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Conditions Affecting Lingzhi or Reishi Medicinal Mushroom *Ganoderma lucidum* (Agaricomycetes) Basidiome Quality, Morphogenesis, and Biodegradation of Wood By-products in Argentina

Francisco Kuhar,[1,2] Pablo Daniel Postemsky,[3,*] & María Virginia Bianchinotti[4,5]

[1]Centro de Investigación y Extensión Forestal Andino Patagónico (CIEFAP), Chubut, Argentina; [2]Universidad Nacional de la Patagonia San Juan Bosco (UNPSJB), Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET); [3]Centro de Recursos Renovables de la Zona Semiárida (CERZOS), Universidad Nacional del Sur (UNS), CONICET, Laboratorio de Biotecnología de Hongos Comestibles y Medicinales, Buenos Aires, Argentina; [4]Laboratorio de Micología y Control Biológico, CERZOS, UNS, CONICET, Buenos Aires, Argentina; [5]Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Buenos Aires, Argentina

*Address all correspondence to: Pablo Daniel Postemsky, Laboratorio de Biotecnología de Hongos Comestibles y Medicinales, CERZOS, UNS, CONICET, Camino La Carrindanga Km 7, Bahía Blanca 8000, Buenos Aires, Argentina; pablop@criba.edu.ar

ABSTRACT: Solid-state fermentation (SSF) with the medicinal higher Basidiomycete *Ganoderma lucidum* was studied as a strategy to use pine (*Pinus radiata* D. Don) and poplar (*Populus nigra* L.) wood chips and sawdust. Fruiting bodies were produced and the value of the biotransformed substrate was assessed. The highest mushroom yield (63 g dry weight per kilogram of dry substrate) was obtained with poplar sawdust and wood chips. Immersion of the bioreactors was a simple watering method that obtained suitable yields. Two morphological types were induced using 2 different incandescent light intensities. High light irradiation induced the highest valued mushroom morphology (as a whole product). Time course study of substrate biodegradation and mycelial growth dynamics indicated that the trophophase lasted 20 days and presented laccase activity of 0.01–0.03 units · g^{-1}. The activity at idiophase was 10 times higher. Aqueous and alkali extracts, as well as carbohydrase enzyme profile activity, revealed differences in the properties of the residual substrate; some related to the substrate source are considered to be of concern for further use of this pretreated biomass. In view of the results obtained, we propose use of SSF of pine and poplar with *G. lucidum* to profitably recycle softwood by-products from the timber industry.

KEYWORDS: fungal morphogenesis, *Ganoderma lucidum*, laccase, lignocellulolytic enzymes, medicinal mushrooms, solid-state fermentation

ABBREVIATIONS: **NAGA**, *N*-acetyl glucosamine; **SSF**, solid-state fermentation

I. INTRODUCTION

Pine and poplar plantations compromise 4 million and 80 million hectares worldwide, respectively.[1,2] These trees are among the softwoods most used in timber production, and because of their adaptability they are frequently selected for silviculture under a wide range of climatic conditions. The Food and Agriculture Organization of the United Nations recently stressed the potential of silviculture to alleviate global poverty, including the virtuous uses of processed wood chips and sawdust obtained as by-products, such as in the artificial cultivation of mushrooms.[3,4]

Mushroom farming is a profitable activity with beneficial impacts on the environment[5] and on society by prompting microscale mushroom enterprises that can be economically beneficial to rural populations.[6] Moreover, it can employ idle farmers; in the case of medicinal mushrooms, their products (particularly those that take advantage of medicinal mushroom antibacterial and antimicrobial properties[7,8]) can be used to treat some public health problems.[9]

Among commercial species, basidiomes from the lingzhi or reishi medicinal mushroom *Ganoderma lucidum* (Curtis) P. Karst. (Ganodermataceae, Agaricomycetes), and their spores, are in increasing demand because of their important medicinal properties,[10] and some are showing promising results in clinical trials.[11–13] A considerable market exists for pharmaceutical products based on *G. lucidum* and includes several corporations.[14] The simplest commercial product is powdered mushroom, with a European market price of approximately $200 per dry kilogram. However, the value after culinary, pharmaceutical, or cosmeceutical preparation can increase several times over (e.g., in the case of hydroalcoholic tinctures). Approximately 20 g powdered *G. lucidum* provides 100 mL extract (worth $200).

