Cyttaria hariotii E.Fisch. as a promising source of pullulan and Mn(II)-pullulan complexes for Mn-deficiency remediation in winter cereals

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Pullulan, a water-soluble polysaccharide consisting of maltotriose units used in the preparation of edible films and in drug delivery, is generally produced from starch by *Aureobasidium pullulans* (de Bary & Löwenthal) G.Arnaud fungus. In this article, the characterization of an alternative pullulan source –the stromata of *Cyttaria hariotii* E.Fisch. fungus– by elemental analysis, infrared spectroscopy and thermal analysis techniques is reported. With a view to a possible valorisation of this pullulan and its derivatives as bioactive formulations in agriculture, low-molecular weight pullulan (<7 kDa) complexes with Mn(II), suitable for the remediation of Mn-deficiencies in winter cereal by foliar application, were synthesized and characterized by mass spectrometry.

Keywords: E1204; FTIR spectroscopy; llao-llao; mass spectrometry; thermal analysis

1. Introduction

Cyttaria hariotii E.Fisch. is one of the most common fungi in Andean-Patagonian forests. General information on this fungus is presented in Supplementary Material. The main carbohydrate component of *C. hariotii* is pullulan, an homo-polysaccharide composed by maltotriosyl repeating units and a small number of maltotetraose residues (Shingel 2004). Pullulan is usually biosynthesized by strains of *Aureobasidium pullulans* (de Bary & Löwenthal) G.Arnaud, and has applications in blood plasma substitutes, edible coatings for fruits, additives and cosmetics (Piergiovanni and Mascheroni 2007).

Upon subjection to the action of enzymes or by treatments with chemicals, low-molecular pullulan may be obtained, which can find applications through complexation with divalent transition metal ions (Mitić et al. 2011). Although synthetic procedures for the formation of complexes between M(II) transition metal ions and pullulan are already

available in the bibliography (Mitić et al. 2011; Mitić et al. 2018), the one corresponding to Mn(II)-pullulan complexes has not been reported so far.

The aim of the study presented herein has been to investigate the vibrational and thermal properties of *C. hariotii* stromata, assessing its suitability for its potential use as raw material. Given the large number of active centres in pullulan that are capable of bond formation with M(II) ions, the preparation of Mn(II) ions complexes with low-molecular weight pullulan from *C. hariotii* has also been investigated. Such complexes may find application in foliar Mn fertilization.

2. Results and discussion

2.1. Characterization of pullulan from C. hariotii

2.1.1. Elemental analysis

Experimental values of CHON for *C. hariotii* stromata (36.8% in C, 7.0% in H, 44.5% in O, and 0.6% in N) were in agreement with calculated values on basis of a ~73 wt% content in pullulan.

2.1.2. Vibrational characterization

The infrared spectra of the samples from *C. hariotii* were compared with those of the commercial pullulan and of maltotriose (Figure S2). Band assignments are summarized in Table S2. The spectra of the samples from *C. hariotti* exhibited very similar features to those of commercial pullulan, although the bands at 720 and 755 cm^{-1} (maltotetraose units) and at 1080 cm^{-1} (α-(1→4) glycosidic linkages) were missing. The absence of bands assignable to ester functionality (1653 cm^{-1}) suggests a low esterification or etherification degree, which confers high solubility. Thus, the concurrence of low

contents in α-(1→4)-D-glucosidic bonds and a low esterification degree would explain the moderate-high solubility in water exhibited by pullulan from *C. hariotii*.

2.1.3. Thermal characterization

The thermal curves of *C. hariotii* stromata samples in inert and oxidative conditions (Figure S3), together with their interpretation, are presented in Supplementary Material. A good correspondence with pullulan obtained from *A. pullulans* was found for the TG thermograms and the maxima of the DTG peaks and DSC effects (Table S3) (Ramos Sánchez 1990; Katsikas et al. 1993).

