Bioresource Technology 231 (2017) 85–93

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Pilot-scale bioconversion of rice and sunflower agro-residues into medicinal mushrooms and laccase enzymes through solid-state fermentation with *Ganoderma lucidum*

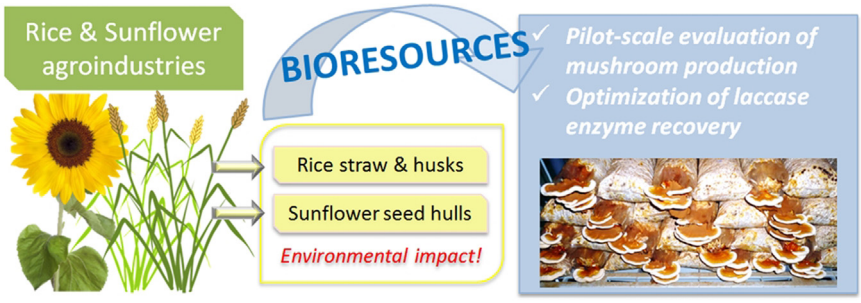
P.D. Postemsky [a,*], M.A. Bidegain [a,b], R. González-Matute [a], N.D. Figlas [a,c], M.A. Cubitto [a,b]

[a] Centro de Recursos Renovables de la Zona Semiárida (CERZOS), Universidad Nacional del Sur (UNS), CONICET, Laboratorio de Biotecnología de Hongos Comestibles y Medicinales, Camino de la Carrindanga Km7, Bahía Blanca (8000), Buenos Aires, Argentina
[b] Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, Bahía Blanca (8000), Buenos Aires, Argentina
[c] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina

HIGHLIGHTS

- Low-cost SSF bioreactors were optimized for mushroom and laccase production.
- Bioprocess turned 5.6% of agro-residues into *G. lucidum* medicinal mushrooms.
- Highest laccase activity was achieved within 5–10 days of SSF.
- At optimal SSF phase, 11,000–16,000 laccase units were obtained per kg of substrate.
- Residual substrates from mushroom culture presented 500–800 U kg^{-1} of laccase.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form 27 January 2017
Accepted 28 January 2017
Available online 2 February 2017

Keywords:
Biomass resources
Ligninolytic enzymes
Low-cost bioreactor
Wastes valorization
White rot fungi

ABSTRACT

Solid-state fermentation was evaluated at the pilot-scale for the bioconversion and valorization of rice husks and straw (RSH), or sunflower seed hulls (SSH), into medicinal mushrooms and crude extracts, with laccase activity. The average mushroom yield was 56 kg dry weight per ton of agro-residues. Laccase activity in crude aqueous extracts showed its maximum value of 10,927 U kg^{-1} in RSH (day 10, *Exudate* phase) and 16,442 U kg^{-1} in SSH (day 5, *Full colonization* phase), the activity at the *Residual substrate* phase being 511 U kg^{-1} in RSH and 803 U kg^{-1} in SSH, respectively. Crude extracts obtained with various protocols revealed differences in the extraction yields. Lyophilization followed by storage at 4 °C allowed the preservation of laccase activity for more than one month. It is proposed that standard mushroom farms could increase their profits by obtaining laccase as a byproduct during the gaps in mycelium running.

1. Introduction

Agricultural crops and their processing generate huge volumes of lignocellulosic materials as residues or by-products which have potential as nutrient sources in a diversity of bioprocesses. Lignocellulosic resources present different degrees of recalcitrant properties in relation to their tridimensional molecular organization, phenolic content and C/N ratio (Oei, 1996; Sindhu et al., 2016).

One bioprocess which is gaining relevance for valorizing this biomass is solid-state fermentation (SSF) using edible and medicinal mushrooms. With this ecofriendly approach, extraordinary

* Corresponding author.
E-mail address: pablop@criba.edu.ar (P.D. Postemsky).

http://dx.doi.org/10.1016/j.biortech.2017.01.064
0960-8524/

volumes of agro-residues can be turned into food, medicines, biomolecules and as biomass pretreatment for further uses (Chen, 2013). In particular, species with white-rot lignocellulolytic metabolism are very efficient when growing directly on raw substrates (Millati et al., 2011). One of them, *Ganoderma lucidum* (common name "reishi" or "lingzhi"), is a medicinal fungus of high-market value used as a tonic or source of biologically active metabolites, particularly the terpenoids (Wasser, 2010). Its spores are also commercialized, as well as the basidiomes, because of their high content in such molecules. Recently, its polysaccharides of high molecular weight gained attention as prebiotic agents capable of preventing dysbiosis and obesity-related disorders in obese individuals (Chang et al., 2015). Moreover, due to the confidence that new farmers are able to grow it by using optimized protocols leads to it being selected in many poverty-alleviation programs (Chen, 2004; Jaramillo, 2005). As well as mushroom production, *G. lucidum* has also been evaluated for industrial and environmental applications, especially those referring to an extracellular oxidative enzyme, the laccase (EC 1.10.3.2) (Table 1), and due to the mechanical resistance of the resulting residual substrate it has also been used for the assemblage of organic pots for horticultural seedlings (Postemsky et al., 2016).

Rice straw, rice husks and sunflower seed hulls are agro-based residues of environmental concern reportedly suitable for cultivating *G. lucidum* (González-Matute et al., 2002; Postemsky et al., 2014; Bidegain et al., 2015). Studies performed under pilot-scale conditions evaluated various aspects of *G. lucidum* production including the effect of copper on the laccase enzyme activity in

Table 1
Production and properties of *Ganoderma lucidum* laccases. Studies on *G. lucidum* laccases obtained from either submerged fermentations (SbF) or from solid state fermentations (SSF) are presented chronologically. Relevant information affecting enzyme activity or extraction yields is highlighted. Remarks stressed by the authors are included.

