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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).

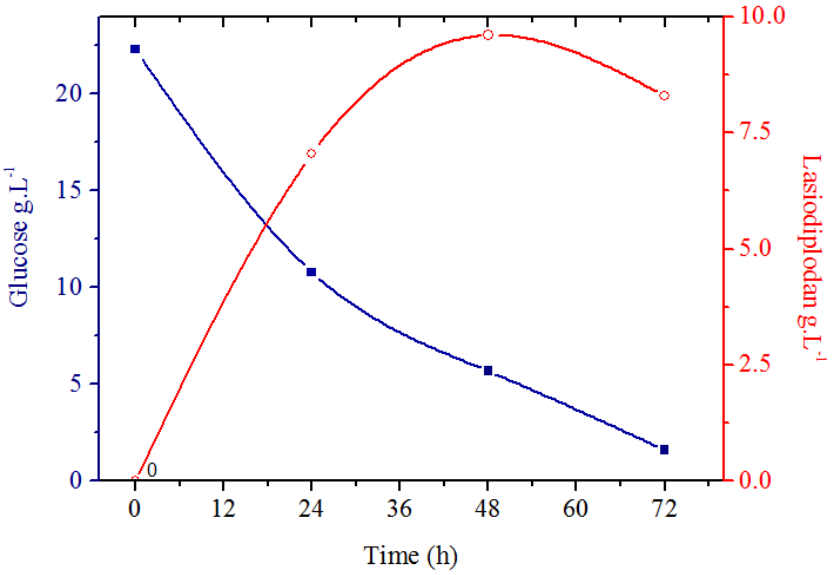

Fig. 1. Fermentative profile of (o) lasiodiplodan production by Lasiodiplodia theobromae MMPI and (■) glucose consumption

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_2HPO_4, 2 g L^{-1} $MgSO_4.7H_2O$ 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 ($Y_{P/S}$) 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_P) was calculated as the ratio of the maximum EPS concentration to the fermentation time. The volumetric productivity of biomass (Q_X) 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 (6×1.5 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 material was dissolved in 100 mL of distilled water 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 from the equation below:

$$Wc = \frac{c \times Mc \times (Vb - Vs)}{m} \times 100$$

$$DS = \frac{Wc \times Ma}{(100\% - Wc)} \times Mc$$

Where:

Wc = The content of carboxymethyl groups in the sample solution (% by mass)
c = The concentration of the HCl solution (0.1 $mol.L^{-1}$) used in the titration
Mc = The molar mass of functional carboxymethyl groups that reacted with LAS (58 $g.mol^{-1}$)
Ma = The molar mass of one anhydrous glucose unit (162 $g.mol^{-1}$)
Vb = The volume of HCl used for titrating the blank (mL)
Vs = The volume of HCl used for titrating the sample (mL)
m = The mass of the sample used in the titration LAS-C (mg)
DS = The degree of substitution of carboxymethyl groups in the sample

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 cupper radiation font (CuKα = 1.5418 Å), 15 mA current, 40 kV voltage, scanning range of 10° to 60° (2θ), a scanning speed of 5° min^{-1} and a step width of 0.02° (2I). 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_2O_2) removal ability was measured according to Liu *et al*. (2010). The reaction mixture consisted of 1 mL of H_2O_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_2SO_4 solution (2 $mol.L^{-1}$) and 7 mL of KI solution (1.8 $mol.L^{-1}$). The mixture was titrated with $Na_2S_2O_3$ (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 $Na_2S_2O_3$ solution used to titrate the control mixture and V_1 represents the volume titrated of the mixture containing the lasiodiplodan samples:

$$\%H_2O_2 scavaging = \frac{(V_0 - V_1)}{V_o} \times 100\%$$

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_2O_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

H_2O_2 (6 mmol.L^{-1}), 0.15 mL of sodium salicylate (20 mmol.L^{-1}) and 1 mL of the lasiodiplodan samples (0.5, 1.0 or 2 mg.L^{-1}). Ascorbic acid was used as a positive control. Absorbance of hydroxylated salicylate complex was measured at 562 nm after a 1 h incubation at 37°C. The percentage of hydroxyl radical scavenging activity was determined from the equation below, where A_1 is absorbance of the lasiodiplodan samples or ascorbic acid, A_0 is control absorbance and A_2 is reagent blank absorbance with sodium salicylate:

$$\%HO^{\bullet}scavenging = \left[1 - \frac{(A_1 - A_2)}{A_o}\right] \times 100$$

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 Müeller-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 (1.5×10^8 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.