2.1.4. Comparison with pullulan obtained from other sources

According to both the literature data and results presented above, stromata of *C. hariotti* can be an excellent source of pullulan, although it exhibits differential characteristics as compared to pullulans from other origins, such as its higher water solubility and lower functionalization degree. In addition, while the M_W of pullulan obtained from *A. pullulans* ranges from thousands to 2,000,000 Da (Rekha and Sharma 2007), pullulan from *C. hariotii* can have a M_W as low as 140 kDa (Oliva et al. 1986). Moreover, some of the drawbacks detected when *A. pullulans* is chosen for pullulan production (Mishra 2017) would be avoided.

2.2. Potential applications of pullulan from C. hariotii: complexes of low-molecular weight pullulan with Mn(II)

As discussed above, low-M_W pullulan can find applications through complexation with divalent transition metal ions (Mitić et al. 2011).

The MALDI-TOF/TOF MS spectra of the Mn(II)-pullulan complexes (Figure S4) showed repeating units of 230 Da and 232 Da, which correspond to Glc*p*$(OH)_4$ and

MnGlc*p*(OH), respectively, where Glc*p* is the anhydro-glucopyranose moiety. Since peaks with *m/z* difference of 54.9 Da (which would to correspond to Mn) did not appear, one may conclude that Mn(II)-RLMP complexes were very stable. According to Mitić et al. (2011), a tentative structure with tetragonal distorted O_h coordination with O ligand atoms can be proposed for the $[Mn(Glcp)_3]$ and $[Mn_2(Glcp)_3(H_2O)_2]$ complexes.

These Mn(II)-pullulan complexes may be useful as a bioactive formulation in agriculture, given that manganese deficiency remains a major unsolved nutritional problem in agricultural plant production. Such deficiency causes substantial yield reductions, especially in the case of winter cereals cultivated on sandy and calcareous soils (Hebbern et al. 2005; Mousavi et al. 2011; Schmidt Sidsel Birkelund et al. 2013), or as a result of increasing phosphorus status due to the application of high levels of animal manure and P-fertilizers (Schjoerring et al. 2011). This deficiency is traditionally corrected by repeated foliar Mn applications (Schmidt Sidsel B. et al. 2016; Ullah et al. 2017). Given the high solubility of the Mn(II)-pullulan complexes reported herein, the pullulan obtained from *C. hariotti* may thus find application in such agricultural practices, using it as a carrier for Mn(II) delivery and expanding its current applications as a carrier in drug delivery (dos Santos and Grenha 2015; Pandurangan et al. 2016; Nasrollahzadeh et al. 2019).

Alternatively, the pullulan for *C. hariotti* may also be used for other previously reported applications in agriculture: for instance, as a binding agent for solid fertilizers, providing higher water solubility and allowing time-released N fertilization (Matsunaga et al. 1977), or in seed coatings (Matsunaga et al. 1978).

3. Conclusions

The present study puts forward the use *C. hariotii* as a cheap source of pullulan, using a

green chemistry technique (sonication) and avoiding some of drawbacks detected when *A. pullulans* is chosen for pullulan production. Moreover, pullulan from *C. hariotii* exhibits higher water solubility and a lower functionalization degree than the pullulan obtained from *A. pullulans* or *R. paludigenum*, making it more suitable for processing. Whereas hydrophobised pullulan is used preferably as a coating material in drug delivery applications, pullulan with higher water solubility and low viscosity, as the one obtained from *C. hariotii*, may have countless industrial applications as a food additive, blood plasma substitute, flocculant and adhesive. Relative to its potential applications in agriculture, complexes of Mn(II) ion with low-M_W pullulan (6 kDa) from *C. hariotii* were synthesized and characterized by MALDI TOF/TOF MS. By repeated foliar spraying, these complexes may be suitable for the timely alleviation of latent Mn deficiency in winter cereals, with a view to ensuring winter survival, and increasing grain yields.

