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Effect of bioactive compounds extracted from *Cordyceps nidus* ANDES-F1080 on laccase activity of *Pleurotus ostreatus* ANDES-F515

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ABSTRACT

Laccases are ligninolytic enzymes produced by different microorganisms, especially by fungi such as the white-rot fungus *Pleurotus ostreatus*. Chemical inductors have been used to promote laccase secretion due to the application of these enzymes in lignocellulosic biomass pretreatment. *Cordyceps nidus* ANDES-F1080 was previously described as a source of bioactive compounds that could influence the enzymatic production system of other fungi. For that reason, this study evaluates the effect of *C. nidus*' ANDES-F1080 extracts on the laccase activity of *P. ostreatus* ANDES-F515. To achieve this objective, *C. nidus* ANDES-F1080 was grown in four different substrates: two artificial-based and two natural-based culture media. Metabolites were extracted from *C. nidus* ANDES-F1080 using water and methanol as solvents. Biochemical characterization of these extracts was performed to complement the analysis of their effect on laccase activity. Our results revealed an enhancement on the laccase activity of *P. ostreatus* ANDES-F515 grown in natural-based cultures when *C. nidus*' ANDES-F1080 extracts were supplemented. The best laccase activities registered values around 10,575 $\pm$ 813 U·L^{-1}.

1. Introduction

The white-rot fungus *Pleurotus ostreatus* (Basidiomycota: Agaricales) produces lignocellulolytic enzymes that are currently employed in the pre-treatment of biomass for bioethanol production [1], pulp bleaching [2], bioremediation [3], food [4], and textiles [5]. *Pleurotus ostreatus* is widely cultivated for its capacity to secrete laccases, including oxidases and peroxidases [6]. Among these ligninolytic enzymes, laccases have occupied a special place in the industry because of their versatility, among other beneficial properties [7]. This multicopper ligninolytic enzyme reduces oxygen and oxidizes phenols, non-phenolic compounds, and complex biopolymers such as lignin [8]. For that reason, high-level production of laccase is desirable for industrial applications.

The yield of laccases from *Pleurotus ostreatus* is a function of culture conditions, therefore, laccase production is susceptible to improvement. Some culture medium characteristics which can increase yield are variation in pH, Carbon-Nitrogen ratio, the presence of inductors, and lignocellulose-containing substrates [9]. Among these factors, inductors have proven to be key for the enhancement of laccase expression because slight amounts of them in the substrate can strongly increase the enzyme production and activity [10]. Both copper sulfate and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) have been shown to promote laccase secretion [11]. Additionally, nucleosides and their analogs are highly bioactive compounds that can be involved as co-substrates and cofactors in biochemical transformations [12]. However, only a few of these compounds have been used in the promotion of enzymatic activities [13], turning this into an opportunity for the enhancement of laccase biosynthesis and its activity.

A worldwide recognized genus that produces beneficial bioactive compounds is *Cordyceps sensu lato* (Ascomycota: Hypocreales), which are arthropod-pathogenic fungi known by their broad application in traditional Chinese medicine [14]. Nucleosides and glycosylamines are the main metabolites associated with their bioactive potential [15]. Nevertheless, the acquisition of these compounds from traditional species on a

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large scale has been hardly developed due to complications in their culture [16]. However, in Colombia, *Cordyceps nidus* ANDES-F1080 is a neotropical species that emerges as a cultivable option. This species has trapdoor spiders as hosts and produces a wide range of bioactive compounds that are highly dependent on the substrate where the fungus is cultivated [17]. In this study, extractions from *C. nidus* ANDES-F1080 cultivated in artificial and natural media were tested in the promotion of laccase activity in *P. ostreatus* ANDES-F515. Biochemical characterizations were also performed to better understand the behavior of this biological system.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this work were acquired in reagent grade or were suitable for cell culture. Agar (bacteriological), BCA Protein Assay Kit, D-(+)-Maltose monohydrate (95 %), L-(+)-Arabinose (99 %), peptone (bacteriological), and yeast extract were supplied by Thermo Fisher Scientific (USA). D-(+)-Xylose (98.5 %), Folin-Ciocalteu's reagent, methanol (99 %), sulfuric acid (98 %), and sodium acetate anhydrous (99.9 %) were obtained from PanReac AppliChem (Barcelona, Spain). Acetic acid (99.7 %), ammonium sulfate (99 %), 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), calcium chloride (99.9 %), copper (II) sulfate anhydrous (99.9 %), gallic acid (97.5 %), L-(-)-Malic acid (99 %), L-(+)-tartaric acid (99 %), magnesium sulfate heptahydrate (99 %), potassium chloride (99 %), potassium phosphate monobasic (99 %), sodium selenite (98 %), succinic acid (99 %), thiamine hydrochloride (99 %), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97 %) were purchased from Merck (Darmstadt, Germany). Glucose POD-GOD kit was obtained from Spinreact (Sant. Esteve de Bas, Girona, Spain). Citric acid (99.5 %), D-(+)-Glucose (97.5 %), and malt extract agar were supplied by Scharlau (Barcelona, Spain).

2.2. Culture and bioactive compound extraction of *Cordyceps nidus* ANDES-F1080

The strain of *Cordyceps nidus* ANDES-F1080 was provided by the strains collection of *Museo de Historia Natural* of *Universidad de Los Andes.* Plugs from a previous culture on Sabouraud Dextrose Agar supplemented with yeast extract (SDAY) at 25 °C were used as inoculum. Four culture media were used as a substrate of *C. nidus* ANDES-F1080 following the same experimental design made by Chiriví et al. for SDAY (2% (w/v) of glucose, 1% (w/v) of peptone, 1% (w/v) of yeast extract, and 1.5 % (w/v) of agar), SDAY-SS (2% (w/v) of glucose, 1% (w/v) of peptone, 1% (w/v) of yeast extract, 1.5 % (w/v) of agar, and 5 ppm sodium selenite), and BR (2% (w/v) of brown rice, and 1.5 % (w/v) of agar) [17]. In this research, a modified version of tarantula agar (TA) [17] was prepared: 1% (w/v) of minced tarantula skin, 1% (w/v) of glucose, 1% (w/v) of peptone, 1% (w/v) of yeast extract, and 1.5 % (w/v) of agar. Because of these characteristics, this medium was named Exuviae Tarantula Medium (ETM) in order to avoid confusion with the formulation proposed by Chiriví et al. [17]. SDAY and SDAY-SS constituted artificial-based substrates, while BR and ETM were established as natural-based substrates. Each culture medium was inoculated with one plug of *C. nidus* ANDES-F1080, then incubated at 25 °C in a shaker (Lab companion, SIF 6000R, JEIO Tech, Daejeon, Korea) without agitation for 15 days in a photoperiod 12/12 of white light. After this period, metabolite extraction was performed following Chiriví et al. [17]. Fractions were obtained using methanol and water, as previously described [17–19]. Methanol fractions were lyophilized and then rehydrated for their use in culture.

