Effect of *Trametes maxima* CU1 supernatants on the bread physical properties

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Abstract: Enzymes are of great interest to the food industry because of their economic benefits in process optimization and the advantages in functional and rheological properties that positively influence foods. Therefore, in the present work, we evaluated the effect of *Trametes maxima* CU1 supernatants on the physical parameters of bread. For this, the supernatants of the 18-day cultures were recovered in a mineral medium supplemented with $CuSO_4$, orange peel, or peanut in combination with wheat straw, in addition to the presence of a co-culture with *Pycnoporus sanguineus* CS2. The titers of laccase, amylases, cellulases, and xylanases were quantified prior to their addition to the bread dough. Once the loaves were obtained, height, hardness, weight loss (%), pores per mm2, and color analysis were determined. The results of the enzyme titers showed a statistically significant difference ($p \leq 0.05$) between the treatments, with the medium with 350 µM $CuSO_4$ highlighting the production of laccase, while the medium supplemented with 5% orange peel presented the four types of activities. In general, the breads showed differences ($p \leq 0.05$) in the parameters evaluated. It should be noted that the bread treated with the supernatant with orange peel had the highest height and color, like the control, in addition to presenting the lowest hardness among the enzymatic treatments. Therefore, these results demonstrate the effect of the culture medium on the enzymatic profiles of the same fungus and its potential application in the baking industry.

Keywords: amylase; cellulases; laccase; *Pycnoporus sanguineus* CS2; xylanases.

Efecto de los sobrenadantes de *Trametes maxima* CU1 en las propiedades físicas del pan

Resumen: Las enzimas son de gran interés para la industria alimentaria debido a sus beneficios económicos en la optimización de procesos y a las ventajas en las propiedades funcionales y reológicas que influyen positivamente en los alimentos. Por ello, en el presente trabajo se evaluó el efecto de los sobrenadantes de *Trametes maxima* CU1 sobre los parámetros físicos del pan. Para ello, se recuperaron los sobrenadantes de los cultivos de 18 días en un medio mineral suplementado con $CuSO_4$, cáscara de naranja o cacahuete en combinación con paja de trigo, además de la presencia de un co-cultivo con *Pycnoporus sanguineus* CS2. Los títulos de lacasa, amilasas, celulasas y xilanasas se cuantificaron antes de su adición a la masa de pan. Una vez obtenidos los panes, se determinaron la altura, la dureza, la pérdida de peso (%), los poros por mm2 y el análisis del color. Los resultados de los títulos enzimáticos mostraron una diferencia estadísticamente significativa ($p \leq 0,05$) entre los tratamientos, destacando en el medio con 350 µM $CuSO_4$ la producción de lacasa, mientras que el medio suplementado con un 5% de cáscara de naranja presentó los cuatro tipos de actividades. En general, los panes presentaron diferencias ($p \leq 0.05$) en los parámetros evaluados. Cabe destacar que el pan tratado con el sobrenadante con cáscara de naranja tuvo la mayor altura y color, al igual que el control, además de presentar la menor dureza entre los tratamientos enzimáticos. Por lo tanto, estos resultados demuestran el efecto del medio de cultivo sobre los perfiles enzimáticos del mismo hongo y su potencial aplicación en la industria panadera.

Palabras clave: amilasa; celulasas; lacasa; *Pycnoporus sanguineus* CS2; xilanasas.

1. Introduction

The baking industry has an excellent social and strategic importance, as it provides essential food products to the population and contributes to food security (Rosell & Dura, 2015). It plays an important role in the stability of society and aims to meet the needs of all segments of the population (Ponte *et al*., 2000). However, negative processes affect the industry in terms of product quality, technical and economic performance, and the availability of raw materials (Rosell & Dura, 2015). In this sense, chemical additives such as emulsifiers, calcium phosphate, and L-ascorbic acid, among others, are known to positively affect the rheological properties of the dough, including water absorption capacity, stability, and energy (Abdullahi *et al*., 2022). However, some additives can cause flavor changes, and some may raise biosafety concerns (Olivieri *et al*., 2020).

