EVALUATION OF LACCASE AND HYDROGEN PEROXIDE PRODUCTION FROM *Penicillium oxalicum* AND *Penicillium* sp.

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ABSTRACT

This investigation was carried out to determine the presence of the laccase enzyme and hydrogen peroxide (H_2O_2) in fungal extracts of *Penicillium oxalicum* and *Penicillium* sp. in different fermentative media. The *P. oxalicum* extract in WM medium contained more joint activity of laccase and H_2O_2 (laccase 77.78 ±0.01 U/L and H_2O_2 8.38 ±1.08 mM after three days) than the monoculture of *Penicillium* sp. in ITM medium, which prioritized only laccase (laccase 194.4 ±19.3 U/L and non-detectable H_2O_2 after two days). There was a negative correlation between biomass and glucose. From the first day of fermentation, the broth showed acidity. It was possible demonstrated that both monoculture extracts contained these two oxyacid compounds, laccase and H_2O_2.

Keywords: Fermentative process. Fungal enzyme extracts. Oxyacid compounds.

1 INTRODUCTION

Fungal enzyme extracts (FEEs) can be generated through liquid or solid fermentation. Extracts obtained from white-rot fungi typically contain oxidative enzymes like laccase, manganese peroxidase, and lignin peroxidase, utilizing hydrogen peroxide (H_2O_2) as the reactive oxygen species (ROS). [1] The specific enzymes and compounds produced, however, vary depending on the fungal species used. They are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various xenobiotic compounds, including dyes and pesticides.[2] In mycoremediation, ROS plays a crucial role, and H_2O_2 acts as a potent oxidant and a precursor for free radicals, participating in advanced oxidation processes which are responsible for the degradation of organic matter such as Fenton and photo-Fenton reactions.[3]

In the soil, exogenous H_2O_2 may be introduced or produced in situ by fungi. Meanwhile, laccases can be constitutive or inductive enzymes, their production generally occurs during secondary metabolism and is subject to complex regulation by nutrients, carbon, nitrogen, and inducers.[4] Numerous studies have shown that the activity of laccase in white-rot or endophytic fungi can be heightened through fungal mono and co-cultures.[5,6] In this investigation, we explored the potential of FEEs produced from monocultures of *Penicillium oxalicum* and *Penicillium* sp. This study encompassed the examination of enzymatic activity (laccase) and the presence of H_2O_2.

2 MATERIAL & METHODS

To standardize the inoculum size in liquid culture experiments, a cell suspension was prepared. Mycelial fragments were extracted from cultures of *Penicillium* sp. and *Penicillium oxalicum* isolated on potato dextrose agar (PDA) (20 g L^{-1} glucose, 15 g L^{-1} agar, 200 g L^{-1} boiled potato broth, pH 7 ±0.2). These fragments were then inoculated into 250 ml Erlenmeyer flasks containing 75 ml of potato dextrose broth (PDB) (20 g L^{-1} glucose, 200 g L^{-1} boiled potato broth, pH 7 ±0.2), which were pre-autoclaved at 121 °C for 15 minutes. These flasks were inoculated with loops filled with fungal spores and placed in an incubator under agitation (120 rpm) at 30 °C for 3 days.

Different media were used for the evaluation of laccase and H_2O_2 production from *Penicillium* sp. and *P. oxalicum*: (TM) - PDB medium adjusted to pH 5, according to Thakhor et al. (2022)[1]; (WM) - Nutrient medium, adapted from Wulandari et al. (2020)[4] composed in g L^{-1} of 3 KH_2PO_4, 2 $MgSO_4.7H2O$, 10 g $MnSO_4$, 25 glucose, 0.1 $CuSO_4$, 10 peptone and 10 malt extract; and (ITM) - Salt medium, from Izcapa-Treviño (2009)[7] with modifications, containing in g L^{-1}: 10 glucose, 1 peptone, 1 $(NH_4)_2SO_4$, 0.5 $MgSO_4.7H_2O$, 0.875 KH_2PO_4, 0.125 K_2HPO_4, 0.1 $CaCl_2.2H_2O$, 0.1 NaCl, 0.02 $MnSO_4.H_2O$ and 0.001 $FeSO_4.7H_2O$, adjusted to pH 5.8.

In summary, 10% (v/v) of the prepared inoculum cell suspension, i.e., 5 mL, was added and cultured in a 125 mL Erlenmeyer flask with 50 mL of medium, subjected to submerged fermentation (agitation at 120 rpm at 30 °C). After 1, 2, 3, and 4 days of cultivation a duplicate sample was collected (two fermentation flasks). The cultures were filtered under vacuum using Whatman No. 1 filter paper at constant weight, the biomass kept at 105 °C until dry weight was stable. The supernatant was designated as FEE. The enzymatic activity (laccase) of FEE was evaluated, as well as pH (using a pHmeter BEL), H_2O_2, and residual glucose.