2.3. *Pleurotus ostreatus* ANDES-F515 culture conditions

The strain of *Pleurotus ostreatus* ANDES-F515 was provided by the strains collection of *Museo de Historia Natural* of *Universidad de Los Andes.* Three plugs retrieved from a previous culture on malt extract agar (MEA) at 25 °C were used as inoculum in a 1 mL broth composed of glucose (40 g L^{-1}), yeast extract (10 g L^{-1}), and peptone (10 g L^{-1}). This broth was incubated at 25 °C for five days in a Barnstead Lab-line 305 Imperial III incubator (Barnstead International, Dubuque, IL, USA) without agitation. On the third day of incubation of *P. ostreatus* ANDES-F515, a solution with 300 μL of each fraction of *Cordyceps nidus* ANDES-F1080 extracts was added. Extracts were added at this moment to assure that *C. nidus'* metabolites interact early with *P. ostreatus*, which is desirable since the composition is highly complex. The resulting samples were used as the inoculum for semi-solid cultures.

2.4. Semi-solid culture and laccase activity

Semi-solid cultures were made in a 250 mL glass flask with 5.3 g of previously milled (1 mm) rice husk as substrate and 17.5 mL basal medium. This solution is composed of glucose (0.5 g L^{-1}), KH_2PO_4 (2 g L^{-1}), $(NH_4)SO_4$ (0.9 g L^{-1}) $CaCl_2$ (0.1 g L^{-1}), KCl (0.5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.25 g L^{-1}), sodium citrate buffer 20 mM (pH 4.5), and a solution of thiamine (0.5 g L^{-1}). This last reactant was filtered and added after autoclave sterilization [20]. The inoculum of *P. ostreatus* ANDES-F515 was added into the flask, then incubated at 25 °C in a shaker (Lab companion, SIF 6000R, JEIO Tech, Daejeon, Korea) without agitation for 26 days with a 12/12 natural photoperiod. After five days of incubation, 95 μL of a solution of 1 M $CuSO_4$ was added [21]. Laccase activity was measured by monitoring the oxidation rate of ABTS (Sigma-Aldrich, St. Louis, MO, USA) (ε 36,000 $M^{-1}\,cm^{-1}$), buffered with 20 mM sodium acetate (pH 5.0). The increment of absorbance values was determined at 436 nm using a UV–vis spectrophotometer (Genesys™ 10S UV–vis, Thermo Scientific, USA). Laccase activity was expressed as $U \cdot L^{-1}$, where one unit of activity was defined as 1 μmol of oxidized ABTS per minute [22].

2.5. Characterization of sugars and organic acids in *Cordyceps nidus* ANDES-F1080 extracts

Five hundred microliters of each polar fraction were diluted in an equal volume of distilled water for High-Performance Liquid Chromatography (HPLC) analysis. The determination was performed using an Aminex HPX-87H column (Bio-Rad, USA), operated by an Agilent Series 1200 HPLC system (Agilent Technologies, Santa Clara, USA), equipped with a reactive index detector (RID) and a diode array detector (DAD). Sulfuric acid at 5 mM was used as a mobile phase consisting of a flow rate of 0.6 mL min^{-1} at 25 °C injecting 20 μL of each sample. Monosaccharides (D-glucose, D-maltose, L-arabinose, and D-xylose) and organic acids (citric acid, malic acid, succinic acid, and tartaric acid) were used as standards at ranges of 0.25–5.00 mg mL^{-1} and 2.00–5.00 mg mL^{-1}, respectively.

2.6. Total protein content, total phenolic content (TPC), and antioxidant capacity in *Cordyceps nidus* ANDES-F1080 extracts

Using a BCA Protein Assay Kit (ThermoScientific™), samples were measured for total protein content using 0.05 mL of extract in 1 mL of BCA reagent. After that, samples were incubated in a Barnstead Lab-line 305 Imperial III incubator (Barnstead International, Dubuque, IL, USA) for 30 min at 37 °C, and absorbance values were measured at 562 nm using a UV–vis spectrophotometer (Genesys™ 10S UV–vis, Thermo Scientific, USA) [23]. The TPC of

the extracts was determined using a modified version of the Folin-Ciocalteu's method [18] with a respective calibration curve done using gallic acid in a concentration range between 0.1 mg L^{-1} and 1 mg L^{-1}. Results were expressed in milligrams of gallic acid equivalents (mg GAE) per gram of dried mycelium. Furthermore, antioxidant capacity was determined by scavenging free radicals of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), measuring the variation of absorbance of the samples after 6 min [24].

2.7. Glucose concentration

A sample of 60 μL of the supernatant from the semi-solid culture medium of *P. ostreatus* ANDES-F515 was taken two times per week until completing the incubation period (23 days) and then stored at −20 °C until spectrophotometric analysis of enzymatic activity and glucose concentration were performed. Free glucose concentration was measured using a commercial kit (Glucose POD-GOD, Spinreact, St. Steve de Bas, Gerona, Spain) [25].

