Exocellular (1→6)-β-D-Glucan (Lasiodiplodan): Carboxymethylation, Thermal Behavior, Antioxidant and Antimicrobial Activity

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Abstract: β-Glucans are biomacromolecules of industrial interest for its biological and technological properties, including ability to modify the rheology of food systems and different biological functionalities. In this study, lasiodiplodan an exocellular polysaccharide of the (1→6)-β-D-glucan type produced by the fungus Lasiodiplodia theobromae MMPI was obtained in a bench-scale bioreactor operated in discontinuous mode. Lasiodiplodan produced was derivatized by carboxymethylation and characterized by Fourier Transform Infrared spectroscopy (FT-IR), Thermogravimetry and Differential Thermal Analysis (TG-DTA), X-Ray Diffraction (XRD), scan electron microscopy (SEM) and their antioxidant and antimicrobial potential also was assessed. Two strong absorption bands in the regions of 1422 to 1598 cm⁻¹, resulting from symmetric and asymmetric stretching vibrations of the -COO⁻ group respectively, were observed in the FT-IR spectrum of the derivatized sample and indicated the carboxymethylation of the macromolecule. TG-DTA curves indicated that native (LAS-N) and carboxymethylated (LAS-C) lasiodiplodan did not suffer significant changes in relation to the heating rates. Native and carboxymethylated lasiodiplodan demonstrated high thermal stability considering the usual standards of the pharmaceutical industry. The final temperature of thermal decomposition increased when the heating rate was increased and less number of steps for thermal decomposition was observed when air was substituted for nitrogen. XRD analysis showed the LAS-N and LAS-C have no crystalline structure, but carboxymethylation led to the arising of regions with certain molecular orientation in biopolymer structure. SEM analysis showed that the carboxymethylation promoted changes in biopolymer macrostructure, including breaking of the polymeric structure and arising of bubbles on the surface area. Carboxymethylation contributed to improving the polysaccharide’s antioxidant capacity and LAS-C demonstrated antimicrobial activity against Candida tropicalis.

Keywords: Exopolysaccharide, Bioactivity, Chemical Derivatization, Macromolecules, Lasiodiplodia theobromae

Introduction
Several microorganisms, including seaweeds, bacteria and fungi are able to produce polysaccharides and some can secrete these outside the cell in soluble forms (Wang et al., 2008). The microbial production of exocellular polysaccharides has aroused much interest by research groups worldwide considering that these
carbohydrate biopolymers are high-valued and have a broad spectrum of industrial applications (Wang et al., 2008). Microbial Exopolysaccharides (EPS) have been used by chemical, pharmaceutical and food sectors as bio-flocculants, bio-adsorbents, agents to remove heavy metals, for control-release of drugs and as gelling, stabilizer and food thickening agents (Wang et al., 2008; Schultheis et al., 2009). Some of these EPS’s and especially the fungal β-D-glucans, present biological activities, which have been demonstrated to possess antioxidant (Schultheis et al., 2009), antimutagenic (Cho et al., 2006), antiviral (Miranda et al., 2008), anticoagulant and antithrombotic (Yuan et al., 2009), hypoglycemic and hypocholesterolemic (Miranda-Nantes et al., 2011) properties, as well as exhibiting an anti-proliferative effect on breast cancer cells (Cunha et al., 2012). These pharmacological properties confer on these biomacromolecules important applications as nutraceuticals and present a promising market for new commercial products.

β-Glucans are naturally-occurring polysaccharides as constituents of the cell walls of yeasts and fungi and are also found in cereal grains (esp., wheat, barley and oat) as soluble fibers (1→3;1→4)-β-D-glucan (Xiu et al., 2010; Mitsou et al., 2010). Altogether they have attracted attention as bioactive substances that can promote human health benefits (Kivela et al., 2010).

Fungal β-D-glucans have been described of the following β-(1→3), β-(1→3;1→6), or β-(1→6) types. The β-(1→3) and β-(1→3;1→6)-glucans can be secreted into the culture medium by a relatively large number of filamentous fungi (Bohn and BeMiller, 1995; Selbmann et al., 2003; Vasconcelos et al., 2008). Exocellular (1→6)-β-glucans are less common and only a few reports have described their production and isolation (Cunha et al., 2012). Research from our group has described lasiodiplodan as an exocellular (1→6)-β-glucans produced by Lasiodiplodia theobromae isolated from Annona squamosa (a tropical fruit commonly named of pinha) (Vasconcelos et al., 2008). This biomacromolecule is composed of glucose units linked by (1→6)-β-glucosidic bonds (Fig. 1) and can easily be recovered from the culture medium by simple methods such as precipitation with ethanol, centrifugation and drying by lyophilization (Kagimura et al., 2015a).

β-Glucans have demonstrated notable physiological effects and have as such received much attention. They belong to a group of physiologically active compounds named biological response modifiers (Bohn and BeMiller, 1995). Studies have shown that β-glucan has significant effects on different immune reactions, on reducing stress, lowering cholesterol, regulating blood sugar levels and protection against the negative effects of environmental toxicity (Richter et al., 2015).

Some biological properties have been identified for lasiodiplodan, including antiproliferative activity on MCF-7 breast cancer cells mediated by oxidative stress, AMP-Activated Protein Kinase (AMPK) and the Forkhead transcription factor (FOXO3a) (Cunha et al., 2012; Queiroz et al., 2015), hypoglycemic activity (Túrmina et al., 2012), antioxidant ability in vitro (Giese et al., 2015; Kagimura et al., 2015b) and protective activity against DNA damage induced by doxorubicin (Mello et al., 2016).