Conflicts of interest

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Cyttaria hariotii E.Fisch. as a promising source of pullulan and Mn(II)-pullulan complexes for Mn-deficiency remediation in winter cereals

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Keywords: E1204; FTIR spectroscopy; llao-llao; mass spectrometry; thermal analysis

General information about *Cyttaria hariotii* E.Fisch. fungus

Cyttaria is a genus of ascomycete fungi that encompasses 14 species found in South America and Australia. Among these, the "llao-llao" fungus, *Cyttaria hariotii* E.Fisch., is one of the most common fungi in Andean-Patagonian forests. Originally described by mycologist M.J. Berkeley in 1842, it owes its name to Greek *cyttarion* = socket, because of the apothecial cavities of its stroma (Figure S1).

Cyttaria spp. exclusively parasite *Nothofagus* spp. trees: ‘ñire’ (*Nothofagus antarctica* (G.Forst.) Oerst.), ‘lenga’ (*Nothofagus pumilio* Krasser), ‘coihue’ or ‘coigüe’ (*Nothofagus dombeyi* (Mirb.) Oerst.), ‘guindo’ (*Nothofagus betuloides* (Mirb.) Oerst.) and ‘coihue’ or ‘coigüe de Magallanes’ (*Nothofagus nitida* Krasser).

Cyttaria fructifications (stromata) form annually in spring and can reach the size of golf ball (Figure S1). In particular, the fruiting bodies of *C. hariotii* typically have a distinctive yellow to orange colour and were used by indigenous people of the region (the mapuches) for the production of an alcoholic drink (‘chicha’).

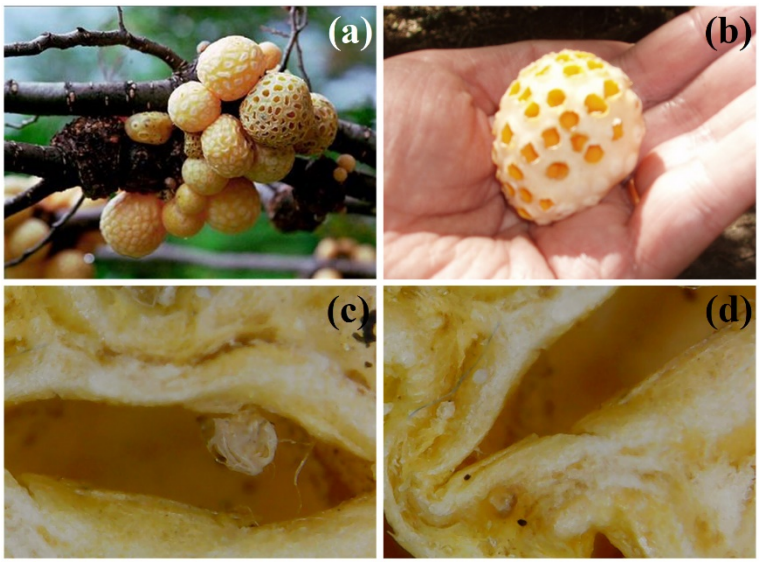

Figure S1. Stromata of *Cyttaria hariotii* showing the immersed apothecia: (a,b) photos, and (c,d) micrographs at 500× magnification.

Previously reported analyses of *Cyttaria* spp. showed that their crude lipid and ash contents were similar to those of other edible fungi, but amino acid analyses revealed that proteins were deficient in methionine and cysteine, and that the contents in valine, isoleucine, leucine, and lysine amino acids were below the WHO recommended values (Schmeda-Hirschmann et al. 1999). As regards their carbohydrate content, *Cyttaria* spp., when mature, show up to 10 % w/v of simple sugars (Lederkremer and Ranalli 1967) and up to 73% of polysaccharides. Other bromatological analysis results for *C. hariotii* are reported in Table S1.

Table S1. Bromatological analysis of *Cyttaria hariotii* collected from Fuerte de Bulnes, Chile (expressed in g per 100 g of dry weight) (Schmeda-Hirschmann et al. 1999).

Sample	Moisture	Crude protein	Crude lipids	Crude fiber	Ash	NNE	Phosphate (mg%)
Cyttaria hariotii	10.0	7.50	2.10	8.00	7.00	75.40	100.0

NNE: non-nitrogenated elements (carbohydrates).

