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THE VALORIZATION OF TEQUILA INDUSTRY AGRICULTURAL WASTE THROUGH ITS USE AS A SUBSTRATE FOR LACCASE PRODUCTION IN A FLUIDIZED BED BIOREACTOR

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ABSTRACT

Agave tequilana Weber leaves and fiber were studied as substrates for laccase production by *Pleurotus ostreatus* in a submerged culture, in order to take advantage of these agro-industrial wastes, which are generated during agave harvesting for tequila production. The optimal conditions for laccase production in submerged fermentation were met by a substrate concentration of 1% (w/v) at 120 h, producing 276.36 and 169.25 U L^{-1} for leaves and fiber, respectively. The addition of glucose did not significantly ($p>0.05$) affect enzyme production. A medium consisting of only water and agave leaves was the best culture broth for enzyme production, and this medium was selected for the study with the fluidized bed bioreactor scale. The specific activity for kinetic studies in a flask (50 ml working volume) was 14.68 U mg^{-1}. In the reactor, the specific activities were 10.78, 1.09 and 1.74 U mg^{-1} for the first, second and third cycles of operation, respectively. Thus, the leaf residues of *A. tequilana* generated by the tequila industry can be used as a substrate by *P. ostreatus* for laccase production without co-substrate addition.

KEYWORDS: *Pleurotus ostreatus,* laccase, *Agave tequilana* Weber, submerged fermentation

1. INTRODUCTION

Laccase (EC 1.10.3.2) is a multi-copper extracellular oxidase that is widely distributed in plants, insects and bacteria; however, the most important source of this enzyme are white-rot fungi [1, 2]. Laccase plays a role in various biological processes, such as sporulation, pigment production, rhizomorph formation, pathogenesis, the formation of fruiting bodies, and lignin degradation [3]. This enzyme also catalyzes the oxidation of various aromatic compounds (particularly phenols), diamines and aromatic amines by single electron transfer using molecular oxygen as the electron acceptor; this process leads to the reduction of oxygen to water [1, 4].

The main biotechnological applications of laccases have traditionally been in the textile industry for effluent treatment and in the paper industry for the bleaching process. However, the use of laccase has more recently spread to the areas of food, nanotechnology, and the detoxification of wastewater. One of the specific uses of laccase is that as a biosensor for the detection of aromatic compounds that exhibit affinity for this type of enzyme [5, 6].

Although only a small percentage of all white rot fungi, including *P. ostreatus*, has been studied for the laccase production, it is known that a single species may express distinct iso-forms of extracellular laccase. The particular iso-form expressed depends on the fungal growth conditions, such as temperature, pH, agitation, amount of inoculum, trace mineral concentration, carbon and nitrogen concentrations, medium composition, use of inducers, and type of fermentation [5, 7, 8]. Therefore, the expression of a given laccase iso-form is determined by environment factors during growth, including the fungal species, and each isoform exhibits different molecular properties [9, 10].

Currently, compounds that have the ability to function as inducers of laccase synthesis are being studied in relation to several species of fungi. For instance, the synthesis of extra-cellular laccase by the fungus *Coriolus hirsutus* was induced with 0.11 µM syringaldazine; an increase i n enzyme activity of close to 1,000% was reached, and two isoforms (I_1 and I_2) with molecular weights of 69 and 67 kDa were obtained [4]. Sonden and Dobson [11], for their part, used various aromatic compounds (ferulic acid, veratrum acid, 2,5-xylidine and hydroxybenzotriazole) and copper as inductors for *P. sajor-caju*, leading to an expression of the lac genes Psc 1, 2, 3 and 4. The results showed

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that ferulic acid and 2,5-xylidine increased the transcription of Psc 1, 2 and 4, whereas the Psc 3 gene was expressed constitutively.

Inducing the white-rot fungi *P. ostreatus* [12, 13], *Trametes trogii* [14], *Trametes hirsute* [15] and *Trametes versicolor* [16] with copper has been found to increase laccase production. Palmieri *et al.* [8] also reported that the production of laccase isoforms is regulated by the presence of copper ions in *P. ostreatus*. The few studies that have reported laccase activity when conjugating the inducers, 2,5-xylidine and copper ions, [17, 16] found that laccase production was increased from 190 to 360 U L^{-1} using 0.075 mmol L^{-1} copper sulfate and 0.030 mmol L^{-1} 2,5-xylidine, respectively. Adding inducer compounds to cultures of some species initiates an extra-cellular biosynthesis that results in new forms of laccase, indicating that constitutive forms are continuously produced [17].