H_2O_2 was measured according to the methodology of Oliveira et al. (2001)[8] through the reaction in an acidic medium of the vanadate ion and hydrogen peroxide, forming the peroxovanadium cation, which has a reddish color. An indicator solution of ammonium vanadate was prepared by dissolving 1.17 g NH_4VO_3 in 5.56 mL of 9 mol L^{-1} H_2SO_4 and completing the volume up to

100 mL with water. Subsequently, a calibration curve was required. In a test tube, 2 mL of the sample at the predetermined concentration and 1 mL of the indicator solution of NH_4VO_3 were placed. After 10 minutes, the mixture was taken to the spectrophotometer for absorbance measurement at 446 nm. The calibration curve was then constructed based on the absorbance measurements. Sample readings were conducted in the same manner as in the construction of the calibration curve. To determine the concentration of H_2O_2 based on the collected absorbance, the equation obtained from the calibration curve was employed:

$$C = (6{,}1708 \times A + 0{,}1223) \times F \quad (1)$$

where, C is the concentration of H_2O_2 in mM; A is the absorbance; F is the dilution factor.

The determination of Laccase enzymatic activity was conducted as adapted from Teng et al. (2019)[9]. A mixture containing 200 µL of 5 mM ABTS and 1.7 mL of pH 5 sodium acetate buffer (50 mM) was added to 100 µL of culture supernatant diluted 100 times. Laccase activity was determined by monitoring the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm for 5 minutes, with readings taken every 30 seconds, at room temperature (25-30 °C), using a molar extinction coefficient of 36,000 $mol^{-1}cm^{-1}$. One unit of laccase activity (U) was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute. Bovine catalase was added 30 minutes before the enzymatic activity assay to eliminate any hydrogen peroxide that might be present. The experiment was conducted in triplicate. The laccase activity in U/L was calculated according to the following formula:

$$U/L = \frac{10^6 \times V \times \Delta A}{\varepsilon \times v \times \Delta t \times d} \quad (2)$$

where, ε is the molar extinction coefficient of the substrate; ΔA is the increase in absorbance at the specific wavelength; Δt is the reaction time in minutes; V is the total volume of the reaction in mL; v is the volume of crude laccase in mL; d is the optical path length of the cuvette in cm.

The residual glucose concentration was measured using High-Performance Liquid Chromatography (HPLC) with a Shimadzu LC-20A Prominence model and a SUPELCOGEL C-610H column. Phosphoric acid (0.1%) was employed as the mobile phase, with a flow rate of 0.5 mL/min, oven temperature set at 32 °C, and an injection volume of 20 µL. The calculations were performed with the assistance of previously prepared standard curves.

3 RESULTS & DISCUSSION

In all conditions studied, the production of compounds with acidic content was measured, as can be seen from the pH values measured (Figure 1A). Maximum biomass production was observed by incubation of *Penicillium* sp. in TM medium (Figure 1B). The increase in biomass corresponded to the decrease in glucose, that is, there was a negative correlation between biomass and glucose consumption throughout fermentations (Figure 1C).

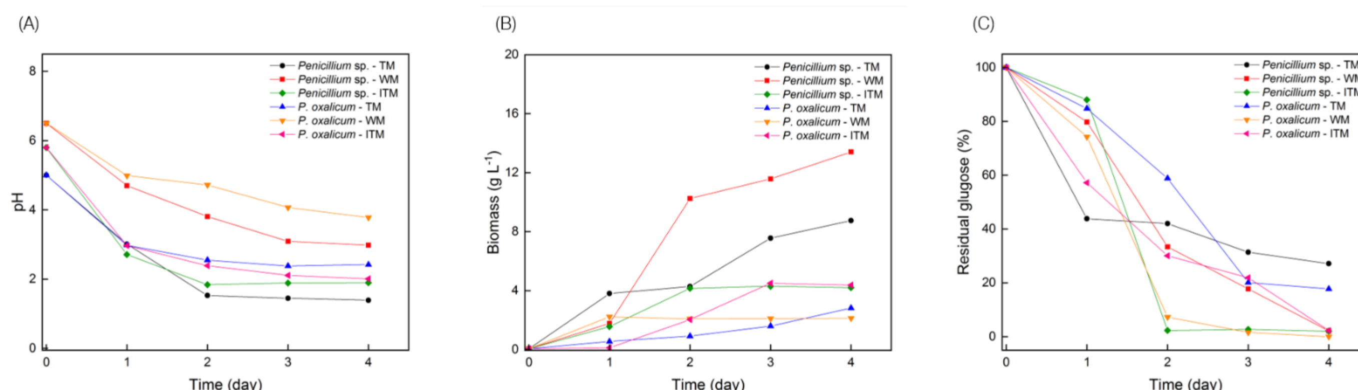

Figure 1 (A) pH, (B) biomass growth, and (C) residual glucose in cultures of *Penicillium* sp and *P. oxalicum* cultivated in TM, WM, and ITM media.