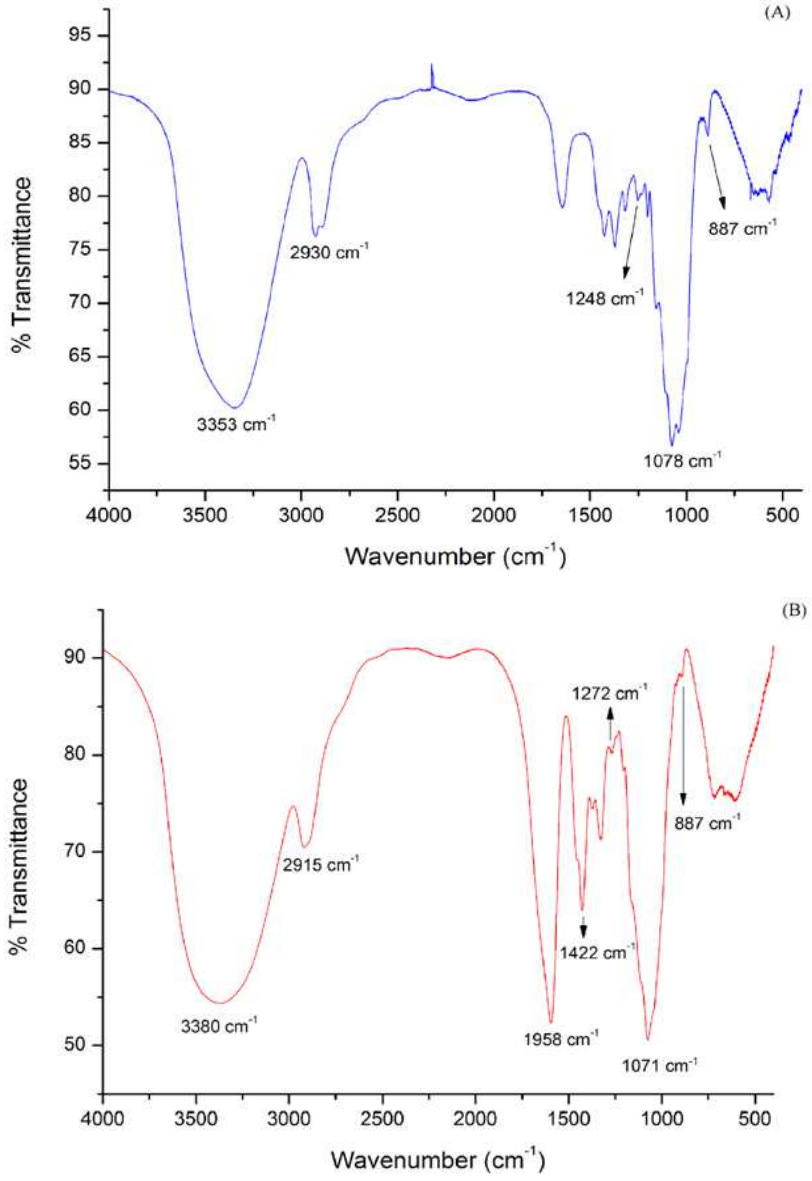

Fig. 2. FI-IR spectra of native (LAS-N) and carboxymethylated (LAS-C) lasiodiplodan