The agricultural industry is the main source of lignocellulosic materials, such as roots, stems, leaves and other plant parts; these materials are usually a by-product of agricultural processes and represent a source of contamination in fields [18]. Approximately one million tons of *A. tequilana* Weber plant material are produced and processed annually for tequila production. During this process, the leaves, which are removed while harvesting the stem (the structure used to produce tequila), are left in the fields; the leaves, therefore, represent a waste product of tequila manufacturing [19].

Currently, several agro-industrial residues, such as straw (from wheat, barley, rice and rye), crop (including corn and beans), bagasse (from sugar cane) and coffee pulp, are used as substrates for laccase production. The lignocellulosic composition of these residues induces the production and secretion of ligninolytic enzymes which, in turn, cause the degradation of lignin, leading to its complete mineralization and the production of carbon dioxide and water [20, 21].

Because of the composition of *A. tequilana* Weber leaves (64.8% cellulose, 5.1% hemicellulose, 15.9% lignin, 14.0% extract) [22] and the ecological problems resulting from the large amounts of leaves discarded during the agave harvest, the use of *A. tequilana* Weber leaves and fiber is proposed herein for the first time as a natural substrate for laccase production. In this way, agave residues that currently represent a burden for the tequila industry, could be used productively.

2. MATERIALS AND METHODS

2.1 Fungal strain

The *P. ostreatus* strain used was kindly donated by the Laboratory of Applied Biotechnology at the Pontificia Universidad Javeriana of Colombia. The fungus was reactivated on Rose Bengal Agar plates (J.T. Baker) at 29 °C for 8 days, and then maintained on solid medium with wheat bran extract agar containing 175 g L^{-1} natural wheat bran (Maxilu), 10 g L^{-1} glucose (J.T. Baker), 5.0 g L^{-1} casein peptone (Bioxon), 2.0 g L^{-1} yeast extract (Bioxon), 0.1 g L^{-1} KH_2PO_4 (Mallinckrodt), 0.05 g L^{-1} $MgSO_4 \cdot 7H_20$ (Mallinckrodt), 0.076 g L^{-1} $MnSO_4$ (Mallinckrodt), 18.0 g L^{-1} bacteriological agar (Bioxon), and 0.1 g L^{-1}chloramphenicol (Merck) at 29 °C for 8 days.

2.2 Preparation of fungal inoculum

The inoculum was obtained by seeding one *P. ostreatus* mycelial plug, which was taken from colonies grown on wheat bran extract agar using a sterile cork borer (5 mm diameter) on a wheat bran extract agar plate incubating it at 29 °C for 8 days [23].

2.3 Conditioning of *Agave tequilana* Weber leaves

A. tequilana Weber leaves were collected from 8-year-old plants grown in Tequila, Jalisco, Mexico. The leaves were washed, cut into squares (0.5 x 0.5 cm), dried at 60 °C in an oven to constant weight, and then packed in polyethylene bags. The remaining leaves were used for fiber production according to a traditional method, which consists of removing the fleshy part of the leaves using a sharp instrument to expose and remove the fiber. The fiber was then rinsed and dried in the sun [5], cut into small pieces (0.5 cm long), and packed into polyethylene bags. The bags with the leaves and the bags containing fiber were both sterilized using gamma irradiation (^{60}Co) at a dose of 21 to 38 kGy [24], at the Instituto Nacional de Investigaciones Nucleares of Mexico (ININ).

2.4 Chemical analyses of the *A. tequilana* Weber leaves

The reducing sugar contents of the *A. tequilana* leaves were characterized using the 3,5-dinitrosalicylic acid technique [25]. Total nitrogen [26], total phosphorus [26] and humidity [26] were also assessed.

2.5 Laccase activity

Laccase enzymatic activity was assayed by measuring the oxidation of ABTS (2,2-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]) (Sigma A1888) at 436 nm for 3 min in a reaction volume of 1 ml [27]. The assay mixture contained 0.1 ml of 100 mM sodium acetate buffer (pH 4.5), 0.8 ml of enzyme extract and 0.1 ml of 5 mM ABTS. One unit of laccase activity was defined as the activity required to oxidize 1 μmol of ABTS per min and 1 L of extract.

2.6 Protein concentration determination

Protein concentration was determined using the Bradford method, for which a standard curve was constructed (100 to 1,000 μg ml^{-1}) using bovine serum albumin as a standard [28].