In recent years, enzymes have emerged as powerful tools for bakers seeking to improve product quality, optimize processes, and address sustainability concerns. Among the most commonly, laccase, xylanase, and cellulose are particularly promising to revolutionize the baking landscape, highlighting their impact on texture, flavor, shelf life, and nutritional value (Lončar *et al*., 2016). Laccase (a multicopper oxidase) possesses oxidizing capabilities with benefits for baked goods. Thanks to its ability to attack phenolic compounds, it cross-links arabinoxylans (AX) in wheat flour, strengthening the structure of the dough and giving rise to a softer crumb and reduced elasticity, providing better texture (Selinheimo *et al*., 2007). In whole wheat bread, a reduction in the aging rate of bread, better gluten cross-linking, and flavor have been observed (Wang et al., 2023). For their part, xylanases are enzymes that catalyze the random hydrolysis of the β-(1 → 4) glycosidic bonds of heteroxylan and convert them into xylooligosaccharides, weakening the structure of xylan (Sharma *et al*., 2019). They are characterized by reducing the dryness and rigidity of the dough, as well as greater elasticity, extensibility, and coherence, as well as an increase in volume and a decrease in the density of the bread, resulting in greater moisture retention and better sensory attributes of the bread (Ahmad *et al*., 2014). Therefore, prospecting studies are essential to find enzymes with robust operational and functional properties that improve bakery products' quality, texture, and shelf life. Thus, the objective of this research was to evaluate the effect of the supernatants of *Trametes maxima* CU1 cultured in a mineral medium supplemented with inducers of inorganic and organic origin, in addition to the effect of a co-culture with *Pycnoporus sanquineus* CS2, on the physical parameters of the bread.

2. Materials and Methods

Strains

The ones used were Trametes maxima CU1 and Pycnoporus sanguineus CS2, which are preserved in the Laboratorio de Ciencias Naturales of the Facultad de Agronomía and preserved by periodic replanting every three months in medium potato dextrose agar (PDA).

Culture medium

The production of enzymes was carried out in modified Kirk medium (KM) whose composition was glucose (10 g/l), yeast extract (0.5 g/l), peptone (10 g/l), NH4SO3 (0.72 g/l), KH2PO4 (1 g/l), MgSO4·7H2O (1 g/l), KCl (0.5 g/l) and 10 ml of 100X trace element solution: FeSO4·7H2O (0.01 g/l), ZnSO4·7H2O (0.028 g/l) and CaCl2·2H2O (0.033 g/l). CuSO4 (350 M) was used as an inorganic inducer, and wheat straw and orange peel were used as natural inducers. Table 1 shows the treatment and production conditions used.

Table 1. Inducers used in the production of the enzymes.

Tt	Inductors	Strain
T1	Orange peel 5%	*Trametes maxima* CU1
T2	Peanut shell 3% + wheat straw 0.5%	*Trametes maxima* CU1
T3	CuSO4 350 mM	*Trametes maxima* CU1
T4	Orange peel 5% + wheat straw 0.5 %	*Trametes maxima* CU1 + *Pycnoporus sanguineus* CS2

Tt= Treatment, * Growth in co-culture. The base medium used was modified Kirk (Pozdnyakova et al., 2004)

All media were inoculated with three 0.5 cm diameter cylinders from the periphery of a culture with 5 days of growth on PDA. Subsequently, they were incubated at 28 oC, under shaking conditions at 150 rpm in an orbital shaker for 18 days. The supernatants were recovered by filtration, using Whatman No. 1 paper and frozen at -20 oC until use.

Laccase assay

Laccase activity was determined by measuring the oxidation of A BTS [2,2'-Azinobis(3-Ethylbenzothiazoline-6-Sulphonic Acid)] at 405 nm [ε_{405} (molar extinction coefficient) = 36,000 M^{-1} cm^{-1}]. The reaction mixture was prepared in sodium acetate buffer (100 mM), adjusted to pH 3.5 with 2 mM ABTS (Heinzkill et al., 1988). Enzymatic activities were expressed in units (U) defined as the amount of enzyme required to produce 1 µmol of product per minute. Enzymatic reactions were carried out in triplicate at 25 °C on a Shimadzu UV-Vis 1800 spectrophotometer (Japan).