![Fig. 1. Fermentative profile of (o) lasiodiplodan production by Lasiodiplodia theobromae MMPI and (■) glucose consumption](Image)
Considering that the biological properties of polysaccharides are associated with their chemical structures, specific chemical modifications in the primary structure of these macromolecules may contribute to the potentiation of their functionalities (Kagimura et al., 2015b). In this context, in the present study lasiodiplodan was derivatized by carboxymethylation and characterized by FT-IR spectroscopy, carboxymethylation degree (DS), TG-DTA thermal analysis, X-ray diffraction and Scanning Electron Microscopy (SEM). Biological abilities as antioxidant capacity and antimicrobial potential also were assessed.

Materials and Methods

Microorganism and Inoculum Preparation

A fungal strain of Lasiodiplodia theobromae MMPI (Saldanha et al., 2007) was maintained through periodic transfer on Sabouraud-agar plates containing chloramphenicol at 4°C. Inoculum preparation from mycelium and its standardization for inoculation purposes were essentially as described previously (Barbosa et al., 2003; Cunha et al., 2012).

Lasiodiplodan Production in a Bench-Scale Stirred-Tank Bioreactor (STBR)

Discontinuous fermentations were carried out in a bench-scale bioreactor (Biostat B, B. Braun Biotech International, Germany) equipped with a 2 L fermentation vessel. The bioreactor was loaded with 1000 mL nutrient medium and 50 mL standardized inoculum and operated at an agitation speed of 180 rpm, a temperature of 28°C and an air flow rate of 0.8 vvm. The pH, temperature, agitation speed and airflow were monitored during the fermentation run. The nutrient medium comprised (20 g L\(^{-1}\) glucose, 2 g L\(^{-1}\) K\(_2\)HPO\(_4\), 2 g L\(^{-1}\) MgSO\(_4\)·7H\(_2\)O and 2 g L\(^{-1}\) yeast extract (Oxoid); initial pH 5.5).

Isolation of Lasiodiplodan and Fermentative Parameters in the Production of Lasiodiplodan

Fungal biomass was recovered from the fermentation broth by centrifugation (1,350 x g, 30 min), washed twice with distilled water and dried in an oven to constant weight at 50°C for gravimetric measurement. The exopolysaccharide (lasiodiplodan) present in the supernatant was precipitated with four volumes of absolute ethanol (overnight at 4°C), recovered by filtration and solubilized by stirring in distilled water at 60°C, followed by exhaustive dialysis (cellulose membrane dialysis tubing (MWCO E 12,000 Da; 1.3 in. diam., Sigma-Aldrich, USA) against distilled water and then dried by lyophilization for subsequent chemical derivatization. The amount of lasiodiplodan obtained during the fermentation run was estimated gravimetrically after drying in an oven to constant weight at 50°C the precipitate obtained by dehydration with ethanol.

Fermentative parameters to evaluate the production profile of L. theobromae MMPI included the yield of EPS, volumetric productivity of EPS, volumetric productivity of fungal biomass, rate of substrate consumption and specific yield and were calculated according the equations described by Cunha et al. (2012). The conversion yield of glucose to EPS (Y\(_{EP}\)) was calculated as the amount of EPS produced from the substrate (glucose) consumed. The conversion yield of glucose into cellular biomass (Y\(_{X/S}\)) was calculated as the amount of cellular biomass produced from the substrate (glucose) consumed.

The volumetric productivity of EPS (Q\(_{EP}\)) was calculated as the ratio of the maximum EPS concentration to the fermentation time. The volumetric productivity of biomass (Q\(_{B}\)) was calculated as the ratio of the maximum biomass concentration to the fermentation time. The total rate of substrate consumption (Q\(_{S}\)) was calculated as the ratio of the glucose consumed to the fermentation time. The percentage of substrate consumed (Y\(_{C}\)) was calculated as the glucose consumed based on the initial glucose concentration. Glucose consumption during the fermentation run was determined with a glucose oxidase Kit (Glicose PAP Liquiform, Labtest Diagnóstica SA, Brazil).

Derivatization of Lasiodiplodan by Carboxymethylation

Carboxymethylation of lasiodiplodan was performed adopting the protocol described by Wang et al. (2012) with some adjustments. Lyophilized lasiodiplodan (1.5 g) was suspended in 65 mL of isopropanol with vigorous stirring for 15 min. Then, 25 mL of NaOH 20% (w.v\(^{-1}\)) was added dropwise and the mixture was stirred for 3 h at room temperature. Thereafter, a solution of monochloroacetic acid (concentration of 8.75 g dissolved in 12.5 mL of 20% NaOH (w.v\(^{-1}\)) and 31.5 mL of isopropanol) was added slowly under stirring. The reaction was maintained at room temperature for 3 h and the temperature was then raised to 60°C for 30 min. Monochloroacetic acid solution (as described above) was again added and the reaction mixture was maintained at 60°C under stirring for 1 h. The reaction was terminated by cooling to room temperature and neutralization with 1.5 mol.L\(^{-1}\) hydrochloric acid solution to pH 7. Isopropanol was removed from the mixture by using a rotary evaporator. The resulting solution containing the carboxymethylated lasiodiplodan (LAS-C) was
dialyzed against distilled water for 7 d in dialysis tubes (MW 12,000 Da, Sigma-Aldrich) and lyophilized.