References	Culture condition	Medium	Extraction and characterization	Remarks
D'Souza et al. (1999)	SbF, 125 mL Erlenmeyers, static culture, 7 days	Pine and poplar chips, sterilized	Centrifugation, 50 μkatals L^{-1}, (ABTS, pH 3), 2 isoforms (40–66 KDa)	Activity was enhanced by a nitrogen additive and pine/poplar mixture
Ko et al. (2001)	SbF, 330 ml L^{-1}	Synthetic medium, sterilized	Ion exchange column, km = 3.7 μM, Vmax = 0.0142 OD min^{-1}. unit (ABTS, pH 3.5), 3 isoforms (65–68 KDa)	Optimum conditions were pH 3.5 and 25 °C, stability decreases over 50 °C
Songulashvili et al. (2006)	SbF, 250 mL flask, 140 rpm, 11 days	Wheat bran, KNO_3 supplemented	Centrifugation, 110,000 U L^{-1} (ABTS, pH 3.8)	*G. lucidum* 447 showed higher laccase activity among 18 basidiomycetes
Punnapayak et al. (2009)	Semi-solid medium	Eucalyptus paper pulp	Dialysis, 507 U L^{-1} (DMP)	Purified laccase degrades several polycyclic aromatic hydrocarbons
Murugesan et al. (2007, 2009, b, 2010)	SSF, 150 mL bottles, 30 °C, 7 days	Wheat bran, glucose, sterilized	Dialysis, ion exchange and FPLC2,540,000 U kg^{-1} substrate (ABTS, pH 4), 1 isoform (43 KDa)	Optimal at 60 °C; tolerance of metal ions, triclosan detoxification, wheat bran were natural enhancers
Asgher et al. (2010)	SSF, flasks, pH4, 35 °C, 75% moisture	Rice straw, sterilized	Aqueous, 338 U mL^{-1} (ABTS)	Laccase production was stimulated by Tween-80 and SDS
Simonić et al. (2010)	SSF, 100 mL flasks, 7 days	Lignocellulosic residues, NH_4NO_3	Aqueous, centrifugation130 U L^{-1} (ABTS pH 6.0)	Strains showed important differences according to the substrate
da Silva Coelho et al. (2010)	SSF, 125 mL Erlenmeyers, 75% moisture, 7 days	Corn cob, sterilized	Aqueous, 1000 U L^{-1} (ABTS, pH 4.5)	*G. lucidum* was able to remove both bentazon and diuron
Li et al. (2011)	SbF, *Ganoderma* and *Candida* 0.5 L flask, 4 days	Synthetic medium, sterilized	Centrifugation, filtration10,000 U L^{-1} (ABTS)	Glycerol promoted laccase activity
Sun et al. (2012)	SbF, recombiant *Pichia pastoris*, laccase gene of *G. lucidum*	Synthetic medium, sterilized	Centrifugation, km = 0.97 mM, Vmax = 3024 μM $mg{\cdot}min^{-1}$, 1 isoform (58 KDa)	Enhanced by K, Na, Cu(II) mannitol and amino acids, inhibited by Fe(II and III), sodium hidrosulphite/azide
Ting et al. (2011)	SbF, 250 mL Erlenmeyers, 10 days	Synthetic medium, sterilized	Filtration, 120 U L^{-1} (ABTS, pH 3)	Additives differentially enhanced or inhibited PAHs degradation
Zilly et al. (2011)	SSF, 250 mL Erlenmeyers, 16–30 days	Yellow passion fruit waste, sterilized	96,000 U L^{-1}, 1,640,000 U kg^{-1} DW (ABTS), 1 isoform (43 KDa)	Textile dyes effluent treatment, Na_2SO_4 stimulates activity, NaCl inhibits activity
Hailei et al. (2013)	SbF, 100 L bioreactor, 8 days	Synthetic medium, sterilized	38,000 U L^{-1} (ABTS), 2 isoforms	Glucose limitation induces laccase production
Kuhar and Papinutti (2013, 2014)	Semisolid culture, SbF, 20 days	Synthetic medium, sterilized	Dilution, 400 U kg^{-1} (ABTS), 2 isoforms (95 and 120 KDa)	Cu(II) and ferulic acid were inducers, thermostability increased with pH
Manavalan et al. (2013)	SbF, 5 L bioreactor, 7 days	Synthetic medium, sterilized	2530 U L^{-1} (ABTS)	Ethanol and a complex medium induced laccase activity
Postemsky et al. (2014)	SSF, synthetic logs, 30–40 days	Rice straw and rice husks, pasteurized	Aqueous, 267 U kg^{-1} DW (SYR, pH 5.0).	Cu(II) enhanced laccase activityLaccase tolerated freeze/thawing
Kuhar et al. (2015)	SSF, flasks, 14 days	Poplar sawdust, sterilized	2200 U kg^{-1} (DMP, pH 3.6)	Co-cultivation with *Trametes versicolor* enhanced laccase activity
Postemsky et al. (*actual study*)	SSF, synthetic logs, 5–7 days	Rice and sunflower residues, pasteurized	Aqueous crude extracts 16,442 U kg^{-1} DW (ABTS, pH 5.0)	Sunflower seed hulls prompted higher laccase activity

SSF: solid state fermentation.
SbF: submerged fermentation.
ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).
DMP: 2,6-Dimethoxyphenol.
SYR: 3,5-Dimethoxy-4-hydroxybenzalazine.
Aqueous: extraction in water.

the residual substrate (Postemsky et al., 2014; Bidegain et al., 2015). In this study, we deal with a new level of optimization of those protocols on rice straw and rice husks (RSH) or on sunflower seed hulls (SSH), focusing on facility optimization. Indeed, a close observation of the process (*substrate decontamination*, *inoculation and mycelium running*, *primordia initiation and basidiome development*) shows the occurrence of a 12 day gap (c.a. every 50 days) when the pasteurization and inoculation infrastructure is unused. This breach was thought to be a good opportunity for making new bioreactors for laccase enzymes production. Thus, this study evaluated laccase production associated with medicinal mushroom production at the pilot-scale. The laccase extraction performance and the analysis of the optimal culture phase for enzyme extraction were also studied. Considering that these agro-residues arise from two different sources, i.e. sunflower is mainly produced in cold-temperate sub-humid areas whereas rice is cultivated in warm-temperate humid ones, so it is expected that issues covered in this research will contribute to a broad agricultural context.

2. Materials and methods

2.1. Fungal source

Ganoderma lucidum E47 (from CERZOS-uNS-CONICET, Bahía Blanca, Argentina) was cultivated in MYSA medium (composition per liter: malt extract 20 g, yeast extract 2 g, sucrose 10 g, and agar 20 g, pH 6) at 25 °C, in darkness for 7 days and kept at 4 °C until use.

2.2. Solid-state fermentation

SSF using optimized substrates, bioreactor assemblage and conditions for rice agro-based residues (RSH) and sunflower agro-based residues (SSH) was carried out according to Postemsky et al. (2014) and Bidegain et al. (2015), respectively.

Inoculum (spawn) was prepared using 59.1% wheat or rice grains, 0.1% $CaCO_3$, 0.8% $CaSO_4$ and 40% water, by weight. Following sterilization (121 °C, 90 min) and inoculation (10% spawn rate) incubation was performed for 10–15 days, at 25 °C, in darkness.

2.2.1. Preparation of RSH and SSH bioreactors

The previously optimized substrate formulas for the RSH bioreactor consisted of 26.2% rice straw, 9.4% rice husk and 1.9% rice bran; whereas for the SSH bioreactor it consisted of 32.5% sunflower seed hulls and 5.0% barley (*Hordeum vulgare*). Substrates were imbibed in 0.5% $CaCO_3$, 2.0% $CaSO_4$ and enough water to achieve a final concentration of 60%, by weight. Additives were 1% olive oil and Cu (II) (100 ppm in RSH and 60 ppm in SSH) on a fresh weight basis. After pasteurization (85 °C for 2 h), the substrates were inoculated with grain spawn (8% rate). Low-cost bioreactors were then assembled by introducing the inoculated substrate under aseptic conditions in polyethylene bags (100 μm thick, 40 cm long × 8 cm diameter, 2 L) at a density of 0.46–0.54 g cm^{-3}. The open end of each bag was tied with a plastic ribbon seal. Both ends of the bags were punctured with needles (c.a. 4 punctures/cm^2) using an *ad hoc* device to facilitate gas exchange (Fig. 1A). Experimental units were 96 in number in RSH and 79 in SSH which were sorted for laccase extraction and mushroom production.

2.2.2. SSF conditions

The bioreactors were arranged horizontally on shelves and incubated at 25 ± 3 °C, in darkness. In order to prevent moisture accumulation and thermogenesis inhibitory events on mycelium growth, the substrate humidity (revealed by drop condensations on the inner side of polyethylene bag) and temperature were periodically checked during mycelium running.

Samples for laccase activity (see detail in Table 2) were collected at the *Full colonization* phase, when all bag surfaces turned white, at the *Exudates* phase, when yellow-brownish colorations appeared on the colonized substrate surface, at the *Primordia* phase, when globose masses started to appear and protrude out from the mycelium on the substrate surface and at the *Residual substrate* phase (as is next described). The bioreactors were characterized by their water content, pH and the apparent intensity of core colonization (considered incipient +, covered ++ or intense +++).

