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Research Article

Production of Enzyme for *Pleurotus pulmonarius* by Solid-State Fermentation on Peach-Palm and Cocoa Waste

Mateus Pereira Flores Santos[1], Camila Oliveira Bezerra[1], Geni Alves Casteliano[1], Ana Paula Trovatti Uetanabaro[1], Erik Galvão Paranhos da Silva[2], Andréa Miura da Costa[1*]

[1]Department of Biological Sciences, State University of Santa Cruz, Ilhéus, Bahia, Brazil
[2]Department of Exact and Technological Sciences, State University of Santa Cruz, Ilhéus, Bahia, Brazil
E-mail: amcosta@uesc.br

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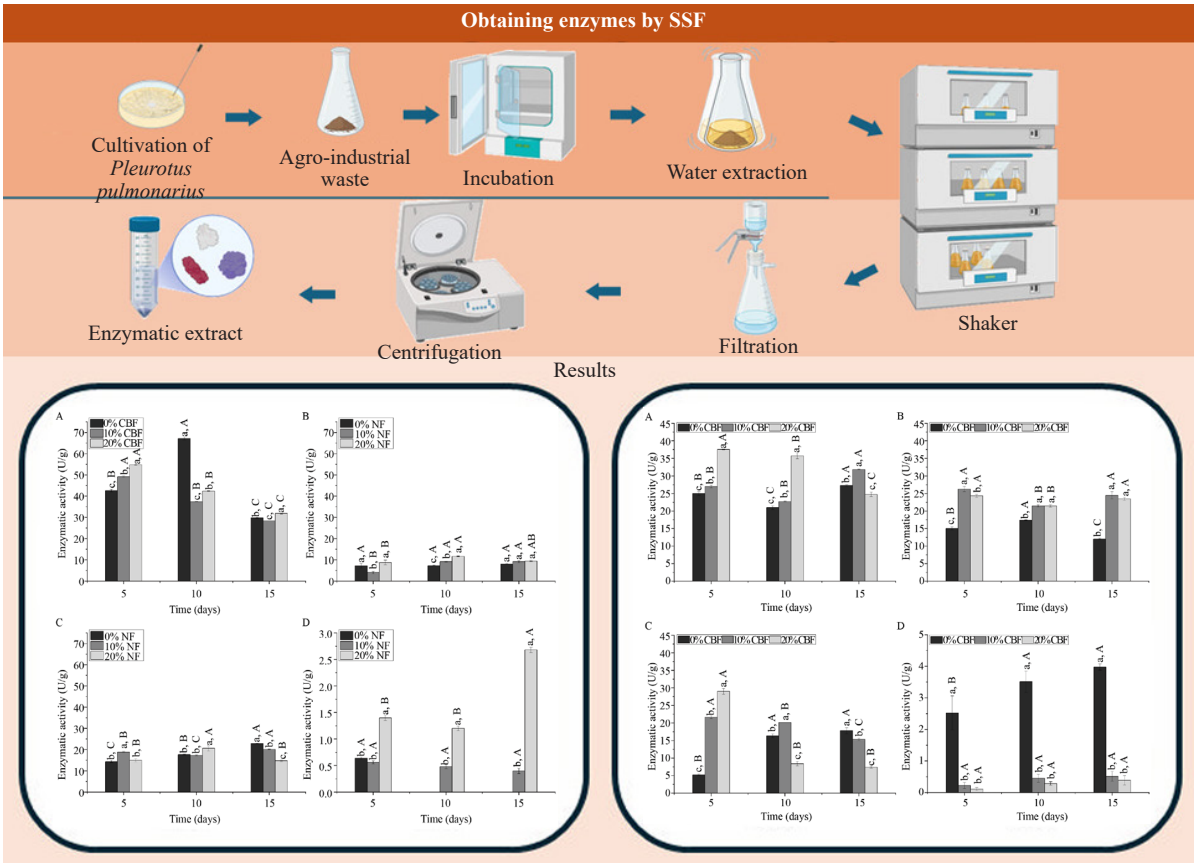