The Degree of Substitution (DS) of LAS-C sample was estimated by the titration method according to Tatongjai and Lumdubwong (2010). LAS-C (150 mg) was dissolved in 100 mL of ultrapure water (Milli-Q), homogenized vigorously for 3 min and centrifuged. The macromolecule present in the supernatant in the form of a salt (LAS-Na-C) was converted to the acid form (H-C-LAS) by percolation through an ion-exchange column (Amberlite IR-120, Sigma-Aldrich) at a flow rate of 3 mL min\(^{-1}\). The column consisted of a 10 mL syringe packed with the resin (6 x 1.5 cm). After elution of the LAS-C-H solution, the column was washed with 400 mL of ultrapure water (Milli-Q) and all was collected (500 mL). The collected solution was subjected to lyophilization and the resulting solid was dissolved in 100 mL of distilled water (Milli-Q) and mixed with 3 drops of phenolphthalein, 2 mL of methanol and 15 mL of NaOH 0.1 mol.L\(^{-1}\). The resulting mixture was titrated with HCl 0.1 mol.L\(^{-1}\). Water was used as the blank. The DS was estimated by the titration method according to Liu et al. (2010). The reaction mixture consisted of 1 mL of H\(_2\)O\(_2\) (0.1 mmol.L\(^{-1}\), freshly prepared), 1 mL of sample suspension (0.5, 1.0 or 2 mg.L\(^{-1}\)), 0.1 mL of ammonium molybdate (3% w.v\(^{-1}\)), 10 mL of H\(_2\)SO\(_4\) solution (2 mol.L\(^{-1}\)) and 7 mL of KI solution (1.8 mol.L\(^{-1}\)). The mixture was titrated with Na\(_2\)S\(_2\)O\(_4\) (5 mmol.L\(^{-1}\)) until the disappearance of a yellow color. Scavenging ability was calculated from the equation below, where \(V_0\) represents the volume of Na\(_2\)S\(_2\)O\(_4\) solution used to titrate the control mixture and \(V_t\) represents the volume titrated of the mixture containing the lasiodiplodan samples:

\[
\%H_2O_2\text{scavaging} = \left(\frac{V_0-V_t}{V_0}\right) \times 100\%
\]

**FT-IR Spectroscopy**

Fourier Transform-Infrared Spectroscopy (FT-IR) of native, unmodified Lasiodiplodan (LAS-N) and carboxymethylated (LAS-C) samples were conducted on a FT-IR Spectrometer Frontier (Perkin Elmer, USA) in the region of 4000-400 cm\(^{-1}\), 4 cm\(^{-1}\) resolution and 32 accumulated scans were obtained using the KBr disc method.

**TG and DTA Thermal Analysis**

Thermogravimetric (TG) and Differential Thermal Analysis (DTA) was performed using a SDT Q600 instrument (TA Instruments, USA). The loss of weight was registered over the range 30-500°C at heating rates of 5, 10 or 20°C min\(^{-1}\). The analysis of the samples was conducted in dynamic atmosphere of air (synthetic) or nitrogen at a flow rate of 100 mL min\(^{-1}\).

**X-ray Diffraction and Scanning Electron Microscopy**

X-ray diffractogram patterns were recorded on a Rigaku MiniFlex600 Diffractometer, using copper radiation font (CuKa = 1.5418 Å), 15 mA current, 40 kV voltage, scanning range of 10° to 60° (2\(\theta\)), a scanning speed of 5° min\(^{-1}\) and a step width of 0.02° (2\(\theta\)). Micrographs of native and derivatized lasiodiplodan were acquired in a scanning electron microscope (Hitachi TM3000, USA) using lyophilized samples and images were taken at magnifications of 200, 400 and 1500 times.

**Antioxidant Capacity**

**Hydrogen Peroxide Scavenging Activity Assay**

The hydrogen peroxide (H\(_2\)O\(_2\)) removal ability was measured according to Liu et al. (2010). The reaction mixture consisted of 1 mL of H\(_2\)O\(_2\) (0.1 mmol.L\(^{-1}\), freshly prepared), 1 mL of sample suspension (0.5, 1.0 or 2 mg.L\(^{-1}\)), 0.1 mL of ammonium molybdate (3% w.v\(^{-1}\)), 10 mL of H\(_2\)SO\(_4\) solution (2 mol.L\(^{-1}\)) and 7 mL of KI solution (1.8 mol.L\(^{-1}\)). The mixture was titrated with Na\(_2\)S\(_2\)O\(_4\) (5 mmol.L\(^{-1}\)) until the disappearance of a yellow color. Scavenging ability was calculated from the equation below, where \(V_0\) represents the volume of Na\(_2\)S\(_2\)O\(_4\) solution used to titrate the control mixture and \(V_t\) represents the volume titrated of the mixture containing the lasiodiplodan samples:

\[
\%H_2O_2\text{scavaging} = \left(\frac{V_0-V_t}{V_0}\right) \times 100\%
\]

**Hydroxyl Radical Scavenging Activity Assay**

Hydroxyl radical (HO•) scavenging activity was assessed according to Liu et al. (2010) with subtle modification. Hydroxyl radicals were generated from FeSO\(_4\) and H\(_2\)O\(_2\) and detected by their ability to hydroxylate salicylate. The reaction mixture (2 mL) contained 0.5 mL of FeSO\(_4\) (1.5 mmol.L\(^{-1}\)), 0.35 mL of...
Reducing Power

Reducing Power was evaluated according to Liu et al. (2010) using a mixture of 2.5 mL of the lasiodiplodan samples or ascorbic acid solutions (0.5, 1.0 or 2 mg L\(^{-1}\)) incubated with 2.5 mL of potassium ferricyanide (1% w.v\(^{-1}\)) at 50°C for 20 min. The reaction was terminated by adding of 2.5 mL of trichloroacetic acid (10% w.v\(^{-1}\)) followed by the addition 5 mL of distilled water and 1 mL of ferric chloride (0.1% w.v\(^{-1}\)). Absorbance was measured at 700 nm. Higher absorbances of the mixture indicates greater reducing power of the sample.