2.8. Potential bioactivity of nucleosidic compounds

In order to determine all the metabolites from *C. nidus* ANDES-F1080 that can potentially improve the laccase activity of *P. ostreatus* ANDES-F515, we compared our results with the metabolomic profiles obtained by Chirivi et al. [17]. From the results of Chirivi et al. [17], we selected the metabolites showing positive values for the mass intensity in the extracts where a higher laccase activity was shown. Then, the list was filtered again to look for nucleosides. The selected nucleosides from Chirivi et al. [17] were then compared to the metabolites characterized in our study to finally identify all the nucleosides with a potential enhancing effect on the *P. ostreatus* ANDES-F515 laccase activity.

2.9. Statistical analysis

Data were analyzed through a two-way analysis of variance (ANOVA) using Minitab® 17 statistical software. This analysis included interactions within the factors studied (culture media and solvent), and the measured response variable was the maximum laccase activity. The Shapiro-Wilk test for normality was used to check whether the variables were normally distributed. Tukey's pairwise comparisons were employed to determine differences in laccase yield between the levels of each factor. All assays were done by triplicate, and a significance level of 5 % was used. An experimental control set-up was made by replacing the extract with water.

A correlation analysis was performed to assess the relationship between the different response variables. Pearson's product-moment correlation was calculated using R studio statistical software (RStudio v.1.2.1335). In addition, a Principal Component Analysis (PCA) was applied to cluster data from sugars, organic acids, total protein content, TPC, and antioxidant capacity. Originally measured response variables were standardized and transformed into principal components, and the respective eigenvalues (>1) allowed for the identification of the number of dimensions needed for data analysis. Minitab® 17 statistical software was employed to obtain the contribution of the variables to the principal components and the graphical representation of the PCA analysis.

3. Results

3.1. Natural-based substrates induced high content of sugars and organic acids in C. nidus ANDES-F1080

The sugar composition of *C. nidus* ANDES-F1080 extracts is presented in Table 1. Overall, water fractions achieved higher sugar concentration in comparison to methanol fractions. Extracts derived from natural-based substrates exhibited the highest concentration of glucose and other sugars in water and methanol fractions. Arabinose was not detected in methanol fractions derived from natural-based substrates, but it was present in artificial-based substrates and registered its highest concentrations in water fractions obtained from natural-based media (Table 1).

Likewise, the concentration of organic acids is shown in Table 2. In general, the highest concentration values of citric acid and malic acid were found in extracts derived from artificial-based substrates, which is contrary to what was observed in sugars. Nevertheless, water fractions from fungal extracts derived from ETM presented an equally high concentration of succinic acid as was found for the results derived from SDAY. In the methanol fractions, succinic acid was only identified in extracts derived from ETM.

3.2. Artificial-based substrates produced equivalent values of protein content, TPC, and antioxidant capacity than C. nidus ANDES-F1080 grown in a host-based substrate

Results from the experimental design indicate that both factors (culture media and solvent) and their interaction are statistically significant (p-value < 0.05) for total protein content and TPC. As shown in Fig. 1A for protein content analysis, water fractions retrieved more protein than methanol fractions. Notably, water fractions obtained from extracts derived from SDAY-SS presented the maximum protein content of the experimental set-up (65.42 ± 2.99 mg g^{-1}), followed by water fractions from ETM ($56-14 \pm 5.05$ mg g^{-1}) and SDAY-SS (51.96 ± 1.44 mg g^{-1}). Conversely, extracts derived from BR showed the lowest protein content (14.53 ± 2.44 mg g^{-1}). In all cases for TPC analysis, water fractions achieved the highest values (Fig. 1B). Nevertheless, a

Table 1
Sugar content of water and methanol extracts of *C. nidus* ANDES-F1080.

Culture medium	Solvent	Maltose [mg/g]	Glucose [mg/g]	Arabinose [mg/g]	Others* [mg/g]
SDAY	Water	n. d.	14.63 ± 0.62^{a}	15.59 ± 0.84^{a}	12.20 ± 1.45^{a}
SDAY-SS	Water	n. d.	16.00 ± 0.2^{b}	12.96 ± 0.06^{b}	10.07 ± 0.19^{b}
BR	Water	n. d.	22.85 ± 0.90^{c}	26.42 ± 0.76^{c}	76.21 ± 0.25^{c}
ETM	Water	n. d.	14.46 ± 0.25^{a}	56.38 ± 1.45^{d}	22.40 ± 1.49^{d}
SDAY	Methanol	n. d.	7.00 ± 0.16^{d}	6.67 ± 0.01^{e}	13.03 ± 0.22^{a}
SDAY-SS	Methanol	6.30 ± 0.07^{a}	6.56 ± 0.11^{e}	5.75 ± 0.10^{f}	9.81 ± 0.05^{e}
BR	Methanol	n. d.	11.33 ± 0.73^{f}	n. d	20.11 ± 1.67^{d}
ETM	Methanol	6.15 ± 0.29^{a}	$13.30 \pm 1.35^{a, f}$	n. d	68.87 ± 1.21^{f}

Different letters in the same column indicate a significant difference (p-value<0.05), data are mean ± SD, n = 3. n. d: non-detectable.
* Amount of xylose, galactose, fructose, and rhamnose. These sugars have the same retention time in HPLC conditions described above.

Table 2
Organic acids content of methanol and water extracts of *C. nidus* ANDES-F1080.

Culture medium	Solvent	Citric acid [mg/g]	Malic acid [mg/g]	Succinic acid [mg/g]
SDAY	Water	15.17 ± 0.55^{a}	15.49 ± 0.53^{a}	32.20 ± 1.96^{a}
SDAY-SS	Water	15.06 ± 1.88^{a}	13.91 ± 0.32^{b}	23.33 ± 0.31^{b}
BR	Water	14.61 ± 0.62^{a}	n. d.	13.35 ± 0.57^{c}
ETM	Water	12.67 ± 0.72^{b}	7.97 ± 1.84^{c}	31.89 ± 0.14^{a}
SDAY	Methanol	12.74 ± 0.35^{b}	23.32 ± 0.86^{d}	n. d.
SDAY-SS	Methanol	$13.87 \pm 0.16^{b, d}$	27.21 ± 0.37^{e}	n. d.
BR	Methanol	10.66 ± 0.70^{c}	n. d.	n. d.
ETM	Methanol	10.32 ± 0.49^{c}	5.00 ± 0.06^{f}	1.31 ± 0.06^{d}

Different letters in the same column indicate a significant difference (p-value<0.05).n. d.: non-detectable.In the evaluated conditions, tartaric acid was not detected.