H_2O_2 production and laccase activity were shown in Figure 2. *P. oxalicum* in WM medium was the microorganism with the best performing for both oxyacid compounds with optimal production after three days of fermentation. In contrast, the highest laccase production was 194.4 ±19.3 U/L on the second day of incubation in culture with ITM medium (Figure 2B). When the laccase activity was evaluated separately, it was observed that the optimal fermentation time was the second day for most of the cultures evaluated.

These fermentations were conducted without the addiction of contaminants or inducers. Other authors, such as Henn et al (2020)[5] evaluated oxyacid compounds in the degradation of organic molecules by fungal fermentations. In this case, the authors concluded that laccase was induced by increasing pesticide atrazine levels, reaching an activity level 4 times higher in 50 mg L-1 of the herbicide, compared to the incubation control. This induction indicates that in joint fermentation experiments with harmful substances, greater productions of laccase and H_2O_2, oxyacid compounds, can be achieved. According to Wulandari et al. (2020)[4], an inducer would be causing stress oxidative of fungal as a consequence over-production of laccase. Thakhor et al. (2022)[1] and Izcapa-Treviño et al. (2009)[7] when dye and pesticide were added as contaminants (Table 1) also found promising results.

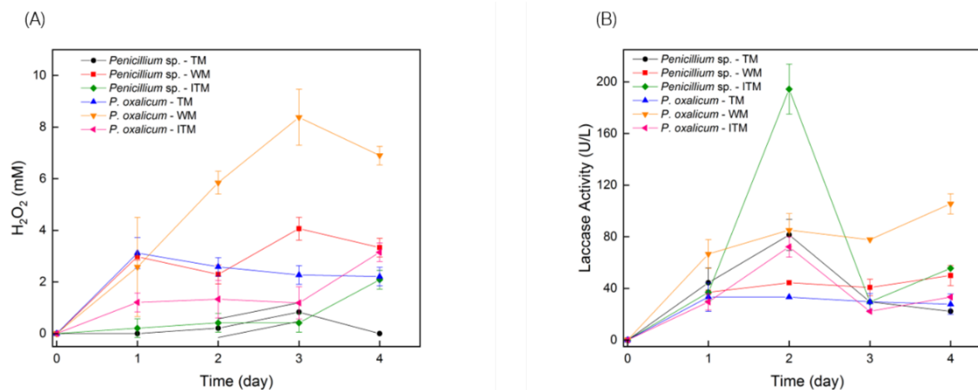

Figure 2 (A) H_2O_2 and (B) Laccase enzymatic activity in the extracts of *Penicillium* sp. and *P. oxalicum* cultivated in TM, WM, and ITM media.

Table 1 Comparison of the best results with the authors of the evaluated media: TM, WM and ITM.

	Thakhor et al. (2022)[1]	Wulandari et al. (2020)[4]	Izcapa-Treviño et al. (2009)[7]	This study (2024)	This study (2024)
Fungi	*P. oxalicum*	*Penicillium* sp.	*Penicillium* sp.	*P. oxalicum* (WM)	*Penicillium* sp. (ITM)
Laccase (U/L)	8332 ±0.23[b]	343[a]	ND	77.78 ±0.01[a]	194.4 ±19.3[a]
H_2O_2 in situ (mM)	NE	NE	3[a] / 5[c]	8.38 ±1.08[a]	ND
Time (day)	0,5	4	2	3	2

[a]Control fermentation; [b]Fermentation with dye addition; [c]Fermentation with pesticide addition; NE: non-evaluable; ND: non-detectable.

4 CONCLUSION

The monoculture extracts of *P. oxalicum* and *Penicillium* sp. were capable of producing H_2O_2 and presenting enzymatic activity related to laccase. The fermentation time for higher laccase activity by *Penicillium* sp. in the ITM medium was shorter than that of *P. oxalicum* in the WM medium, although the latter contained higher levels of laccase and H_2O_2 at the same time. The experiments were satisfactory when the objective was to assess the production capacity of oxyacid compounds, but the study of the addition of inducers to achieve overproduction is recommended. Furthermore, the results indicated that *Penicillium oxalicum* and *Peniccilium* sp. are effective candidates for detoxification or degradation of organic compounds.

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