Carbohydrate active enzymes (CAZyme)

Cellulases, xylanases and amylases were determined by the reducing sugar quantification method established by Miller et al. (1959). The reaction mixtures consisted of 0.5 ml of sodium citrate buffer solution (50 mM), adjusted to pH 5.0, 0.3 ml of 1% (w/v) substrate depending on the activity to be determined (CM-cellulose, D-xylan or starch) and 0.2 ml of sample. The reactions were incubated for 15 min at 60 °C. For the quantification of reducing sugars, 0.1 ml of the reaction mixture was added to 0.1 ml of dinitrosalicylic acid (DNS). The mixtures were boiled for 5 min at 100 °C and subsequently immersed in an ice bath at -4 °C. Afterwards, to photometrically determine the absorbance of the samples, 1 ml of double-distilled water was added, and it was read at a wavelength of 540 nm in a Shimadzu UV-Vis 1800 spectrophotometer. For the quantification of cellulases and amylases, a glucose curve in a range of 0 to 1 mg, for xylanases a xylan curve in the same range was used. One enzyme unit (U) was defined as the amount of enzyme required to release 1 µmol of glucose or xylan per minute. Enzymatic reactions were carried out in triplicate at 25 °C.

Preparation and characterization of bread

In the preparation of the bread, wheat flour, distilled water, salt and sugar, extra virgin olive oil, yeast and enzyme extracts selected from the quantification of the enzymatic activity present (lacase, amylase, cellulase and xylanase) at 5% were used. (Table 2).

Table 2. Effect of *Trametes maxima* CU1 supernatants on breads

Ingredients	Control	Treatments		
		T1	T2	T3
Wheat flour (g)	310.00	310.00	310.00	310.00
Warm water (ml)	175.00	145.00	145.00	145.00
Salt (g)	8.00	8.00	8.00	8.00
Sugar (g)	12.00	12.00	12.00	12.00
Olive oil (ml)	20.00	20.00	20.00	20.00
Yeast (g)	5.50	5.50	5.50	5.50
Supernatants (ml)	0.00	30.00	30.00	30.00

Tt=Treatment;, CON: water, T1= supernatants of the medium supplemented with 5% orange peel, T2= supernatant of the medium with 3% peanut shell + 0.5% wheat straw, T3= supernatant of the medium with orange peel 5 % + wheat straw 0.5% in co-culture with *Pycnoporus sanguineus* CS2. a-c Means (µ) ± standard deviation (σ) in columns with different superscripts indicate statistical difference (P≤0.05).

To make the bread, 5.5 g of yeast was hydrated with 12 g of sugar (Table 2). 30 ml of the enzyme extract, 20 ml of olive oil, and 310 g of wheat flour were added. The ingredients were homogenized in a Hamilton pedestal mixer (USA)

at low speed for 5 minutes, after which water was added and mixed for 7 minutes at maximum speed. The dough was left to rest for 30 minutes at room temperature (25 °C), and 6 g dough balls were made, on which elasticity tests were carried out. Before cooking, they were distributed randomly in silicone molds and placed in a convection oven heated to 185 °C for 30 minutes. After cooking, the hardness, weight loss, number of pores per mm2, height of the bread, and colorimetric characteristics were evaluated.

The height of the bread was measured with a digital Vernier Caliper with a precision of 0.01 mm (Mexico) after cooling the bread, taking the bread in half to have a standardized measurement in all the breads and thus be able to compare them. The texture analysis of the bread was carried out using a previously calibrated TA-XT2i texturometer (RHEO Stable Micro Systems). A 100 mm compression disc was used, and the parameters used were 5 mm/s. Likewise, a compression in millimeters (mm) corresponding to 50% of the average height of the bread was used for each treatment, and a force of 0.0493 N was applied. Weight loss was expressed as a percentage term and was determined by weighing three loaves of each treatment on an analytical balance of 0.1 mg sensitivity. Weight loss was reported as the difference found between the initial weight of the dough before baking and the weight after baking using the following formula:

$$Weightloss\ (\%) = (1 - (\frac{Final\ weight\ (g)}{Starting\ weight\ (g)})) * 100$$

To estimate the pores per mm2, the bread was divided in half, and the number of pores that existed in 1 mm2 was counted with the help of a digital Vernier Caliper with a precision of 0.01 mm (Mexico). Color analysis was previously calibrated with a CR 400 Minolta Camera Co. Ltd (Osaka, Japan) colorimeter. The data obtained after the measurement were L*, a, and b as luminosity, tendency to red color, and tendency to yellow color, respectively.

Statistic analysis

The data collected were expressed as means of five samples ± standard deviation, so the samples were analyzed in the Minitab software under the statistical significance of the analysis of variance (ANOVA), as well as with the comparison of means through the Tukey test, to be able to test the hypothesis at a 5% (p ≤ 0.05), all under the Experimental Design of Randomized Complete Blocks (DBCA).