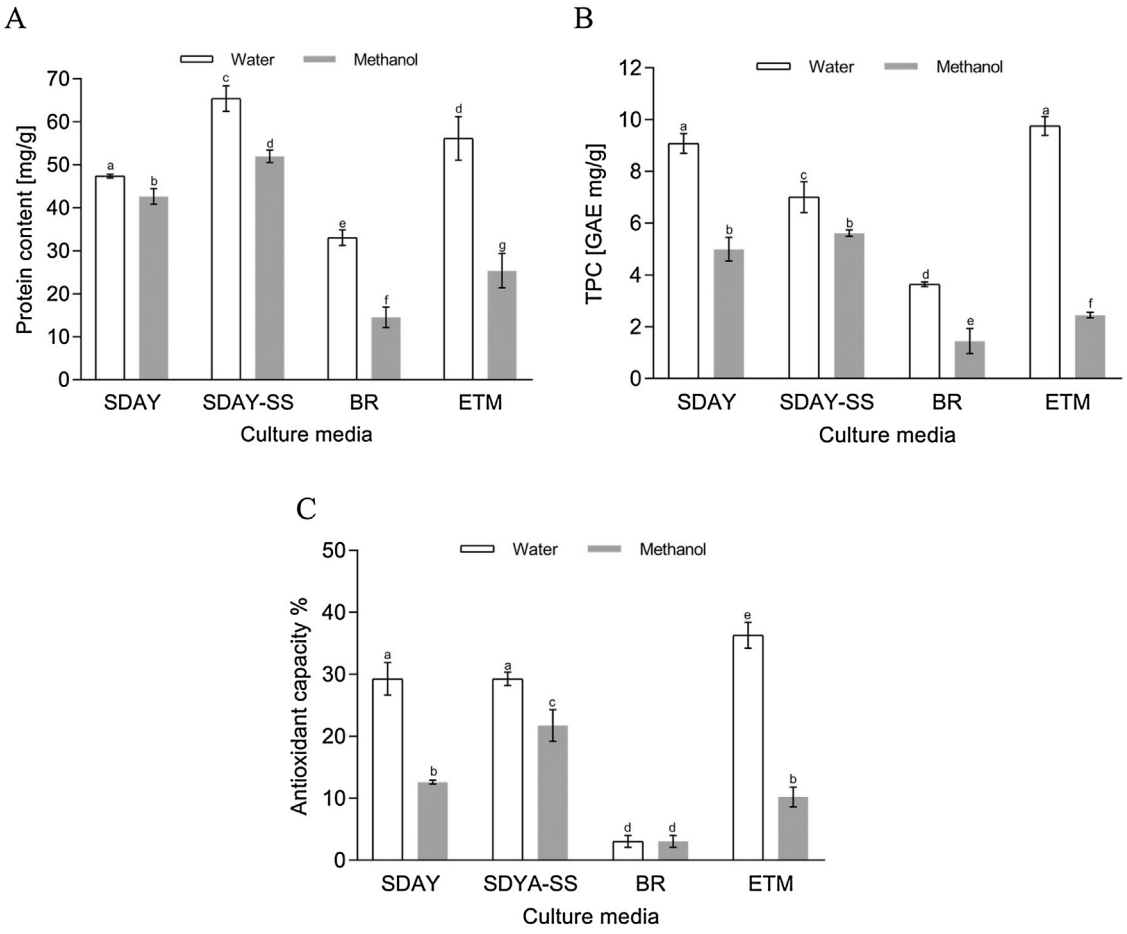

Fig. 1. A) Total protein content B), Total Phenolic Content (TPC), and C) Antioxidant capacity measured with ABTS assay in *Cordyceps nidus* ANDES-F1080. Different letters indicated a significant difference (p-value<0.05).

different pattern was found in those analyses where water fractions of extracts derived from ETM presented the highest GAE concentration, sequentially followed by extracts derived from artificial-based substrates and from BR. The same described profile pattern found in TPC analysis was obtained in the determination of antioxidant capacity (Fig. 1C).

3.3. Metabolites obtained from C. nidus ANDES-F1080 growth on natural-based substrates induce laccase activity of P. ostreatus ANDES-F515

Maximum laccase activity obtained in each profile was graphed in bars to compare the effects of adding each *C. nidus* ANDES-F1080 extract into *P. ostreatus* ANDES-F515 culture (Fig. 2). From these results, the highest maximum laccase activity was found in cultures supplemented with *Cordyceps nidus* ANDES-F1080 extracts derived from natural-based substrates. Additionally, treatments supplemented with water fractions always reached higher maximum laccase activities in comparison to the control. The profile of glucose concentration of each treatment was also graphed to further understand the effect of metabolite extracts on laccase activity of *P. ostreatus* ANDES-F515 (Supplementary data). Glucose concentration was typically consumed through time and was independent of the substrate treatment and solvent used. Although the variation was high in almost all days of sampling, treatments derived from natural culture tended to be higher than treatments derived from artificial culture media. The glucose profiles for artificial-based culture media were significantly lower

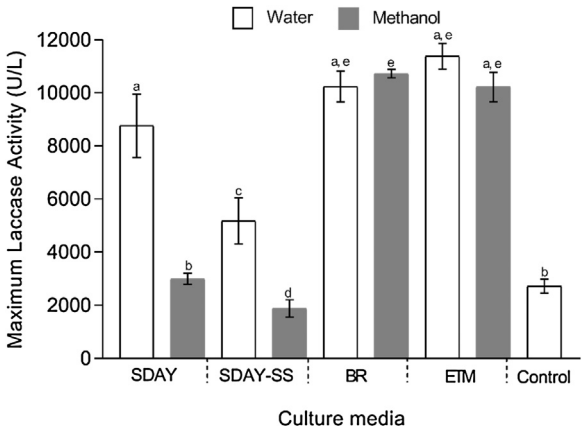

Fig. 2. Maximum laccase activity of *P. ostreatus* ANDES-F515 in different culture media using *C. nidus* ANDES-F1080 extracts with water and methanol as solvents.

and, in most cases, similar to the control condition. The two-way ANOVA indicated that both culture media and solvent have a significant effect on the maximum laccase activity.

