Biopulp from Pineapple Leaf Fiber Produced by Colonization with Two White-Rot Fungi: *Trametes versicolor* and *Pleurotus ostreatus*

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Trametes versicolor and *Pleurotus ostreatus* were used for the biopulping from pineapple leaf fiber (PALF). PALF substrate was subjected to *T. versicolor* for 2 to 6 weeks and to *P. ostreatus* for 4 to 8 weeks. The yields, holocellulose and lignin contents, and extractives in ethanol-toluene mixture and in sodium hydroxide (NaOH) solution were evaluated. Fourier transform infrared spectroscopy (FTIR) spectra, thermogravimetric analysis (TGA), and color studies by *L*a*b** systems were used for sample analysis. The results showed that the pulp yield was 55% to 70% with *P. ostreatus* and 35% to 50% with *T. versicolor*. Longer colonization periods increased the amount of holocellulose and decreased the amount of lignin and extractives in ethanol-toluene and NaOH solution. TGA showed an increase in intensity associated with cellulose, and the observed inflexion was attributed to lignin, which showed a tendency to fade. The FTIR spectrum showed high intensity between 3100 cm^{-1} and 3600 cm^{-1} (cellulose) and decreased intensity at 1730 cm^{-1} (lignin). For both fungi, the pulp color produced an increase in *L** color parameter and decreased in yellowness, while little variation was observed in redness. The most appropriate colonization period was 5 weeks for *P. ostreatus* and 4 weeks for *T. versicolor*.

Keywords: Fungus; Agricultural waste; Bioprocess; Paper; Degradation

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INTRODUCTION

Pineapple (*Ananas comosus*) is an agricultural crop usually grown in tropical regions (Moya and Camacho 2014). In Costa Rica, this species was introduced in 1970 (Canapep 2012), and by 2014 there were approximately 37660 ha of pineapple-sown fields (INEC 2015). However, the great amount of waste generated after processing the fruit is a major issue. Nearly 300 tons of stubble are produced per pineapple-sown hectare (Canapep 2012). In Costa Rica, as in other countries, this waste has not yet received adequate management (Moya and Solano 2012). Because this is a slow-decaying material, the use of toxic herbicides such as N, N′-dimethyl-4,4′-bipyridinium dichloride (Paraquat) is commonly used; paraquat is a soil contaminating herbicide with accumulative toxicity (Canapep 2012).

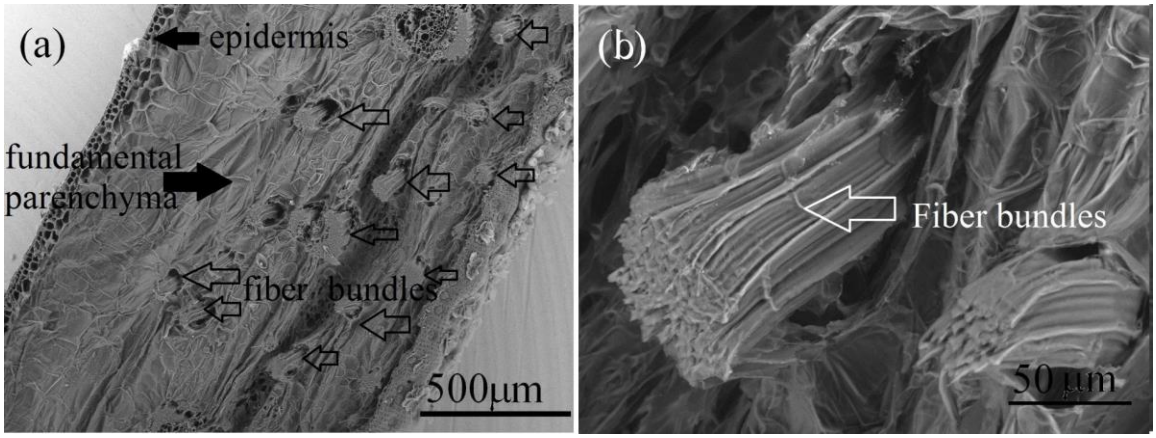

Fig. 1. Pineapple plant leaf. (a) Cross-section of leaf showing epidermis, fundamental parenchyma, and fiber bundles. Source: Moya *et al.* 2013

Pineapple is a bromeliad perennial crop. Spawning from a short and fleshy stem forming a rosette, pineapple leaves are succulent, sessile, and superposed. These leaves are surrounded by a layer of epidermis (Fig. 1a), which encloses the fundamental parenchyma tissue with thin-walled cells (Fig. 1b); fiber bundles of varying diameters are found inside this tissue (Fig. 1b) (Moya *et al.* 2013).