To induce mushroom morphogenesis, the bioreactors were moved after the *Primordia* phase to an environmentally controlled

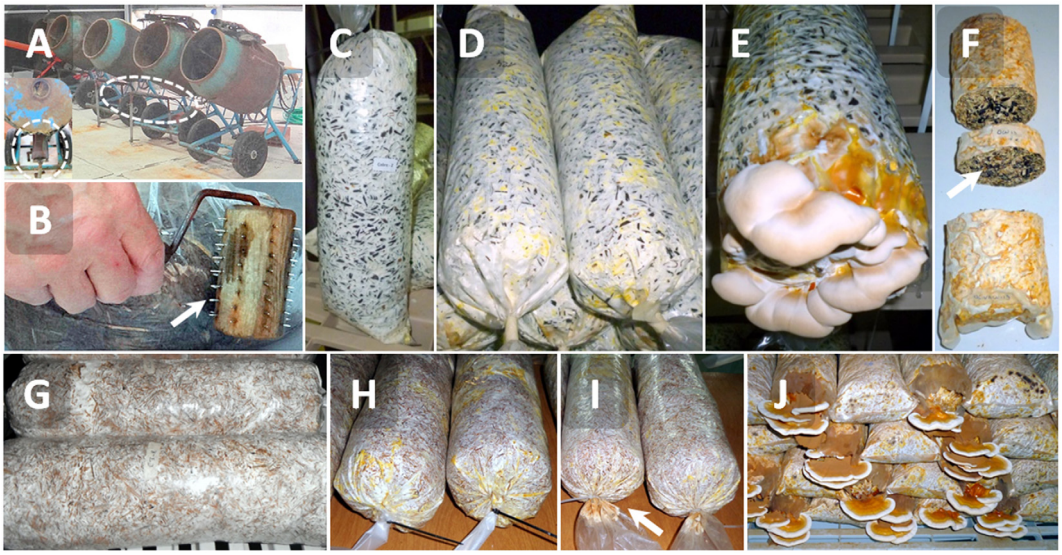


Fig. 1. Setup and different phases of the solid-state fermentation process. A: Substrate pasteurization system. Hydrated substrate (20–35 kg, 60–65% water content) is enclosed in an adapted concrete mixer and heated for 2.5 h with the aid of a gas burner (A, dotted lines) and orbital movements. B: *Ad hoc* micro perforation device performed with 4 needles per cm^2 (B, arrow). SSH at full colonization (C), exudates (D), primordia (E) and residual substrate (F) phases. The arrow in F illustrates a section of substrate used for determination of laccase activity. RSH at full colonization (G), exudates (H), primordia (I) and residual substrate (J) phases. The arrow in I shows a protruding primordia from a small hole at the ribbon-sealed end.

Table 2
Solid-state fermentation (SSF) for production of laccase enzymes from Rice Straw and Husks (RSH) and Sunflower Seed Hulls (SSH) biomass. Enzyme activity was studied at different phases of biotransformation with *Ganoderma lucidum*. SSF is characterized by the days for sample extraction, the number of analyzed experimental units, the extent of colonization areas inside the core of bioreactor (+: loose,++: filled,+++: intense), the water content in the residual substrate (WC), pH of aqueous extract, and soluble protein content (P: mg of protein per kg of SSF substrate on a dry weight basis). Laccase activity is expressed as enzymatic units (U) of oxidized syringaldazine (Syr.) or ABTS per kg of dry weight SSF substrate. Values within a column bearing the same letter are not significantly different ($P > 0.05$) according to Tukey's test.

Biomass source	SSF phase	Bioreactor traits						Enzyme activity	
		Day	n	Core colonization	WC (%)	pH*	P (mg/kg)	U kg^{-1} (Syr.)	U kg^{-1} (ABTS)
Rice straw and rice husks	*Full colonization*	7	17	+	58 ± 3 b	4.5 ± 0.2 ab	14 ± 6 c	337 ± 111 c	1026 ± 465 c
	Exudates	10	19	++	60 ± 2 ab	4.4 ± 0.2 b	24 ± 5 b	**5865 ± 2197** a	**10927 ± 3770 a**
	Primordia	15	20	+++	62 ± 4 a	4.5 ± 0.1 ab	23 ± 5 b	1651 ± 543 b	3875 ± 842 b
	Residual substrate	40	18	+++	60 ± 3 ab	4.5 ± 0.2 a	39 ± 8 a	186 ± 76 d	511 ± 188 d
Sunflower seed hulls	*Full colonization*	5	17	++	58 ± 6 b	4.5 ± 0.4 a	32 ± 9 c	**9359 ± 3703 a**	**16,442 ± 6032 a**
	Exudates	7	17	+++	65 ± 2 a	4.1 ± 0.1 b	80 ± 18 a	4550 ± 2369 b	**12,066 ± 9665 a**
	Primordia	12	15	+++	64 ± 2 a	4.4 ± 0.1 ab	32 ± 6 c	1232 ± 1124 c	3530 ± 3989 b
	Residual substrate	30	18	+++	63 ± 2 a	4.2 ± 0.1 b	42 ± 6 b	341 ± 102 d	803 ± 213 c

area. There, they were exposed to 25 ± 3 °C, with plenty of air renovation (more than 8 air room volumes per day, <400 ppm CO_2), high relative humidity (80–100%) and a 12 h photoperiod (100–500 lx irradiated from white fluorescent lights). The experiment was performed in spring with average temperatures <25 °C, in consequence the heating system was operational more frequently than the cooling system and a range of 23-25 °C was recorded inside the environmentally controlled area.

Basidiomes were cropped and biomass conversion into medicinal mushrooms was calculated as Biological Efficiency (%BE: kg of fresh mushrooms of each flush/dry weight substrate × 100), Accumulated Biological Efficiency (%ABE: kg fresh mushrooms from all flushes/dry weight substrate × 100), Productivity (Prod.%/d = ABE/days from inoculation) and the Rate of ABE (% RABE: [BE of the first flush/ABE] × 100) was calculated for an estimation of the efficacy of the first flush. Since *G. lucidum* basidiomes present different water contents between experiments and also they are commercialized in a dry state, mushroom yield parameters were also compared considering the basidiome dry weight using the Mushroom Production parameter for the first flush (%MP = kg of dry mushrooms of first flush/dry weight substrate × 100) and Total Mushroom Production (%TMP = kg of dry mushrooms from all flushes/dry weight substrate × 100).

2.3. Laccase enzyme assessment

The solubilized protein content was analyzed using the Bradford method as previously described by Postemsky et al. (2014). For laccase activity, 0.01–0.2 mL crude enzyme extract were mixed with 0.1 M sodium acetate buffer (0.8–0.99 mL pH 5.0) and the enzyme-mediated reaction was initiated by adding 25 μl of either syringaldazine ([N,N'-bi(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)], Sigma-Aldrich) in ethanol (0.5 mM final concentration) or ABTS ([2,20-azino bis (3-thylbenzthiazoline-6-sulphonic acid)], Sigma-Aldrich) in water (0.5 mM final concentration). After 1 min at 25 °C, absorbance readings were obtained at λ525 nm for syringaldazine (ε525 = 65,000 M^{-1} cm^{-1}) and at λ420 nm for ABTS (ε420 nm = 36,000 M^{-1} cm^{-1}). Laccase activity unit (U) was defined as the amount of enzyme that oxidizes 1 mmol of syringaldazine or ABTS per min.

2.3.1. Enzyme extractions

The standard procedure for obtaining crude extracts consisted in an abrasive disruption of the tighten mycelium-substrate solid phase at the time that it was solubilized in water, as an environmentally friendly option previously explored (Postemsky et al., 2014). The procedure was carried out as follows: two fresh weight samples (6.0 g each) were sectioned from the bioreactor nuclear zone (see sampling details in Table 2). One sample was used for the gravimetric determination of the water content and the other was placed in a 30 mL glass flat bottom tube with 10 mL distilled water (pH 5.5). The imbibed sample was chopped for 0.5 min with a scalpel and allowed to stand for 16 h at 4 °C. The extractive mixture was compressed (5 kg cm^{-2}) and the liquid was collected and the volume recorded. Aliquots (1.0 mL) (n = 3) were centrifuged (6000*g*, 3 min) and the supernatant was recovered to obtain the crude enzyme extract.