3. Results and Discussion

Enzymatic content of the supernatants

The different conditions studied were established to evaluate the effect of natural inducers (provided by agroindustrial waste), as well as the presence of another fungus (co-culture), in comparison with the enzyme levels in the medium supplemented with copper sulfate (inorganic inducer). Figure 1 shows the laccase titers after 18 days of culture, where the medium supplemented with the combination of peanut shell and wheat straw (T2) had the highest levels with 3757 U/l, while the rest of the treatments had a production of less than 1000 U/l. The chemical nature of the inducers can explain these differences since it has been reported that the addition of wheat straw and copper sulfate as inducers can significantly increase the volumetric activity of laccase in *Pycnoporus sanguineus* (Eugenio *et al.*, 2010). Copper sulfate can induce laccase production in basidiomycete fungi, although its effectiveness may vary depending on the specific fungal species and growth conditions (Rodriguez *et al.*, 2019). *Trametes versicolor*, *Trametes suaveolens*, *Daedaleopsis confragosa*, *Fomes fosterius* and *Trametes gibbosa* showed maximum production between day 15 and 18 in the presence of inducers based on covers (Vrsanska *et al.*, 2016), with titers similar to those observed in the supernatants used in this research. On the other hand, when using agricultural waste as inducers of laccase activity, it has been reported that its effect depends on the type of waste used as a substrate, the fermentation parameters, and the addition of carbon and nitrogen sources (Pinheiro *et al.*, 2020). In this regard, wastes such as orange peel, pumpkin peel, and rice straw have been described as effective substrates for laccase production (Wang et al., 2019; Zhao et al., 2017). It is important to mention that the mechanism of action of natural inducers obtained from plant materials in the production of laccase involves the activation of metabolic pathways and regulation of gene expression in fungi that produce this enzyme. According to a study conducted by Elisashvili *et al.* (2009), natural inducers derived from plant materials can stimulate laccase synthesis by interacting with fungal signaling systems, leading to an increase in the expression of genes related

to the production of this enzyme. Janusz *et al*. (2017) suggests that natural inducers of plant origin can trigger physiological responses in fungi, promoting the synthesis of ligninolytic enzymes such as laccase. For its part, the co-culture of basidiomycetes is considered a promising strategy to increase the production of individual enzymes. However, there needs to be more information on the production of lignin-modifying enzymes in the co-culture of basidiomycetes, mainly in media based on plant raw materials (Ijoma *et al*., 2021). However, the co-culture evaluated in the present investigation presented the lowest laccase titers. This may be due to the interspecies interactions that they establish in the culture medium, causing a negative effect as has been reported for other species (Kachlishvili *et al*., 2021).
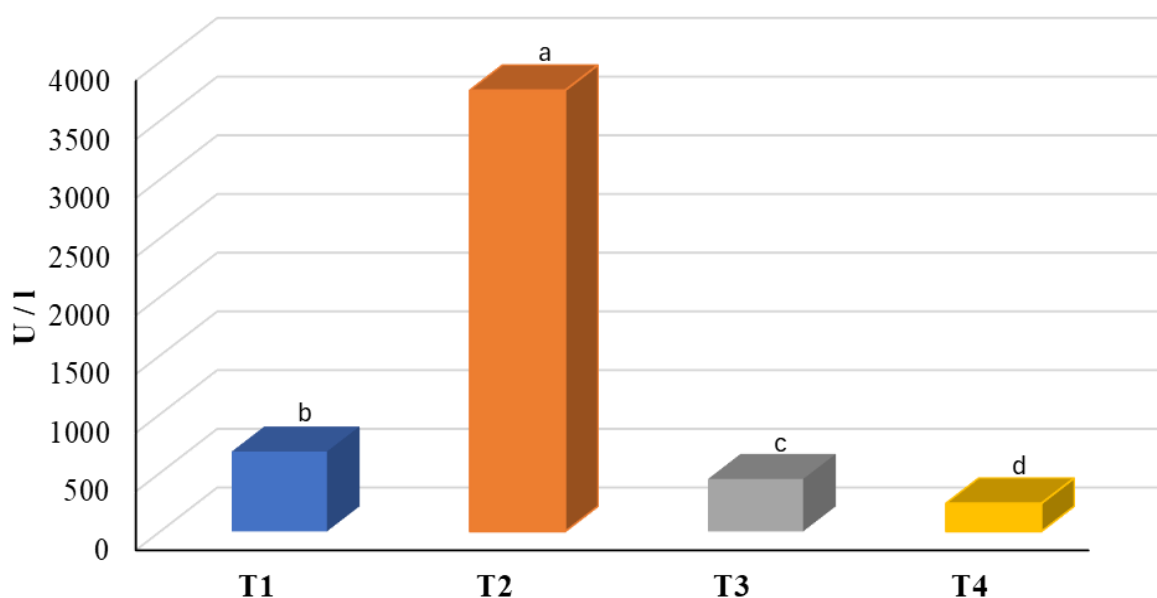
Figure 1. Laccase titers in 18-day supernatants. Where T1 corresponds to the medium supplemented with 5% orange peel, T2 to the medium with 3% peanut shell and 0.5% wheat straw, T3 with 350 mM $CuSO_4$ as an inorganic inducer, and T4 medium with 5% orange peel and 0.5% wheat straw, in co-culture *with Pycnoporus sanguineus* CS2.

