hydroxide and sequential nitric acid-sodium hydroxide pretreatment. *Biomass Convers. Biorefinery* **2020**, *10*, 813–822. [CrossRef]
19. Martiniano, S.E.; Philippini, R.R.; Chandel, A.K.; Rosa, C.A.; Pagnocca, F.C.; Da Silva, S.S. Evaluation of Rice Bran Extract as a Nitrogen Source for Improved Hemicellulosic Ethanol Production from Sugarcane Bagasse by New Xylose-Fermenting Yeast Strains Isolated from Brazilian Forests. *Sugar Tech.* **2013**, *16*, 1–8. [CrossRef]
20. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]
21. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
22. Vogel, H.J. A convenient growth medium for Neurospora (Medium N). *Microb. Genet. Bull.* **1956**, *13*, 42–43.
23. Gomes, F.M.; Pereira, F.M.; Silva, A.F.; Silva, M.B. Multiple response optimization: Analysis of genetic programming for symbolic regression and assessment of desirability functions. *Knowledge-Based Syst.* **2019**, *179*, 21–33. [CrossRef]
24. Vazquez-Rodriguez, A.; Vasto-Anzaldo, X.G.; Perez, D.B.; Garza, E.V.; Villanueva, H.C.; García-Rivas, G.; Garza-Cervantes, J.A.; Gómez-Lugo, J.J.; Gomez-Loredo, A.E.; Gonzalez, M.T.G.; et al. Microbial Competition of Rhodotorula mucilaginosa UANL-001L and E. coli increase biosynthesis of Non-Toxic Exopolysaccharide with Applications as a Wide-Spectrum Antimicrobial. *Sci. Rep.* **2018**, *8*, 798. [CrossRef]

25. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
26. Guerra, A.; Mendonça, R.; Ferraz, A. Molecular weight distribution of wood components extracted from Pinus taeda biotreated by Ceriporiopsis subvermispora. *Enzym. Microb. Technol.* **2003**, *33*, 12–18. [CrossRef]
27. Gmoser, R.; Sintca, C.; Taherzadeh, M.; Lennartsson, P.R. Combining submerged and solid state fermentation to convert waste bread into protein and pigment using the edible filamentous fungus N. intermedia. *Waste Manag.* **2019**, *97*, 63–70. [CrossRef] [PubMed]
28. Karr-Lilienthal, L.K.; Grieshop, C.M.; Merchen, N.R.; Mahan, D.C.; Fahey, G.C. Chemical Composition and Protein Quality Comparisons of Soybeans and Soybean Meals from Five Leading Soybean-Producing Countries. *J. Agric. Food Chem.* **2004**, *52*, 6193–6199. [CrossRef]
29. Wang, J.; Salem, D.R.; Sani, R.K. Synthesis of Biopolymers from a Geobacillus sp. WSUCF1 Using Unprocessed Corn Stover. *ACS Sustain. Chem. Eng.* **2020**, *8*, 9483–9496. [CrossRef]
30. López, J.C.; Pérez, J.S.; Sevilla, J.M.F.; Fernandez, F.G.A.; Grima, E.M.; Chisti, Y. Production of lovastatin by Aspergillus terreus: Effects of the C:N ratio and the principal nutrients on growth and metabolite production. *Enzym. Microb. Technol.* **2003**, *33*, 270–277. [CrossRef]
31. Hitzenhammer, E.; Büschl, C.; Sulyok, M.; Schuhmacher, R.; Kluger, B.; Wischnitzki, E.; Schmoll, M. YPR2 is a regulator of light modulated carbon and secondary metabolism in Trichoderma reesei. *BMC Genom.* **2019**, *20*, 211. [CrossRef] [PubMed]
32. Félix, C.; Libório, S.; Nunes, M.; Félix, R.; Duarte, A.S.; Alves, A.; Esteves, A.C. Lasiodiplodia theobromae as a producer of bio-technologically relevant enzymes. *Int. J. Mol. Sci.* **2018**, *19*, 29.
33. Ko, Y.-S.; Kim, J.W.; Lee, J.A.; Han, T.; Kim, G.B.; Park, J.E.; Lee, S.Y. Tools and strategies of systems metabolic engineering for the development of microbial cell factories for chemical production. *Chem. Soc. Rev.* **2020**, *49*, 4615–4636. [CrossRef]
34. Synytsya, A.; Novak, M. Structural analysis of glucans. *Ann. Transl. Med.* **2014**, *2*. [CrossRef]
35. Johansson, L.; Virkki, L.; Maunu, S.L.; Lehto, M.; Ekholm, P.; Varo, P. Structural characterization of water soluble β-glucan of oat bran. *Carbohydr. Polym.* **2000**, *42*, 143–148. [CrossRef]
36. Oliveira, K.S.; Di Bastiani, M.; Cordeiro, L.M.; Costa, M.F.; Toledo, K.A.; Iacomini, M.; Barbosa, A.M.; Dekker, R.F.H.; Nascimento, V.M.G. (1→6)-and (1→3)(1→6)-β-glucans from Lasiodiplodia theobromae MMBJ: Structural characterization and pro-inflammatory activity. *Carbohydr. polym.* **2015**, *133*, 539–546. [CrossRef]
37. Luna, W.N.S.; Santos, V.A.; Teixeira, S.D.; Barbosa-Dekker, A.M.; Dekker, R.F.H.; Cunha, M.A.A. O-Acetylated (1→6)-β-D-Glucan (lasiodiplodan): Chemical derivatization, characterization and antioxidant activity. *J. Pharm. Pharmacol.* **2018**, *6*, 320–332.
38. Kagimura, F.Y.; da Cunha, M.A.A.; Theis, T.V.; Malfatti, C.R.M.; Dekker, R.F.; Barbosa, A.M.; Teixeira, S.D.; Salome, K. Carboxymethylation of (1→6)-β-glucan (lasiodiplodan): Preparation, characterization and antioxidant evaluation. *Carbohydr. Polym.* **2015**, *127*, 390–399. [CrossRef]
39. Uchiyama, H.; Dowaki, M.; Kadota, K.; Arima, H.; Sugiyama, K.; Tozuka, Y. Single-stranded β-1,3–1,6-glucan as a carrier for improved dissolution and membrane permeation of poorly water-soluble compounds. *Carbohydr. Polym.* **2020**, *247*, 116698. [CrossRef]