2.3.2. Influence of the extraction procedure on laccase activity

Modifications of the physical and chemical conditions of the standard procedure (described in 2.3.1) were studied in samples obtained from RSH at the *Exudates* phase (n = 7) and at the *Residual substrate* phase (n = 8), and those from SSH were evaluated at the *Full colonization* phase (n = 7) and at the *Residual substrate* phase (n = 6).

Sonication and chopping were applied for 0.5, 1.0 or 2.0 min using distilled water as the solvent. Combinations of both treatments (in a sonication-chopping order) were also evaluated for 0.5:0.5 min, 1.0:1.0 min and 2.0:2.0 min. Sonication was carried out with a Branson equipment (mod. Bransonic 220, 50/60 Hz).

Moreover, solvents as a buffer (100 mM sodium acetate buffer, pH 5.0) or surfactant (0.1% Tween 20) were employed alone or in combination. In this case, chopping (0.5 min) was used as a physical treatment.

2.4. Storage conditions effects on laccase activity

The effect of three different storage conditions of laccase activity was studied over a four-month period. Extracts were obtained by chopping 60 g fresh weight of substrate (with a known dry weight) in 100 mL distilled water with a home mixer (200 mL capacity). Then the mixture was filtered, its volume recorded and centrifuged (6000*g*, 3 min). Aliquots (0.4 mL) were frozen at −18 °C or lyophilized (Ricifor, mod. L-T4-A-B3) and then stored at 4 °C or at 25 °C. Samples consisted of 4 experimental units (n = 16) from each of the four treatments described in 2.3.2.

2.5. Effect of biodegradation on biochemical traits in residual substrate

A representative portion of raw materials and residual substrates were dried, ground (1 mm grid size) and pooled. Samples were studied for their apparent density, pH, electrical conductivity (EC), fiber fractions (organic matter, cellular content, hemicellulose, cellulose and lignin) and the mineral composition as previously described (Postemsky et al., 2011; Postemsky and Curvetto, 2015). Fibers analyses were done by the Van Soest acid detergent fiber method and mineral composition was obtained using inducted coupled plasma mass spectrometry.

2.6. Data analysis

Mushroom production, laccase enzyme activity and the protein content in extracts were analyzed with one way-ANOVA. The effect of the storage condition on enzyme stability (2.4) was analyzed with a two way-ANOVA. Mean differences were detected using Tukey's ($\alpha = 0.05$) and/or Fisher's ($\alpha = 0.10$) test in the case of 2.3.2 (effects of physical procedures on the extraction of laccases). Data were analyzed using the Infostat software (Di Rienzo et al., 2011).

3. Results and discussion

3.1. Biomass conversion into medicinal mushrooms

The bioprocess efficiency of 5.6 kg of dry *G. lucidum* mushrooms produced by 100 kg of either RSH or SSH (dry weight) was achieved (Table 3). These results were obtained in spring and they were 1.4 and 1.3 times higher than others obtained previously during spring-summer conditions (Postemsky et al., 2014; Bidegain et al., 2015, see technical comment on 2.2.2). A longer crop time was required and the first flush presented a lower efficiency of 49–48% whereas in the previous evaluations the first flush accounted for 90–80% of the total yield (Table 3). SSF performance considering the fresh weight of mushrooms revealed similar ABE values between RSH and SSH (29.5% and 30.1%, respectively, Table 3), which were 1.3–1.5 times more efficient than those reported previously for coffee agro-based residues by Jaramillo et al. (2010) (24.2% in two flushes), or with poplar sawdust-wheat bran by Erkel (2009) (20.9% in three flushes). Now considering the time required for the production of two flushes, shorter values (of 50 and 78 days in RSH and SSH, respectively) were obtained in comparison with those from coffee agro-based residues (120 days) or poplar sawdust-wheat bran (60 days). Summing up, the bioconversion of rice and sunflower agro-based residues into mushrooms was demonstrated to have a great performance producing high yields of product in short production cycles.

White rot fungal SSF performed with low cost bioreactors present some advantages regarding automatized solid-state fermenters which have the capacity to treat larger volumes of agro residue with small units (hand-carryable), the requirement of aseptic but not of axenic conditions, higher tolerance to environmental oscillations and a lower cost operation in terms of materials, equipment and energy. However some drawbacks are their high variability in the bioprocess, including variations in the content of *G. lucidum* medicinal compounds when cultivated on either RSH or SSH (unpublished results), risk of mycelium fail by flooding or thermogenesis and the huge volumes of discarded plastic bags.

3.2. SSF monitoring for an optimal laccase recovery phase

Selection of the optimal time for enzyme recovery was guided by visual traits of the bioreactors (Fig. 1). The *Full colonization* phase refers to the moment that the total mycelium extension is achieved. The *Exudates* phase concerns the pigmentation of the bioreactor surface with orange-brown exudates. This feature is peculiar to some fungal species-strains (including *G. lucidum* E47) exhibited when moving from a primary to a secondary metabolism (Postemsky and Curvetto, 2015). The *Primordia* phase is recognized by the prompting of a globose mass of cells out of the bioreactor and indicates the time at which the biodegrading activity lessens and the translocation of nutrients becomes important. Thus, by considering this mycelium metabolic phase, the variance expected in pilot-scale conditions is avoided if, for example, the time since inoculation is used.

The progression of the bioreactors phases elapsed faster in SSH than in RSH (Table 2). Both bioreactors presented adequate gas-exchange conditions revealed by the conservation of water content and the extent of core colonization (Table 2); also they showed media acidification during the whole process as the protein content increased steadily in RSH whereas in SSH this happened sharply and then decreased, implying that nutrient translocation worked more efficiently in the latter. The organic matter in the residual substrate at the first flush was more reduced in RSH (c.a. −6%) than in SSH (c.a. −2%, Table 4), supporting a more efficient biotransformation of organic matter into mycelial biomass in the latter.

Regarding laccase activity, both syringaldazine and ABTS assays were consistent in indicating the optimal phase for enzyme recovery (Table 2). The *Exudates* phase in RSH reached by day c.a. 10 showed a maximum in enzymatic units of 7000–14,000 U per kg of dry substrate which, calculated by volume, are 300–600 U per L of the crude extract. In the case of the *Full colonization* phase of the SSH bioreactor, a higher activity of 10,000–23,000 U kg^{-1} was obtained 5 days earlier which, calculated by volume, represented 1000–3500 U per L of crude extract.

When compared with other studies regarding *G. lucidum* laccases, these current values appear significant (Table 1). Indeed, this was the first report made on pilot-scale conditions among the others obtained with SSF at lab-scale conditions. Furthermore, the experiments were carried out in two days of pasteurization and inoculation proceedings comparable to a professional medium size mushroom enterprise (LBHCyM mushroom cultivation facilities at CERZOS-uNS-CONICET are of c.a. 300 m^2) and they were performed by experienced cultivators. However, when compared with concentrated or partially purified crude extractions the values are well below those reported, for example, by Zilly et al. (2011) of 1,640,000 U kg^{-1} or 96,000 U L^{-1} (Table 1). Interactions of the strain-substrate influencing enzymatic yields, shown by the current results, were consistent with these phenomena described by Simonić et al. (2010) in other strains of *G. lucidum* using corn stem and oak sawdust as substrates. Other studies involving sunflower seed hulls by Rodríguez Couto and co-workers (2009) highlighted the capacity of sunflower seed hulls as the supporting material to induce laccase activity in other white-rot-fungi species.