G. lucidum is a white-rot species that decomposes substrates. Cultivable strains can biodegrade a wide range of lignocellulosic substrates, implying outstanding catabolic performance.[15,16] In the case of the strain evaluated in this study (*G. lucidum* E47), its biodegradative potential seems to be comparable to that of highly ligninolytic strains of *Pleurotus ostreatus*.[17] Fiber analysis has shown that the physiology of its catabolic activity acts differentially on hemicellulose or cellulose fractions, exerting low or high ligninolytic activity according to the lignocellulosic nature of the substrate.[18]

The artificial cultivation of *G. lucidum* basidiomes is more complex than for other commercial mushroom species, as they may require more time to colonize the substrate under accurately controlled conditions, including light quality,[19] in order to properly induce basidiome development.[20] Although *G. lucidum* is currently produced on a large scale, scientific studies of the large-scale production of this fungus have been reported for only a few lignocellulosic materials, such as rice or sunflower.[18,21,22] Moreover, exploitation of the residual substrate is also desirable to create a profitable farming protocol. Indeed, putative applications for *G. lucidum* E47 residual substrates are laccase enzymes (EC 1.10.3.2)[18] and biodegradable pots for horticultural seedlings.[23]

Knowledge of conditions for culturing *G. lucidum* on pine- or poplar-based substrates is scarce, as is knowledge of other features concerning the exploitation of residual substrates by *G. lucidum* enzymes. This study evaluated the recycling of industrial pine and poplar by-products in solid-state fermentation (SSF) with *G. lucidum* strain E47 for both mushroom and enzyme production. This study also focused on some changes produced in the lignocellulosic biomass by the white-rot biodegrading activity of the mycelia in order to evaluate them as a potential source of enzymes of industrial interest. Solubilized fractions of the basidiomes were also quantified to estimate the yield from extracts of compounds of potential biomedical interest, such as β-glucans. The proposed methodology for basidiome development is suitable for production ranging from a small to a large scale, requires no expensive aspersion devices, and requires light intensity easily achieved with natural or incandescent light. These factors, together with the low cost of the substrate, make this an interesting alternative for use in family-scale economies as well as for medium- to large-scale producers.

II. MATERIALS AND METHODS

A. Mushroom Material

G. lucidum strain E47, deposited in the culture collection of CERZOS-UNS-CONICET (Bahía Blanca, Argentina), was maintained on malt extract agar (20 g malt extract, 2 g yeast extract, 10 g sucrose, and 20 g agar per liter; pH 6) at 25°C in darkness. Research on morphological and molecular taxonomies is currently underway to accurately describe this strain, taking into account the emerging discussion on the nomenclature of the genus *Ganoderma* P. Karst.[24]

B. Solid-State Fermentation

1. Inoculum Production

Inoculum (spawn) was prepared using whole oat (*Avena sativa*) grains. Hydrated grains (500 g; 60% initial water content) and sterilized grains (121°C for 1 hour) were inoculated with agar explants from Petri dishes and incubated at 24°C in darkness for 15–30 days.

2. Bioreactors Sembling

Pine (*Pinus radiata* D. Don) and poplar (*Populus nigra* L.) sawdust (particle size, 1–2 mm) and wood chips (particle size, 0.5–3 cm) were obtained from a local timber factory. Four substrate treatments were studied for mushroom production: 100% pine or poplar sawdust, 100% pine or poplar chips, and respective mixtures of 50% sawdust/50% chips.