The production of amylases, xylanases, and cellulase is shown in Figure 2. The co-culture *Trametes maxima* CU1 and *Pycnoporus sanguineus* CS2 (growing in the medium supplemented with orange peel and wheat straw; T4) had the highest activity of amylases and xylanase (46.7 and 41.9 mmol/min/l, respectively), although no cellulase activity was detected. At the same time, T1 showed the highest cellulase activity and the second highest amylase and xylanase activity (42.7 and 35.6 9 mmol/min/l). These values are similar to those reported for Lentinus edodes and Pleurotus spp., grown in different plant materials (Elisashvili et al., 2008). It is worth mentioning that, in the work above, it was observed that different strains of the same genus have different titers and combinations of enzymes produced, depending on the composition of the medium and fermentation conditions (Elisashvili et al., 2008). The same behavior was observed in *Trametes maxima* CU1 in the different fermentation media and conditions, which in this case included the presence of a co-culture with *Pycnoporus sanguineus* CS2. Therefore, in future studies, the optimal conditions for maximum enzyme production will be studied.
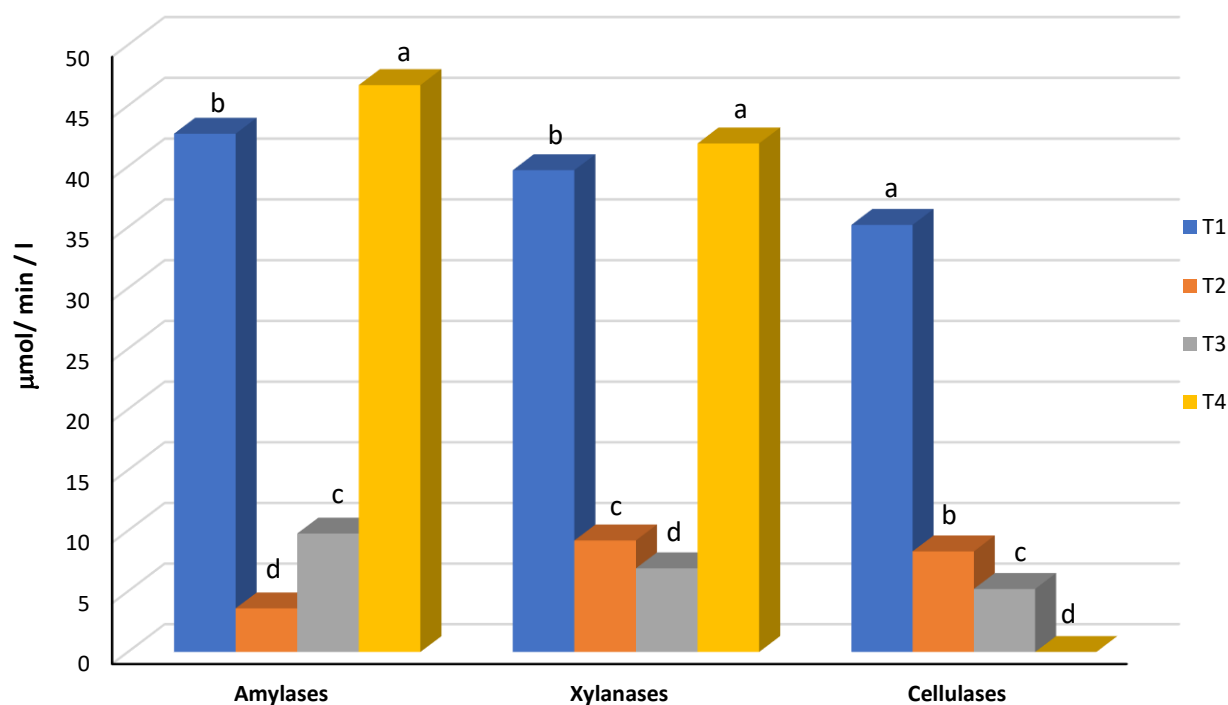
Figure 2. CAZymes titers in 18-day supernatants. Where T1 corresponds to the medium supplemented with 5% orange peel, T2 to the medium with 3% peanut shell and 0.5% wheat straw, T3 with 350 mM CuSO$_4$ as an inorganic inducer, and T4 medium with 5% orange peel and 0.5% wheat straw, in co-culture with *Pycnoporus sanguineus* CS2.