Antimicrobial Potential Assessment

Antimicrobial activity of LAS-N and LAS-C was evaluated against the bacteria: Escherichia coli (ATCC 25922), Salmonella bongori (ATCC 43975), Salmonella enterica serovar Typhimurium (ATCC 0028), Staphylococcus aureus (ATCC 25923) and the yeasts: Candida albicans (ATCC 18804) and Candida tropicalis (ATCC 13803).

Antimicrobial activity was evaluated by (MIC) minimum inhibition concentration determination, through the microdilution method in culture broth, following the procedure described by the Clinical and Laboratory Standards Institute- CLSI (2008; 2015; Krichen et al., 2015) with subtle modifications. One hundred microliters of Müller-Hinton broth, 100 µL of different concentrations of LAS-N and LAS-C (7.5-17.5 mg L\(^{-1}\)) and 5 µL of the cell suspension (different microbial strains) previously standardized in 0.5 McFarland standard (1.5x10\(^{8}\) CFU mL\(^{-1}\)), were distributed in a microtiter plate well. MIC was defined as the lowest samples concentration that completely inhibited the strain growth after incubation at 37°C/24 h (bacteria) and 28°C/48h (yeasts). Microbial viability was evaluated by a pink color being developed after addition of 2,3,5-triphenyltetrazolium chloride. Positive results (absence of color) to microbial inhibition were submitted to evaluation of Minimal Bactericide Concentration (MBC) and Minimal Fungicidal Concentration (MFC), through inoculation in BHI (Brain Heart Infusion) agar (bacteria) and Sabouraud dextrose agar (yeast), following the incubation step under the same conditions cited above. Norfloxacin (antibacterial agent) and fluconazole (antifungal agent) was used as negative control, both at concentrations of 1 mg L\(^{-1}\). Peptone water 0.1% (w v\(^{-1}\)) was used as positive control.

Results

Figure 1 shows the profiles of glucose consumption and lasiodiplodan production by submerged cultivation with L. theobromae carried out in a bench-scale bioreactor. As can be seen, the ascomycete L. theobromae was effective in assimilating the glucose substrate during the course of fermentation and presented high exopolysaccharide production capacity. A maximum production of lasiodiplodan was observed at 48 h of cultivation and high yield and volumetric productivity in EPS were also observed at the end of the bioprocess (Table 1).

FT-IR spectroscopic analysis allowed the characterization of the structure of lasiodiplodan by identifying specific absorption bands of chemical groups and bonds presents in the macromolecule. The FT-IR spectra of native (LAS-N) and carboxymethylated (LAS-C) lasiodiplodan are shown in Fig. 2 (A and B, resp). Both spectra were similar to each other and exhibit bands of absorption characteristics of polysaccharide macromolecules between 4000 and 400 cm\(^{-1}\). Chemical derivatization of lasiodiplodan by carboxymethylation was confirmed by the presence of two strong absorption bands in the regions of 1422 to 1598 cm\(^{-1}\), being attributable to carboxymethyl groups, whereas these were absent in LAS-N.

Thermogravimetry (TG), Differential Thermogravimetry (DTG) and Differential Thermal Analysis (DTA) and Differential Scanning Calorimetry (DSC) are useful techniques for the rapid analysis of polymeric compounds using small amounts of solid and liquid samples (Ramos-Sánchez et al., 1991). TG and DSC have been used to provide information of drug and molecules regarding the physicochemical properties such as stability, purity and formulation compatibility, among others (Yoshida et al., 2010). Application of TG presents information on the thermal stability of the molecule, behavior of thermal decomposition and the degree of hydration; important parameters affecting the stability of polymeric molecules (Lucas et al., 2001). Figure 3 shows the thermal behavior of LAS-N and LAS-C.
over the range 30 to 500°C submitted at different heating rates (5, 10 or 20°C min⁻¹) and differing conditions of dynamic atmospheres (air or nitrogen). The relationship between the logarithmic heating rate and the reciprocal temperature, as well as the half-life estimated according to time of LAS-N and LAS-C are presented in Fig. 4. Table 2 shows the kinetic parameters obtained by the multiple heating rate method. Both samples (LAS-N and LAS-C) exhibited relatively high thermal stability with respect to temperatures involved in processes of the pharmaceutical industry. Las-N and LAS-C presented similar initial decomposition temperatures, close to 230°C. The thermal behavior after the first decomposition step was different between the native and carboxymethylated samples.

The crystallographic profile of the carboxymethylated sample (LAS-C) was similar to the profile of the native sample (LAS-N). However, carboxymethylation promoted change in intensity of some peaks, especially in peaks observed at 21.1 (2θ) and 23.8° (2θ), which were more intense and pronounced in the LAS-C (Fig. 5).