One possible application for the pineapple leaf fiber (PALF) has been the extraction of natural fibers for rope and textiles manufacturing, among other products (Moya and Camacho 2014). For example, Paul *et al.* (1998) and Banik *et al.* (2011) have determined that fibers from *A. comosus* possess high textile potential. Moreover, fibers from *A. comosus* have suitable properties for mixing with cotton, jute, and ramie fibers, as well as artificial fibers (Banik *et al.* 2011). Furthermore, this fiber has potential for paper production. Nayan *et al.* (2014) mentioned that the abundance and low extraction cost of lignocellulosic natural fibers, such as PALF in tropical countries, offer a unique opportunity to explore their potential use as low-cost raw materials for paper pulp.

Chemical methods that dissolve and grind fibers during paper-pulp manufacture result in pollution (Singh and Singh 2014). For this reason, some companies have resorted to more environmentally friendly methods such as biopulping, where fungal treatments are applied to reduce the use of chemicals, minimize the associated costs, and diminish health and environmental hazards (Singh *et al.* 2010). Another eco-friendly treatment is the retting process; however, retting causes large variations in fiber quality, leading to coarser and lower quality of fiber; it has limitations regarding geographical regions with appropriate temperature and moisture, high labor costs, and occupation of agricultural land for several weeks (Chauhan *et al.* 2013).

During biopulping, the lignocellulosic material, which is composed of lignin, hemicellulose, and cellulose, is subjected to microbial decomposition, especially by white-rot fungi, which colonizes and selectively degrades lignin. Different types of fungi have been tested for this process (Singh *et al.* 2010, 2015; Razak *et al.* 2015a); *Ceriporiopsis subvermispora* has been most often used (Singh *et al* 2010). However, other species have gained popularity recently, namely *Trametes versicolor* (Singh *et al.* 2013) and some *Pleurotus* species (Cohen and Persky 2002).

Few studies have been carried out to obtain PALF pulp for the subsequent manufacture of products. For example, Nayan *et al.* (2014) and Razak *et al.* (2015) fabricated paper and obtained mercerised pulp for cardboard production by employing the fungus *Ceriporiopsis subvermispora* for 25 days in a biopulping process using PALF. However, there is a limited information about biopulping process in PALF with two important fungus in tropical area: *T. versicolor* and *Pleurotus ostreatus*. Although biopulping can be carried out in bioreactors, several requirements of the particular microorganism would have to be met to achieve optimal results (Ferraz *et al.* 2008). It follows that with knowledge of the optimal biopulping conditions, a lignocellulosic material such as PALF can be processed effectively.

Considering the importance of finding a solution for handling *A. comosus* plants after harvesting, this work poses an alternative use of PALF in biopulping processing using two types of white-rot fungi: *T. versicolor* and *P. ostreatus*. The following parameters were evaluated in this study: biopulp for each fungus at five different fungal colonization moments (2, 3, 4, 5, and 6 weeks for *T. versicolor* and 4, 5, 6, 7, and 8 weeks for *P. ostreatus*); and their performances; state of the fiber bundles from PALF; content of cellulose, lignin, and extractives in ethanol-toluene and sodium hydroxide solution; chemical evaluation using the Fourier transform infrared spectroscopy (FTIR); thermogravimetric behavior; and color, using *L*a*b** color systems.

EXPERIMENTAL

Source of Materials

Materials were obtained from two *Ananas comosus* plantations in Costa Rica. The first one was located in the humid tropical zone on the Caribbean Coast; the second plantation was located in the dry tropical zone on the Central-Pacific Coast. Details about the source of the materials and the procedure used for extraction of the fibers are described in Moya *et al.* (2013) and Moya and Camacho (2014), wherein a small model (prototype) for industrialization of *A. comosus* fibers was developed, and samples from approximately 150 plants were obtained.