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Abstract: Agro-industrial waste, which is generated during the process of food production, has the potential to contaminate significant quantities of natural resources. Concurrently, the cultivation of edible mushrooms from waste materials that have been previously discarded is undergoing exponential growth. This is due to the nutritional value of the mushrooms and their biotechnological potential in the production of enzymes of industrial interest. The present study aims to evaluate the production of lignocellulolytic and hydrolytic enzymes by the edible mushroom *Pleurotus pulmonarius* on cocoa and peach-palm wastes, with or without the addition of cocoa bean film (CBF) as a growing medium. The solid-state fermentation methodology was employed, with the proportion of CBF varying between 0, 10 and 20% for a duration of 5, 10 and 15 days at 28 °C. The enzymatic activities of amylase, xylanase, pectinase and laccase were analysed. For amylases, the most optimal results were achieved through the utilisation of cocoa residue, yielding a maximum activity of 97.16 U/g (0% CBF and 10 days of fermentation). In the case of xylanase, pectinase and laccase, the optimal activities were observed with peach-palm waste, with levels of 29.01 U/g (20% CBF and 5 days of fermentation), 26.25 U/g (10% CBF and 5 days of fermentation) and 3.98 U/g (0% CBF and 15 days of fermentation), respectively. The results obtained demonstrate the efficient utilisation of this waste for the production of microbial enzymes with potential biotechnological applications. This is achieved through the employment of cost-effective techniques to yield high-value-added outputs.

***Keywords*:** edible mushrooms, microbial enzymes, fungi, agro-industrial waste

1. Introduction

The quest for methodologies to repurpose agro-industrial by-products has been the focal point of numerous research endeavours. This is primarily attributable to two interrelated factors: the expanding global population and the escalating demand for foodstuffs. These dynamics have precipitated a substantial augmentation in agricultural output, concomitantly generating copious amounts of agro-industrial waste.[1-3] One potential avenue for the utilisation of this waste material is as a substrate in bioprocesses with the objective of producing various commercially relevant enzymes.[4-8] In addition to being economically viable, this approach helps to alleviate the environmental problems that arise from the improper disposal and accumulation of such waste in the natural environment.[7] As Siqueira et al.[9] have observed, the agribusiness sector plays an important role in the Brazilian economy, generating approximately 291 million tons of waste per year. Inadequate management of this waste can pose significant social and environmental risks. It is also noteworthy that this amount of agricultural waste biomass has been growing in parallel with increasing agricultural and agro-industrial activities in many countries.

In Brazil, particularly in the southern region of Bahia, the cacao tree (*Theobroma cacao* L.) and the peach-palm tree (*Bactris gasipaes* Kunth) are prominent among the numerous crops cultivated in the region, yielding a substantial amount of waste.[8,10] According to Quelal-Vásconez et al.,[11] it is estimated that chocolat processing produces around 54% of waste (cocoa shells and cocoa skins), which have no specific purpose and are, in most cases, incinerated or discarded in nature.[12] The peach-palm tree is extensively utilised for palm heart cultivation in the region. This tropical tree is distinguished by its multi-stemmed nature and its ability to produce two food crops (namely palm heart and fruit), which are suitable for human and animal consumption.[13,14] Despite the financial viability of this crop for the agricultural sector, the process of production gives rise to a substantial amount of waste, constituting approximately 80-90% of the total gross weight of the crop. It is estimated that for every 400 g of palm heart extracted, 13 kg of waste is generated.[15] The slow rate of degradation of fibrous waste, coupled with the common practice of leaving it exposed at the harvesting site without proper or adequate disposal has led to the exploration of its use or reuse in the synthesis of new products with high added value as one of the primary alternatives for its utilization.[16] According to Ansari et al.,[17] this agro-industrial waste can contain a variety of substances, particularly lignocellulosic substances (cellulose, hemicellulose and lignin) as well as proteins, lipids and pectin. Furthermore, the peach-palm residue has been found to contain significant quantities of fermentable sugars (4.4 $g \cdot g^{-1}$) and fibre (2.9 $g \cdot g^{-1}$).[8] A similar nutritional profile is exhibited by cocoa waste, which is abundant in protein, fibre and carbohydrates.[18] The employment of suitable technology, has been shown to enable the transformation of these materials into commercial products or raw materials for secondary processes with high added value.[19,20]