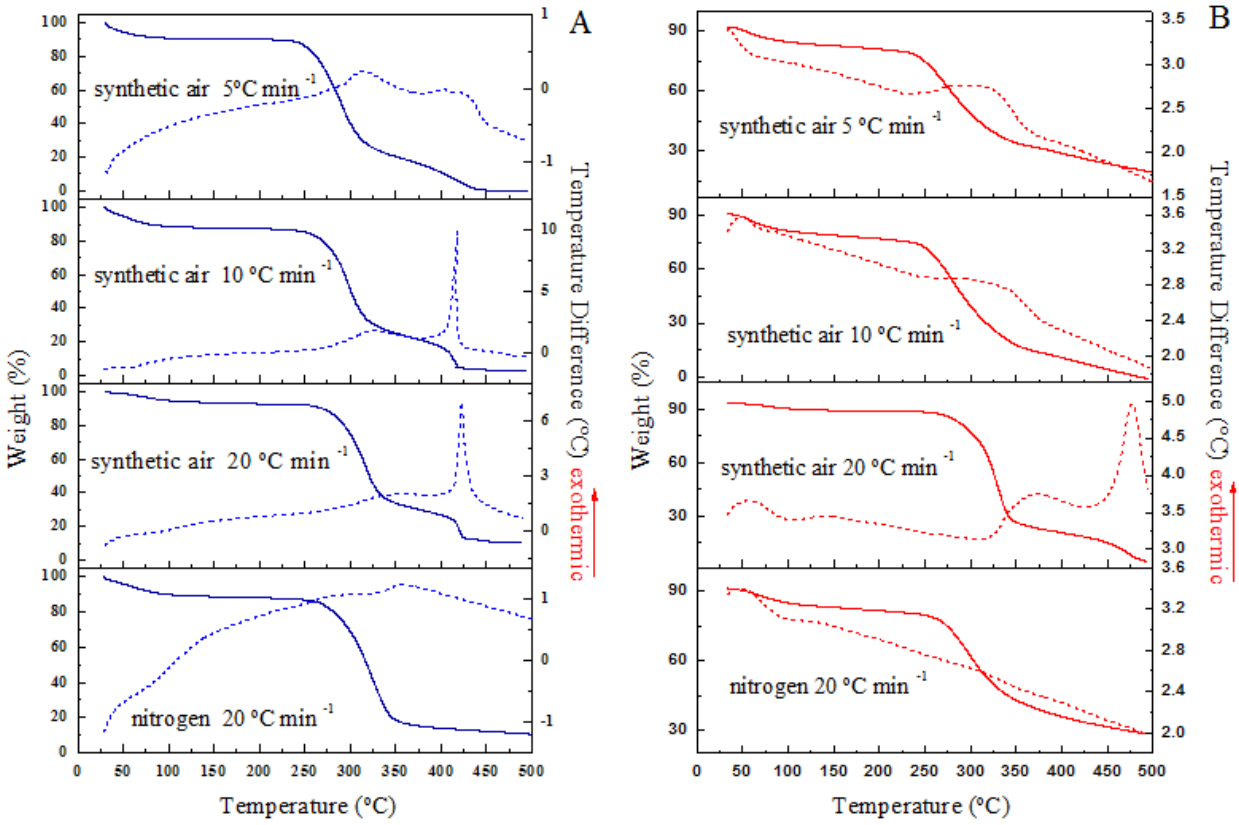

Fig. 3. TG (solid line) DTA (dotted line) curves of the LAS-N (A) and LAS-C (B) obtained under different atmospheric and heating rate conditions

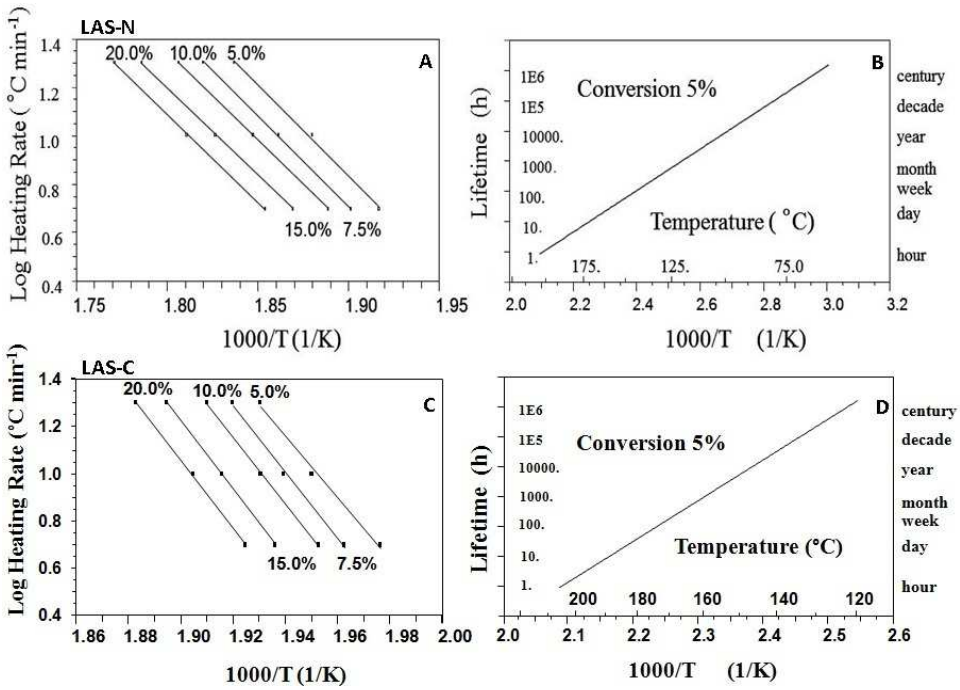

Fig. 4. Relationship between logarithmic heating rate and reciprocal temperature (A, C). The half-life estimated according to time (B, D) of LAS-N and LAS-C