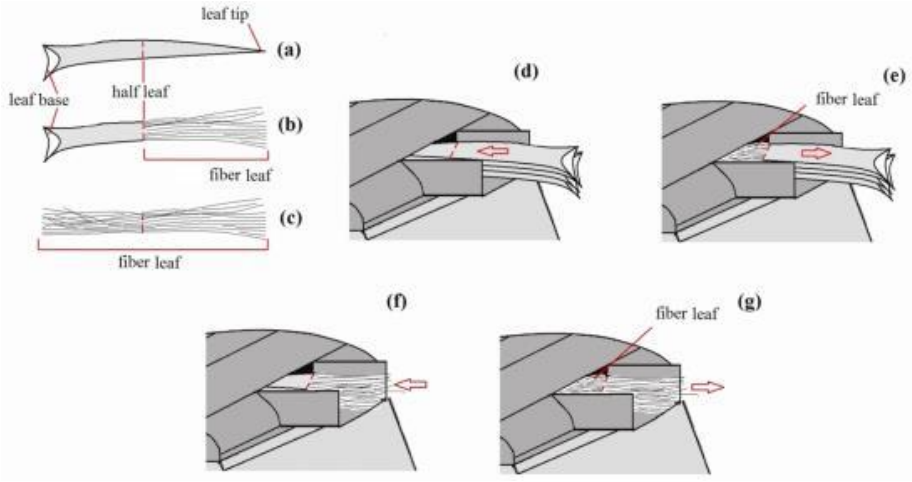

Fig. 2. Machine removal of fibers from *A. comosus* leaves. Source: Moya and Camacho (2014)

Fiber Extraction and Processing

Extraction of the PALF was performed according to Moya and Camacho (2014), who describe the processing of the leaves of the plant. With the leaf-tips facing forwards, about 4 to 6 pineapple leaves were introduced into the machine (Fig. 2a). Once the leaves were inserted up to approximately half of their length (Fig. 2d), they were pulled backwards (Fig. 2e). At this moment, the pineapple leaf fibers were stripped of their parenchymal tissue (Fig. 2b). The operator then held the leaf by the fiber-free end (Fig. 2f) and again introduced it from the base-side until reaching the exposed fibers (Fig. 2g). At this point, the leaves were removed, thus completely extracting the PALF (Fig. 2c). The PALF was washed with water to remove any remaining chlorophyll (Moya and Solano 2014) and then stored in humid conditions until fungal inoculation.

Fungal Inoculation

The PALF was first cut into small pieces of 1-cm length. It was then separated into 30 samples of 60 g each and stored inside autoclave-resistant Polipet plastic bags. A small sample of approximately 1 g was used for determining the moisture content with a thermobalance. The samples were slightly moistened to guarantee an adequate moisture content (50% humidity, approximately) followed by sterilization in an autoclave for 1 h at 120 °C. Subsequently, 15 samples were inoculated with *Trametes versicolor* (L.) Lloyd and the other 15 with *Pleurotus ostreatus* (Jacq.) P. Kumm. These fungi were selected due to their aggressiveness in lignin degradation.

Both fungi were previously cultivated in a petri dish in malt agar for 10 d, until the mycelium reached ¾ of the petri dish diameter. Once the cultures were grown, 2.5 cm diameter of mycelium segments. These segments were left to grow for 14 d in 250 mL flasks with 50 mL of a malt-extract-based liquid medium.

Biopulping of Pineapple Leaf Fibers

The flasks containing the inoculated fungi were kept intact to avoid the formation of mycelial lumps. Once enough mycelial mass was obtained, the samples were placed in 2000 mL beakers and blended at 3000 rpm with an electric manual whisk previously sterilized with alcohol. This enabled the rupture of the mycelia to attain a greater contact surface. The liquid medium containing the fungus was then poured into each one of the plastic bags containing PALF, which were then stored in a dark environment at 26 °C with 80% relative humidity during the fungal colonization period. For *Trametes versicolor*, the colonization intervals were 2, 3, 4, 5, and 6 weeks, whereas for *Pleurotus ostreatus* the intervals were 4, 5, 6, 7, and 8 weeks. This is because the former has a greater colonization speed than the latter.

Pulp Preparation

At the end of the fungal colonization period, 3 bags of pulp were taken per week and per fungus, which were then washed with distilled water to eliminate the mycelia grown on the PALF. Subsequently, 500 mL of water was blended with PALF-mycelia for 2 min to totally separate the pulp. Finally, the mixture was washed with water several times until the fungal mycelium disappeared. The pulp was then placed on a mesh sieve (0.40 mm) and manually pressed to eliminate excess water, after which it was stored at 3 °C. Before storage, 3 samples of approximately 3 g each were taken to determine moisture content.