Dry material (~300 g) was soaked with 1.5 L water (80% initial water content) and placed in polypropylene bags (10 cm wide, 30 cm long). Sterilization was performed by autoclaving at 15 psi for 2 hours. Once cooled, the sterile substrate was inoculated at 10% by weight. Aeration was provided using a section of plastic pipe (5 cm in diameter) and a cotton plug; this is considered here to be a bioreactor. The mycelia were incubated at 24°C in darkness for 30 days.

3. Mushroom Production

After total colonization of the substrate, basidiomes were induced by transferring the bioreactors to a humid chamber (90% relative humidity, 28°C). Samples were randomly divided to receive 1 of 3 moistening treatments (n = 10 samples per treatment): aspersion (extra daily moistening with a handheld sprinkler), immersion (the basal section of the plastic bag was removed and the bioreactors were submerged in water [25°C] for 10 minutes), and the control (exposed only to humid air). Results were expressed as biological efficiency, calculated as the dry weight of mushrooms (grams) ÷ weight of fresh substrate (kilograms).

The effect of light on basidiome morphology was evaluated in fully colonized substrates exposed randomly (n = 12) each day to either 8 hours of low-intensity (50 mM · m^{-2} · s^{-1}) or high-intensity (350 mM · m^{-2} · s^{-1}) light. Light was provided by incandescent lamps placed at varying distances. The level of radiation was controlled using a Cole Parmer 9811-50 radiometer.

C. Time Course Study of Biodegradation and Mycelial Growth

The biodegradation and mycelial growth dynamics of the substrate were evaluated on the basis of dry weight loss, *N*-acetyl glucosamine (NAGA) content, laccase enzyme activity, and lignin content from day 1 to day 28 of SSF in randomly selected bioreactors.

Dry weight loss was measured gravimetrically using a stove at 105°C. NAGA content was analyzed in dried samples (100 mg) milled in a mortar and hydrolyzed with 6 N HCl (3 mL) at 100°C for 4 hours. The hydrolyzed extract was centrifuged (4000 × *g*, 10 minutes), and 0.5 mL supernatant was alkalinized with 1.5 mL 3 N NaOH and 1.5 mL 0.2 M Na_2HPO_4 (2.78 g per 100 mL), reaching pH 8. The colorimetric reaction consisted of the incubation of 0.5 mL alkalinized sample with 1 mL acetylacetone and 50 mL 0.25 N Na_2CO_3 at 100°C for 20 minutes. After cooling, 3 mL ethanol was added, mixed, then incubated (at 65°C for 10 minutes) with 0.5 mL *p*-methyl benzaldehyde (2.67 g in 100 mL HCl [37%] and ethanol [1:1] at 96°).

To extract extracellular enzymes, 500 mg solid medium from cultures was stirred with 2.5 mL distilled water at 20°C for 30 minutes, then centrifuged and filtered as described by Kuhar et al.[25] Supernatant aliquots were stored at −20°C until needed for assays.

Laccase enzyme activity was measured according to Majcherczyk[26] using 2,6-dimethoxyphenol as substrate. Enzymatic units are expressed as micromoles per minute. Activities of manganese peroxidase (EC 1.11.1.13) and lignin peroxidase (EC 1.11.1.14) enzymes were measured according to Orth et al.[27] and Archibald,[28] respectively.

Carbohydrases (endoxylanase, exoglucanase, endoglucanase, and polymethylgalacturonase) were studied in the residual substrate (on day 30 after inoculation) according to the reducing sugars released after reaction with the specific substrate, following Magnelli and Forchiassin.[29] Enzyme activity was expressed as milligrams per minute per milliliter.

D. Residual Substrate and Basidiome Composition Analyses

Aqueous and alkali-soluble extracts were studied in pine and poplar bioreactors with the initial substrate, the residual substrate, and their respective basidiomes. Representative and pooled materials were ground and each 100-mg sample was solubilized in cold water (25°C for 1 hour), hot water (90°C for 1 hour), and 1 M NaOH (25°C for 1 hour). After extraction, the insoluble matter was dried and the weight loss was calculated. Results were expressed in relation to the total dry matter.