As an alternative to the disposal of this waste, a number of studies have been carried out on the utilisation of the waste in the production of materials with high added value due to its composition which is rich in nutrients and minerals.[8] These materials have been found to be effective as substrates in solid-state fermentation (SSF).[18,21-23] This fermentation process involves the fermentation of a substrate, which is typically insoluble agro-industrial waste, with sufficient moisture, but without free water, to facilitate microbial development and the formation of the desired product.[20,22] Agro-industrial waste can be used as a substrate in SSF to produce various enzymes of commercial interest, including amylases, cellulases, xylanases, invertases, inulinases, hemicellulases, laccases, pectinases and proteases, amongst others.[16,21-26] The enzyme produced is contingent on the type of waste used as a substrate and the microorganism employed. A variety of microorganisms, including bacteria, yeasts and filamentous fungi, can be employed in this fermentation process; however, filamentous fungi are particularly noteworthy for their enhanced adaptability to this method, particularly species of the genus *Pleurotus* which are capable of effective degradation of lignocellulosic material.[10,27]

In the domain of mycology, the employment of edible mushrooms in SSF has emerged as a subject of notable interest. These organisms, when employed in the fermentation process, yield a range of enzymes of commercial significance, including hydrolases and oxidases.[21,28] A distinguishing feature of these enzymes is their edibility, a property that facilitates their development and subsequent marketing. This duality in generating economically viable products is a testament to the versatility and potential of this fungal application.[10,28,29] Furthermore, SSF provides a destination for this waste through its reuse, thereby contributing to environmental issues.[30] Yan et al.[31] state that various types of edible mushrooms, such as *Pleurotus florida*, *Pleurotus ostreatus*, *Ganoderma lucidum*, *Pleurotus cistidiosus*, *Agaricus brasiliensis*, among others, have been/are being studied in the production of different classes of enzymes that are already widely used in various industrial sectors, thus reducing the costs associated with their production. In addition, Ekundayo et al.[32] emphasise that mushrooms of the genus *Pleurotus*, with a particular focus on *P. pulmonarius*, possess the capacity to degrade lignin, cellulose and hemicellulose, along with other constituents of agro-industrial waste. This ability is attributed to their ability to produce a range of extracellular enzymes, including cellulase, xylanase, amylase, pectinase, MnP, and laccase, among others.[28,29] In addition, it is emphasised that the utilisation of these macrofungi in the production of extracellular enzymes by SSF is economically advantageous, with a low cost of enzyme production and a high yield.[18,21] In view of the aforementioned findings, the objective of this work was to evaluate the production of hydrolytic and oxidative enzymes using *P. pulmonarius* in cocoa and peach-palm wastes with or without the addition of cocoa bean film.

2. Methodology

2.1 *Obtaining and maintaining the microorganism*

P. pulmonarius was obtained from the culture collection of the Instituto de Botânica de São Paulo (CCB) and the Laboratory of Biochemistry of Microorganisms and Food of the Universidade Estadual de Maringá, Paraná, Brazil. The cultivation of the fungi was undertaken in potato dextrose agar (PDA) at 26 °C for 8 days. Thereafter, the fungi were maintained at 4 °C until utilization. The reagents employed included 3,5-dinitrosalicylic acid (DNS), citric pectin, beechwood xylan, 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), all of which were obtained from Sigma-Aldrich (Chemical Abstracts Service (CAS)), while the starch was from Synth. All of the other chemical reagents were of analytical grade.

2.2 *Solid-state fermentation (SSF) and the extraction of enzymes*

The fermentation process was conducted in 125 mL Erlenmeyer flasks, each containing a specific substrate: 4 g of peach-palm waste or 6 g of cocoa shells waste. Cocoa bean film was added to the substrate at concentrations of 0, 10 and 20% of the residue. The humidity of the cultures was adjusted to 80% with tap water and following sterilisation, the residues were inoculated with four 8 mm mycelial plugs of the fungus previously grown in PDA medium. The cultures were then subjected to incubation at 28 °C, without agitation, in the dark, and were interrupted at intervals of 5, 10 and 15 days.[18]

2.3 *Enzyme activity assay*

2.3.1 *Amylase*

Enzymatic activity was determined by measuring the amount of reducing sugars formed using 3,5-dinitrosalicylic acid (DNS).[33] Soluble starch at a concentration of 1% (w/v) in 0.1 M sodium phosphate buffer at pH 7.0 was utilised as a substrate for amylase activity. The enzymatic reaction was carried out at 50 °C for 10 min after which the absorbances were measured at 540 nm on a spectrophotometer (BEL Photonics 2000UV) to determine the reducing sugars.[26] The definition of one unit (UI) of enzymatic activity was established as the amount of enzyme necessary to produce 1 µmol of reducing sugar per minute under the conditions tested and expressed in U/g.