Determination of Pulp Yield Percentage

About 30 samples were weighed before and after fungal colonization. The following equation was used to determine the performance of PALF production by fungal digestion (Eq. 1).

$$Yield\ of\ pulp\ (\%) = \frac{Dry\ weight\ before\ digestion - Dry\ \ Weight\ after\ digestion}{Dry\ weight\ before\ digestion} * 100 \quad (1)$$

Moisture present in the PALF samples was considered for both conditions of dry weight, before and after colonization (Eq. 2). The samples for determination of moisture content were weighed (green weight), then placed in an oven at 103 °C for 24 h and weighed again afterwards (dry weight).

$$Moisture\ Content\ (\%) = \frac{Green\ weight\ (g) - Dry\ weight\ (g)}{Green\ weight\ (g)} * 100 \quad (2)$$

Determination of Pulp Characteristics

Chemical analyses of the pulp were conducted to determine the amount of lignin and holocellulose and the amount of extractives in hot and cold water, ethanol-toluene (1:1), or 1% sodium hydroxide. Moreover, thermogravimetric analysis (TGA), FTIR analysis, and color analysis of the dried pulp were performed. These characteristics were determined for every week of fungal colonization period.

Chemical Analyses

The TAPPI T222 om-02 (2002) standard was used to measure the lignin and holocellulose contents. Extractives were determined by the method proposed by Seifert (1960). ASTM D-1109-84 (2003a) was used to determine extractives in sodium hydroxide (NaOH), and ASTM D-1107-96 (2003b) was used to measure extractives in ethanol-toluene (1:1). For each chemical analysis, 3 samples were taken per week of growth or digestion.

Thermogravimetric Analysis

Thermogravimetric analysis was conducted on two 4 to 5 mg samples of the pulp exposed to fungal activity. This test was carried out at a speed of 20 °C/min in a nitrogen atmosphere (N_2 UAP), reaching a temperature of 700 °C in approximately 15 min, using a NBR model TGA 5000 thermogravimetric analyzer (TA Instruments, New Castle DE, USA) and TA Instruments Universal Analysis 2000 software (New Castle DE, USA). This analysis renders the weight loss rate as a function of temperature by means of the differential thermogravimetric spectrum (DTG) curves. In addition, the remnant mass at the end of maximum decomposition was shown throughout the fungal colonization period, as well as the remnant mass at maximum mass loss rate.

FTIR Spectra

A sample of approximately 0.1 mg was taken from the surface of the pulp and analyzed on Thermo Scientific infrared spectrometer (Nicolet 380, Madison, WI, USA).

All data were recorded at room temperature, in the spectral range of 4000 to 700 cm^{-1}, by accumulating 64 scans with a resolution of 1 cm^{-1}.

The obtained IR spectra were processed on Spotlight 1.5.1, Hyperview 3.2, and Spectrum 6.2.0 software (Perkin Elmer, Waltham, MA, USA). Base-line correction was applied at 600 to 1800 cm^{-1} and the main peaks of these vibrations were identified.

Color

The color of the pulp was evaluated in a sample of paper. For this purpose, two 20 cm diameter paper sheet samples were prepared per week of fungal colonization. To manufacture the paper, 17 g of the pulp (at 75 to 78% moisture content) were added to about 500 mL of distilled water, and this mixture was blended for 5 min using a blending machine (Waring Commercial, Intertek, USA). The mixture was left to hydrate for 3 days, after which 500 mL of more water were added and blended again for 15 min to segregate all the fibers. The mixture was poured into a circular 20 cm-diameter mold, drained, and oven-dried at 50 °C for 2 h. The estimated weight of the paper was 80 g/m^2.