2.7 Laccase production in submerged fermentation

2.7.1 Laccase production kinetics

Submerged fermentation (SmF) was carried out in 250-ml Erlenmeyer flasks by adding 1 g of fiber or leaves,

2.5 g L^{-1} glucose and 10 mycelial plugs to 50 ml of sterile mineral medium (SMM) containing 2.0 g $L^{-1}KH_2PO_4$, 0.03 g L^{-1} NH_4Cl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.1 g L^{-1} thiamine, and a trace element solution (10 ml L^{-1} of medium) comprising 0.5 g L^{-1} $MnSO_4$, 0.1 g L^{-1} $FeSO_4 \cdot 7H_2O$ and 0.1 g L^{-1} $ZnSO_4 \cdot 7H_2O$ [29]. The flasks were incubated in the dark on a rotatory shaker (120 rpm) at 29 °C for 168 h, and enzyme activities and protein concentrations were measured every 24 h. A control sample was processed under the same conditions, but using the fungus as free biomass and without agave. The experiments were carried out in triplicate.

The time that yielded the highest laccase production was considered for the following experiments.

2.7.2 Effect of agave concentration on laccase production

The effect of agave concentration on laccase production was studied in 250-ml Erlenmeyer flasks by adding 50 ml of SMM and 2.5 g L^{-1} glucose to 0.2, 0.6, 1.0, 2.0 and 3.0% w/v suspensions of fiber or leaves and incubation at 29 °C and 120 rpm for 120 h. Laccase activities and protein concentrations were measured at the end of the incubation period. The control experiment was processed under the same conditions, but using the fungus as free biomass and without agave. The experiments were conducted in triplicate, and the concentration that induced the highest enzyme activity was used for the following experiment.

2.7.3 Effect of medium composition on laccase production

A study of the effects of agave fiber and leaves as substrate and medium composition (adding sterile distilled water or SMM to the culture medium) was carried out according to the experimental design presented in Table 1. The glucose concentration was 2.5 g L^{-1} for treatments 1 and 4, and the concentration of fiber and leaves used in all cases was 1.0% (w/v). In treatments 3 and 6, sterile water was used as the culture medium instead of SMM, and 1% w/v of fiber or leaves was employed as the substrate. The controls used for this test were as follows: fungus used as free biomass including glucose in the SMM and without agave (treatment 7), fungus used as free biomass without including glucose in the SMM and without agave (treatment 8), dead biomass including glucose in the SMM and without agave (treatment 9), leaves without including glucose in the SMM and without inoculum (treatment 10), and fiber without including glucose in the SMM and without inoculum (treatment 11). All of the treatments were performed at 29 °C and 120 rpm for 120 h. Enzyme activities and protein concentrations were measured as previously described, and the experiments were conducted in triplicate. The results of this experiment were considered when scaling up laccase production.

2.8 Scale up of laccase production in a fluidized bed bioreactor

This test was performed using two fluidized bed bioreactors (1.0 and 1.5 L) with an effective volume of 50%. The working conditions were as follows: 20 mycelial plugs in100 ml of medium, agave leaves as substrate (1% w/v), sterile distilled water at 29 °C as medium, a saturation pressure of 4 kg/cm^2 and an air flow-rate of 1 ml min^{-1}. There were 3 fermentation cycles of 120 h each. At the end of each cycle, triplicate samples were taken, and enzyme activity and protein concentration were determined in each.

2.9 Statistical analysis

Analyses of variance and Tukey comparison of means ($p \leq 0.05$) between treatments were performed using SAS® statistical software, version 8.0 (SAS Institute, Cary, USA).

3. RESULTS AND DISCUSSION

3.1 *A. tequilana* Weber leaf composition

Agave leaves contained 4.40% reducing sugars (w/w), 0.0014% total nitrogen (w/w) and 0.260% (w/w) total phosphorus (dry matter). Therefore, these leaves were a rich source of nutrients, mainly reducing sugars; due to their organic nature, and these sugars are easily metabolized by microorganisms, resulting in a more economical process [6].

TABLE 1 - Experimental design used to determine the effects of the medium composition on laccase production by *P. ostreatus* using *Agave tequilana* Weber waste as substrate.