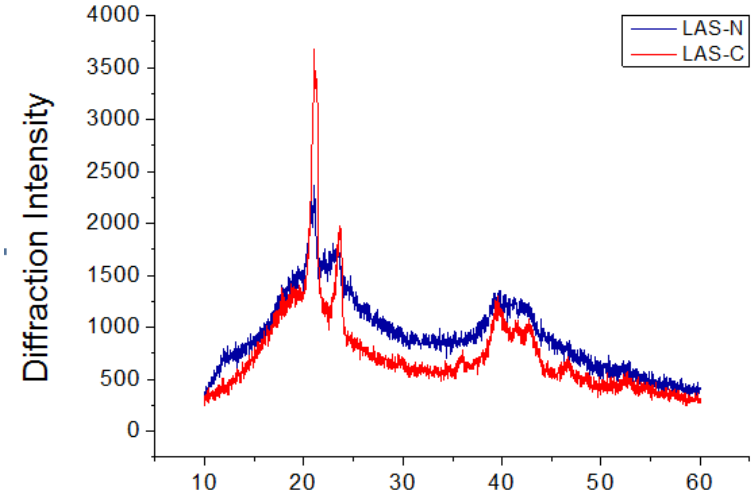

Fig. 5. Standard X-ray diffraction profiles of LAS-N and LAS-C samples

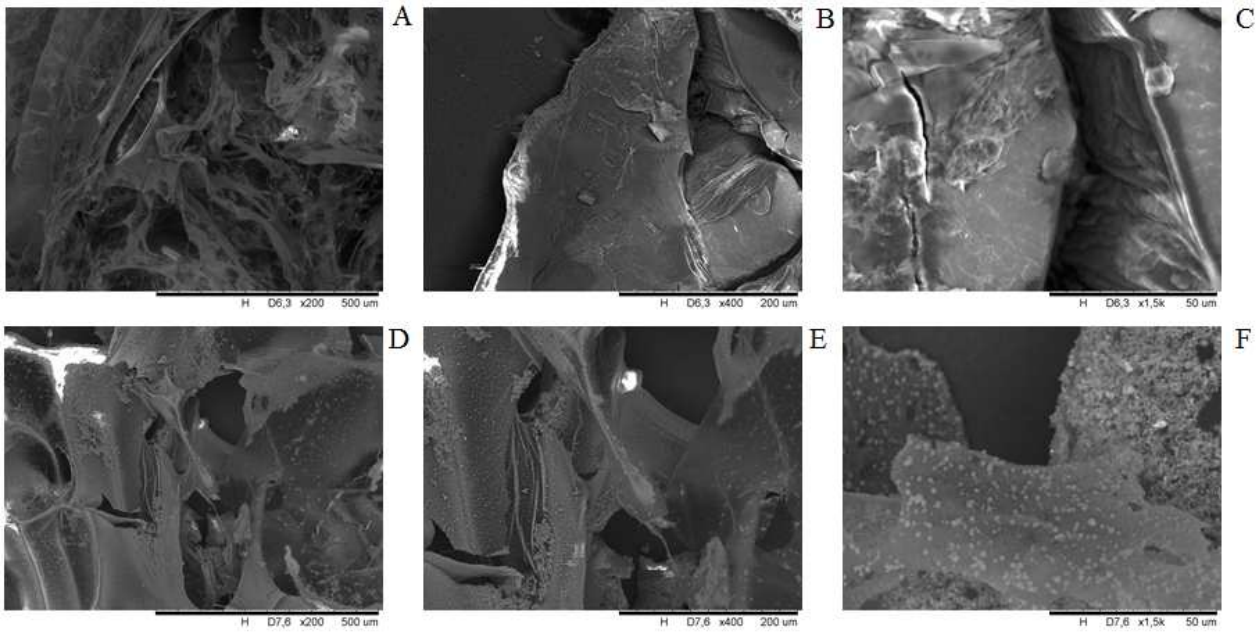

Fig. 6. Micrographs of LAS-N and LAS-C obtained by scanning electron microscopy: (A) LAS-N at 200× magnification, (B) LAS-N at 400× magnification and (C) LAS-N at 1500×magnification. (D) LAS-C at 200× magnification, (E) LAS-C at 400× magnification and (F) LAS-C at 1500× magnification

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
$Y_{P/S}$ ($g.g^{-1}$)	0.40±0.04
$Y_{X/S}$ ($g.g^{-1}$)	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. (E_a: Activation energy; Log Z: Pre-exponential factor; Temperature: Temperature of half-time).

Conversion (weight %)	E_a^* (kJ mol^{-1})		Log Z^{**} (min^{-1})		Temperature*** (half-life at 60 min) (°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})	
	LAS-N	LAS-C
Staphylococcus aureus (ATCC 25923)	> 17.5	> 17.5
Salmonella enterica Typhimurium (ATCC 0028)	> 17.5	> 17.5
Escherichia coli (ATCC 25299)	> 17.5	> 17.5
Salmonella bongori (ATCC 43975)	> 17.5	> 17.5
Candida tropicalis (ATCC 13803)	> 17.5	17.5*
Candida albicans (ATCC 18804)	> 17.5	> 17.5