Pullulan: structural information, properties and obtaining methods

Pullulan is an extracellular homo-polysaccharide composed by maltotriosyl repeating units (*α*-(1→4)Glc*p*-*α*-(1→4)Glc*p*-*α*-(1→6)Glc*p*-), connected by *α*-(1→6) linkages of linear *α*-(1→6) linked maltotriose units and a small number of randomly distributed maltotetraose (*α*-(1→4)Glc*p*-*α*-(1→4)Glc*p*-*α*-(1→4)Glc*p*-*α*-(1→6)Glc*p*-) residues (Shingel 2004) (Glc*p*=D-glucopyranose). Despite its linear structure, it exhibits good flexibility. The *α*-(1→6) linked maltotriose repeat unit interferes with hydrogen bonding and crystallization, and confers higher water solubility on pullulan (unlike cellulose and other well-known polysaccharides). Another characteristic of pullulan associated with its solubility is its esterification or etherification degree: it is known that partial esterification or etherification of the polymer chain can reduce its solubility in water and that a complete esterification or etherification results in insolubility (Yuen 1974).

Important properties of pullulan are its high resistance to fats, its ability to act as good barrier to gases and being non-toxic, biodegradable, non-mutagenic and non-carcinogenic.

Pullulan is usually biosynthesized by strains of the yeast-like polymorphic fungus *Aureobasidium pullulans* (de Bary & Löwenthal) G.Arnaud, and is more expensive (*ca*. 25 $/kg) than other exopolysaccharides, which is a major limiting factor for its effective application. The high production costs for pullulan production by *A. pullulans* may be reduced by the alternative use of other potential sources: the saprophytic (sometimes mycoparasitic) fungus *Tremella mesenterica* Retz. (Fraser and Jennings 1971); the fungal agent of chestnut blight, *Cryphonectria parasitica* (Murrill) M.E.Barr (Corsaro 1998); the obligate tree parasitic fungi *C. hariotii* and *Cyttaria darwinii* Berk. (Oliva, Cirelli, et al. 1986; Oliva, Fernandez Cirelli, et al. 1986); the lichenized ascomycete *Teloschistes flavicans* (Sw.) Norman (Reis 2002); *Rhodotorula bacarum* (Buhagiar) Rodr.Mir. & Weijman (Chi and Zhao 2003) and, recently, *Rhodosporidium paludigenum* Fell & Tallman (Singh and Kaur 2018).

Experimental

The samples of “llao-llao” (*C. hariotii*) under study were collected in the Bernardo O'Higgins National Park (50°S, 74°W), Chile, in December 2018. The identification was based on morphological data and host associations.

Standard pullulan from *A. pullulans* (CAS 9057-02-7) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) for comparison purposes. Maltotriose (CAS 1109-28-0; ≥90%), also used for comparison purposes, was supplied by Henan DaKen Chemical Co. Ltd. (Zhengzhou City, China).

Pullulan from *C. hariotii* was extracted from “llao-llao” stromata by ultrasonic assisted digestion in water (200 mg/10 mL) for 2 hours. A UIP1000hdT (Hielscher, Teltow, Germany, 1000 W, 20 kHz) ultrasonic bath was used to promote the extraction of pullulan. The ultrasound bath equipment was filled with water and a one-by-time glass centrifuge tube was placed, with the aid of a base, in the centre of the bath to perform the ultrasound-assisted extraction. A Labolan (Esparza de Galar, Navarra, Spain) centrifuge with 50 mL centrifuge tubes was used to separate the solid from the liquid phase.

Hydrolysis of pullulan from *C. hariotii* was carried out by addition of citric acid (CAS 77-92-9, ≥99.5%) and hydrogen peroxide (CAS 7722-84-1, 30 wt.% in H_2O), purchased from Sigma-Aldrich. Optional release of protein remains from the *C. hariotii* hydrogel was attained by adding 1 mL of 10% v/v Neutrase® (E.C.3.4.24), a zinc metallo endo-protease from *Bacillus amyloliquefaciens* that randomly hydrolyses peptide bonds, supplied by Strem Chemicals Inc. (Newburyport, MA, USA).