Scanning electron microscopy showed that the carboxymethylation of lasiodiplodan led to changes in the morphological structure of the biopolymer, breaks being observed of the original macrostructure as can be seen in Fig. 6.

Fig. 2. FI-IR spectra of native (LAS-N) and carboxymethylated (LAS-C) lasiodiplodan
Fig. 3. TG (solid line) DTA (dotted line) curves of the LAS-N (A) and LAS-C (B) obtained under different atmospheric and heating rate conditions.

Fig. 4. Relationship between logarithmic heating rate and reciprocal temperature (A, C). The half-life estimated according to time (B, D) of LAS-N and LAS-C
Fig. 5. Standard X-ray diffraction profiles of LAS-N and LAS-C samples

Fig. 6. Micrographs of LAS-N and LAS-C obtained by scanning electron microscopy: (A) LAS-N at 200× magnification, (B) LAS-N at 400× magnification and (C) LAS-N at 1500× magnification. (D) LAS-C at 200× magnification, (E) LAS-C at 400× magnification and (F) LAS-C at 1500× magnification

Table 1. Kinetic parameters for the production of lasiodiplodan by Lasiodiplodia theobromae MMPI cultivated in a stirred-tank bioreactor for 72 h

| Fermentation parameters | Observed values |
|-------------------------|-----------------|
| $P_p$ (g.L$^{-1}$)      | 8.30±0.76       |
| $P_x$ (g.L$^{-1}$)      | 5.25±0.67       |
| $Y_{P_0}$ (g.g$^{-1}$)  | 0.40±0.04       |
| $Y_{X_0}$ (g.g$^{-1}$)  | 0.25±0.005      |
| $Q_p$ (g.L$^{-1}$h$^{-1}$) | 0.12±0.01 |
| $Q_x$ (g.L$^{-1}$h$^{-1}$) | 0.07±0.002 |
| $Q_s$ (g.L$^{-1}$h$^{-1}$) | 0.28±0.007 |
| $Y_c$ (%)               | 93.0±0.76       |
Table 2. Kinetic parameters obtained by the multiple heating rate method. (Eₐ: Activation energy; Log Z: Pre-exponential factor; Temperature: Temperature of half-time).

| Conversion (weight %) | Eₐ (kJ mol⁻¹) | Log Z (min⁻¹) | Temperature (°C) |
|-----------------------|---------------|---------------|------------------|
|                       | LAS-N         | LAS-C         | LAS-N            | LAS-C            | LAS-N | LAS-C |
| 5.0                   | 130.3         | 262.0         | 11.37            | 25.51            | 238.1 | 225.5 |
| 7.5                   | 135.9         | 273.8         | 12.02            | 26.76            | 235.5 | 225.5 |
| 10.0                  | 139.4         | 267.0         | 12.42            | 26.04            | 234.0 | 225.3 |
| 15.0                  | 141.5         | 267.4         | 12.68            | 26.05            | 232.3 | 225.9 |
| 20.0                  | 142.2         | 260.5         | 12.78            | 25.33            | 231.3 | 225.9 |

Table 3. Minimal inhibitory concentration of LAS-N and LAS-C against different bacterial and yeast strains (*Fungistatic effect)

| Microorganism                        | Minimal inhibitory concentration (mg.L⁻¹) |
|--------------------------------------|--------------------------------------------|
| Staphylococcus aureus (ATCC 25923)   | > 17.5                                     |
| Salmonella enterica Typhimurium (ATCC 0028) | > 17.5                                     |
| Escherichia coli (ATCC 25299)        | > 17.5                                     |
| Salmonella bongori (ATCC 43975)      | > 17.5                                     |
| Candida tropicalis (ATCC 13803)      | > 17.5                                     |
| Candida albicans (ATCC 18804)        | > 17.5                                     |
|                                      |                                            | *Fungistatic effect                            |

Fig. 7. Antioxidant activity of LAS-N, LAS-C, glucose (GLU) and ascorbic acid (AA) (positive control) measured by (A) Hydrogen peroxide scavenging, (B) HO⁻ scavenging and (C) reducing power

Substances which at low concentrations retard or prevent the oxidation of a substrate, are called antioxidants (Huang et al., 2005). Antioxidant substances have the ability to remove free radicals (scavenging activity) and can provide protection against diseases. In vitro assays are commonly used to estimate...
the antioxidant potential of different compounds and extractives. However, due to different types of free radicals and their different ways of actuation on living organisms, there is hardly a simple and universal method by which antioxidant activity can be measured accurately (Alves et al., 2010). In this sense, it is important that more than one method of analysis be conducted to evaluate the antioxidant ability of a substance. In the present study, three different tests were used to evaluate the antioxidant ability of native and carboxymethylated lasiodiplodan, as hydrogen peroxide and hydroxyl radical scavenging assays and reducing power.

The results of evaluating the antioxidant potential of LAS-N and LAS-C by the different methods of assessment are presented as histograms in Fig. 7. Ascorbic acid has been studied as the standard antioxidant (positive control) and the antioxidant ability of glucose, which is the monomeric unit of the macromolecule lasiodiplodan, was also evaluated. The introduction of carboxymethyl groups in the chemical structure of lasiodiplodan promoted an increase of its antioxidant ability and reducing power potential.

Table 3 shows the results of the minimal inhibitory concentration of LAS-N and LAS-C against gram positive and negative bacteria and yeasts strains. LAS-N showed minimal inhibitory concentration higher than 17.5 mg L\(^{-1}\) to all tested microorganisms. Similar behavior was observed for LAS-C for all strains tested, except for C. tropicalis that showed minimal inhibitory concentration and fungistatic effect at a concentration of 17.5 mg L\(^{-1}\).