Additionally, from the secondary data analyzed from Chiriví et al. [17], mass intensity values of nucleosidic compounds detected in extracts derived from BR were chosen for the analysis, given that these values were higher than those derived from artificial culture media. Seventeen nucleosidic compounds were obtained based on the described search process (Supplementary data).

3.4. Correlation between measured response variables

Pearson's product-moment correlation results are summarized in Fig. 3. Several direct and significantly related relationships among variables were found. It is apparent from these data that there is a strong correlation between protein content, TPC, and antioxidant capacity. The most striking observation to emerge from the correlation analysis is that laccase activity is possibly influenced by the presence of glucose, xylose, and malic acid.

3.5. Principal component analysis (PCA)

A PCA was implemented with the purpose of detecting possible relationships and differences within the studied variables. As shown in the plot of component loadings in Fig. 4, data were grouped into principal components, with the first two dimensions (PC1 and PC2) accounting for most of the variance of all measured variables (46.6 % and 27.6 %, respectively). As seen in these results, evident regions of clustering occur and are separable along PC1 and PC2. The behavior of PC1 differentiates between natural media (ETM and BR) and artificial media (SDAY and SDAY-SS). If we turn to PC2, there is a partition between water extracts and methanol extracts. Thus, there is a clear class separation between culture media and the solvent used for extraction.

Overall, methanol extracts supplemented in SDAY and SDAY-SS pertain to a separated group with a tendency to have high values of succinic acid, antioxidant capacity, total phenolic content, and protein content. A separate group consisting of methanol extracts supplemented in BR is distinguished by its arabinose content. In the case of water extracts added to SDAY and SDAY-SS, another

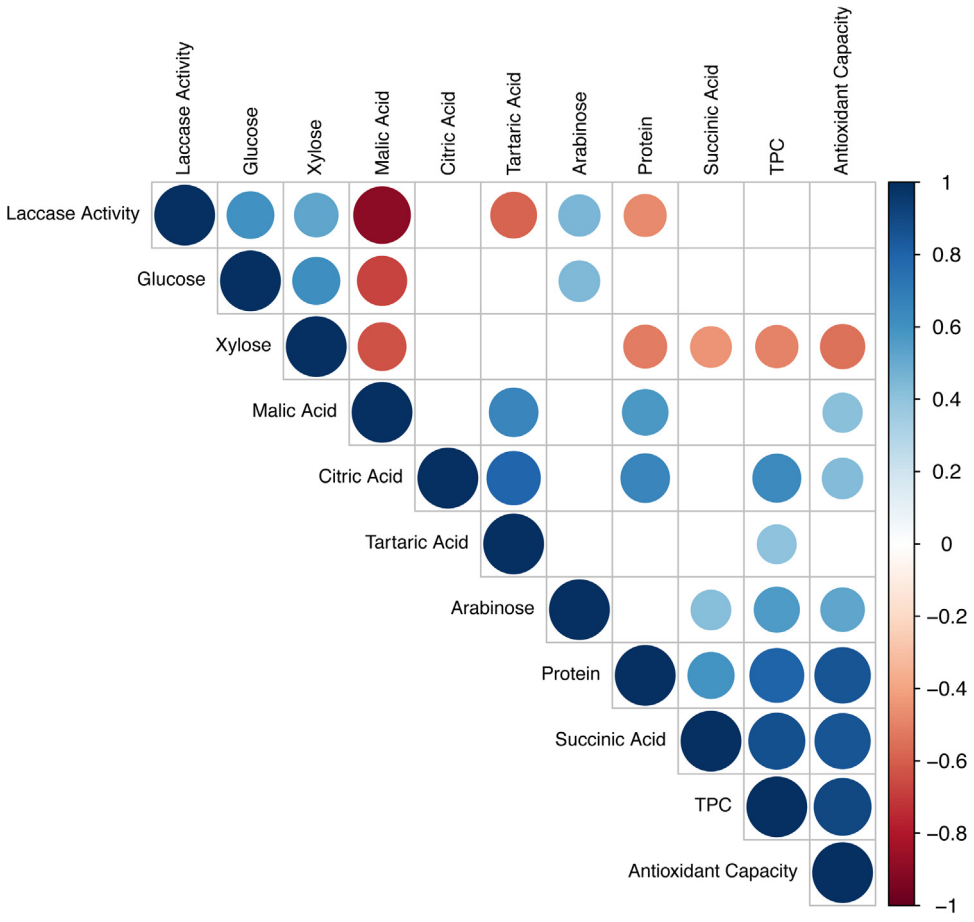

Fig. 3. Pearson's correlation coefficients obtained for the studied response variables.

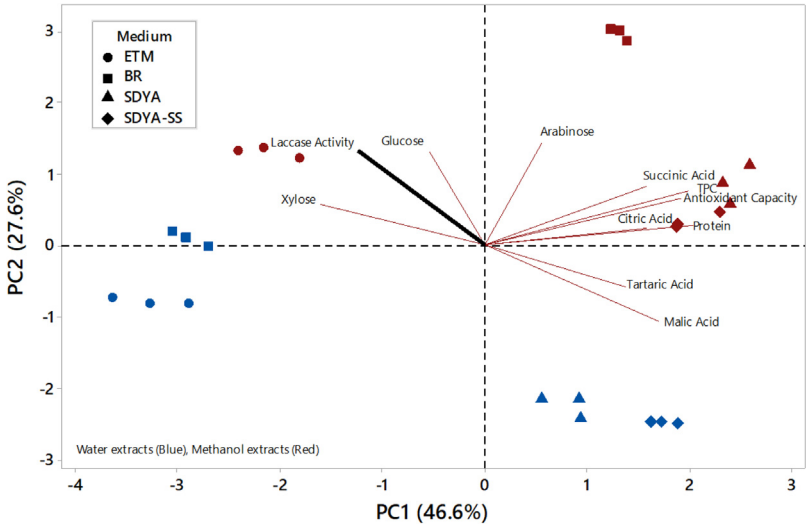

Fig. 4. Principal component analysis (PCA) for maximum laccase activity, total protein content, TPC, antioxidant capacity, sugars, and organic acids obtained with *C. nidus* ANDES-F1080 extracts using water and methanol as solvents.

cluster characterized by high levels of malic acid and tartaric acid is formed. From this data, the most interesting arrangement is the one composed by water extracts of ETM and BR, and methanol extracts of ETM; in this group, high contents of glucose, xylose, and low amounts of malic acid are evidently associated with high laccase activity.