For the preparation of the bread, the supernatants of the medium supplemented with 5% orange peel, 3% peanut shell + 0.5% wheat straw, and the supernatant from co-culture with *Pycnoporus sanguineus* CS2 in medium with 5% orange peel + 0.5% wheat straw due to the combination of enzymes they presented, discarding the KM supernatant. Since it presented low values of the four enzymes, like co-culture and given the inorganic nature of the inducer.

Physical characterization of bread

Table 3 shows the results of height, hardness, weight loss (%), and the number of pores per mm2. The control and the T1 treatment presented the highest bread height (p ≤ 0.05). The results obtained had a behavior similar to those reported by Benejam *et al*. (2009), who evaluated the effect of adding enzymes in panettone samples and observed no differences between the control and treatments with amylase and xylanase. For their part, Hernández (2014) mentions that the excessive addition of this type of enzyme can cause a dough that is too soft or sticky, which generates a deterioration in the quality of the bread. It is also important to mention that baking time is a critical factor in this parameter because a longer baking time results in a greater volume and height of the bread. The effect of laccase, xylanase, and amylase on bread height depends on the specific enzyme used and the dough formulation (Salinas-Sánchez *et al*., 2022). Thus, using xylanases can influence the degradation of hemicellulose present in flour, which can affect gas retention during fermentation and, consequently, the final height of the bread (Sheikholeslami *et al*., 2021). Similarly, amylases can influence the degradation of starch, which affects the dough's viscosity and, therefore, the final structure of the bread. As for laccase, its promising potential in the baking industry could be related to its ability to modulate the network of polysaccharides present in flour, which could influence bread's texture and height (Niño-Medina et al.., 2017).

Table 3. Effect of *Trametes maxima* CU1 supernatants on breads

Tt	Height (mm)	Hardness (N)	Weight loss (%)	Pores by mm^2
Control	27.0 ± 1.00 [a]	58.21 ± 7.72 [c]	1.03±0.50 [a]	14.0±2.64 [ab]
T1	28.7 ± 0.58 [a]	67.16 ± 9.06 [b]	0.93±0.45 [ab]	19.3±3.21 [a]
T2	24.3 ± 1.15 [b]	77.16 ± 4.77 [a]	0.00±0.00 [b]	7.0±1.00 [b]
T3	22.7 ± 0 .58 [c]	68.05 ± 11.71 [b]	0.00±0.00 [b]	9.3±2.08 [b]

Tt=Treatment, WITH: water, T1= supernatants of the medium supplemented with 5% orange peel, T2= supernatant of the medium with 3% peanut shell + 0.5% wheat straw, T3= supernatant of the medium with orange peel 5 % + wheat straw 0.5% in co-culture with Pycnoporus sanguineus CS2. a-cMeans (μ) ± standard deviation (σ) in columns with different superscripts indicate statistical difference (P≤0.05).

The treatments had a statistically significant difference ($p \leq 0.05$), with the control having the lowest hardness, reflected in the best weight loss and the more significant number of pores per mm2. This behavior was similar to what Hernández (2014) observed, who observed no differences between the hardness of the control and the treatments associated with starch gelation. However, in bread made with the purified laccase of *Tramete maxima* CU1, the hardness of the bread was reduced by 17.71%, contrary to what was observed with the supernatants (Niño-Medina *et al*., 2017). This could be explained by the combination of enzymes and their activity titers.