Treatment	Substrate	Medium
1	Fiber	With glucose in the SMM
2	Fiber	Without glucose in the SMM
3	Fiber	Sterile water
4	Leaves	With glucose in the SMM
5	Leaves	Without glucose in the SMM
6	Leaves	Sterile water
Controls		
7	Fungus with glucose in the SMM and without agave	
8	Fungus without glucose in the SMM and without agave	
9	Dead biomass with glucose in the SMM and without agave	
10	Leaves without glucose in the SMM and without biomass	
11	Fiber without glucose in the SMM and without biomass	

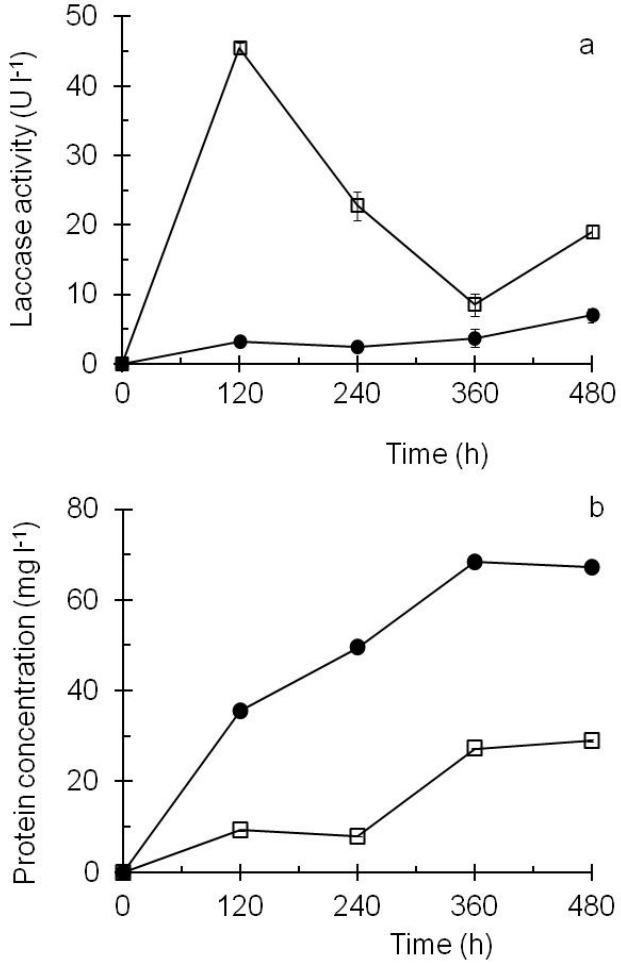

FIGURE 1 - The production of laccase (a) and protein content (b) by *P. ostreatus* using *Agave tequilana* Weber waste in solid-state fermentation (fiber, leaves; T= 29 °C).

3.2 Laccase production in submerged fermentation

3.2.1 Laccase production kinetics

The time required for the production of maximum laccase activity in this culture system when using either fiber or leaves as substrate was 120 h; the maximum activities were 306.21 and 217.91 U L^{-1} for fiber and leaves, respectively. After this time, enzyme activity decreased sharply. For example, at 168 h, laccase activities were 190.41 and 148.89 U L^{-1} for fiber and leaves, respectively (Fig. 2a). In contrast, the treatment with the fungus as free biomass and without agave exhibited 17.38-fold lower activity than the treatment when using fiber as the substrate, and exhibited 12.37-fold lower activity than when using the treatment with leaves as the substrate. These values indicate that agave is a suitable substrate that can induce high enzyme activity.

Commercially, the mostly used natural substrate to culture *P. ostreatus* is wheat straw [30]. However, given the broad scope that has been proposed for the possible uses of laccases, other natural wastes, such as coffee pulp, straw, cotton, or barley straw could also be employed. In each case, fermentation factors such as medium or substrate composition, carbon source, pH, temperature, particle size and aeration can be controlled to increase enzyme production [1, 31]. However, not all agricultural wastes are efficient substrates for laccase production.