4. Discussion

This work is the first evaluation of the effect of a biochemical complex derived from a fungus, a *Cordyceps* species, on the enzymatic activity of another fungus, the white-rot species *Pleurotus ostreatus*. These results contribute to the existing knowledge about the industrial applications of laccases, such as biological delignification and bioremediation. It was demonstrated that natural-based substrates are the best culture media to induce metabolites in *Cordyceps nidus* ANDES-F1080 promoting laccase activity in *P. ostreatus* ANDES-F515. For any of the previously mentioned cases, water was the best solvent to extract bioactive metabolites in *C. nidus* ANDES-F1080. Nevertheless, artificial-based substrates could also be used to produce metabolites with different bioactivity.

It was found that natural-based substrates induced the synthesis of organic compounds as evident in BR and ETM (Tables 1–2, and Fig. 4) presenting a high concentration of sugars and organic acids. Overall, these results are similar to the ones found by Chiriví et al. in their metabolomic study [17]. The main chemical difference in the composition of natural-based and artificial-based substrates is the absence of glucose in BR and the presence of a complex carbon source in both natural-based substrates. These differences confer important properties that could explain the profile of organic compounds that was achieved. A possible explanation for the behavior of *C. nidus* ANDES-F1080 in the studied culture media is that glucose allows the fungus to access a primary metabolic pathway as being a simple carbon source [26,27]. Its absence can induce the fungus to reach secondary metabolic pathways and to store essential metabolites [28]. Moreover, the presence of biopolymers can induce specific metabolic pathways mediated by catabolic repression [29].

In this regard, high concentration values of glucose derived from BR (Table 1) can be explained by the biomass saturation of the fungus and consequent carbohydrate bioaccumulation, as was previously described for the closely related species *Beauveria bassiana* which presented glucose and other simple carbon-based compounds, as a storage product [30]. Andersen et al. (2006) described a difference in glucose bioaccumulation into the conidia of entomopathogenic fungi when they were cultured on different substrates [31], a pattern that could be expected for mycelia as well. Similarly, the accumulation of glucose was also observed in the fruiting body and mycelium of *Pleurotus ostreatus* from a composting system, a carbohydrate degrading system similar to our own [32]. In this sense, the availability of glucose from the beginning of artificial-based cultures provides an easily available carbon source. As a result, *C. nidus* ANDES-F1080 consumes glucose in its early stages of growth, which allows the fungus to synthesize and to accumulate other metabolites, such as the organic acids shown in Table 2. Glucose and xylose are the main constituents of cellulose and hemicellulose, which are present in substrates where *P. ostreatus* grows [33], and they are available in high concentrations in the studied natural media. In the natural wood decay system, glucose and xylose are found ten times more often on the surface compared to the center of the plank where *P. ostreatus* as a secondary decomposer is mostly located [34,35]. This fact agrees with the effect of the carbon concentration associated with laccase genes promoter regions found in *P. ostreatus* [11]. Tinoco et al. (2011) also found that the addition of xylose in the substrate of the fungus has a positive effect on the laccase activity [36]. Regarding ETM, this condition is an intermediate case in comparison to the other media studied and is more closely associated with BR. Although the fungus can use glucose as an available carbon source from the beginning, the fungus can produce enzymes, among other molecules, to exploit the tarantula exuviae once glucose is totally assimilated. In this sense, a high concentration of some compounds in ETM is possibly explained by the phenomenon of catabolic repression [28].

In relation to protein content, TPC, and antioxidant capacity, a different response was obtained where extracts derived from artificial-based substrates presented higher values in comparison to BR. The TPC measured in *C. nidus* ANDES-F1080 extracts (3–10 mg of GAE per gram of dried mycelium or 80 mg/L to 450 mg/L) is in the same order of magnitude to those reported as inductors of laccase activity on *Pleurotus spp*. by other authors in the literature with values between 85 mg/L and 1400 mg/L of phenolic compounds [37–39]; however, the induction of laccase activity was not

observed in our study. By contrast, these results disagree with Pezzella et al. (2013) who found a positive effect from the addition of phenolic compounds in the laccase activity; however, in that study, ferulic acid was added in the culture media as a phenolic compound but in a different concentration and its effect was only measured in the transcriptional levels of laccase genes [40]. On the other hand, Lo et al. (2001), who studied the effect of several phenolic monomers on the laccase activity of *Pleurotus sajor-caju*, found that the presence of different phenolic monomers resulted in specific laccase isoforms that induce laccase activity [37]. The addition of phenolic compounds, such as 4-hydroxybenzaldehyde and vanillin, was found to be more effective to improve laccase activity than the addition of vanillic acid, syringic acid, ferulic acid, caffeic acid, and vanillin. This means that phenolic compounds could act as inducers, but not all of them may produce the same enhancing effect on laccase activity. As seen in Fig. 3, the phenolic compounds that could have been present on *C. nidus* ANDES-F1080 extracts may not have a significant effect on the laccase activity of *P. ostreatus* ANDES-F515.

According to the solvent used for extraction, the results revealed that water was the best solvent for the organic compounds studied in this research, which was expected due to a higher polarity in comparison to methanol [41]. Nevertheless, this fact partially agreed with the metabolomic determination of *C. nidus* ANDES-F1080, where methanol extracted higher quantities in specific cases [17]. Particularly for those compounds found in this study, a similar pattern related to the use of water or methanol for their extraction was found. Therefore, based on previous findings of nucleosides extraction [17,19], water is suggested as the first solvent option for the extraction of bioactive organic compounds from *C. nidus* ANDES-F1080.