Manganese(II)-pullulan complexes were synthesized at neutral or slightly alkaline pH from pullulans of certain MW produced by partial hydrolysis of *C. hariotii* pullulan with citric acid, successive fractionation with 96% (vol.) ethanol, subsequent alkalinisation with NaOH and finally, by dropwise addition of 100 mM $MnCl_2$.

Optical microscopy images were acquired with an Avangard Optics (China) AN-E500 iScope 500x USB digital microscope.

Carbon/Hydrogen/Nitrogen (CHN) elemental analysis was carried out with a LECO (St. Joseph, MI, USA) CNH628 apparatus. A non-dispersive infrared absorption system was used for detection of C and H, and a thermal conductivity (TC Cell) system was used for detection of N.

The infrared vibrational spectrum was characterized using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 Fourier-Transform Infrared (FTIR) spectrometer, equipped with an in-built diamond attenuated total reflection (ATR) system. The spectrum was collected in the 400-4000 cm^{-1} region at room temperature, with a 1 cm^{-1} spectral resolution; a total of 64 scans were co-added and the resulting interferogram was averaged.

Thermal analysis was performed with a Mettler Toledo (Columbus, OH, USA) TG-DSC2 device, heating the samples from 50 to 600 °C, under both N_2 and air flow (20 $cm^3 \cdot min^{-1}$), with a heating rate of 20 °C·min^{-1}.

MALDI-TOF/TOF MS characterization was performed using the “dried droplet" method to prepare the samples. 2.0 µL of matrix (10 mg/mL 2,5-dihydroxybenzoic acid, DHB) and 2.0 µL of pullulan complex (1 mg/mL) were mixed, and then 0.5 µL of the obtained mixture was applied on a MALDI target plate of polished steel (Bruker Daltonik GmbH, Bremen, Germany) and allowed to dry for 15 min at room temperature. The plate was inserted into a Bruker Autoflex LRF speed mass spectrometer operating in linear positive mode in the *m/z* range of 450-10000 Da. A solid-state, Nd:YAG 355 run SmartBeam laser, with a frequency of 1000 Hz was used for ionization. Spectra were obtained by accumulating 2000 laser shots from 4 different spots. The MS spectra were acquired using the FlexControl software.

Vibrational characterisation results

The infrared spectra of the samples from *C. hariotii* and of the commercial pullulan used as a reference are compared in Figure S2 and Table S2. The strong absorption at 3300 cm^{-1} indicates that both *Cyttaria*-derived samples and commercial pullulan have some –OH repeating units. The other strong absorption bands at 2920 cm^{-1} and 2849

cm^{-1} indicate that a sp^3 C–H bond of alkane compounds exists. The band at 1683 cm^{-1} may result from chelation between a C=O group and a neighbouring O–H group. In the 1560–650 cm^{-1} region, which is characteristic for the pullulan molecule as a whole, the spectra of the samples from *C. hariotti* exhibited very similar features to those of commercial pullulan. The absorption at around 1558 cm^{-1} represents C=C stretching vibration indicative of the lignin. Absorption bands at 1638, 1370 and 1150 cm^{-1} should be assigned to O–C–O stretching, C–O–H bending and C–O–C stretching, respectively.

Absorption at 920 cm^{-1} is indicative of the presence of α-(1→6)-D-glucosidic bonds, while the absorptions at 755 and 720 cm^{-1} are associated with the presence of α-(1→4)-D-glucosidic bonds (Thirumavalavan et al. 2009). The weak band at 835 cm^{-1} is also associated with α-D-glucopyranoside units.