Klason lignin was determined gravimetrically after hydrolyzation of the samples with 72% sulfuric acid at different stages of SSF, according to the Tappi T 222 om-98 test method.

E. Statistical Analysis

The *t* test was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). Sigmoidal adjustments were made with SigmaPlot (Systat Software Inc., San Jose, CA).

III. RESULTS

A. SSF for Mushroom Production

Sterilized pine and poplar chips and sawdust were successfully biodegraded by *G. lucidum* under SSF within a period of 20–25 days. Primordia were abundant throughout the bags. Time until cropping was similar for both substrates, and the first flush was achieved at day 30.

The maximum mushroom production (63 g dry mushroom per kilogram fresh substrate) was achieved with poplar residues (mixture of both the chips and sawdust) (Fig. 1A). The mixture of sawdust with chips resulted in a 2-fold increase in mushroom yield (Fig. 1A). In this situation, the density was reduced from 0.8 to 0.5 $g \cdot cm^{-3}$, with a concomitant increase in the porosity of the substrate. In terms of production (dry weight) per 100 kg substrate (dry weight), the results varied from 10 kg in pine-based substrate to 16 kg in poplar.

Air exchange and the humidification procedure affected mushroom induction (Fig. 1B). Yields were larger when plenty of air was used and when the bioreactors were immersed in sterile water. To the contrary, yield was considerably affected under control conditions (high humidity in a confined chamber with low air exchange; Fig. 1B).

With regard to basidiome response to light conditions, induction with 50 $mM \cdot m^{-2} \cdot s^{-1}$ incandescent light favored stipe elongation: after 7 days, mushrooms presented a thick pileus that developed with

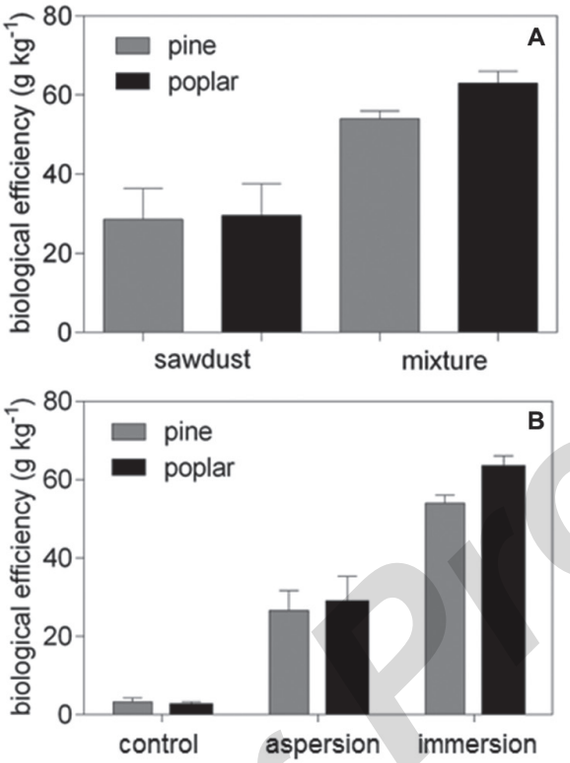

FIG. 1: *Ganoderma lucidum* production yields in pine- and poplar-based bioreactors. (A) Biological efficiency in sawdust and a sawdust–wood chip mixture. (B) Effects of humidification on biological efficiency: "control" corresponds to high humidity in a confined chamber with low air exchange, "aspersion" is adequate air exchange and aspersion with sterile water, and "immersion" refers to the introduction of sterile water at 25°C to bioreactors for 10 minutes.

the hymenium oriented upward (Fig. 2A). On the other hand, when exposed to a higher light intensity (350 mM · m^{-2} · s^{-1}), white mycelia proliferated consistently along the margins, with flattened lateral growth a more common feature of the hymenium and shape of the basidiome (Fig. 2B and C). Only globose masses developed in darkness; these were covered by a resupinate, poroid hymenia that did not elongate.