Regarding the weight loss results, the treatments had a statistically significant difference ($p \leq 0.05$), with the control having the highest percentage (1%). However, the values were lower than those reported in other investigations (Niño-Medina *et al*., 2017). It is worth mentioning that the behavior of the treatments was also different from what was observed in breads treated with laccase, xylanase, and lipase (Vega Castro et al., 2015). This could be explained by the fact that despite the different levels of laccase activity in the supernatants, this is the main enzyme and could act on the phenolic compounds and proteins present in the dough, coupled with the activity of the enzymes active on carbohydrates, affecting the absorption and distribution of water in the dough, therefore, also the porosity. In future research, the optimal temperature and cooking time conditions for doughs treated with the supernatants of *Trametes maxima* CU1 will be evaluated.

For its part, the color analysis showed a statistically significant difference ($p \leq 0.05$) in the values of L* and a* between the treatments, except the results of b* (Table 4). It is worth mentioning that T1 and the control did not show a statistically significant difference ($p > 0.05$), which can be explained by the levels and activities in the supernatants. The supernatant obtained from the medium with orange peel showed amylase, cellulase, xylanases, and laccase activity, while the supernatant from the medium supplemented with peanut shell and straw had the highest laccase activity but the lowest CAZyme activity. As for the supernatant obtained from co-culture with *Pycnoporus sanguineus*, CS2 showed the maximum concentration of amylases and xylanases with the lowest production of laccase. This suggests that high and low titers of laccase activity result in hard bread and different colors in correlation with CAZyme levels. Thus, it has been reported that the combination of laccase and xylanase gives bread a darker color and a crispier crust (Niño-Medina *et al*., 2017). In the case of laccase, it is an enzyme with great potential to improve the properties of wheat flour dough and the quality of bread, due to its ability to improve the structure, resistance, and stability of the dough, in addition to improving the volume and texture of the bread (Rosell & Dura, 2015). The above is due to its mechanism of action, as it catalyzes the oxidation of various aromatic compounds, which can produce semiquinones associated with the reduction of molecular oxygen to water, while the free radicals generated can lead to the polymerization of semiquinones to form brown or black pigments (Figueroa-Espinoza and Rouau, 1998). However, its synergy with xylanase improves the color of the bread since it action on the xylan fibers in fractions weakens the structure of bread and allows browning in less time (Rosell & Dura, 2015). In the case of amylase, it is an enzyme that breaks down starch into simpler sugars, which can increase the number of sugars available for the Maillard and caramelization reactions during bread baking, contributing to the color of the bread, in addition to increasing the volume of the bread and crumb texture. (Lončar *et al*., 2016). Therefore, the effect of the enzymatic cocktail of laccase, amylase, xylanase, and cellulase on the color of wheat flour bread may vary depending on several factors, such as the concentration of enzymes used, the

fermentation, and cooking time, among others. In addition to influencing the quality and nutritional value of bread, it offers alternatives to the use of chemical additives and the development of biofunctional foods.

Table 4. Color analysis of breads

Tt	L*	a*	b*	View
Control	39.01±5.9855[b]	27.25±1.9126[a]	42.29±3.6220	
T1	47.50±10.0986[b]	22.43±3.84[a]	42.32±4.4571	
T2	67.25±4.3486[a]	10.63±1.6127[b]	43.15±2.2315	
T3	72.04±1.7225[a]	5.22 ±0.5729[c]	37.98±1.8278	

Tt= Treatment, CON: water, T1= supernatants of the medium supplemented with 5% orange peel, T2= supernatant of the medium with 3% peanut shell + 0.5% wheat straw, T3= supernatant of the medium with orange peel 5 % + wheat straw 0.5% in co-culture with Pycnoporus sanguineus CS2. a-cMeans (μ) ± standard deviation (σ) in columns with different superscripts indicate statistical difference (P≤0.05). L*: Luminosity; a*: the tendency to red; b*: the tendency to yellow.

4. Conclusions

Different profiles (combinations) and titers of laccase, amylases, xylanases, and cellulases were observed in the culture media evaluated, with $CuSO_4$ being the best laccase inducer and orange peel at 5% for enzymes active on carbohydrates (amylases, xylanases, and cellulases) and laccase. At the same time, the bread obtained with the orange peel supernatant had the highest height and color, like the control, in addition to presenting the lowest hardness among the enzymatic treatments. Therefore, these results demonstrate the effect of the culture medium on the enzymatic profiles of the same fungus and its potential application in the baking industry.

6. Acknowledgments

7. Conflicts of Interest

The authors declare no conflict of interest.

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