Absorptions intensities at 1080 and 1105 cm^{-1} inform on the proportion of α-(1→6) and α-(1→4) glycosidic linkages in the pullulan chains. The disappearance or a decrease in intensity of the band at 1080 cm^{-1} in favour of the one that appears at 1105 cm^{-1} implies that the α-(1→6)/α-(1→4) ratio is higher than that expected from the strict sequence of two α-(1→6) linkages and one α-(1→4) in the pullulan structure.

In polysaccharides, when a band appears at 1045 cm^{-1}, it is characteristic of a more organized structure, but when a band at 1020 cm^{-1} is shown, as it occurs for all the studied samples, an amorphous structure occurs.

In the spectrum of pullulan from *C. hariotii* treated with Neutrase® for purification purposes, a strong absorption band at 1148 cm^{-1} (C–O; C–C stretching) was observed, together with the absence of the bands at 1636 cm^{-1} and at 1420–1436 cm^{-1} (COO^- stretching vibration, attributed of carboxyl groups) (Nejatzadeh-Barandozi and Enferadi 2012), and at 1254 cm^{-1}. Since carboxyl constitutes the main functional moiety

involves in flocculation (Zheng et al. 2008; Wan et al. 2013), its entire removal from pullulan would not be positive, advising against the use of this treatment.

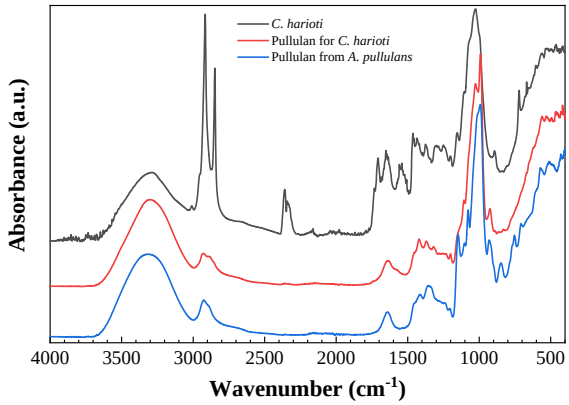

Figure S2. FTIR spectra of *Cyttaria hariotii*, pullulan obtained from *C. hariotii* and pullulan obtained from *A. pullulans*.

Table S2. Comparison of the absorption bands in the FTIR spectra of "llao-llao" (*Cyttaria hariotii*) fungus, of its main component, of commercial pullulan obtained from *A. pullulans* and of maltotriose.

C. hariotii	Pullulan from			Maltotriose	Assignation
	C. hariotii	*C. hariotii*†	*A. pullulans*		
3291	3300	3351	3312		OH stretching vibrations
3010					intrahydrogen bonds
2916	2927	2938	2926	2926	C–H stretch
2849					C–H stretch
2360					C-O stretch (carboxylic group)
1733					C=O stretch (o-acetyl esters)
1707		1712			C=O (carboxyl)
1684					C=O and O-H chelation / amide I
1653			1654		C–O stretch (esters)
1647			1645		O–H bending / O–C–O
1636	1636		1637	1637	asymmetrical COO^- stretch
1558					C=C stretch / COO^-, amide II
1540					–NH deformation
1507					C=C aromatic
1464			1454		C–H stretch / C–O stretch and O–H bend
1436	1420		1423	1416	C–O–H bend / C=O str (sym) of COO^-
1375	1371	1397	1355-68	1367	C–O–H bend / S-O stretch
1301	1320	1332			CH_2 twisting
1250	1254		1250-56	1255	ω-carboxyalkanoates / o-acetyl esters
1201	1201	1202	1206	1201	C–O–C stretch in esters
1154		1148	1158	1160	C–O–C stretch / exocyclic C–O stretch
1104	1106	1105	1077		C–O stretching / C–O–H bending
1024	1027	1015	1019	1029	C–O stretching / O–C=O
	991		992		CH_2 twisting
893	924	896	931	928	both α-(1→4) and α-(1→6) linkages
837	831		832-50	847	α-D-glucopyranoside units
		773	755	769	α-(1→4) in (1→4)(1→6)-D-glucans
721			711-720		α-(1→4)-D-glucosidic linkages

† After Neutrase® treatment

Thermal characterisation results

The TG, DTG and DSC curves of *C. hariotii* stromata samples in inert and oxidative conditions are shown in Figure S3.