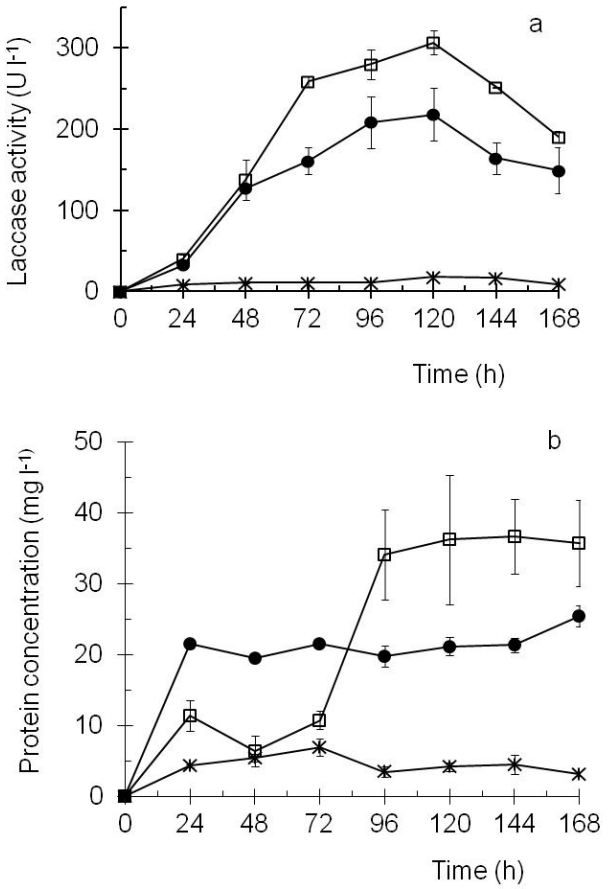

FIGURE 2 - The production of laccase (a) and protein content (b) by *P. ostreatus* using *Agave tequilana* Weber waste in submerged fermentation (free fungus, fiber, leaves. T= 29 °C; 120 rpm).

Guillen *et al.* [32] evaluated the production of ligninolytic enzymes obtained from *P. ostreatus* using SmF in a reactor using a synthetic medium including yeast extract and glucose (0.5 g L^{-1}). The values for laccase activity obtained were similar to those obtained herein when using agave fiber. In the aforementioned study, an activity of 307 U L^{-1} was observed at 85 h of incubation, compared to an activity of 306.21 U L^{-1} after 120 h of incubation with fiber found herein.

For *P. ostreatus* strain 494, the maximum activity (256 U l^{-1}) was observed in a submerged culture after 120 h of fermentation using only mandarin orange peel as substrate [33]. This value is similar to that achieved in this investigation using the same fungal species but a different waste as substrate.

On the other hand, laccase activity obtained with rice bran in a liquid medium was 222 U l^{-1} after 360 h of cultivation, whereas activities obtained with wheat bran, glucose and rice straw meal in liquid media were 90.0, 10.0 and 10.0 U l^{-1}, respectively [1]. These values are lower than

those obtained in the present study at 120 h using fiber or leaves, indicating that the expression of laccase by fungi is influenced by the culture conditions, including the nature of the carbon source used [34].

The particle size is an important factor influencing ligninolytic enzyme production. Indeed, enzymatic action on the substrate depends mainly on the following factors: particle size, material properties, accessible area, surface area, and porosity [31]. This could explain the lower enzyme activity found when using leaves (217.91 U L^{-1}) rather than fiber (306.21 U L^{-1}) as a substrate, because leaves have a larger particle size and their geometry is more complex, leading to lower enzyme activity.

Thus, lignin molecules might be exposed in the fiber particles, which were washed during the production process. If true, this means that laccase induction would be favoured [31]. As expected, in the culture using the fungus as free biomass without any lignin available, the amount of enzyme induced was significantly lower ($p \leq 0.05$), reaching a maximum protein concentration of 6.93 mg L^{-1} at 72 h (Fig. 2b). Considering that the production achieved in SmF was better than that obtained with SSF, all further experiments were carried out using the former system.

3.2.2 Effect of agave concentration on laccase production

One of the factors leading to increased enzyme production is the use of high concentrations of lignocellulosic materials as substrates and the subsequent reduction of glucose. According to the present results, enzyme activity increases with fiber concentration. Conversely, maximum laccase activity was reached at 1% (w/v) with agave leaves.

The enzyme activities achieved with 0.0, 0.2, 0.6, 1.0, 2.0 and 3.0% fiber were 17.61, 25.41, 105.28, 169.25, 279.96 and 541.71 U L^{-1}, respectively, whereas the activities achieved with leaves were 17.61, 156.05, 202.88, 276.36, 187.00 and 256.92 U L^{-1} at the same percentages (Fig. 3a).

Some authors have evaluated the effects of 0.5, 1.0 and 2.0% (w/v) of rice bran in liquid medium on laccase production by the fungus *Coriolus versicolor* and found that the productivity of laccase per g substrate was higher at 1% w/v (17.0 U g^{-1}) than that obtained when using 2% w/v of substrate (9.5 U g^{-1}) [1]. In the present study, laccase production using 1% w/v of leaves as substrate was 62.52% higher than that found when using 1% w/v of rice bran, whereas laccase productivity with 2% w/v of leaves was 1.58% less than that achieved when using 2 % w/v of rice bran.