FIG. 2: Basidiome shape in response to light conditions. (A) Beginning of primordia expansion under low light intensity (50 mM · m^{-2} · s^{-1}). (B) Primordia development induced with high light intensity (350 mM · m^{-2} · s^{-1}). (C) A fully developed basidiome induced under high light intensity.

B. Time Course Biodegradation and Mycelial Growth Dynamics

Analyses were performed to study the biotransformation process in poplar sawdust–based bioreactors. Time course biodegradation and mycelial growth dynamics, expressed as the loss of dry weight and NAGA accumulation in the substrate, respectively (Fig. 3A and B), exhibited a logistic model (R^2 = 0.88 and 0.93, respectively), with a maximum velocity at days 11–12 and a plateau at day 20. By that time, 13–17% dry weight had been lost and NAGA content was 250 mg · g^{-1}. These growth dynamics were for the immersion procedure (see 3.1), which became ideal at the end of the lineal growth phase at days 18–20 (Fig. 3A and B).

Laccase was the only ligninolytic enzyme detected, showing an increase in activity around day 20 (Fig. 4A). Hence it is considered to be a response to the start of secondary metabolism during idiophase. Laccase recovery showed the highest values (0.3 units · g^{-1}) at days 25–30. However, more extensive delignification was detected between days 10 and 20 (Fig. 4B). In this case, lignin was progressively degraded in a quadratic dynamic (R^2 = 0.89). Therefore, the *G. lucidum* E47 strain at low titles of ~0.02 units · g^{-1} laccase activity in the trophophase (Fig. 4A) could delignify poplar sawdust.

C. Composition Analyses and Chemical Characterization

Changes in substrate modification as a result of fungal activity were assessed by comparing differential solubilization in the initial and residual substrates in poplar- and pine-based bioreactors. Aqueous extracts at ambient temperature were found to be higher in poplar-based substrate (Fig. 5A), whereas hot water

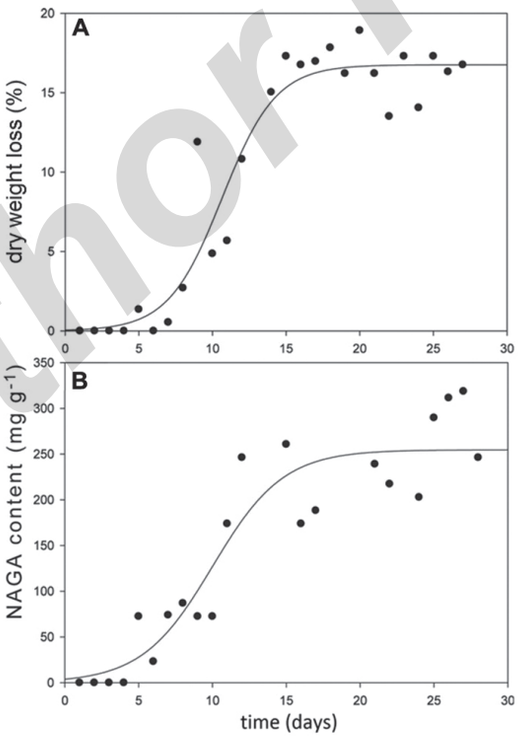

FIG. 3: Time course biodegradation and mycelial growth dynamics in a poplar sawdust–based bioreactor. Graphs show dry weight losses (A) and *N*-acetyl glucosamine (NAGA) content (B) (R^2 = 0.88 and 0.93, respectively). Each point corresponds to an independent culture.