2.3.2 *Pectinase*

Enzymatic activity was measured by the presence of reducing sugars, using the DNS method.[33] Citric pectin 1% (w/v) diluted in sodium acetate buffer pH 4.5 to 50 mM was used as the substrate for pectinase activity. The enzymatic reaction was carried out at 40 °C for 30 minutes after which the absorbance was read at 540 nm in a spectrophotometer (BEL Photonics 2000UV) to determine the amount of reducing sugars. One unit (IU) of enzymatic activity was established as the quantity of enzyme necessary to yield 1 µmol of reducing sugar per minute under the conditions evaluated and this value is expressed in units per gram of dry substrate (U/g).[34]

2.3.3 *Xylanase*

Xylanase activity was assayed according to the method outlined by Carvalho et al.,[8] with the amount of reducing sugars released following the incubation of the enzyme solutions with 1% (w/v) beechwood xylan in 50 mM acetate buffer (pH 5.0) at 50 °C for 10 minutes. The amount of reducing sugars was determined by the dinitrosalicylic acid (DNS) method as described by Miller.[3] The activity of one unit of xylanase was defined as the amount of enzyme capable of releasing 1 µmol of d-xylose per minute under the conditions of the assay.

2.3.4 *Laccase*

Enzyme activity was measured in accordance with the methodology outlined by Martin et al.,[35] employing a 10 mM 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.05 M sodium acetate buffer pH 5.0. In summary, 1,700 µL of buffer was added to glass tubes followed by the addition of 100 µL of enzyme extract. The reaction was initiated following the addition of 200 µL of ABTS and was continued for a period of 5 minutes at a temperature of 40 °C. Absorbance was measured at 420 nm using a spectrophotometer (BEL Photonics 2000UV). The controls for the colourimetric reaction comprised the substrate blank and the enzyme blank. The enzyme unit was defined as the amount of enzyme required to oxidise 1 µmol of ABTS and expressed as a unit per gram of substrate (U/g).

2.4 *Statistical analysis*

All experiments and analyses were performed in triplicate. Subsequently, the data were subjected to analysis of variance and the Tukey test ($p < 0.05$) was used to evaluate the differences between the mean hydrolytic capacities averages at the times studied. The Statistical Analysis System (SAS, University Edition) package was used for the analyses.

3. Results and discussion

Following the extraction process of the fermentation extracts, enzyme activity assays were conducted in order to verify the types of extracellular enzymes produced by *P. pulmonarius* in the various substrates that were tested. The results obtained for the enzyme activities demonstrated that the fungus was capable of producing the hydrolytic enzymes (amylase, pectinase, and xylanase) and oxidative enzymes (laccases) tested, using solely the agro-industrial residues of cocoa and peach-palm.

As demonstrated in Figure 1, we observe the temporal production of the enzymes was observed for a period of up to 15 days (5, 10, and 15 days), with varying concentrations of cocoa shells at 0, 10, and 20% in the peach-palm residue. The optimal conditions for enzyme production were identified for amylase with 10 days of fermentation without supplementation yielding an activity of 67.16 U/g, for xylanase, with 15 days without supplementation achieving an activity of 22.87 U/g, for pectinase, with 10 days of fermentation supplemented with 20% cocoa shells resulting in an activity of 11.63 U/g and for laccase, with 15 days of fermentation supplemented with 20% cocoa shells yielding an activity of 2.68 U/g.