From the TG curve, at the end of the heating cycle (600 °C) and in N_2 atmosphere, about 25.3% carbonaceous residue was found, a value higher than that obtained in an oxidative atmosphere (16.3%). For comparison purposes, the heating of commercial pullulan at 650 °C in N_2 has been reported to produce *ca*. 20% residue (Katsikas et al. 1993). The difference between the two values should be ascribed to differences both in the final heating temperature and in the heating rates (provided that the formation of char is heating rate dependent, with more residue being produced at lower heating rates).

In DTG curves, two decomposition peaks were observed at around 223.5 and 281–284 °C, both in oxidative and non-oxidative conditions (Table S3). These peaks were followed by another five peaks above 440 °C, albeit only in oxidative conditions. While the decomposition under 285 °C would be representative of long chain scission, the features at higher temperatures would be associated with the decomposition of the glucose ring. For pullulan obtained from *A. pullulans*, the maxima of the DTG peaks reported by Katsikas et al. (1993) were at 300 and 310 °C, in oxidative and non-oxidative conditions, respectively. However, the authors also referred a small shoulder at about 270 ºC in both the non-oxidative and oxidative case, which was ascribed to the decomposition of lower molecular weight fractions of the sample. The same study also referred that oxidative DTG curves of pullulan had a second maximum at 445 °C.

Regarding the DSC curves, a good correspondence with the DTG curves was observed, both in oxidative and non-oxidative conditions, for the effects at ~225 °C, at ~275 °C, and the four exotherms that appeared at above 460 °C (at 461, 557, 581 and 590 °C). The DSC curves also showed two effects not shown in the DTG curves: one at 178 °C, close to the one exhibited by pullulan obtained from *A. pullulans* at 171.6 °C (Ramos Sánchez 1990); and another effect at 314–317 °C, in reasonably good correspondence with that exhibited by pullulan at 304.3 °C in air (at a heating rate of 10 ºC/min) (Ramos Sánchez 1990).

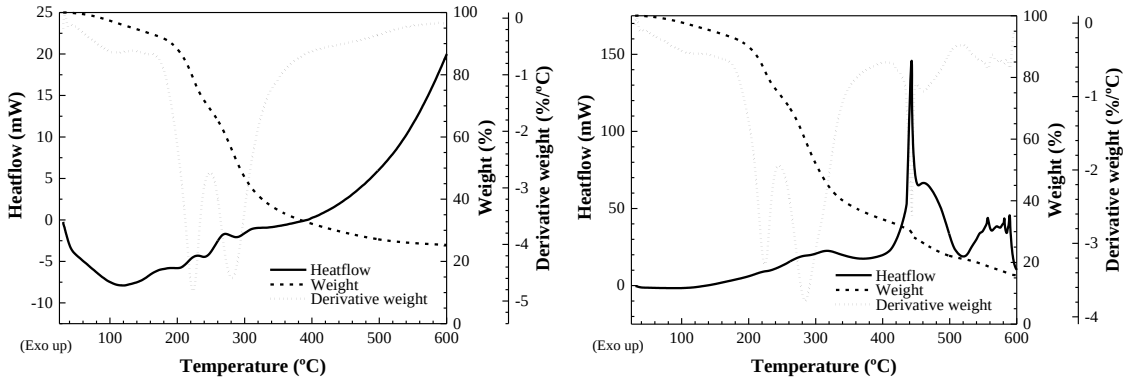

Figure S3. DSC (*dotted line*, y-axis on the left side of the graph), TG (*solid line*, first y-axis on the right side of the graph) and DTG (*dashed line*, second (rightmost) y-axis on the right side of the graph) curves for *Cyttaria hariotii* stromata in: (*left*) inert and (*right*) oxidative conditions.