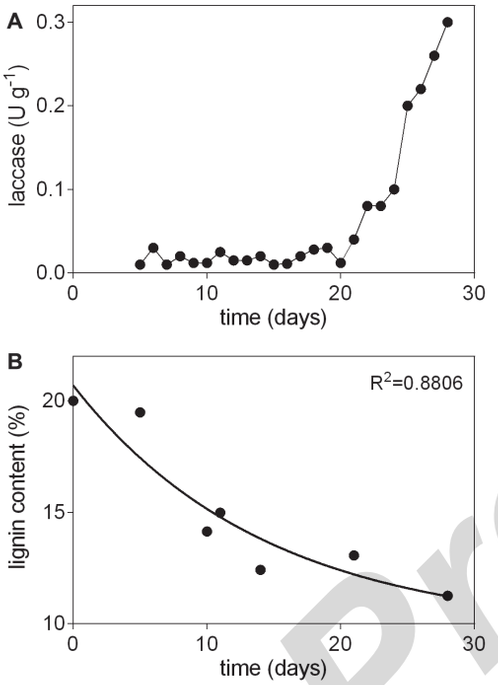

FIG. 4: Activity of laccase and the degradation of lignin in poplar sawdust–based bioreactors over time. Laccase activity (standard deviation < 5%) (A) and Klason lignin content (B) were followed for 30 days.

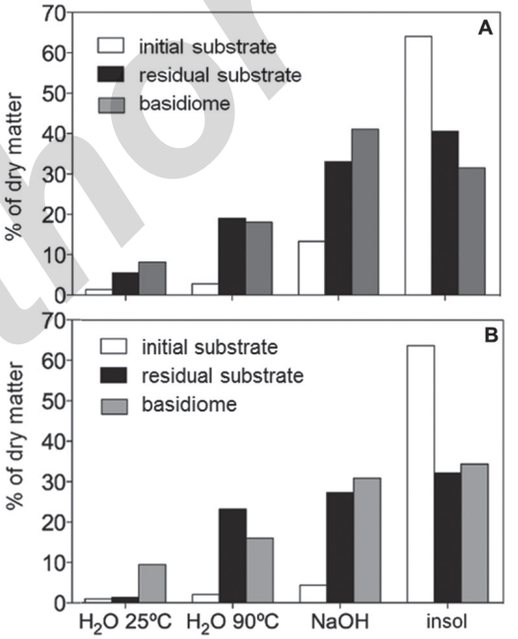

FIG. 5: Aqueous and alkali-soluble extracts obtained from the initial substrate, residual substrate, and basidiomes. Water extraction was performed at 25°C and 90°C; the alkaline solution consisted of 1 M NaHO and was extracted at 25°C. Insoluble matter is also presented. Poplar-based (A) and pine-based (B) bioreactors were studied. Standard deviation of the pool sample was < 5%.

extracts in pine-based substrate (Fig. 5B). The insoluble fraction was smaller in the pine than in the poplar substrate.

Basidiome analysis did not show any differences in either cold or hot water extractions. However, a 30% increase in the alkali extracts was present in the poplar substrate.

Regarding carbohydrase exoenzyme activity, the results presented in Fig. 6 reveal that residual substrate in the poplar-based bioreactors presented higher exoglucanase, endoglucanase, and polymethyl galacturonase activity at day 30. On the other hand, endoxylanase exhibited higher activity in the pine-based bioreactors (Fig. 6).

IV. DISCUSSION

A. SSF for Mushroom Production

Mushroom cultivation is a 3-step agricultural practice that involves (1) SSF of a substrate in which the mycelia accumulate nutrients; (2) the start of nutrient translocation into a developing primordium, a process that continues until the basidiomes are fully mature; and (3) induction of primordia under specific environmental conditions to obtain marketable basidiomes.[17,30] Some advantages of the SSF process in comparison with submerged fermentation are that SSF uses a large volume of by-products, uses water sparingly, and is a low-cost technology that requires only a short time to train operators. A significant aspect is that under SSF conditions, microorganisms behave in a way that is similar to that when they are in their natural environment; thereby, they robustly express secondary metabolism in terms of the amount and quality of natural products.

In this study, sterilized pine and poplar chips and sawdust were successfully biodegraded by *G. lucidum* within a relatively short period. Primordia production was delayed 5–7 days when compared with previous results from the same strain on optimized substrates of sunflower seed hulls[22,31] or rice straw and husks,[21] but we noticed no differences in basidiome production.