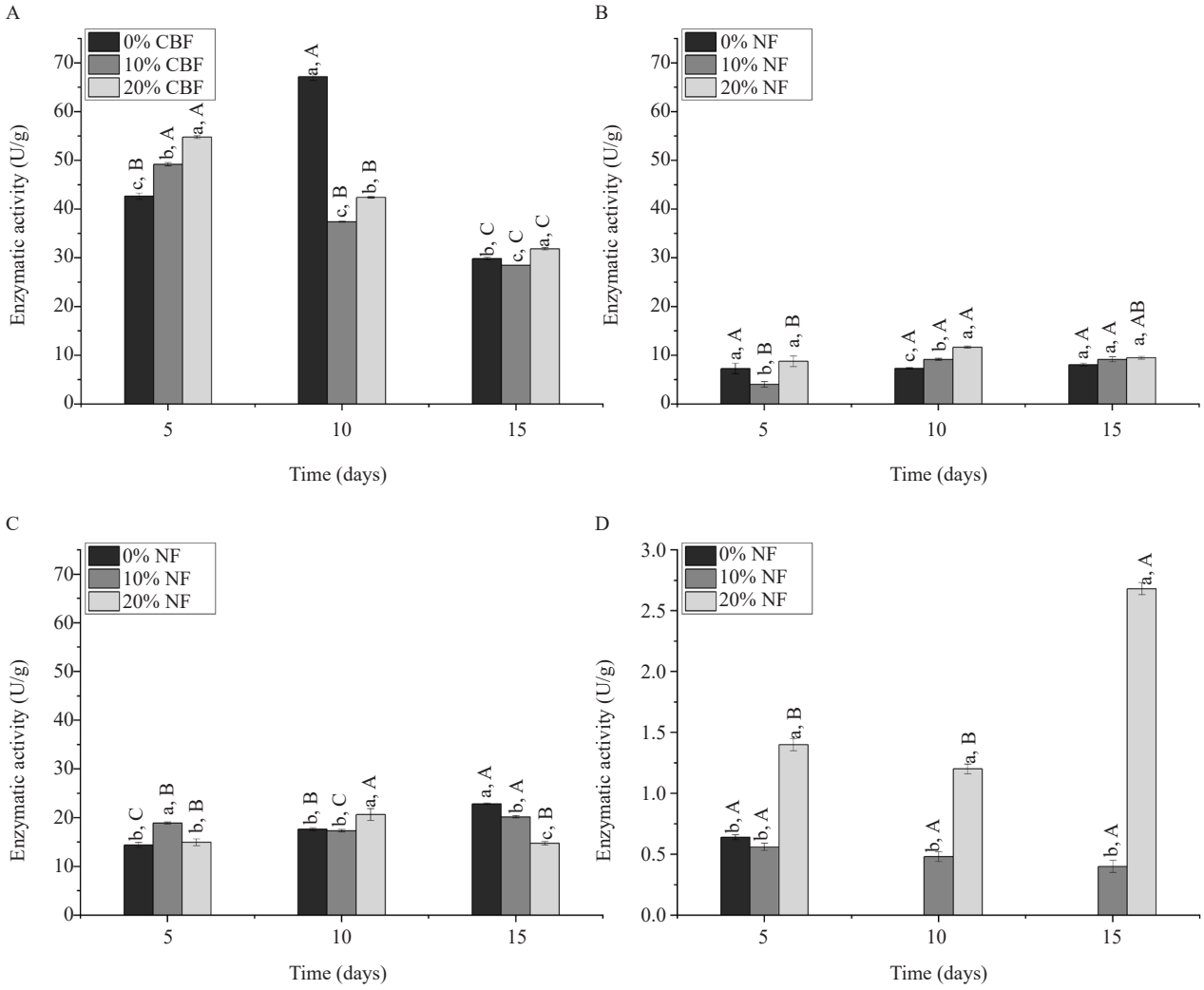

Figure 1. Enzymatic production of amylase (A), pectinase (B), xylanase (C), and laccase (D) after solid-state fermentation using cocoa waste as substrate for 5, 10 and 15 days, varying the concentration of cocoa bean film (CBF) at 0, 10, and 20%
*Means followed by the same lowercase letter are not different with respect to fermentation time and means followed by the same uppercase letter are not different with respect to cocoa shell content, Tukey test at 5%