**Discussion**

The profile of glucose consumption and production of lasiodiplodan by *L. theobromae* MMPI during submerged fermentation in a bench-scale bioreactor is shown in Fig. 1. Lasiodiplodan concentration reached a maximum level of 9.6±0.07 g L\(^{-1}\) at 48 h of cultivation. The lasiodiplodan concentration at 72 h of cultivation, however, was 13.5% less than at 48 h. The reduction in the lasiodiplodan concentration was most likely attributable to the production of \(\beta\)-1,6-glucanases by the fungal isolate, which at 72 h most likely led to the enzymatic hydrolysis of lasiodiplodan and the end-products released (glucose and gluco-oligosaccharides) were consumed by the fungus to sustain growth. As shown in Fig. 1 and Table 1, glucose was almost totally consumed (93.0±0.76%) after 72 h of cultivation. The global rate of glucose consumed was 0.28±0.007 g L\(^{-1}\) h\(^{-1}\), the volumetric productivity of lasiodiplodan was 0.12±0.01 g L\(^{-1}\) h\(^{-1}\) and the yield of fungal biomass was 0.25±0.005 g g\(^{-1}\).

The kinetic parameters of fermentation (Table 1) indicated that the fungal isolate was effective in converting glucose into lasiodiplodan. The production of lasiodiplodan using glucose at an initial concentration of 20 g L\(^{-1}\) and a turbine agitation speed of 400 rpm was 1.45-fold higher than when using glucose at 40 g L\(^{-1}\) and an agitation speed of 180 rpm as previously described in the STBR (Cunha et al., 2012). Similarly, the volumetric productivity of lasiodiplodan (Q\(_{Pb}\)) was 2.4-fold higher under the cultivation conditions described here (i.e., 20 g L\(^{-1}\) and 400 rpm). Glucose was more effectively consumed (93.0%) at an initial concentration of 20 g L\(^{-1}\) glucose and an agitation speed of 400 rpm than compared to when the fungus was cultivated on 40 g L\(^{-1}\) glucose and 180 rpm (69.5%) (Cunha et al., 2012). The conversion yield of glucose to lasiodiplodan (Y\(_{PS}\)) was 90% higher. On the other hand, the yield of biomass on consumed glucose (Y\(_{X/S}\)) was less on 20 g L\(^{-1}\) glucose and 400 rpm by comparison to 40 g L\(^{-1}\) glucose and 180 rpm (0.25 g g\(^{-1}\) Vs. 0.43 g g\(^{-1}\)) (Cunha et al., 2012), as well as the volumetric productivity of fungal biomass (Q\(_{X}\)) (0.07 g L\(^{-1}\) h\(^{-1}\) Vs. 0.10 g L\(^{-1}\) h\(^{-1}\)). The best kinetic parameters for lasiodiplodan production (final production and conversion yield of glucose to lasiodiplodan) obtained in the present work in comparison with that previously reported (Cunha et al., 2012), could be related to the inhibition of the enzymatic system involved in lasiodiplodan synthesis because of the higher substrate concentration that had been employed.

Characterization of native (LAS-N) and carboxymethylated (LAS-C) lasiodiplodan by Fourier transform-infrared spectroscopy is shown in Fig. 2. The strong absorption band at 3353 cm\(^{-1}\) (LAS-N) and 3380 cm\(^{-1}\) (LAS-C) is attributed to stretching vibration of O-H (Xu et al., 2009; Hu et al., 2011; Souza et al., 2015). The peak in the region 2930 cm\(^{-1}\) (LAS-N) and 2915 cm\(^{-1}\) (LAS-C) is attributed to the symmetric stretching vibration of C-H bond of the CH\(_2\) groups (Kagimura et al., 2015a; Liu et al., 2012). The symmetric stretching vibrations of the bonds C-O-C (characteristic group of sugars) are assigned to bands at 1078 cm\(^{-1}\) (LAS-N) and 1071 cm\(^{-1}\) (LAS-C). The bands at 1246 cm\(^{-1}\) (LAS-N) and 1276 cm\(^{-1}\) (LAS-C) correspond to asymmetric vibrations of C-O-C bonds (Xu et al., 2009; Kagimura et al., 2015a). In the region of 887 cm\(^{-1}\) there is a band with low intensity indicating \(\beta\)-configuration of lasiodiplodan (Corradi da Silva et al., 2008; Kagimura et al., 2015a; Ren et al., 2007; Thomas, 2015). Carboxymethylation of the polymer is indicated by the appearance of two strong absorption bands in the regions of 1422 cm\(^{-1}\) and 1598 cm\(^{-1}\) observed in the LAS-C spectrum resulting from symmetric and asymmetric stretching vibrations of the -COO- group, respectively (Xu et al., 2009; Wang and Zhang, 2009).

The Degree of Substitution (DS) represents the average number of functional groups introduced onto a glucose unit. The evaluation of this parameter is important for optimization of the reaction conditions for
substitution, besides providing an understanding of the new structure (Heinze and Koschella, 2005; Stojanovic et al., 2005; Liu et al., 2014). The derivatized sample presented a substitution degree (DS) of 0.45, which indicates that under the operational conditions employed in carboxymethylation, a relatively low degree of substitution of hydroxyls by carboxymethyl groups was obtained. In previous work we had obtained carboxymethylated lasiodiplodan with a higher DS (1.27) than observed in this study (Kagimura et al., 2015a). It is important to note that the substitution degree is a parameter of great relevance with relation to biological potential of the macromolecule, but a high value of DS does not mean a larger or smaller biological potential. It is the combination of various physico-chemical parameters, including the substitution degree that will define the potential of the macromolecule.