Extracts derived from *C. nidus* ANDES-F1080 cultured in natural-based substrates had a significant promoting effect of the *P. ostreatus* ANDES-F515 laccase activity registering values around $10{,}575 \pm 813$ U·L^{-1} (Fig. 2). The enhancement reached values almost four times higher than the one found in the control (2717 ± 370 U·L^{-1}) and was independent of the fraction used (Fig. 2). *P. ostreatus* has been reported to achieve maximum laccase activity values (using ABTS) similar to our control condition grown on rice husk: 3000 U·L^{-1} in a semisynthetic basal medium with glucose and yeast extract [42], 3100 U·L^{-1} in alfalfa stem, and 4400 U·L^{-1} in corn straw [43]. Another species of the genus *Pleurotus*, *Pleurotus nebrodensis*, has been reported to produce a maximum laccase activity around 2800 U·L^{-1} when it is grown in a mandarin peels based medium [44]. These values are considerably lower than the maximum laccase activity achieved in this work using *C. nidus* ANDES-F1080 extracts. As explained above, complex structures such as tarantula exuviae and plant polysaccharides in BR induce several metabolites in fungi which can present different or higher bioactivity compared to those produced when the medium is less complex, such as the artificial-based substrates. This is supported by the correlations obtained and the PCA (Figs. 3–4), where the bioaccumulation of different compounds by *C. nidus* ANDES-F1080 in natural culture media can cause a positive increment in maximum laccase activity.

Due to the importance of laccases in the industry, it is necessary to consider whether the best conditions found in our study for laccase activity ($10{,}238 \pm 1012$ U·L^{-1} using water extracts from

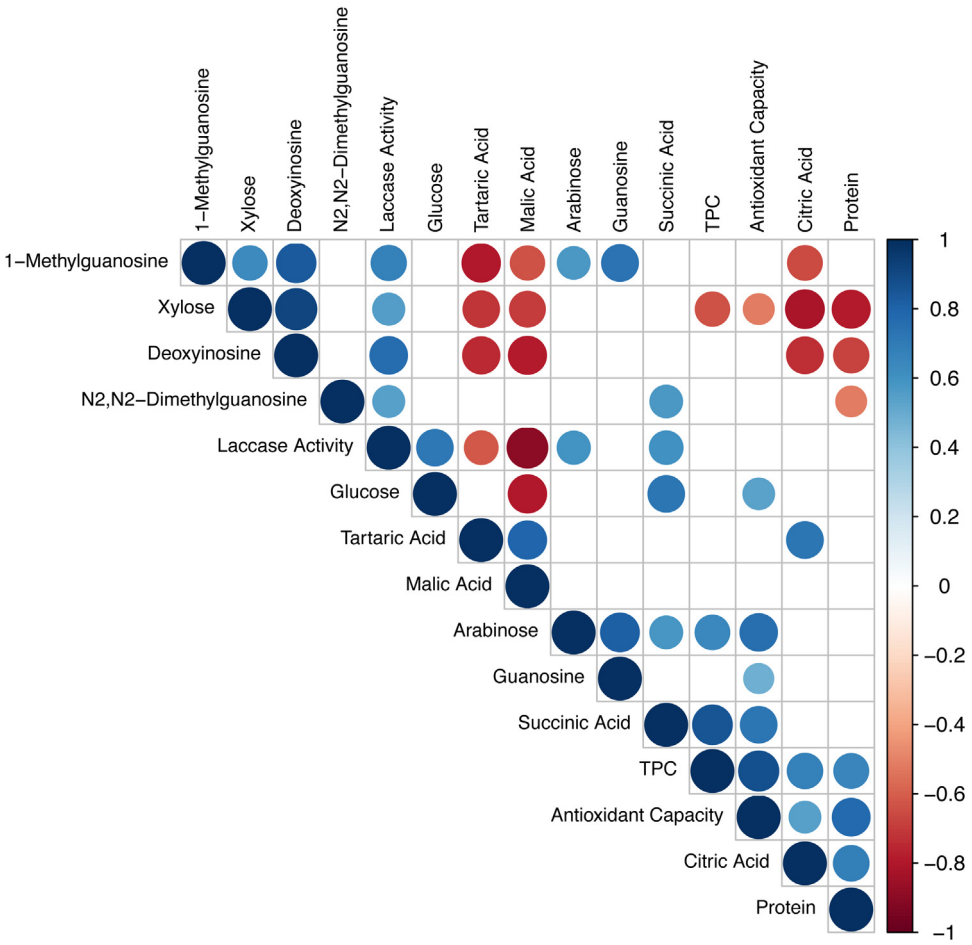

Fig. 5. Pearson's Correlation coefficients obtained including selected nucleosides from Chiriví et al. [17].

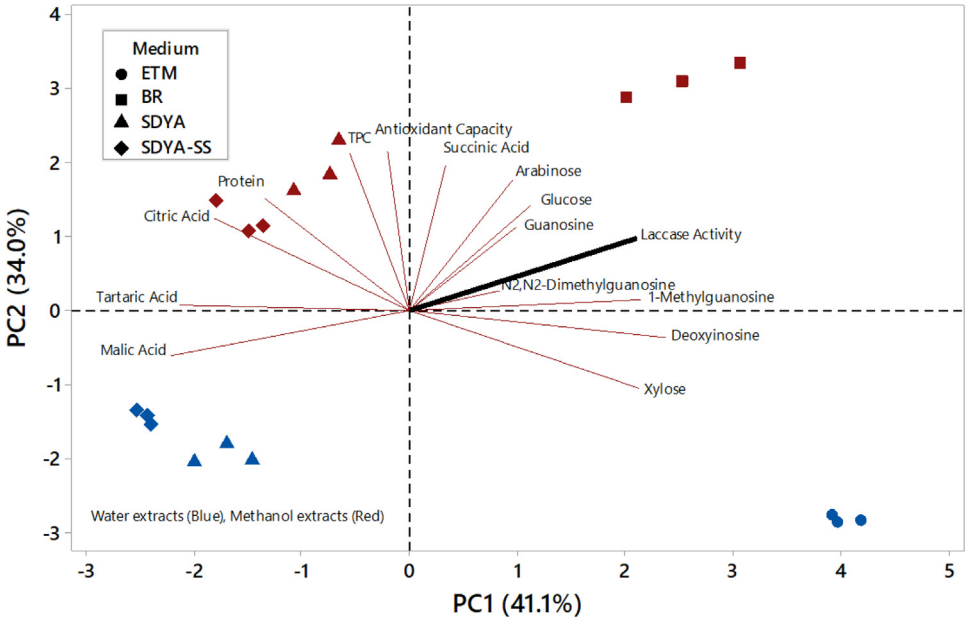

Fig. 6. Principal component analysis (PCA) including selected nucleosides from Chiriví et al. [17].