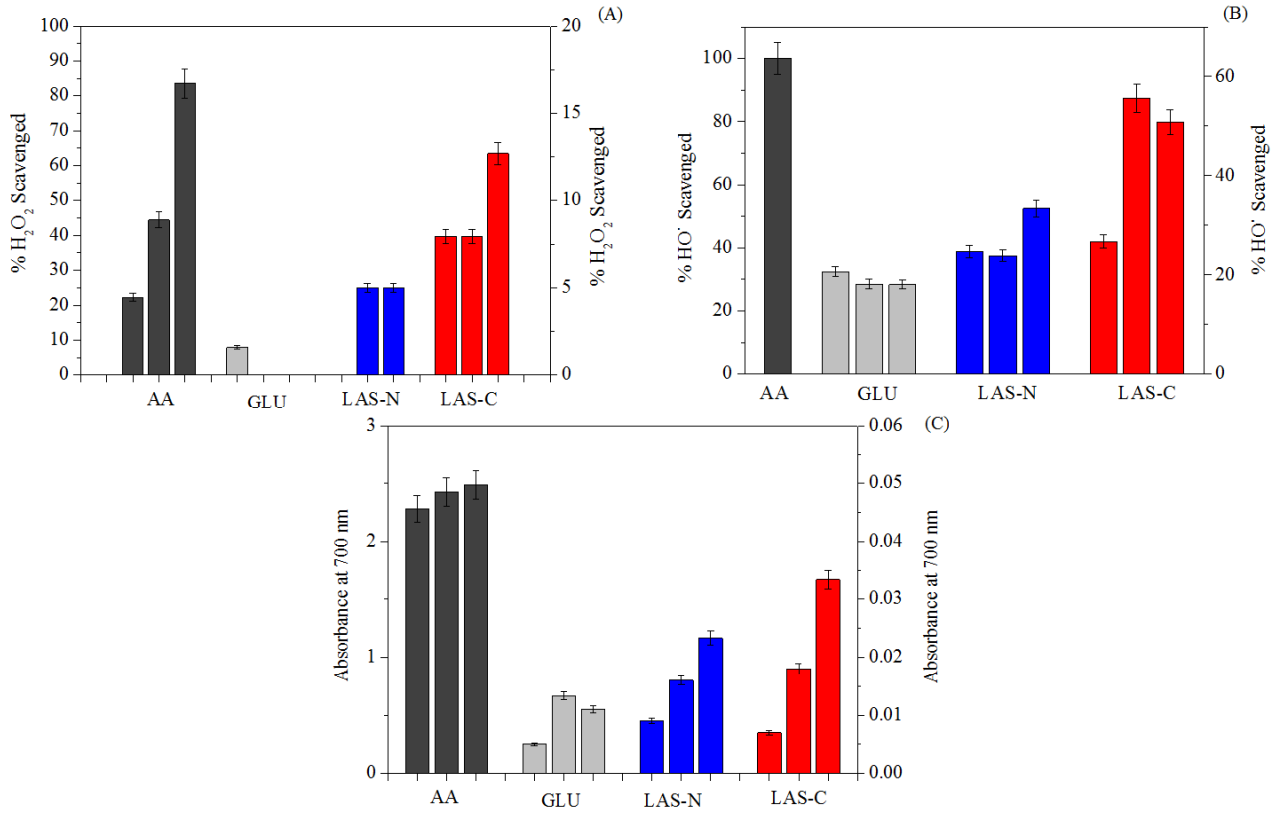

Fig. 7. Antioxidant activity of LAS-N, LAS-C, glucose (GLU) and ascorbic acid (AA) (positive control) measured by (A) Hydrogen peroxide scavenging, (B) HO• scavenging and (C) reducing power

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 β-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} 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 concentration of 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_P) 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} of glucose and 180 rpm (69.5%) (Cunha *et al*., 2012). The conversion yield of glucose to lasiodiplodan ($Y_{P/S}$) 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 β-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 2θ 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 2θ) in β-glucan nanoparticle samples, which were attributed to a crystalline nature. The peaks observed at 21.1°, 23.8° (2θ) 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 (2θ), 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 sample (Fig. 6F) at magnification of 1500 x, where bubbles were observed across the all surface area of the polymeric macrostructure and the disruption of the plates. Similarly to what was observed in this study, Kagimura *et al*. (2015a) also reported structures in form of irregular plates in micrographs of native lasiodiplodan. Furthermore, they observed that the carboxymethylation led to changes in surface macrostructure, including cracks along the surface of the plates.

Figure 6 shows the antioxidant potential of LAS-N and LAS-C. LAS-N and LAS-C demonstrated the ability of scavenge hydrogen peroxide and the concentration of the macromolecule was shown to have an influence on the antioxidant capacity. The H_2O_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_2O_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_2O_2 scavenging. Furthermore, higher concentration of LAS-C (2 mg.mL^{-1}) led to greater H_2O_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 $OH^{\bullet}$ 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.