Figure 3 shows the TG-DTA curves obtained under different heating rates and atmospheres. Under purging conditions with air, native lasiodiplodan presented three steps of mass loss. Similarly, Fonseca et al. (2011) related three steps of mass loss by thermogravimetric analysis of botryosphaeran, an exopolysaccharide of the (1→3;1→6)-β-D-glucan type and concluded that both steps of mass loss was attributable to removal of water of hydration. In the present work, we observed that the first step of mass loss of native lasiodiplodan occurred up to 100°C, which was attributed to elimination of water of hydration of the exopolysaccharide and was indicated by an endothermic peak at 65°C in the DTA curve. The temperature of endothermic peaks relate to the loss of water was higher for botryosphaeran (134-149°C) (Fonseca et al., 2011) than that obtained with lasiodiplodan (65°C). This behavior can be attributed to differences in the chemical structures between these polysaccharides. Lasiodiplodan appears to be a molecule less hydrophilic than botryosphaeran. In fact, lasiodiplodan consists of a linear chain of (1→6)-β-D-linked glucopyranosyl residues with no ramifications; while botryosphaeran comprises a backbone of β-(1→3)-linked glucose residues with 22% branching with β-(1→6)-linked glucose residues (Barbosa et al., 2003). The branched structure of botryosphaeran makes the molecule more hydrophilic than lasiodiplodan because of the higher number of free hydroxyl groups available to interact through hydrogen bonding with water.

LAS-N was stable up to 230°C, where it began its thermal decomposition. Under a dynamic synthetic air atmosphere, two weight loss steps were observed and were attributable to oxidative decomposition of the dehydrated polysaccharide, which was indicated by exothermic peaks in the DTA curves. Under a nitrogen dynamic atmosphere, a single weight loss step by the dehydrated polysaccharide was verified and was attributed to volatilization of compounds generated by LAS-N thermal decomposition.

Regarding carboxymethylated sample, the TG curves indicated similar behavior of the sample under the different heating rates under a dynamic synthetic air atmosphere. A single weight loss step under heating rates of 5 and 10°C.min⁻¹ was verified and attributed to oxidative decomposition of the anhydrous polysaccharide. At a heating rate of 20°C.min⁻¹, the anhydrous polysaccharide showed similar behavior to that found with the LAS-N sample, with two weight loss steps attributed to oxidative decomposition and indicated by exothermic peaks in the DTA curve. Under a dynamic nitrogen atmosphere, anhydrous LAS-C also showed only one weight-loss step. It was observed that, regardless of the analysis condition, LAS-C did not establish a plateau at the end of the process (at final temperature of analysis), unlike what occurred with the sample LAS-N. This behavior indicates that the volatilization of compounds generated in the decomposition of LAS-C is slower than compared with the LAS-N sample.

The kinetic parameters presented in Table 2 were obtained by the method of multiple heating rates. Although the start of thermal decomposition occurred at temperatures close in the two anhydrous polysaccharide as found in the thermogravimetric curves (Fig. 3), modifying the molecule led to an increase in the activation energy before the decomposition process begins with LAS-C (Table 2).

As can be seen in Fig. 5, X-ray diffraction analysis of samples of LAS-N and LAS-C indicated diffraction patterns with broad peaks, which are common to β-glucans due to their polymeric structure (Veverka et al., 2014). Broad peaks indicate structures that are different from conventional crystalline structures (Kagimura et al., 2015a). Nevertheless, in both XRD diffractograms, three peaks were observed with diffraction angles at 20 value of about 21.1°, 23.8° and 39.6°, which indicate that lasiodiplodan has regions with certain molecular organization within an amorphous matrix. This can be observed from the high intensity of such peaks. Similarly, Anusuya and Sathiyabama (2014) observed three peaks (23.2°, 32.5°, 46° at 20) in β-glucan nanoparticle samples, which were attributed to a crystalline nature. The peaks observed at 21.1°, 23.8° (20) were more pronounced in the LAS-C sample, which suggests that the introduction of carboxymethyl groups in the native macromolecule may have contributed to some increase in its degree of crystallinity. Peaks that were a little more pronounced appeared at 41.6 and 42.8 (20), can also be assigned to arising from regions with certain crystallinity caused by carboxymethylation.

Scanning electron microscopy revealed that the native lasiodiplodan (Fig. 6A) presented an irregular
surface macrostructure with morphological structures in the form of plates or sheets containing irregularities like folds and twists along the surface area. The SEM scans showed that carboxymethylation leads to physical changes in the macrostructure of this modified biopolymer. Particularly, in the micrograph of LAS-C bubbles were observed across the all surface area of the plates. Similarly to what was observed in this study, polymeric macrostructure and the disruption of the percentage of 82.5% removal at 2 mg.mL$^{-1}$ peroxide at concentrations of 1 and 2 mg.mL$^{-1}$ scavenging capacity, even when used at lower concentration of the macromolecule was shown to have an influence on the antioxidant capacity. The H$_2$O$_2$ scavenging potential of ascorbic acid standard, was likewise influenced by the concentration in the assay. Increasing the concentration of ascorbic acid promoted greater removal activity of H$_2$O$_2$, which reached a percentage of 82.5% removal at 2 mg.mL$^{-1}$. Native lasiodiplodan was able to remove 5% of hydrogen peroxide at concentrations of 1 and 2 mg.mL$^{-1}$, but at 0.5 mg.mL$^{-1}$, it was not able. The carboxymethylation of lasiodiplodan promoted an increase of its hydrogen peroxide scavenging capacity, even when used at lower concentrations (0.5 mg mL$^{-1}$) in the assay, resulting in 7.94% H$_2$O$_2$ scavenging. Furthermore, higher concentration of LAS-C (2 mg.mL$^{-1}$) led to greater H$_2$O$_2$ scavenging potential (12.7%).