Mixing sawdust with wood chips reduced the substrate density and prevented it from clumping, thereby favoring gas exchange. Under this physical condition, aerobic catabolic processes improved and the biological efficiency increased 2-fold, resulting in 6.3 kg mushroom being produced. This is 12–46% over production values previously reported in research using the same strain on sunflower seed hulls (5.6 kg)[22] or rice (4.3 kg).[21] The results obtained with the poplar sawdust and chips mixture emphasize the outstanding capacity of this

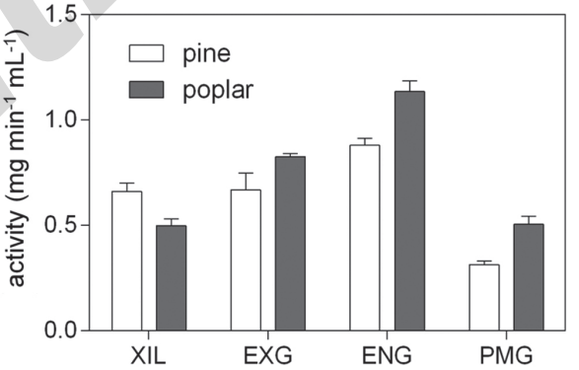

FIG. 6: Carbohydrase activity of aqueous extracts of the residual substrate at day 30. Endoxylanase (XIL), exoglucanase (EXG), endoglucanase (END), and polymethylgalacturonase (PMG) enzymatic activity was expressed as milligrams reducing sugars released per milliliter and per minute. Values are the mean of 3 independent measurements; the bars represent the standard error.

strain to recycle wood. Also, the small size of the bioreactors makes them easily manageable, and they are recommended for small-scale projects. Moreover, considering that herbotherapy doses comprise 2–5 g per day (1 mushroom weighs, on average, 10–15 g), such bioreactors could be used at home in adapted places.

Gas exchange, together with humidification, induced higher mushroom yields when plenty of air was provided and when the bioreactors were immersed in sterile water (instead of high humidity in a confined chamber with low air exchange). In previous reports, such immersion procedures seemed to be unnecessary, but a high rate of air exchange and high humidity seemed to be crucial.[18,21,22,31] *G. lucidum*—like other fungi such as *Lentinus edodes*—showed a positive response to immersion conditions. This should be considered when using substrates with low water retention, such as certain wood chips or sawdust. Indeed, according to an extensive literature survey,[32] the results of immersion of synthetic sawdust logs were the first reported case for *G. lucidum* culture.

Basidiomes showed differences in shape in response to light conditions. Induction under low-intensity incandescent light (50 mM · m^{-2} · s^{-1}) favored stipe elongation, whereas a common kidney-shaped feature formed when light intensity was increased 7 times (350 mM · m^{-2} · s^{-1}). Although this traditional "kidney" shape is desired by whole-mushroom consumers, various products are produced from *G. lucidum* based on "whole" powdered basidiomes. Indeed, it has been reported that stipe sections include more active ingredients with higher tumor inhibitory capacities and immunomodulatory activities,[33,34] and larger amounts of umami and a palatable taste—characteristics[35] that may eventually increase acceptance by consumers. Furthermore, the effect of light on basidiome traits has not been thoroughly studied, although different polysaccharide content and quality were reported in response to light quality,[36] and a notorious increase of vitamin D content was recorded after *G. lucidum* basidiomes were exposed to direct sunlight.[37] These setup possibilities are considered advantageous for obtaining different products to match the specific preferences of the different sectors in the herbotherapy market.

B. Time Course Biodegradation and Mycelial Growth Dynamics

Time course analyses of the biotransformation process in poplar sawdust–based bioreactors indicated that both biodegradation and mycelial growth dynamics exhibited a maximum velocity by days 11–12, whereas the process stabilized by day 20. The sigmoid dynamics evidenced by the NAGA content, and by dry matter loss, indicates that at this time the trophophase ended and idiophasic metabolism began. At this stage, nutrient uptake becomes inefficient and toxic compounds such as tannins[38] and monomeric phenols[39] exceed the threshold of tolerable concentrations. Therefore the immersion procedure might be expected to provide rehydration, metabolic stimulation, and the outflow of undesirable compounds,[40,41] which is still convenient despite the loss of some desirable compounds. In view of the results obtained, weight loss and NAGA content are proposed as growth estimators in future research aiming to optimize the content of supplements and additives to improve mushroom productivity.