C. nidus ANDES-F1080 grown in BR) could result in a process that competes with currently available enzymes. The conventional process to produce laccases at the industrial level uses low-cost substrates (agricultural or food residues) where fungi can be cultivated either in submerged fermentation or solid-state fermentation. This process requires the use of incubators or bioreactors for growth and the use of autoclaves to maintain sterile conditions. Osma et al. proposed a model for the cost analysis of laccase production and evaluated the process using *Trametes pubescens* [45]. In that study, Osma et al. emphasize that the final cost of production depends mainly on the costs of the culture media, the equipment, and the operation. In submerged fermentation, using similar conditions to our study, it was found that the cost of the culture media represents the greatest part of the total cost (~89 % of all cost) which is mainly associated with the use of reagents for the carbon source and the inductors. Osma et al. reported a maximum laccase activity of 2107 $U{\cdot}L^{-1}$ for *T. pubescens* when it was grown in a mandarin peel based medium in submerged fermentation with a final cost of 0.48 cent €/U. This value of laccase activity is in the same order of magnitude as the obtained in our control condition (2717 ± 370 $U{\cdot}L^{-1}$) and other reports for the genus *Pleurotus* grown in several agricultural residues (2800–4400 $U{\cdot}L^{-1}$) [42–44]. The best conditions found in our work for laccase activity using extracts from *C. nidus* ANDES-F1080 are similar to the values obtained by Osma et al. in the solid-state fermentation of sunflower-seed shells using various inductors (soy oil, tannic acid, or coconut oil) with values of laccase activity between 9708 and 10,951 $U{\cdot}L^{-1}$, and a final cost between 0.20 and 0.23 cent €/U. A similar cost could be expected for our process with an additional advantage of using the extracts of *C. nidus* ANDES-F1080 to enhance the laccase activity of *P. ostreatus* ANDES-F515 instead of using expensive reagents. What is more, the scale-up of the process and the valorisation of *C. nidus* mycelium by-product [14] could further reduce the total cost of the process. The proposed methodology could be applied in Colombia and other developing countries where the industry of recombinant laccase production is not available yet. Nonetheless, a rigorous techno-economic analysis must be performed in future work in order to make a fair comparison with the available technologies.

Identification of metabolites with potential bioactivity on laccase activity was performed through a data search in the metabolomic analysis made by Chiriví and co-authors [17], [Supplementary file]. Among these metabolites, the mass intensity obtained from three of them presented higher values in extracts derived from BR than those observed in artificial culture media: 1-Methylguanosine, guanosine, and N2,N2-Dimethylguanosine. In addition, 3′-deoxyadenosine (Cordycepin) was considered due to its importance to assess the quality of *C. nidus* ANDES-F1080 extracts. Nucleosides' mass intensity values were analyzed in conjunction with the results obtained from this work, performing again Pearson's correlation and PCA (Figs. 5–6). From this analysis, it is evident that 1-Methylguanosine, cordycepin, glucose, xylose, and malic acid are strongly correlated with the maximum laccase activity that was achieved using water extracts derived from natural culture media. It is worth mentioning that guanosine and N2,N2-Dimethylguanosine exhibit a moderate correlation with laccase activity, which is not strong enough to validate its influence in the studied process. Interestingly, 1-Methylguanosine and cordycepin have not been reported as production enhancers of lignocellulolytic enzymes [46]. Both 1-Methylguanosine and N2, N2-Dimethylguanosine have been studied in methylation processes of RNA in prokaryotic organisms and eukaryotic plastids [46,47]. Although enhancer activity could result from a combined effect of several compounds, monitoring evaluation of these compounds is recommended due to the bioactivity of nucleosides in different metabolic pathways. For further information about data inspection using PCA, refer to Kohler et al. [48].

5. Conclusions

This research establishes a baseline for the application of extracts from *Cordyceps* species to stimulate the enzymatic activity and other biochemical properties of other fungi. Our results strengthen the research on the production of lignocellulolytic enzymes and are proposed as potential enhancers of laccase activity. Natural-based substrates induce the production of sugars and organic acids and enhance the laccase activity of *P. ostreatus* ANDES-F515. This work confirms the bioactive properties of

C. nidus ANDES-F1080 based on the culture medium used for its growth. From our results and those determined by Chiriví et al. [17], we found a strong correlation of glucose, xylose, malic acid, 1-methylguanosine, and cordycepin with the maximum laccase activity in *P. ostreatus* ANDES-F515. Nevertheless, molecular mechanisms of sugars, organic acids, and nucleosidic compounds need to be further studied to completely understand how laccase activity could be increased without matrix effect interference.

Author statement

We declare that all persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

CRediT authorship contribution statement

Durán-Aranguren D.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Chiriví-Salomón J.S.:** Conceptualization, Methodology, Validation, Writing - original draft. **Anaya L.:** Validation, Investigation, Data curation. **Durán-Sequeda D.:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Cruz L.J.:** Validation, Investigation, Writing - review & editing. **Serrano J.D.:** Investigation. **Sarmiento L.:** Investigation. **Restrepo S.:** Writing - review & editing. **Sanjuan T.:** Methodology, Writing - review & editing. **Sierra R.:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that there are not conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00466.

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