A total of 10 color measurements were performed on the fabricated paper (5 per each paper sample). A miniScan XE Plus spectrophotometer (Reston, VA, USA) was used to obtain the values for the CIE $L^*a^*b^*$ standardized chromatological system, as detailed in Moya *et al.* (2012). Color parameters (L^*, a^*, b^*) were used to calculate the color change of the pulp in relation to the color of pulp processed chemically by kraft-like methods. This change, known as color difference (ΔE^*), was calculated in accordance with the ASTM D 2244-11 (2014) standard (Eq. 3). Color difference (ΔE^*) was determined for the color change that occurred in the pulp after each week and for each fungus, in relation to pulp prepared traditionally using chemical methods,

$$\Delta E* = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2} \quad (3)$$

where ΔE^* is the pulp color difference; $\Delta L = L^*_{biopulp} - L^*_{kraftpulp}$; $\Delta a = a^*_{biopulp} - a^*_{kraftpulp}$; and $\Delta b = b^*_{biopulp} - b^*_{kraftpulp}$. Regarding color change of kraft pulp, the L^*, a^* and b^* values were as follows: $L^* = 84.06$, $a^* = -0.024$, and $b^* = 12.58$. These values were obtained from 10 color measurements in 2 different sheets.

Analysis of Results

A variance analysis verified significant differences among the averages of cellulose, lignin, and extractives contents ($P < 0.05$). Tukey's test was carried out to determine the statistical differences among fungi, for the mean value of each of the above mentioned values. Variation of color parameters with colonization time was analyzed by means of a regression analysis, in which the model with the highest coefficient of correlation was selected; it was a second degree polynomial type for all cases. Pearson's correlation coefficients were used to determine the relationship between color parameters and chemical components (cellulose, lignin, and extractives contents). Statistical analysis was conducted using SAS 8.1 software (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Yield Analysis

The percentage yield in pulp production with fungal colonization of *Pleurotus ostreatus* was higher than *Trametes versicolor* (Fig. 3). For the former, the yield ranged between 55% and 70%, while for the latter, the degradation of the fibers varied between 35% and 50%. However, a statistically confirmed reduction in the yield percentage of the pulp was observed as colonization time of the fungus on the pineapple fiber increased, which was a common factor in both types of fungus (Fig. 3).

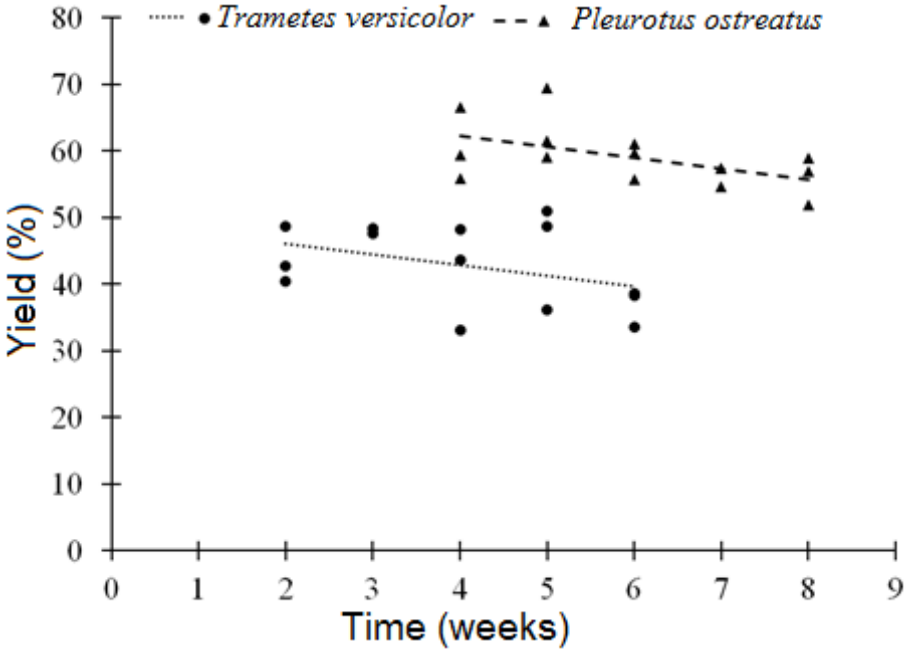

Fig. 3. Yield percentage of pineapple fiber colonized by white-rot fungi *Trametes versicolor* and *Pleurotus ostreatus* throughout 8 weeks