Table 3
Bioconversion at pilot-scale conditions of Rice Straw and Husks (RSH) and Sunflower Seed Hulls (SSH) based substrates into *Ganoderma lucidum* medicinal mushrooms. Bioconversion of biomass into medicinal mushrooms traits are presented for each flush as Biological Efficiency (BE) and water content (WC); values for the analysis of total production are Accumulated Biological Efficiency (ABE), Total Mushroom Production (TMP) and Productivity (Prod.); for a comparison of the efficacy of the first flush results are presented as Rate of ABE (RABE) and Mushroom Productivity (MP). First flush was obtained at 30–40 days from inoculation; second and third flushes were produced within a 15–25 days period. Data are mean values ± standard deviation (n = 40 in RSH; n = 30 in SSH). Values within a column bearing the same letter are not significantly different ($P > 0.05$) according to Tukey's test.

Biomass source	1st flush		2nd flush		3rd flush		Total production values			Efficacy of 1st flush	
	BE (%)	WC (%)	BE (%)	WC (%)	BE (%)	WC (%)	ABE (%)	TMP (%)	Prod. (%/d)	RABE (%)	MP (%)
Rice straw and rice husks	14.4 ± 2.4 a	83 ± 2	11.4 ± 2.1 a	82 ± 4	3.7 ± 1.6 a	77 ± 5	29.5 a	5.6 a	0.30 a	49 a	2.2 ± 0.6 a
Sunflower seed hulls	14.4 ± 1.9 a	82 ± 2	10.0 ± 1.7 a	82 ± 3	5.7 ± 1.2 b	79 ± 2	30.1 a	5.6 a	0.31 a	48 a	2.5 ± 0.3 a

Table 4
Biochemical traits following solid-state fermentation of rice and sunflower agro residues by *Ganoderma lucidum*. Fiber analysis and mineral content in raw materials and in residual substrates of RSH and SSH bioreactors are presented. Apparent density, pH, electrical conductivity (EC) and fiber analyses are expressed as the range within the mean+/− SD values (n = 3). Mineral composition is the mean value of three determinations of the same digested sample (coefficient of variation is less than 15%). Increments or decrements in mineral content in residual substrates with regards of initial values which resulted higher than ×2 or lower than ×0.5 folds are shown with its respective value in lowercase letters.

Physicochemical properties	Rice straw and rice husks		Sunflower seed hulls	
	Initial	Residual substrate	Initial	Residual substrate
Apparent density (g/cm^3)	0.15–0.18	0.12–0.16*	0.20–0.25	0.17–0.23*
pH 1:6 (v/v)	6.7–6.8	4.8–5.2*	5.9–6.0	4.2–4.4*
EC 1:6 (v/v) (mS/cm)	1.0–1.2	1.0–1.4*	1.4–1.6	1.4–1.8*
Fiber analysis				
Organic matter (%)	76–81	73–76	91–93	81–88
Cellular components (%)	17–20	25–31	20–21	25–26
Hemicelluloses (%)	36–40	21–24	22–24	31–28
Cellulose (%)	13–23	18–24	21–24	15–18
Lignin (%)	6–10	1–5	24–28	8–11
Mineral composition				
C (%)	37.5	30.9	36.1	35.9
Ca (%)	0.29	1.11 $_{\times 4}$	0.34	2.10 $_{\times 6}$
K (%)	*nd*	1.91	*nd*	1.24
Mg (%)	0.13	0.28 $_{\times 2}$	0.11	0.36 $_{\times 3}$
N (%)	0.79	1.17	0.72	1.68 $_{\times 2}$
P (%)	0.16	0.28	0.08	0.17 $_{\times 2}$
S (%)	0.06	0.47 $_{\times 8}$	0.06	1.21 $_{\times 2}$
Al (mg/kg)	224	119	509	642
As (mg/kg)	1.52	1.36	1.76	1.00
B (mg/kg)	*nd*	13.1	*nd*	19.3
Cd (mg/kg)	0.047	0.014 $_{\times 0.3}$	0.051	0.014 $_{\times 0.3}$
Co (mg/kg)	0.21	<0.5	0.021	<0.5
Cr (mg/kg)	1.1	1.4	1.9	5.2 $_{\times 3}$
Cu (mg/kg)	2.7	2.4	3.2	8.5 $_{\times 2}$
Fe (mg/kg)	229	270	410	250
Hg (mg/kg)	<0.01	<0.01	<0.01	<0.01
Mn (mg/kg)	630	805	780	61 $_{\times 0.1}$
Mo (mg/kg)	2.3	0.5 $_{\times 0.2}$	3.5	1.2 $_{\times 0.3}$
Na (mg/kg)	54	464 $_{\times 9}$	117	593 $_{\times 5}$
Ni (mg/kg)	0.49	0.74	0.56	0.95
Pb (mg/kg)	0.96	0.46 $_{\times 0.5}$	1.64	0.67 $_{\times 0.4}$
Se (mg/kg)	<0.1	<0.1	<0.1	<0.1
Zn (mg/kg)	14.9	22.8	12.2	16.3

nd: not determined.
* Ranges are the mean ± SD (n = 3) adapted from Postemsky et al. (2016).

Considering the timing of the current conditions under continuous bioprocessing, a gap of c.a. 12 days is presented until new space is available. This is conceived as an opportunity for using the pasteurization and inoculation facilities to elaborate new RSH or SSH bioreactors. Based on the outcomes of this research, this constitutes a strategic opportunity to increase profits by producing bioreactors at their highest laccase activity with no further investment. Besides, and from an environmental point of view, the recycling-rate would increase, for example, more than five times, essentially depending on the design of the pasteurization and inoculation areas.

3.3. Extraction procedure effects on laccase activity

Selection of the finest downstream conditions deals with the challenge to employ solvents able to extract the laccase enzymes (and its natural mediators) selectively from large volumes of treated biomass. Also they have to be environmentally safe considering possible further uses for bioremediation or food processing. To meet this purposes water, a saline buffer and a bio compatible surfactant were considered. In addition, due to the mechanical resistance and superficial hydrophobicity of the resulting organic matrix shaped by the *G. lucidum* E47 mycelium net (Postemsky et al., 2016), such a solubilization process has to be physically aided. In this study, shredding (easily adapted on mushroom farms) and sonication (of industrial nature and energetically efficient) were evaluated.

Laccase activity obtained in RSH at the *Exudates* or in *Residual substrate* phases revealed that humble extraction treatments (#1, #4, Table 5A) for enzyme recovery gave the same activity as the more complex ones. However, the specific activity revealed that with surfactant (#10, Table 5A), the extraction efficiency increased by 1.85 and 2.11-fold in the *Exudates* and *Residual substrate* phases, respectively.

Dealing with SSH, no significant increments were found in the enzyme activity or specific activity over the reference method (#1, Table 5B). However, a higher tendency was observed in the case of 0.5 m sonication using water as the solvent at the *Full colonization* phase (#4, Table 5B).

These results provide preliminary data about the extraction efficiency when using ecofriendly and low-cost methods. The outcomes are expected to be useful in further downstream optimization studies involving enzyme purification through an aqueous two phase system, lyophilization and ultrafiltration.

3.4. Storage conditions effects on laccase activity

Fig. 2 shows the storage effect on the initial laccase activity monitored over four months. In view of the fact that no significant interactions were detected in the effect of storage conditions with

Table 5
Effects of physical procedures on extraction of laccases. RSH and SSH based solid-state fermented substrates and residual substrates after 1st flush were extracted using chopping and sonication at different times and using Tween 20 and/or sodium-acetate buffer as solvents. Treatment 1 is considered as the reference treatment. Experimental units were 7, 8, 7, 6, for RSH SSF substrate, RSH Residual substrate, SSH SSF substrate, SSH Residual substrate, respectively. Specific activity of laccases is expressed as units of laccase activity per milligram of solubilized protein (U mg^{-1}). Values within a column bearing the same letter are not significantly different ($P > 0.10$) according to Fisher's test. DMS values for Tukey at ($\alpha = 0.05$) and of Fisher's tests ($\alpha = 0.10$) are indicated. Treatment 1 was the reference of the standard method also used in Table 2.