References

Alves, C.Q., J.M. David, J.P. David, M.V. Bahia and R.M. Aguiar, 2010. Métodos para determinação de atividade antioxidante *in vitro* em substratos orgânicos. Química Nova, 33: 2202-2210. DOI: 10.1590/S0100-40422010001000033

Anusuya, S. and M. Sathiyabama, 2014. Preparation of β-D-glucan nanoparticles and its antifungal activity. Int. J. Biol. Macromolecules, 70: 440-443. DOI: 10.1016/j.ijbiomac.2014.07.011

Barbosa, A.M., R.M. Steluti, R.F.H. Dekker, M.S. Cardoso and M.L. Corradi da Silva, 2003. Structural characterization of Botryosphaeran: A (1→3;1→6)-β-D-glucan produced by the ascomyceteous fungus *Botryosphaeria* sp. Carbohydrate Res., 338: 1691-8. DOI: 10.1016/S0008-6215(03)00240-4

Bohn, J.A. and J.N. BeMiller, 1995. (1→3)-β-D-Glucans as biological response modifiers: A review of structure-functional activity relationships. Carbohydrate Pol., 28: 3-14. DOI: 10.1016/0144-8617(95)00076-3

Cho, E.J., J.Y. Oh, H.Y. Chang and J.W. Yun, 2006. Production of exopolysaccharides by submerged mycelial culture of a mushroom *Tremella fuciformis*. J. Biotechnol., 127: 129-140. DOI: 10.1016/j.jbiotec.2006.06.013

CLSI, 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts-M27-A3. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

CLSI, 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically-M7-A10. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Corradi da Silva, M.L.C., E.K. Fukuda, A.F.D. Vasconcelos, R.F.H. Dekker and A.C. Matias *et al*., 2008. Structural characterization of the cell walld-glucans isolated from the mycelium of *Botryosphaeria rhodina* MAMB-05. Carbohydrate Res., 343: 793-798. DOI: 10.1016/j.carres.2007.12.021

Cunha, M.A.A., J.A. Turmina, R.C. Ivanov, R.R. Barroso and P.T. Marques *et al*., 2012. Lasiodiplodan, an exocellular (1→6)-β-D-glucan from *Lasiodiplodia theobromae* MMPI: Production on glucose, fermentation kinetics, rheology and anti-proliferative activity. J. Industr. Microbiol. Biotechnol., 39: 1179-1188. DOI: 10.1007/s10295-012-1112-2

Fonseca, P.R., R.F.H. Dekker, A.M. Barbosa, J.L. Silveira and A.F. Vasconcelos *et al*., 2011. Thermal and rheological properties of a family of botryosphaerans produced by *Botryosphaeria rhodina* MAMB-05. Molecules, 16: 7488-7501. DOI: 10.3390/molecules16097488

Giese, E.C., J. Gascon, G. Anzelmo, A.M. Barbosa and M.A.A. da Cunha *et al*., 2015. Free-radical scavenging properties and antioxidant activities of botryosphaeran and some other β-D-glucans. Int. J. Biol. Macromolecules, 72: 125-30. DOI: 10.1016/j.ijbiomac.2014.07.046

Heinze, T. and A. Koschella, 2005. Carboxymethyl ethers of celulose and starch-A review. Macromolecular Symposia, 223: 13-39. DOI: 10.1002/masy.200550502

Hu, W., S. Chen, Q. Xu and H. Wang, 2011. Solvent-free acetylation of bacterial cellulose under moderate conditions. Carbohydrate Pol., 83: 1575-1581. DOI: 10.1016/j.carbpol.2010.10.016

Huang, D., O.U. Boxin and R.L. Prior, 2005. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem., 53: 1841-1856. DOI: 10.1021/jf030723c

Kagimura, F., M.A.A. da Cunha, T.V. Theis, C.R.M. Malfatti and R.F.H. Dekker *et al*., 2015a. Carboxymethylation of (1→6)-β-glucan (lasiodiplodan): Preparation, characterization and antioxidant evaluation. Carbohydrate Pol., 127: 390-399. DOI: 10.1016/j.carbpol.2015.03.045

Kagimura, F.Y., M.A.A. Da Cunha, A.M. Barbosa, R.F.H. Dekker and C.R.M. Malfatti, 2015b. Biological activities of derivatized D-glucans: A review. Int. J. Biol. Macromolecules, 72: 588-598. DOI: 10.1016/j.ijbiomac.2014.09.008

Kivela, R.L., L. Pitkänen, P. Laine, V. Aseyev and T. Sontag-Strohm, 2010. Influence of homogenisation on the solution properties of oat β-glucan. Food Hydrocolloids, 24: 611-668. DOI: 10.1016/j.foodhyd.2010.02.008

Krichen, F., W. Karoud, A. Sila, B.E. Abdelmalek and R. Ghorbel *et al*., 2015. Extraction, characterization and antimicrobial activity of sulfated polysaccharides from fish skins. Int. J. Biol. Macromolecules, 75: 283-289. DOI: 10.1016/j.ijbiomac.2015.01.044