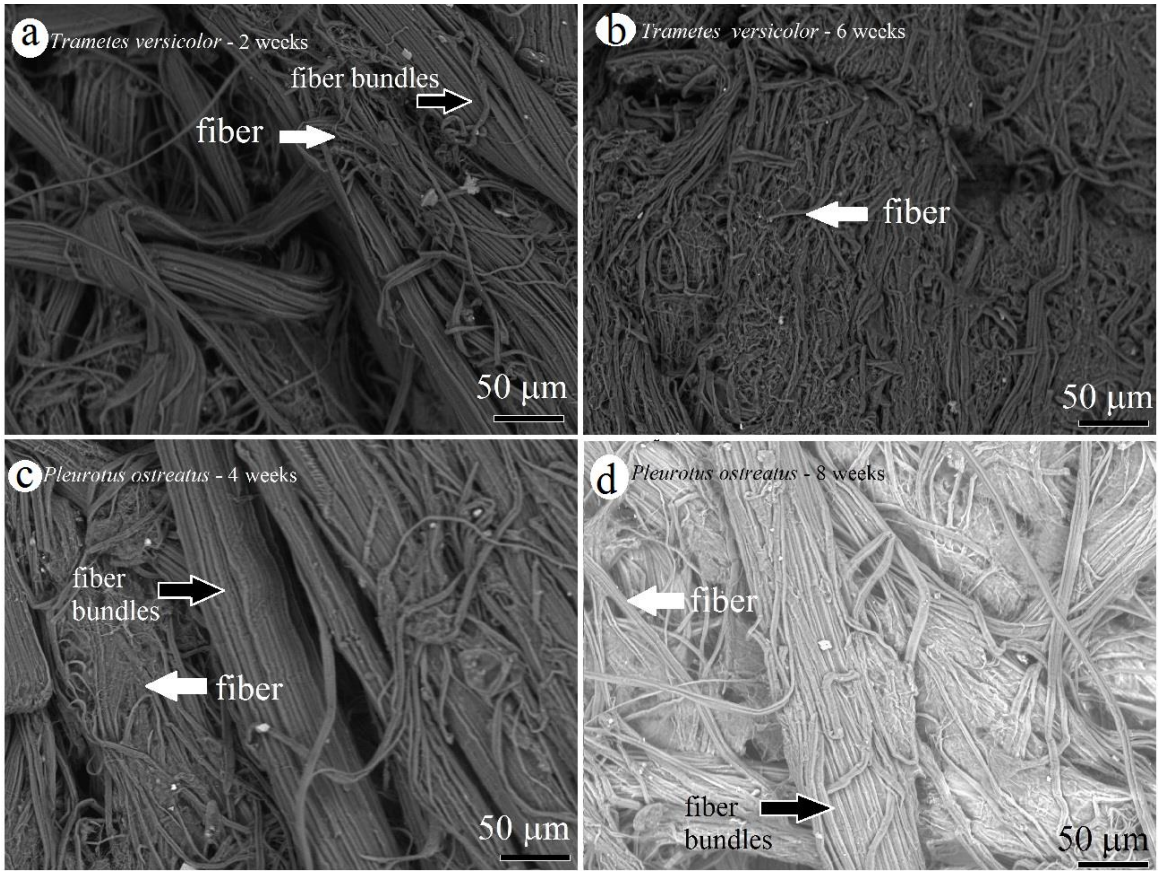

Fig. 4. Pineapple leaf fibers exposed to (a) *Trametes versicolor* for 2 weeks, (b) *Trametes versicolor* for 6 weeks, (c) *Pleurotus versicolor* for 4 weeks, (d) and *Pleurotus versicolor* for 8 weeks

The behavior of PALF subjected to colonization from white-rot fungi was evaluated by weight loss. After two weeks of colonization with *T. versicolor* (Fig. 4a), the fiber bundles remained joined to one another, separating and breaking apart as time passed (Fig. 4b). For *P. ostreatus*, lignin degradation initiated at week 4, but at this time fiber bundles were still joined together (Fig. 4c), separating at week 8 (Fig. 4d).

Lignin is the secondary component of pineapple leaf fibers and may represent 15.34% of total chemical components (Table 1). The structural features of this heterogeneous polymer impose unusual restrictions on its biodegradability (Mishra *et al.* 2004). White-rot fungi have, nonetheless, the ability to penetrate the complex structure of lignin (Isroi *et al.* 2011). This type of fungus acts on the pineapple leaf fiber first by penetrating at the extracellular level *via* enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP9 and laccase) (Cohen *et al.* 2002), a complex known as the ligninolytic system (Isroi *et al.* 2011). In the first weeks of colonization with both types of fungus, the enzymes are barely beginning to have an extracellular action on lignin (Cohen and Persky 2002).