In contrast, there was no significant difference ($p > 0.05$) in enzyme production between using either 1 or 3% w/v of fiber; therefore, the concentration achieving maximum use of the substrate was 1% w/v.

3.2.3 Effect of medium composition on laccase production

Another important factor for laccase expression is the concentration of the carbon source used as the primary substrate. Thus, to obtain effective laccase expression, it is essential to optimize all culture conditions, including medium composition, to facilitate the most economical design of the full-scale fermentation operation system [4, 34].

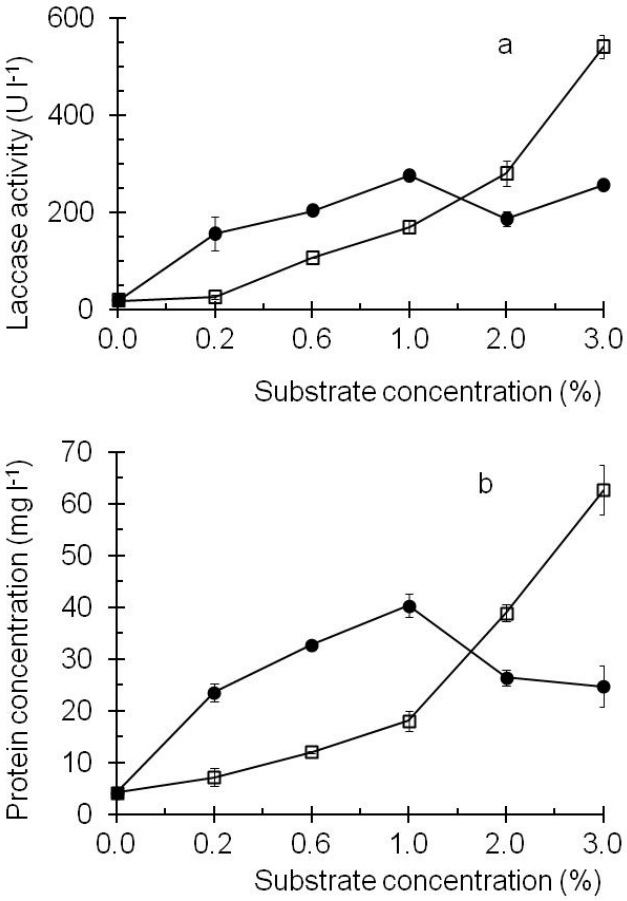

FIGURE 3 - The production of laccase (a) and protein content (b) by *P. ostreatus* using various *Agave tequilana* Weber waste concentrations in submerged fermentation (□ fiber, ● leaves, T= 29 °C, 120 rpm).

In previous studies, the carbon source used in the medium for the production of ligninolytic enzymes has been found to be important [33]. Mansur *et al.* [35] showed that the use of fructose rather than glucose resulted in a 100-fold increase of specific laccase activity in basidiomycetes. Likewise, in the present study, the enzyme activity was significantly different ($p \leq 0.05$) when glucose was added to the culture medium, regardless of whether fiber or leaves were used as substrate. With fiber, a 29.90% increase in laccase activity was observed when glucose was added (treatments 1, 2), whereas with leaves, a 46.19% increase was observed (treatments 4, 5).

It should be noted that the conditions that prevailed in treatment 4 (leaves and glucose in the SMM) promoted the highest enzyme activity (Fig. 4a). The reducing sugars contained in the leaves (4.4% w/w) as well as the carbon source supplied by adding glucose to the medium probably aided in the accumulation of biomass in the culture medium, positively affecting laccase production [36].

Interestingly, when leaves were the sole carbon source suspended in sterile distilled water (treatment 6), enzyme production was not significantly different ($p>0.05$) from that in the medium that contained fiber, glucose and minerals (treatment 1). These results suggest that reducing sugars and minerals are released from the leaves during the first hours of fermentation, and that both of these elements are probably used by the fungus as easily assimilated nutrients for the production of biomass and laccase [37].

For *P. ostreatus*, Guillen *et al.* [32] found an enzyme activity of 307 U L^{-1} after 85 h in SmF using a culture medium comprising yeast extract and glucose (5 g). This level of activity is slightly greater than that obtained herein, most likely because the glucose concentration was twice that employed by us.