A) Rice straw and husks		Exudates			Residual substrate		
		Protein (mg/kg)	Laccase activity (U kg^{-1})	Specific activity (U mg^{-1})	Protein (mg/kg)	Laccase activity (U kg^{-1})	Specific activity (U mg^{-1})
1	Chopping 0.5 min	17 ± 11 ab	1256 ± 584 a	87 ± 35 b	74 ± 20 a	601 ± 161 a	9 ± 4 b
2	Chopping 1.0 min	16 ± 8 ab	1358 ± 822 a	105 ± 61 ab	74 ± 25 a	639 ± 162 a	10 ± 5 b
3	Chopping 2 min	22 ± 12 a	1494 ± 831 a	113 ± 122 ab	68 ± 21 a	610 ± 167 a	10 ± 6 ab
4	Sonication 0.5 min	19 ± 9 ab	1305 ± 348 a	102 ± 94 ab	77 ± 33 a	598 ± 132 a	11 ± 9 ab
5	Sonication 1.0 min	23 ± 8 a	1553 ± 700 a	72 ± 34 b	71 ± 21 a	549 ± 195 a	9 ± 5 b
6	Sonication 2.0 min	18 ± 8 ab	1403 ± 437 a	99 ± 64 ab	81 ± 22 a	644 ± 243 a	9 ± 6 b
7	Chop.: Son. 0.5: 0.5	17 ± 9 ab	1618 ± 478 a	126 ± 91 ab	77 ± 25 a	655 ± 457 a	10 ± 9 ab
8	Chop.: Son. 1.0: 1.0	16 ± 9 ab	1538 ± 326 a	120 ± 53 ab	70 ± 23 a	653 ± 327 a	11 ± 8 ab
9	Chop.: Son. 2.0: 2.0	14 ± 7 b	1555 ± 510 a	135 ± 73 ab	77 ± 21 a	686 ± 384 a	11 ± 9 ab
10	Water + surfactanct	16 ± 9 ab	1619 ± 468 a	161 ± 157 a	68 ± 31 a	617 ± 344 a	19 ± 31 a
11	Buffer	21 ± 7 ab	1684 ± 385 a	92 ± 40 ab	74 ± 19 a	653 ± 252 a	11 ± 9 ab
12	Buffer + surfactanct	21 ± 6 ab	1291 ± 313 a	64 ± 18 b	73 ± 26 a	721 ± 273 a	10 ± 4 ab
	DMS Fisher $\alpha = 0.10$	8	491	72	22	255	10
	DMS Tukey $\alpha = 0.05$	16	996	146	45	516	21
B) Sunflower seed hulls		Full colonization			Residual substrate		
		Protein (g/kg)	Laccase activity (U kg^{-1})	Specific activity (U mg^{-1})	Protein (g/kg)	Laccase activity (U kg^{-1})	Specific activity (U mg^{-1})
1	Chopping 0.5 min	42 ± 12 ab	11,624 ± 4034 ab	293 ± 134 ab	47 ± 11 bc	555 ± 178 a	12 ± 3 a
2	Chopping 1.0 min	48 ± 11 ab	10,317 ± 7899 ab	205 ± 127 bcd	44 ± 12 c	556 ± 112 a	14 ± 5 a
3	Chopping 2 min	42 ± 13 ab	9846 ± 6398 ab	233 ± 122 abcd	52 ± 7 abc	633 ± 213 a	12 ± 4 a
4	Sonication 0.5 min	42 ± 14 ab	12,618 ± 5330 a	315 ± 111 a	50 ± 14 abc	631 ± 149 a	14 ± 5 a
5	Sonication 1.0 min	47 ± 14 ab	7496 ± 2791 b	166 ± 69 d	54 ± 18 abc	597 ± 174 a	13 ± 7 a
6	Sonication 2.0 min	48 ± 12 ab	9185 ± 4388 ab	205 ± 127 bcd	54 ± 6 abc	634 ± 174 a	12 ± 3 a
7	Chop.: Son. 0.5: 0.5	39 ± 14 ab	10,304 ± 4094 ab	273 ± 79 abc	55 ± 18 abc	718 ± 294 a	14 ± 5 a
8	Chop.: Son. 1.0: 1.0	42 ± 6 ab	7766 ± 4743 b	183 ± 97 cd	57 ± 17 abc	608 ± 199 a	11 ± 4 a
9	Chop.: Son. 2.0: 2.0	38 ± 9 b	8204 ± 3402 b	232 ± 108 abcd	50 ± 17 abc	570 ± 199 a	14 ± 10 a
10	Water + surfactant	45 ± 16 ab	10,598 ± 6073 ab	231 ± 99 abcd	56 ± 12 abc	680 ± 249 a	12 ± 5 a
11	Buffer	43 ± 10 ab	7570 ± 1794 b	184 ± 50 cd	59 ± 14 ab	709 ± 105 a	12 ± 2 a
12	Buffer + surfactant	49 ± 15 a	10,842 ± 3461 ab	226 ± 56 abcd	61 ± 16 a	575 ± 110 a	10 ± 3 a
	DMS Fisher $\alpha = 0.10$	11	4286	91	13	181	5
	DMS Tukey $\alpha = 0.05$	23	8689	184	27	369	10

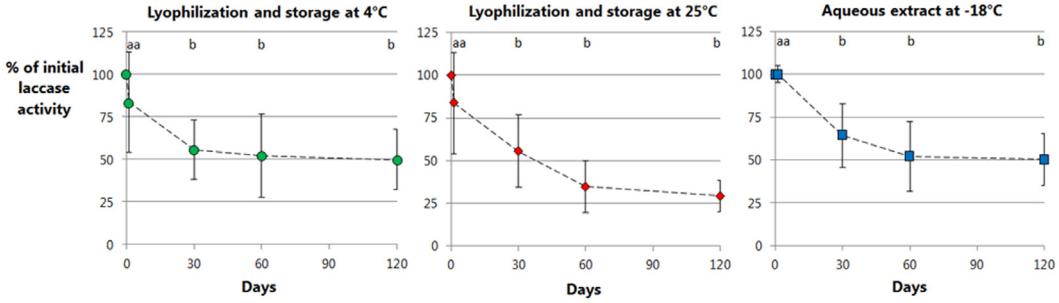


Fig. 2. Storage conditions effects on laccase activity. Laccase activity was monitored during four months. Aqueous crude extractions were preserved at −18 °C or lyophilized and then preserved at either 4 °C or 25 °C. Data are the mean of the percentage of the initial activity ± standard deviation (n = 16). Different letters indicate significant differences between days ($\alpha = 0.05$, Tukey's test).

regards to the enzyme source (RSH vs. SSH, either at optimum phase or in the residual substrate, data not shown), the values were pooled and the storage condition was presented as the main effect. The initial activities of the RSH exudates phase, RSH residual substrate, SSH full colonization and of SSH residual substrate were 1256, 601, 11,624 and 555 U/kg, respectively. After 30 days, both lyophilization followed by storage at 4 °C and freezing at −18 °C reduced the initial activity to 50–60% and no further reduction was then observed until the end of the evaluation at day 120. In lyophilization followed by storage at 25 °C, the initial laccase activity was also reduced to 50%, but in this case then it decreased to 30% by day 120.

Lyophilization can be used for both enzyme conservation and concentration. Preliminary evaluations are necessary since laccases can lose catalytic and stabilization properties when submitted to lyophilization (Morozova et al., 2007). In this study *G. lucidum* laccases were not affected by the lyophilization conditions explored any more than with freezing, hence both storage options were found adequate and their particular convenience has to be considered. An advantage of lyophilization would be that with a dilution,

using less solvent than the original, the loss of enzyme activity per volume can be compensated or even incremented.