LAS-N was also able to scavenge hydroxyl radicals. At concentrations of 0.5 mg.mL$^{-1}$ and 1 mg.mL$^{-1}$, similar hydroxyl radical scavenging activities (24.8 and 23.8%, respectively) were observed. The increase in the concentration of the macromolecule to 2 mg.mL$^{-1}$, promoted an increase of around 37.3% in OH$^-$ scavenging activity, which reached 33.4% radical removal. Carboxymethylation intensified the hydroxyl radical scavenging activity at 1 and 2 mg.mL$^{-1}$ concentrations, when compared to LAS-N at similar concentrations. LAS-C was capable to remove 55.6% of hydroxyl radicals at a concentration of 1 mg.mL$^{-1}$ and 55.6% at 2 mg.mL$^{-1}$.

Both LAS-N and LAS-C demonstrated capacity for potassium ferricyanide reduction to potassium ferrocyanide. Similar reduction potential was observed between native and carboxymethylated lasiodiplodan at concentrations of 0.5 and 1 mg.mL$^{-1}$, but at a higher concentration, only LAS-C (2 mg.mL$^{-1}$) showed a higher reducing power.

Polysaccharides submitted to chemical modifications such as carboxymethylation, can have potentiated or modified biological functionality depending upon the Degree of Substitution (DS) obtained from derivatization (Weng et al., 2011; Kagimura et al., 2015b). In the present study, carboxymethylation of lasiodiplodan with a DS of 0.45, presented fungistatic activity towards Candida tropicalis (ATCC 13803) at a concentration of 17.5 mg.L$^{-1}$. On the otherhand, LAS-N, did not show effective fungistatic or fungicidal activities at this concentration against C. tropicalis. As can be seen in Table 3, an antimicrobial effect was observed only on the yeast, C. tropicalis. The other microorganisms tested were not inhibited by LAS-N nor LAS-C at the concentration evaluated.

**Conclusion**

Carboxymethylated lasiodiplodan with a degree of substitution of 0.45 was obtained using monochloroacetic acid as derivatizing agent under the conditions described. FT-IR spectroscopy analysis confirmed that lasiodiplodan was carboxymethylated, as verified by strong absorption bands in the regions of 1422 to 1598 cm$^{-1}$, resulting from symmetric and asymmetric stretching vibrations of the -COO- group, respectively. The thermal behavior profile evaluated by TG-DTA analysis showed that both native and carboxymethylated lasiodiplodan with DS of 0.45, exhibited relatively high thermal stability considering process conditions usually employed by the pharmaceutical industry. XRD analysis showed the LAS-N and LAS-C have no crystalline structure and that derivatization by carboxymethylation promoted regions with certain molecular orientation in the biopolymer matrix. SEM analysis revealed carboxymethylation promoted morphological changes in the biopolymer macrostructure, including disruption or breaking of structures similar to plates originally observed in the native biopolymer matrix. Antioxidant potential of the native lasiodiplodan was enhanced after derivatization, resulting in high hydroxyl radical removal ability and reducing power. Carboxymethylated lasiodiplodan demonstrated antimicrobial activity against Candida tropicalis yeast with Minimum Inhibitory Concentration (MIC) of 17.5 mg.L$^{-1}$. The results obtained in this study suggest that carboxymethylation of lasiodiplodan could be a strategic tool for the enhancement the macromolecule bioactivity. Further studies should be conducted for understanding and assessment of potential biological properties of lasiodiplodan derivatized by carboxymethylation. Research for evaluation of non-toxicity on cells and organs are also essential to ensure the safety of the new biomacromolecule.

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Author’s Contributions

Thais Vanessa Theis: Conducted experiments on the fermentative production of lasiodiplodan, its chemical derivatization by carboxymethylation and on thermal analysis, FT-IR, X-ray diffraction and scanning electron microscopy (SEM).

Gabrielle Cristina Calegari: Assisted Dr. Queiroz Santos in performing the bioassays on antioxidant and antimicrobial activity.

Vidiany Aparecida Queiroz Santos: Was responsible for conducting the bioassays on antioxidant and antimicrobial activity and in interpreting the data.

Henrique Emilio Zorel Junior: Worked on the treatment and interpretation of thermal analysis data.

Aneli M. Barbosa: Was involved in research discussions and contributed to the writing of the manuscript.

Robert F. H. Dekker: Was involved in research discussions and contributed to the writing and critical revision of the manuscript.

Mário A. Alves da Cunha: Planned, supervised and directed the research project and contributed to the writing and revision of the manuscript, as well as in interpreting the experimental data.

 Ethics

This article is original and the corresponding author confirms that all of the other authors have read and approved the manuscript. There are no conflicts of interest.

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