Laccase increase has been related to the depletion of primary sources of soluble macronutrients. The greater extent of delignification detected by days 10 and 20 demonstrated that even at low titles, the laccase activity of the *G. lucidum* E47 strain at trophophase could delignify poplar sawdust. The final ligninolytic activity was comparable to the laccase values from other efficient wood-biodegrading white-rot fungi.[17,30,42,43]

Although *G. lucidum* showed low laccase activity (the highest laccase activity was 0.3 units · g^{-1}), if the SSF process continues until days 25–30, the matrix will retain maximum enzyme content. This material can be further used as a porous filter to treat liquids or gases.[44] If more activity is desired within the matrix, it is possible to enhance enzyme yield through chemical or biological induction of the studied strain by adding copper or phenolic compounds, or in cocultures with *Trametes versicolor*.[25]

C. Composition Analyses and Chemical Characterization

Differential solubilization in poplar and pine substrates (Fig. 5) was analyzed to show the feasibility of each substrate to be used in large-scale mushroom cultivation. Low-weight aqueous extracts were higher in poplar substrates, whereas hot water extracts were higher in pine substrates. In the latter, the elution of large molecules, such as hemicelluloses, is implied. In the alkaline extract, recalcitrant phenolic compounds are represented, a fraction that is usually higher in coniferous woods.[45] These results indicate that poplar-based substrates are a choice for growing *G. lucidum* in terms of their recalcitrant properties.

Cold and hot water extracts and analyses did not show any differences between basidiomes produced on different substrates. Therefore a similar pattern of hydrosoluble compounds could be expected, including polyphenols, short-chain sugars, and larger molecules such as polysaccharides.[46] The latter is of interest in view of its relation to a mushroom's medicinal properties[47] and as a source of dietary fiber.[48] However, an increase in the alkali extracts, as determined in the poplar substrate, may result in possible differences in medicinal properties in comparison with basidiomes produced on pine. Further research is needed to conclude whether these differential compounds might reflect immunomodulatory activity via the complement pathway, as shown by Yue et al.[34] Some compounds of this fraction are known to participate in particular sensory traits that are considered during the formulation of blended infusions.[35]

Carbohydrase exoenzyme activity covers a wide range of catabolic hydrolytic enzymes and their isozymes, which are secreted in different quantities by mycelia in response to the lignocellulose nature of the substrate. This study revealed a large effect of the mycelial hydrolytic enzyme profile of the substrate source. Carbohydrase exozyme activity evaluated in the residual substrate of poplar-based bioreactors at day 30 indicated (in order of importance) exoglucanase, endoglucanase, and polymethyl galacturonase activity, whereas in the case of pine-based bioreactors, the endoxylanase activity was highest. Such outcomes are expected as a result of chemical differences in hemicellulose between deciduous and coniferous species.[51] Carbohydrases are relevant in many industrial processes that aim to break down plant cell walls for use in a broad field of applications, particularly in food processing and sensory modification.[52] As a consequence, these results are considered relevant for future research on the recovery of *G. lucidum* exoenzymes from residual substrates recycled from by-products of the timber industry.

V. CONCLUSIONS

Industrial pine and poplar wood by-products were suitable for *G. lucidum* production. Immersion of bioreactors incremented mushroom yield, and mushrooms could be induced to have a longer stipe and an expanded pileus. Laccase activity, NAGA accumulation, carbohydrase activity, and lignin reduction are some further uses of the residual substrate, which is a source of, for example, lignocellulolytic enzymes, medicinal polysaccharides, and delignified biomass.

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