Table S3. Temperatures of thermal effects for "llao-llao" (*Cyttaria hariotii*) fungus stromata samples in inert and oxidative conditions.

Atmosphere	DTG							DSC								
N_2	223.5	281						178	228	271	314					
Air	223.5	284	444	461	557	581	590		225	272	317	443	462	557	581	589

MS characterization of complexes of low-molecular weight pullulan with Mn(II)

The MALDI-TOF/TOF MS spectra of the Mn(II)-pullulan complexes (Figure S4) showed repeating units of 230 Da and 232 Da, which correspond to Glc*p*$(OH)_4$ and MnGlc*p*(OH), respectively, where Glc*p* is the anhydro-glucopyranose moiety.

Characteristic peaks were those that occurred at the following *m/z*: 447.8 [$Mn_2(Glcp)(H_2O)_6(OH)_4$], 540.4 [$Mn(Glcp)_3$], 632.3 [$Mn_2(Glcp)_3(H_2O)_2$], 864.7 [$Mn_2(Glcp)_4(H_2O)_5(OH)$], 1050.0 [$Mn_4(Glcp)_3(H_2O)_6$] and 2224.0 [$Mn_{11}(Glcp)_{10}$].

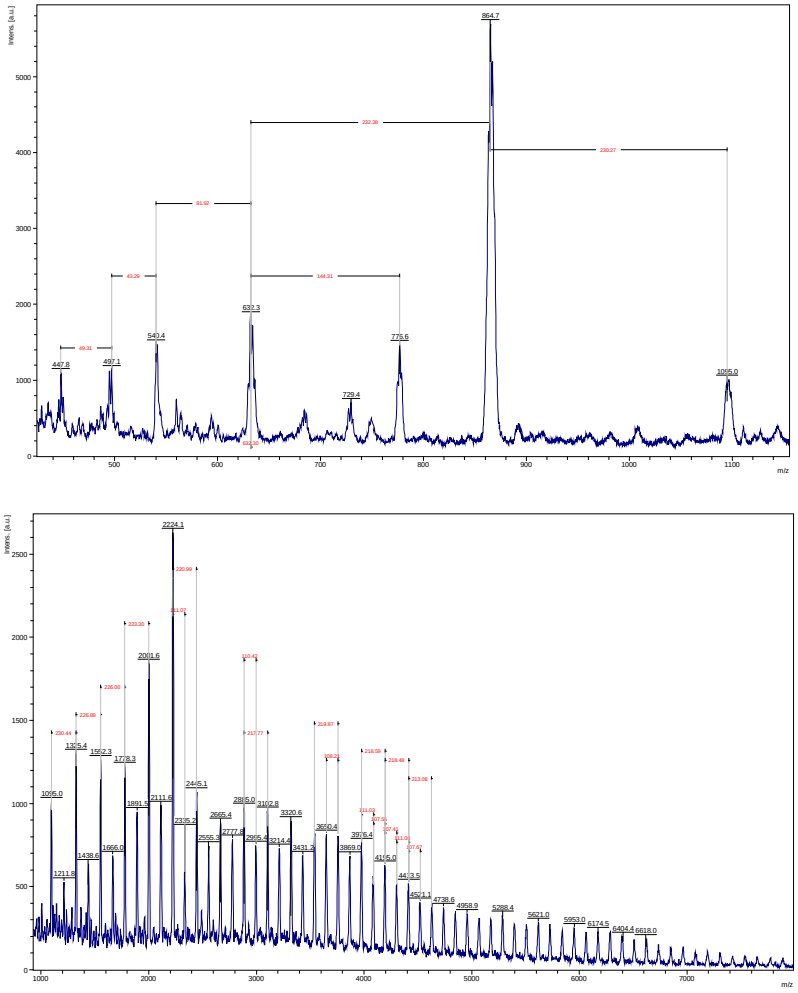

Figure S4. The MALDI-TOF/TOF MS spectra of Mn(II)-low molecular weight pullulan complexes in the 400-1100 Da range (*top*) and in 1000-7000 Da range (*bottom*).