Stajic *et al.* [33] showed that the highest level of laccase activity in *P. eryngii* was obtained when using dry ground mandarin orange peels as the carbon source after 7 days of SmF cultivation (999.5 U L^{-1}), whereas a low level of laccase activity was obtained when using glucose as the carbon source after 7 days of cultivation (4.4U L^{-1}). However, significant levels of laccase activity were evident when using xylan and D-gluconic sodium salt on the 5th day of cultivation (134.4 and 121.5 U L^{-1}, respectively).

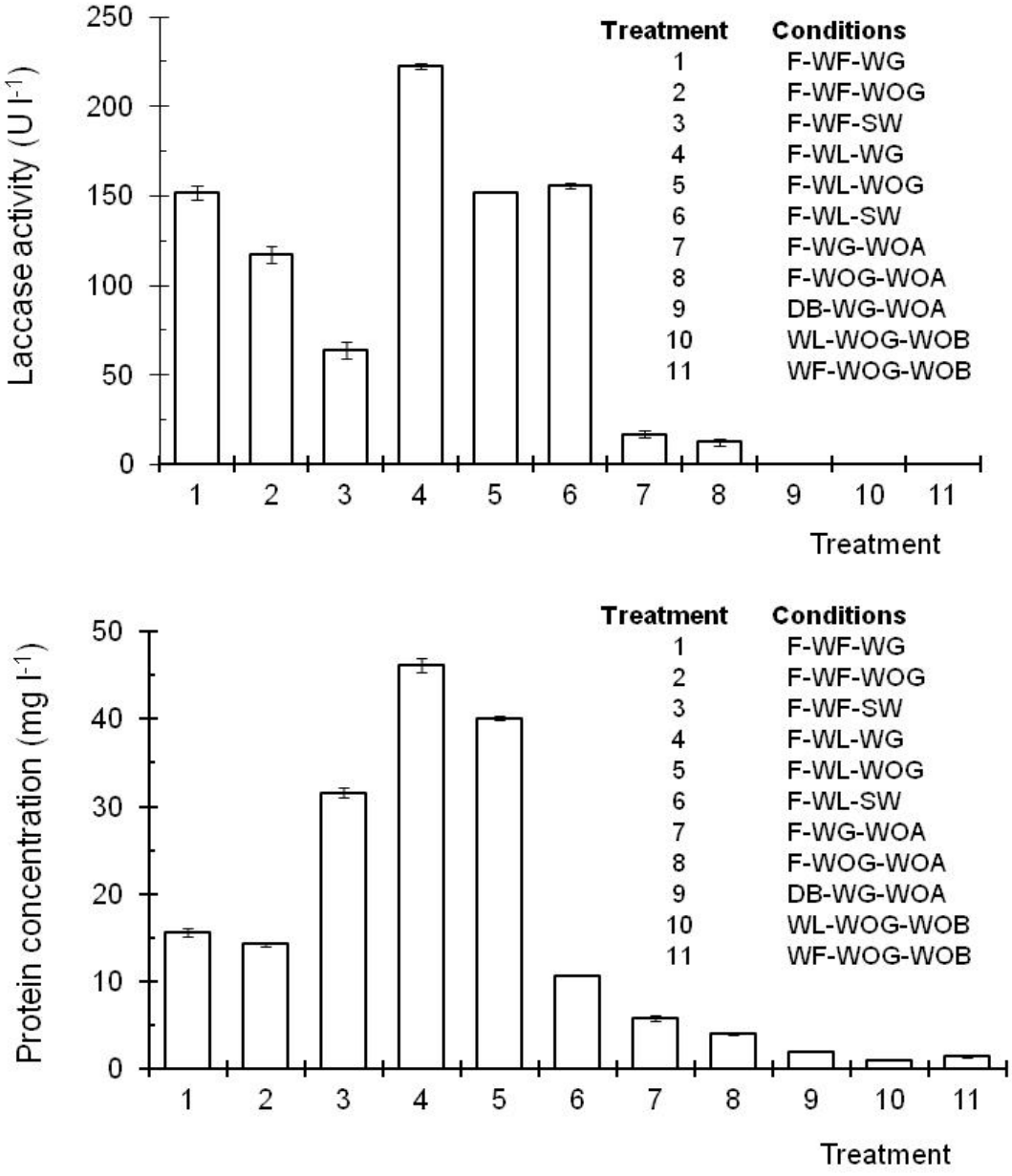

FIGURE 4 - Effect of medium composition on the production of laccase (a) and protein content (b) by *P. ostreatus* using *Agave tequilana* Weber waste in submerged fermentation (DB: dead biomass; F: fungus; SW: sterile water as medium; WF: using fiber as substrate; WG: using glucose in the SMM; WL: using leaves as substrate; WOA: without using agave; WOB: without using biomass; WOG: without using glucose in the SMM).