Following graphical analysis, it can be concluded that in the SSF of cocoa wastes with *P. pulmonarius*, there was a higher amylolytic enzyme activity in comparison to the other hydrolases (pectinase and xylanase). Furthermore, a higher activity of laccase was also observed compared to the cocoa residue. In accordance with the findings of Sahu et al.,[36] the utilisation of diverse residues for amylase production is a viable proposition. Enzyme production by solid-state fermentation (SSF) is regarded as being highly economical in comparison to submerged fermentation (SF), as SSF utilises agricultural residues as a substrate for microorganism growth, thereby simulating their natural environment. In

a separate study, Yossa et al.[37] characterized cocoa shells and found a high carbohydrate content in its composition (75.25 g 100 g^{-1}), as well as protein (8.91 g 100 g^{-1}), dietary fibers (9.02 g 100 g^{-1}), and various minerals. Consequently, the elevated amylase production may be associated with the components present in the cocoa shells. This is in contrast to the low activity levels of pectinase and xylanase, which are possibly attributable to the limited pectin and xylan content.

Costa et al.[38] posit that the higher lignin content of cocoa shells hinders the access of microorganisms to hemicellulose and cellulose, leading to low levels of xylanase activity, with values below 100 U/g. The low amylase activity is attributed to the brief fermentation period. A comparison of the effect of cocoa shells on enzyme production revealed that, while the addition of cocoa shells did not lead to an increase in the production of these enzymes, with the exception of laccase, the presence of cocoa shells did have a significant impact on the activity of these enzymes. The increase in the content of cocoa bean shells resulted in an increase in their enzymatic activity. This increase in activity may be attributed to the elevated carbon and nitrogen content of the substrate. Guo et al.[39] recommend that residues with elevated carbon and nitrogen content are utilised for laccase production.