Liu, W., H. Wang, X. Pang, W. Yao and X. Gao, 2010. Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. Int. J. Biol. Macromolecules, 46: 451-7. DOI: 10.1016/j.ijbiomac.2010.02.006

Liu, J., J. Luo, H. Ye and X. Zeng, 2012. Preparation, antioxidant and antitumor activities in vitro of different derivatives of levan from endophytic bacterium *Paenibacillus polymyxa* EJS-3. Food Chem. Toxicol., 50: 767-772. DOI: 10.1016/j.fct.2011.11.016

Liu, Y., Y. Liu, H. Jiang, L. Xu and Y. Cheng *et al*., 2014. Preparation, antiangiogenic and antitumoral activities of the chemically sulfated glucana from *Phellinus ribis*. Carbohydrate Pol., 106: 42-48. DOI: 10.1016/j.carbpol.2014.01.088

Lucas, E.F., B.G. Soares and E. Monteiro, 2011. Caracterização de Polímeros-Determinação de Peso Molecular e Análise Térmica. 1st Edn., E-papers, Rio de Janeiro, ISBN-10: 8587922254, pp: 366.

Miranda, C.C., R.H.F. Dekker, J.M. Serpeloni, E.A. Fonseca and I.M. Cólus *et al*., 2008. Anticlastogenic activity exhibited by botryosphaeran a new exopolysaccharide produced by *Botryosphaeria rhodina* MAMB-05. Int. J. Biol. Macromolecules, 42: 172-177. DOI: 10.1016/j.ijbiomac.2007.10.010

Mello, M.B., C.S. Machado, D.L. Ribeiro, A.F. Aissa and R.V. Burim *et al*., 2016. Protective effects of the exopolysaccharide Lasiodiplodan against DNA damage and inflammation induced by doxorubicin in rats: Cytogenetic and gene expression assays. Toxicology. DOI: 10.1016/j.tox.2016.05.010

Miranda-Nantes, C.C.B.O., E.A.I. Fonseca, C.T.B.V. Zaia, R.F.H. Dekker and N. Khaper *et al*., 2011. Hypoglycemic and hypocholesterolemic effects of botryosphaeran from *Botryosphaeria rhodina* MAMB-05 in diabetes-induced and hyperlipidemia conditions in rats. Mycobiology, 39: 187-193. DOI: 10.5941/MYCO.2011.39.3.187

Mitsou, E.K., N. Panopoulou, K. Turunen, V. Spiliotis and A. Kyriacou, 2010. Prebiotic potential of barley derived β-glucan at low intake levels: A randomised, double-blinded, placebo-controlled clinical study. Food Res. Int., 43: 1086-1092. DOI: 10.1016/j.foodres.2010.01.020

Queiroz, E.A.I.F., Z.B. Fortes, M.A.A. Da Cunha, A.M. Barbosa and N. Khaper *et al*., 2015. Antiproliferative and pro-apoptotic effects of three fungal exocellular β-glucans in MCF-7 breast cancer cells is mediated by oxidative stress, AMP-Activated Protein Kinase (AMPK) and the Forkhead transcription factor, FOXO3a. Int. J. Biochem. Cell Biol., 67: 14-24. DOI: 10.1016/j.biocel.2015.08.003

Ramos-Sánchez, M.C., A. Rodriguez-Torres, J.A. Leal, F.J. Martín-Gil and J. Martín-Gil, 1991. Thermolytical techniques to characterize fungal polysaccharides and bacterial lipopolysaccharides. Biotechnol. Progress, 7: 526-533. DOI: 10.1021/bp00012a007

Ren, J.L., R.C. Sun, C.F. Liu, Z.N. Cao and W. Luo, 2007. Acetylation of wheat straw hemicelluloses in ionic liquid using iodine as a catalyst. Carbohydrate Pol., 70: 406-414. DOI: 10.1016/j.carbpol.2007.04.022

Richter, J., V. Svozil, V. Král, L.R. Dobiášová and V. Vetvicka, 2015. β-glucan affects mucosal immunity in children with chronic respiratory problems under physical stress: Clinical trials. Ann. Translat. Med., 3: 52-52. DOI: 10.3978/j.issn.2305-5839.2015.03.20

Saldanha, R.L., J.E. Garcia, R.F.H. Dekker, L.A. Vilas-Boas and A.M. Barbosa, 2007. Genetic diversity among *Botryosphaeria* isolates and their correlation with cell wall-lytic enzyme production. Brazil. J. Microbiol., 38: 259-64. DOI: 10.1590/S1517-83822007000200013

Selbmann, L., F. Stingele and M. Petruccioli, 2003. Exopolysaccharide production by filamentous fungi: The example of *Botryosphaeria rhodina*. Antonie van Leeuwenhoek, 84: 135-145. DOI: 10.1023/A:1025421401536