3.5. Recycling of residual substrate for laccase recovery

The residual substrate used for enzyme recovery has gained attention for its valorization potential (Phan and Sabaratnam, 2012). In the case of enzyme recovery from residual substrates the greatest contest is the economical convenience since, as described previously, with less time higher yields of enzyme contained in the crude aqueous extracts can be obtained at optimal phases. In this regard, research on downstream processes highlights the aqueous two phase system among those that are more promising for laccase recovery, mainly because of their environmental compatibility in further applications in bioremediation (Mayolo-Deloisa et al., 2009). Laccases crude extracts from the *Residual substrate* of RSH or SSH presented c.a. 5% of the activity at optimal SSF phases (Table 3). Thereby for a competitive recovery of the laccase enzymes in RSH or SSH, very highly efficient downstream processes would be required. Although other applications for laccases contained/supported within the substrate would be more advantageous, especially considering the ability of these substrates to become implanted in soils (Postemsky et al., 2016; Postemsky and López Castro, 2016). Some relevant applications are, for instance, as organic matrix for the bioremediation of agrochemicals and pollutants from soils, sludges or wastewaters (Lau et al., 2003; Phan and Sabaratnam, 2012).

Biochemical traits in the residual substrate from RSH and SSH bioreactors are presented on Table 4 and analyzed in order to serve as orientation for further uses of the residual substrate. Biodegradation disrupted the fiber components affecting the physical properties as shown by an increase in the AD. In the case of chemical properties, biodegraded materials presented lower pH and increased EC. The latter indicates a higher content of ionic substances, a fact that is also supported by the increment in the cellular component fraction resulting from the fiber analyses, which in this latter case represents solubilized compounds on neutral detergent.

In addition, fiber analyses revealed that *G. lucidum* E47 differed in the catabolic physiology when cultivated on rice or sunflower agro residues. When compared with initial raw materials, in RSH, the hemicellulose fraction was reduced, the cellulose fraction increased and the lignin and insoluble matter fraction did not differ. In the case of SSH, the opposite occurred for hemicelluloses and cellulose while the lignin and insoluble matter fraction was reduced. Thereby the highest laccase activity in SSH was in accordance with the reduction observed only in this case for the lignin related fraction. Moreover, ligninolytic traits of *G. lucidum* E47 were compared with other strains and white rot fungi under *in vitro* conditions (Postemsky and Curvetto, 2016). The study revealed that the actual strain performed better in high-concentrated tannic/gallic acid agarized media. Interestingly, at the lower phenolic concentration present in rice straw and husks, *G. lucidum* E47 used hemicelluloses in preference to the others and left the cellulose exposed, the opposite effect to what occurred with SSH, with a higher phenolic content. Certainly, this result on rice straw and husks is of consideration for further uses in cellulose-derived bioprocesses like biopulping, biofuels or animal feed, among others (Phan and Sabaratnam, 2012).

With regards to the mineral composition, mycelium can absorb and translocate the elements towards the basidiome with a certain degree of selection. Table 4 emphasizes the effect of concentration following the biodegradation of RSH and SSH. Elements that increased more than $\times 2$ or reduced lower than $\times 0.5$ indicates a lower or higher translocation effect, respectively. Consideration of these readily-available nutrients is of interest in further biological recycling process like bio fertilizers in soils (Sindhu et al., 2016). Moreover, special attention has to be paid in the case of Cd, Mo and Pb, those being heavy metals with a tendency to bioaccumulate in basidiomes.

4. Conclusions

SSF was employed to add value to rice and sunflower agro-industrial residues. High yields of medicinal mushrooms were obtained in short periods. Crude extracts with laccase activity obtained from residual substrate presented 5% of the laccase enzyme activity compared to those obtained from the optimal phases. *G. lucidum* laccase recovery at the optimized phase is suggested as an alternative use of mushroom farm equipment during vicious periods. This investigation provides promising and preliminary results with regards to a sustainable technology associated with the mushroom production. Ongoing research lines are focused on enzyme purification, immobilization and their evaluation in biotechnological applications.

Acknowledgements

Authors wish to thank to Devalis R. and Curvetto N. for their helpful collaboration during the execution of this work. This study was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional del Sur (UNS, PGI MAyDS), Argentina, Banco Interamericano de Desarrollo and Ministerio de Ciencia Tecnología e Innovación Productiva (grants BID PICT 2014-2414 and PICT 2010-0271).

References

Asgher, M., Sharif, Y., Bhatti, H.N., 2010. Enhanced production of ligninolytic enzymes by *Ganoderma lucidum* IBL-06 using lignocellulosic agricultural wastes. Int. J. Chem. Reactor Eng. 8 (1), 59–77.

Bidegain, M.A., Cubitto, M.A., Curvetto, N.R., 2015. Optimization of the yield of Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (Higher Basidiomycetes), cultivated on a sunflower seed hull substrate produced in Argentina: effect of olive oil and copper. Int. J. Med. Mushrooms 17 (11), 1095–1105.

Chang, C.J., Lin, C.S., Lu, C.C., Martel, J., Ko, Y.F., Ojcius, D.M., Tseng, S.F., Wu, T.R., Chen, Y.Y.M., Young, J.D., Lai, H.C., 2015. *Ganoderma lucidum* reduces obesity in mice by modulating the composition of the gut microbiota. Nat. Commun. 6, 7489.

Chen, A.W., 2004. Growing *Ganoderma* mushrooms. In: Mushroom Growers' Handbook, Part III: Mushrooms Worldwide, Chapter 11: Mushrooms for the Tropics. MushWorld, Heineart Inc., Korea, pp. 224–234.

Chen, H., 2013. Modern solid state fermentation Chapter 1: Introduction. Springer, Netherlands, pp. 1–21.

D'Souza, T.M., Merritt, C.S., Reddy, C.A., 1999. Lignin-modifying enzymes of the white rot basidiomycetes Ganoderma lucidum. Appl. Environ. Microbiol. 65, 5307–5313.

da Silva Coelho, J., Marques de Souza, C.G., de Oliveira, A.L., Bracht, A., Ferreira Costa, M.A., Peralta, R.M., 2010. Comparative removal of Bentazon by *Ganoderma lucidum* in liquid and solid state cultures. Curr. Microbiol. 60 (5), 350–355.

Di Rienzo, J., Casanoves, F., Balzarini, M., González, L., Tablada, M., Robledo, C., 2011. InfoStat v2011. InfoStat Group, College of Agricultural Sciences, National University of Córdoba, Argentina.

Erkel, E.I., 2009. The effect of different substrate mediums on yield of *Ganoderma lucidum* (Fr.) Karst. JFAE 7 (3), 841–844.

González-Matute, R., Figlas, D., Devalis, R., Delmastro, S., Curvetto, N., 2002. Sunflower seed hulls as a main nutrient source for cultivating *Ganoderma lucidum*. Micol. Aplicada Int. 14, 19–24.

Hailei, W., Chaozhi, T., Guangli, Y., Ping, L., 2013. A novel membrane-surface liquid co-culture to improve the production of laccase from *Ganoderma lucidum*. Biochem. Eng. J. 80, 27–36.

Jaramillo, C., 2005. Mushroom growing project in Colombia. In: Mushroom Growers' Handbook II, Chapter 9: Mushroom Growing Project. MushWorld, Heineart Inc., Korea, pp. 276–287.

Jaramillo, C., Rodríguez, N., Chang, S.T., 2010. Simple methodology for the cultivation of the medicinal mushroom *Ganoderma lucidum* in Colombian coffee farms. In: Martínez-Carrera, D., Curvetto, N., Sobal, M., Morales, P., Mora, V.M. (Eds.), Hacia un Desarrollo Sostenible del Sistema de Producción-Consumo de los Hongos Comestibles y Medicinales en Latinoamérica: Avances y Perspectivas en el Siglo XXI. Red Latinoamericana de Hongos Comestibles y Medicinales: Producción, Desarrollo y Consumo, Mexico, p. 648.