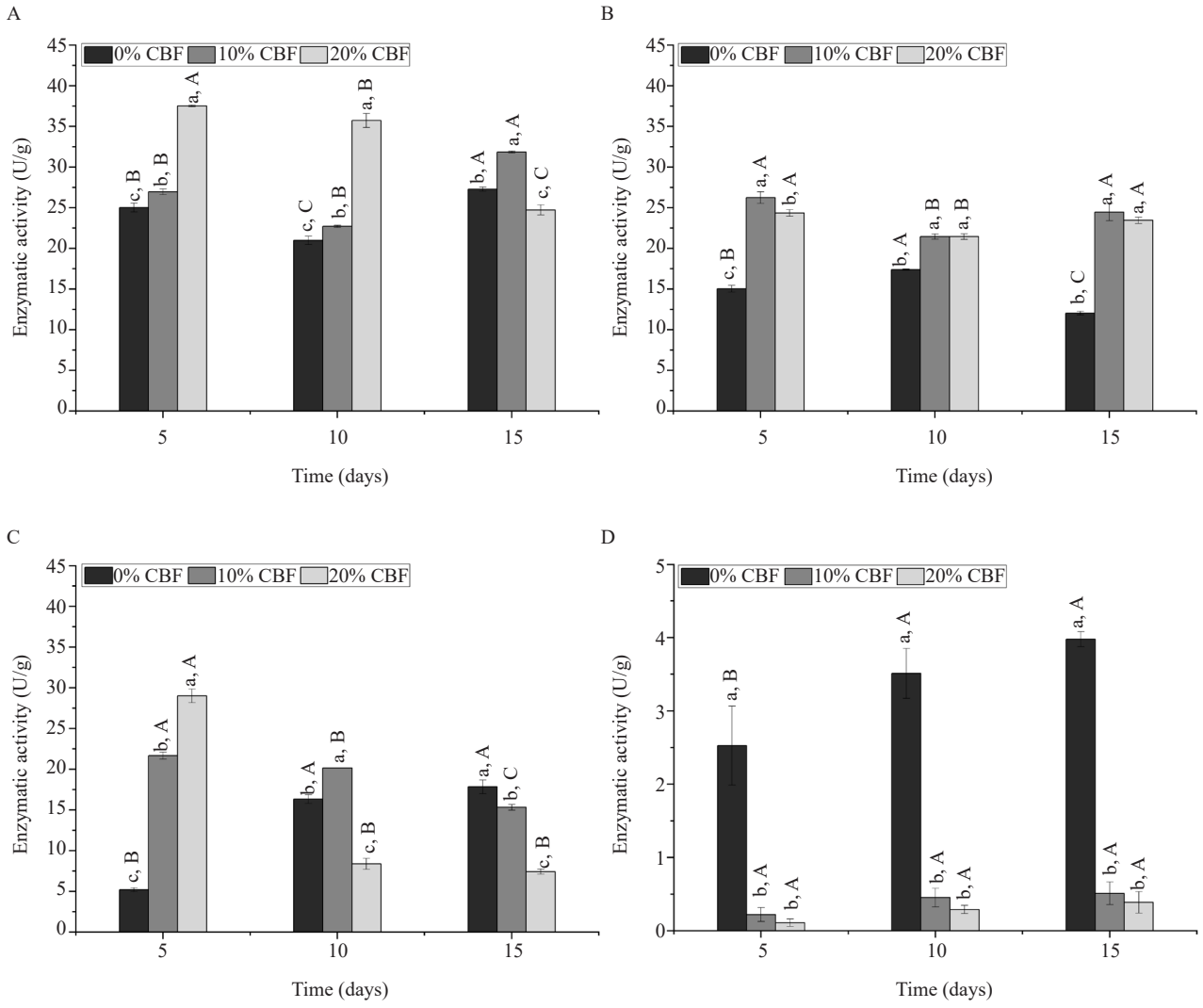

Figure 2. Enzymatic production of amylase (A), pectinase (B), xylanase (C), and laccase (D) after solid-state fermentation using peach-palm waste as substrate for 5, 10 and 15 days, varying the concentration of cocoa bean film (CBF) at 0, 10 and 20%
*Means followed by the same lowercase letter are not different with respect to fermentation time and means followed by the same uppercase letter are not different with respect to cocoa shell content, Tukey test at 5%

In relation to the fermentation time, it was observed that for each enzyme, a specific fermentation time yielded optimal activity. Consistent findings for amylases were reported by Costa et al.,[38] who identified a 4-day duration as the optimal time for production utilising *Aspergillus awamori*. The rationale behind this is that fungi are known to exhibit a higher efficiency in the hydrolysis of carbohydrates when operating at shorter fermentation time.[40] In the case of pectinase, a fermentation time of 10 days is more appropriate due to the lower abundance of the molecule and its greater complexity. In addition, Ruban et al.[41] found that starch is more readily available for microbial degradation and enzyme production than lignocellulosic materials. In contrast, a study by Wong et al.[42] on *Aspergillus fumigatus* R6 found that a period of more than 5 days was the most promising. In contrast, xylanase and laccase, due to their role in the hydrolysis of more resistant lignocellulosic residues, require longer fermentation times for higher enzyme production. Guo et al.[39] in their study of laccase production by *Polyporus sp*. using agro-industrial residues, obtained the highest activity after 14 days of fermentation. In a separate study, Dhaver et al.[43] reported the highest xylanase production after 5 days of fermentation using *Trichoderma harzianum*. However, these differences may be attributable to the residues utilised as substrates and the fungi employed as enzyme producers.

The results of the enzyme production obtained in SSF using peach-palm residues can be observed in Figure 2. In general, the optimal conditions for enzyme production were as follows: amylase, obtained after 5 days of fermentation with 20% cocoa shells supplementation with activity of 37.53 U/g, and xylanase, obtained after 5 days with 20% cocoa shells supplementation with activity of 29.01 U/g, pectinase obtained after 5 days with 10% cocoa shells supplementation with activity of 26.25 U/g, and laccase obtained after 15 days of fermentation without cocoa supplementation with activity of 3.98 U/g.