Similarly, *P. ostreatus* strain 494 showed high laccase production in SmF with mandarin orange peels on the 5th day of cultivation (256 U L^{-1}), whereas laccase was produced in amounts of 45.4 U L^{-1} using glucose and 46.6 U L^{-1} using mannitol as carbon source (after 7 days in both cases) [33].

Elisashvili *et al.* [38] achieved the highest laccase activity in *P. ostreatus* by cultivation on a mannitol-containing medium. Ardon *et al.* [39] showed that *P. ostreatus* cultures treated with cotton stalk extract exhibited both increased laccase activity and enhanced lignin mineralization. Controls (treatments 7 to 11) confirmed that sterilized leaves and fiber did not show any laccase activity.

In the medium with fiber as substrate, the protein produced by the fungus was increased from 14.30 to 31.60 mg l^{-1} when using sterile distilled water (treatment 3) rather than SMM (treatment 2) as the liquid medium (Figure 4b). In contrast, when leaves were used as substrate, the protein content was 4-fold higher in the medium with added minerals (treatment 5) than in the medium with only sterile distilled water (treatment 6).

Although the fungus released high amounts of protein to the medium in the treatment with fiber and sterile distilled water (treatment 3), the laccase activity was lower, indicating that the available carbon sources could induce the production of enzymes other than laccase [1]. Based on these results, laccase production using agave waste depends on culture conditions.

Lastly, the highest specific laccase activity (14.68 U mg^{-1}; $p \leq 0.05$) was achieved when *P. ostreatus* was cultivated with leaves and sterile distilled water (treatment 6). Hence, the scaled up production of laccase at the bioreactor level was performed using only sterile distilled water as the liquid medium and leaves as the substrate.

3.3 Scale up of laccase production in a fluidized bed bioreactor

P. ostreatus growth and laccase production in a bioreactor gave similar results to those found on a small scale (Table 2). However, only the first cycle of fermentation provided a high enzymatic yield of 127.42±1.73 U L^{-1}, a value close to that obtained in flasks with a working volume of 50 ml (156.00±2.50 U L^{-1}). In the second cycle, the enzyme activity decreased significantly ($p \leq 0.05$) and was 11.64-fold less than that in the first production cycle, suggesting that enzymes other than laccase were produced due to changes in the substrate composition [1]. The protein content was similar in both cycles.

For the third cycle, both enzyme activity and protein concentration fell sharply: enzyme activity decreased from 127.42 to 2.28 U L^{-1}, whereas protein concentration decreased from 11.91 to 1.31 mg L^{-1}. Activity changes between fermentation cycles have been explained by differences in the fungal growth phase [32].

These results show that it is feasible to produce laccase using agave leaves as substrate in a fluidized bed bioreactor in a single 120-h cycle. Under these conditions, the protein yield is 11.91 mg L^{-1}, and the specific activity is 10.69 ± 1.27 U mg^{-1}.

TABLE 2 - Enzyme activity and protein concentration of *P. ostreatus* at various cycles of production in a fluidized bed bioreactor with submerged fermentation using *Agave tequilana* Weber leaves as substrate.

Cycle (5 days)	Laccase activity (U L^{-1})	Protein concentration (mg L^{-1})	Specific activity (U mg^{-1})
1	127.42 ± 1.73	11.91 ± 1.25	10.69 ± 1.27
2	10.94 ± 0.46	10.05 ± 0.63	1.09 ± 0.10
3	2.28 ± 0.34	1.31 ± 0.00	1.74 ± 0.26

Hence, the fermentation of *A. tequilana* Weber leaves is an effective way to valorize residues from the tequila industry for laccase production.

4. CONCLUSIONS

A. tequilana Weber waste was shown to be an efficient natural substrate for laccase production by *Pleurotus ostreatus* using a 120-h submerged fermentation with sterile distilled water as the culture medium and agave leaves as the substrate. Scaling up in a bioreactor produced favourable and reproducible results and yielded laccase with a high specific activity.

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The authors have declared no conflict of interest.

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