Schultheis, E., M.A. Dreger, J.M.M. Villegas, J.I. Escalante and E.F. Lara *et al*., 2009. Rheological characterization of culture broth containing the exopolysaccharide PS-EDIV from *Sphingomonas pituitosa*. Biochem. Eng. J., 47: 116-121. DOI: 10.1016/j.bej.2009.07.012

Souza, N.L., J. Bartz, E.D.R. Zavareze, P.D. de Oliveira and W.S.V. da Silva *et al*., 2015. Functional, thermal and rheological properties of oat β-glucan modified by acetylation. Food Chem., 178: 243-250. DOI: 10.1016/j.foodchem.2015.01.079

Stojanovic, Z., K. Jeremic, S. Jovanovic and M.D. Lechner, 2005. A comparison of some methods for the determination of degree of substitution of carboxymethyl starch. Starch, 57: 79-83. DOI: 10.1002/star.200400342

Tatongjai, J. and N. Lumdubwong, 2010. Physicochemical properties and textile utilization of low and moderate substituted carboxymethyl rice starches with various amylose contents. Carbohydrate Pol., 81: 377-384. DOI: 10.1016/j.carbpol.2010.02.019

Thomas, S., 2015. Spectroscopic tools.

Túrmina, J.A., E. Carraro, M.A. Alves da Cunha, R.F.H. Dekker and A.M. Barbosa *et al*., 2012. Toxicological assessment of β-(1→6)-glucan (lasiodiplodan) in mice during a 28-day feeding study by gavage. Molecules, 17: 14298-309. DOI: 10.3390/molecules171214298

Vasconcelos, A.F.D., N.K. Monteiro, R.F.H. Dekker, A.M. Barbosa and E.R. Carbonero *et al*., 2008. Three exopolysaccharides of the β-(1→6)-D-glucan type and a β-(1→3; 1→6)-D-glucan produced by strains of *Botryosphaeria rhodina* isolated from rotting tropical fruit. Carbohydrate Res., 343: 2481-2485. DOI: 10.1016/j.carres.2008.06.013

Veverka, M., T. Dubaj, J. Gallovic, V. Jorík and E. Veverková *et al*., 2014. Beta-glucan complexes with selected nutraceuticals: Synthesis, characterization and stability. J. Funct. Foods, 8: 309-318. DOI: 10.1016/j.jff.2014.03.032

Wang, Y., Z. Ahmed, W. Feng, C. Li and S. Song, 2008. Physicochemical properties of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir. Int. J. Biol. Macromolecules, 43: 283-288. DOI: 10.1016/j.ijbiomac.2008.06.011

Wang, J. and L. Zhang, 2009. Structure and chain conformation of five water-soluble derivatives of a β-D-glucan isolated from *Ganoderma lucidum*. Carbohydrate Res., 344: 105-112. DOI: 10.1016/j.carres.2008.09.024

Wang, Y., Q. Mo, Z. Li, H. Lai and J. Lou *et al*., 2012. Effects of degree of carboxymethylation on physicochemical and biological properties of pachyman. Int. J. Biol. Macromol., 51: 1052-1056. DOI: 10.1016/j.ijbiomac.2012.08.022

Yuan, C., X. Huang, L. Cheng, Y. Bu and G. Liu *et al*., 2009. Evaluation of antioxidant and immune activity of Phellinus ribis glucan in mice. Food Chem., 115: 581-584. DOI: 10.1016/j.foodchem.2008.12.055

Xiu, A., Y. Kong, M. Zhou, B. Zhu and S. Wang *et al*., 2010. The chemical and digestive properties of a soluble glucan from *Agrobacterium* sp. ZX09. Carbohydrate Pol., 82: 623-628. DOI: 10.1016/j.carbpol.2010.05.027

Xu, J., W. Liu, W. Yao, X. Pang and D. Yin *et al*., 2009. Carboxymethylation of a polysaccharide extracted from *Ganoderma lucidum* enhances its antioxidant activities in vitro. Carbohydrate Pol., 78: 227-234. DOI: 10.1016/j.carbpol.2009.03.028

Weng, B.B.C., Y.C. Lin, C.W. Hu, M.Y. Kao and S.H. Wang *et al*., 2011. Toxicological and immunomodulatory assessments of botryosphaeran (β-glucan) produced by *Botryosphaeria rhodina* RCYU 30101. Food Chem. Toxicol., 49: 910-916. DOI: 10.1016/j.fct.2010.10.036

Yoshida, M.I., E.C.L. Gomes, C.D.V. Soares, A.F. Cunha and M.A. Oliveira, 2010. Thermal analysis applied to verapamil hydrochloride characterization in pharmaceutical formulations. Molecules, 15: 2439-2452. DOI: 10.3390/molecules15042439