The graphical analysis indicates that the utilisation of peach-palm residue as a substrate for SSF with *P. pulmonarius* is an effective method for producing enzymes of industrial significance. A notable observation is the predominance of amylolytic enzymes, which exhibited higher activity levels compared to the other hydrolases such as pectinase and xylanase. In addition, a notable laccase production was observed in comparison to cocoa residues. This increase may be attributed to the higher lignocellulosic content of the peach-palm waste, which increases its carbon source, providing this improvement compared to cocoa shells.[39] It is also noteworthy that, akin to cocoa residue, the most prevalent enzyme in peach-palm residue is amylase, a finding that may be associated with the high carbohydrate content inherent in its composition.[44]

As posited by Gillet et al.,[26] peach-palm residue has the potential to produce amylases, with an activity close to 30 U/g. Furthermore, the authors report that supplementation with various nitrogen sources such as cocoa husk, enhances the production of amylases, resulting in an activity of 48.9 U/g. In a separate study, Carvalho et al.[8] investigated the production of xylanase using peach-palm residue in SSF, yielding an activity of 551.5 U/g. This finding is consistent with the results obtained in this study, suggesting that peach-palm waste and cocoa waste can be used as a substrate for the production of various enzymes, and that *P. pulmonarius* has a substantial enzyme production capacity.

In the context of the supplementation of peach-palm residue with cocoa shells, an enhancement in enzyme production was observed, particularly during shorter fermentation times, resulting in a reduction in process costs. This enhancement in the supplementation process may be attributed to the chemical composition of the cocoa shells, which has been shown to increase the carbon and nitrogen content of the substrate, thereby enhancing the fermentation process.[45] The results of this study demonstrate that the incorporation of cocoa shells leads to a substantial production of amylase and pectinase during the fermentation process, which lasts for 15 days. Conversely, the addition of cocoa shells was only effective in enhancing xylanase production after five days of fermentation. This finding suggests that the addition of the residue enhances enzyme production and that a shorter fermentation time is more conducive to xylanase production. However, for laccases, the supplementation of cocoa shells led to a reduction in enzyme production, irrespective of the fermentation time employed.

A five-day fermentation period was identified as the most efficient duration for enzyme production. According to Manan and Webb,[46] the most efficient form of solid-state fermentation should be carried out at shorter times and under milder conditions, due to the greater reduction in costs associated with production. Conversely, a 20-day duration was identified as optimal for laccase production, a finding that may be attributed to the utilisation of lignin, a highly resistant molecule.[39,40]

In general, it can be concluded that the utilisation of *P. pulmonarius* in various residues yielded disparate levels of enzymatic activity. It is evident that the utilisation of cocoa shells resulted in elevated levels of amylase activity.

Conversely, for the other enzymes evaluated (pectinase, xylanase, and laccase), the peach-palm waste exhibited higher activity levels, suggesting that it possesses greater potential for the production of a broader range of enzymes. Ekundayo et al.[32] observed that the maximum activity of several extracellular enzymes was attained within 10 days of fermentation for SSF using agro-industrial waste, whereas for oxidative enzymes, the greatest activity was achieved in fermentations lasting 15 days, attributable to the more intricate nature of the lignocellulosic structure. This discussion corroborates the results obtained where the maximum activity of the enzymes obtained from simpler molecules was obtained between 5 and 10 days of fermentation, while laccase was obtained within 15 days of fermentation, since it is released when the fungus degrades the lignin.

De Carvalho et al.[18] posited that both peach-palm and cacao waste have the potential to produce oxidases due to their lignocellulosic composition. Manan and Webb[47] further posit that the discrepancies in enzyme production capacity are not solely attributable to the fungi employed but also to the composition of the materials utilised as substrates for fermentation. It is noteworthy that certain residues may possess a composition that is more conducive to the fermentation of a specific microorganism than another. Consequently, the optimization of substrates, in conjunction with investigations into the utilisation of novel microorganisms, is imperative to establish more conducive conditions for specific enzymes. The substantial variability in enzyme production and the divergent levels of activity observed for each enzyme are associated with the fermentation stage and are influenced by the substrate composition, moisture content, and the utilized fungus.[47]

4. Conclusion

This study demonstrates that the edible mushroom *P. pulmonarius* has the capacity to produce a series of enzymes with significant added value and biotechnological/industrial interest. Utilising diverse agro-industrial materials as substrates in solid-state fermentation for short incubation periods is a key factor in this process. Furthermore, the study aims to encourage the use of diverse agro-industrial waste materials as substrates in SSF, thereby converting these materials that would otherwise be discarded into high-value products.

Conflict of interest

The authors declare no competing financial interest.

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