Ko, E.M., Leem, Y.E., Choi, H., 2001. Purification and characterization of laccase isozymes from the white-rot basidiomycete Ganoderma lucidum. Appl. Microbiol. Biotechnol. 57 (1–2), 98–102.
Kuhar, F., Papinutti, L., 2013. Protective effect of vanilloids against chemical stress on the white-rot fungus *Ganoderma lucidum*. J. Environ. Manag. 124, 1–7.
Kuhar, F., Papinutti, L., 2014. Optimization of laccase production by two strains of *Ganoderma lucidum* using phenolic and metallic inducers. Rev. Argen. Microbiol. 46 (2), 144–149.
Kuhar, F., Castiglia, V., Levin, L., 2015. Enhancement of laccase production and malachite green decolorization by co-culturing *Ganoderma lucidum* and *Trametes versicolor* in solid-state fermentation. Int. Biodeterior. Biodegrad. 104, 238–243.
Lau, K.L., Tsang, Y.Y., Chiu, S.W., 2003. Use of spent mushroom compost to bioremediate PAH-contaminated samples. Chemosphere 52 (9), 1539–1546.
Li, P., Wang, H., Liu, G., Li, X., Yao, J., 2011. The effect of carbon source succession on laccase activity in the co-culture process of *Ganoderma lucidum* and a yeast. Enzyme Microb. Technolo. 48 (1), 1–6.
Manavalan, T., Manavalan, A., Thangavelu, K.P., Heese, K., 2013. Characterization of optimized production, purification and application of laccase from *Ganoderma lucidum*. Biochem. Engineering J. 70, 106–114.
Mayolo-Deloisa, K., Trejo-Hernández, M.D.R., Rito-Palomares, M., 2009. Recovery of laccase from the residual compost of *Agaricus bisporus* in aqueous two-phase systems. Process Biochem. 44 (4), 435–439.
Millati, R., Syamsiah, S., Niklasson, C., Cahyanto, M.N., Ludquist, K., Taherzadeh, M.J., 2011. Biological pretreatment of lignocelluloses with white-rot fungi and its applications: a review. BioResources 6 (4), 5224–5259.
Morozova, O.V., Shumakovich, G.P., Gorbacheva, M.A., Shleev, S.V., Yaropolov, A.I., 2007. "Blue" laccases. Biochemistry (Moscow) 72 (10), 1136–1150.
Murugesan, K., Nam, I., Kim, Y., Chang, Y., 2007. Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture. Enzyme Microbi. Technol. 40 (7), 1662–1672.
Murugesan, K., Kim, Y.M., Jeon, J.R., Chang, Y.S., 2009. Effect of metal ions on reactive dye decolorization by laccase from *Ganoderma lucidum*. J. Hazard. Mater. 168 (1), 523–529.
Murugesan, K., Yang, I.H., Kim, Y.M., Jeon, J.R., Chang, Y.S., 2009. Enhanced transformation of malachite green by laccase of *Ganoderma lucidum* in the presence of natural phenolic compounds. Appl. Microbiol. Biotechnol. 82 (2), 341–350.
Murugesan, K., Chang, Y.Y., Kim, Y.M., Jeon, J.R., Kim, E.J., Chang, Y.S., 2010. Enhanced transformation of triclosan by laccase in the presence of redox mediators. Water Res. 44 (1), 298–308.
Oei, P., 1996. Mushroom Cultivation; With Special Emphasis on Appropriate Techniques for Developing Countries. Tool Publications, Leiden, Netherlands, p. 192.
Phan, C.W., Sabaratnam, V., 2012. Potential uses of spent mushroom substrate and its associated lignocellulosic enzymes. Appl. Microbiol. Biotechnol. 96 (4), 863–873.
Postemsky, P.D., Curvetto, N.R., 2015. Solid-state fermentation of cereal grains and sunflower seed hulls by *Grifola gargal* and *Grifola sordulenta*. Int. Biodeterior. Biodegrad. 100, 52–61.
Postemsky, P.D., Curvetto, N.R., 2016. *In vitro* studies of secondary metabolite-related responses in some species of genus *Grifola* (Agaricomycetes) from Argentina. Int. J. Med. Mushrooms 18, 355–363.
Postemsky, P., López Castro, R., 2016. *Aplicaciones de sustrato residual del cultivo de hongos en la producción hortícola*. Applications of mushrooms residual substrate in horticultural production. Horticultura Argentina 35 (86), 44–63.
Postemsky, P.D., Palermo, A.M., Curvetto, N.R., 2011. Protective effects of new medicinal mushroom, *Grifola gargal* Singer (Higher Basidiomycetes), on induced DNA damage in somatic cells of *Drosophila melanogaster*. Int. J. Med. Mushrooms 13 (6), 583–594.
Postemsky, P.D., Delmastro, S.E., Curvetto, N.R., 2014. Effect of edible oils and Cu(II) on the biodegradation of rice by-products by *Ganoderma lucidum* mushroom. Int. Biodeterior. Biodegrad. 93, 25–32.
Postemsky, P.D., Marinangeli, P.A., Curvetto, N.R., 2016. Recycling of residual substrate from *Ganoderma lucidum* mushroom cultivation as biodegradable containers for horticultural seedlings. Sci. Hortic. 201, 329–337.
Punnapayak, H., Prasongsuk, S., Danmek, K., Messner, K., Lotrakul, P., 2009. Polycyclic aromatic hydrocarbons (PAHs) degradation by laccase from a tropical white rot fungus *Ganoderma lucidum*. Afr. J. Biotechnol. 8, 5897–5900.
Rodríguez-Couto, S., Osma, J.F., Toca-Herrera, J.L., 2009. Removal of synthetic dyes by an eco-friendly strategy. Eng. Life Sci. 9 (2), 116–123.
Simonić, J., Vukojević, J., Stajić, M., Glamoclija, J., 2010. Intraspecific diversity within *Ganoderma lucidum* in the production of laccase and Mn-oxidizing peroxidases during plant residues fermentation. Appl. Biochem. Biotechnol. 162 (2), 408–415.
Sindhu, R., Binod, P., Pandey, A., 2016. Biological pretreatment of lignocellulosic biomass – an overview. Bioresour. Technol. 199, 76–82.
Songulashvili, G., Elisashvili, V., Wasser, S., Nevo, E., Hadar, Y., 2006. Laccase and manganese peroxidases activities of *Phellinus robustus* and *Ganoderma adspersum* grown on food industry wastes in submerged fermentation. Biotechnol. Lett. 28, 1425–1429.
Sun, J., Peng, R.H., Xiong, A.S., Tian, Y., Zhao, W., Xu, H., Liu, D.T., Chen, J.M., Yao, Q.H., 2012. Secretory expression and characterization of a soluble laccase from the Ganoderma lucidum strain 7071–9 in Pichia pastoris. Mol. Biol. Rep. 39 (4), 3807–3814.
Ting, W.T.E., Yuan, S.Y., Wu, S.D., Chang, B.V., 2011. Biodegradation of phenanthrene and pyrene by *Ganoderma lucidum*. Int. Biodeter. Biodegrad. 65 (1), 238–242.
Wasser, S.P., 2010. Medicinal mushroom science: history, current status, future trends, and unsolved problems. Int. J. Med. Mushrooms 12 (1), 1–16.
Zilly, A., Coelho-Moreira, J., Bracht, A., da Souza, C.G.M., Carvajal, A.E., Koehnlein, A., Peralta, R.M., 2011. Influence of NaCl and Na_2SO_4 on the kinetics and dye decoloration ability of crude laccase from *Ganoderma lucidum*. Int. Biodeterior. Biodegrad. 65, 340–344.