However, white-rot fungi produce enzymes that degrade other fiber components such as hemicellulose, which is easily degraded (Isroi *et al.* 2011). This behavior of the fungi explains why performance declines to low values in the last weeks as, in addition to degrading lignin, the fungi also degrades hemicellulose and cellulose in the fibers.

In *T. versicolor*, together with its high lignin degradation capacity, there is a high ability to degrade hemicellulose and cellulose in the fibers (Singh *et al.* 2013), superior to that of *P. ostreatus*, hence its low performance in the biopulping process. This was evidenced in the SEM photographs (Fig. 4). According to Saha *et al.* (1990), the degree of dissociation of the fibers in bundles is related to degradation of wax, gum, pectin, and the lignin itself. Treatment with *P. ostreatus* showed less degradation than with *T. versicolor*, hence the greater degradation of fiber bundles of the latter (Fig. 4b).

Evaluation of Holocellulose, Lignin, and Extractives Content

In the evaluation of the percentage of holocellulose, lignin, and extractives in the pulp for the different fungal colonization periods, the amount of holocellulose increased considerably, whereas the amount of lignin and extractives in ethanol-toluene and NaOH solution decreased (Table 1). However, the main change occurred at 5 weeks with the *P. ostreatus* fungus, with the exception of extractives in ethanol-toluene, wherein this change occurred at 6 weeks (Table 1). With *T. versicolor*, the holocellulose and lignin contents changed at 4 weeks, while in the extractives in ethanol-toluene and NaOH, the greatest change occurred after one week of colonization (Table 1).

Before the fungal colonization of PALF with both fungal specimens, holocellulose and lignin values obtained were at the upper end of the ranges reported by other authors. Nadirah *et al.* (2012) and Khalil *et al.* (2006), for example, reported holocellulose content close to 80% and lignin content of 10.5%, values which are slightly inferior to those found in this study (Table 1). Furthermore, in a study of 12 pineapple leaf fiber varieties from Brazil, slightly inferior values to those found in the present work were also reported (Neto *et al.* 2015). Meanwhile, Daud *et al.* (2014) reported holocellulose and lignin contents of 85.7% and 4.7%, respectively. In this study, the holocellulose was similar, but the lignin content was higher (15.45%). Finally, regarding the content of extractives in ethanol-toluene and NaOH solution, these were similar to those reported by Khalil *et al.* (2006), Nadirah *et al.* (2012), and Daud *et al.* (2014) in Malaysia and Neto *et al.* (2015) from the 12 Brazilian varieties.

Table 1. Holocellulose, Lignin, and Extractives in Different Solvents for Pineapple Leaf Fibers Colonized by *Pleurotus ostreatus* and *Trametes versicolor*

Fungus	Time of colonization (Weeks)	Holocellulose (%)	Lignin (%)	Ethanol-toluene (%)	NaOH Solution (%)
Pineapple leaf fibers	0	84.45^{A}	15.45^{A}	17.62^{A}	29.81^{A}
Pleurotus ostreatus	4	94.70^{B}	9.85^{B}	11.92^{B}	22.45^{B}
	5	94.14^{B}	9.53^{B}	12.05^{B}	21.63^{B}
	6	90.71^{C}	6.53^{C}	11.16^{B}	17.98^{C}
	7	90.08^{C}	5.01^{C}	8.88^{C}	18.18^{C}
	8	90.67^{C}	5.62^{C}	8.71^{C}	16.80^{C}
Trametes versicolor	2	95.49^{B}	14.18^{B}	13.91^{B}	30.13^{A}
	3	95.14^{B}	13.85^{B}	11.03^{C}	25.44^{C}
	4	95.56^{B}	11.43^{BC}	11.18^{C}	25.24^{C}
	5	93.42^{C}	10.07^{C}	9.42^{C}	24.62^{C}
	6	92.66^{C}	10.97^{C}	10.09^{C}	25.78^{C}

Legend: Average values identified with different letters are statistically different at α= 99%.

As shown in this study, the white-rot fungi degraded lignin in PALF (Isroi *et al.* 2011). The amount of lignin contained in the pulp decreased with colonization time, as happened to the amount of extractives in various solvents (Table 1); hence the low lignin content of the generated pulp — a desirable condition when producing paper (Razak *et al.* 2015). The maximum holocellulose amounts were observed during the first weeks, reaching nearly 95% in the pulp generated by fungal colonization; this amount, however, decreased as colonization progressed (Table 1). As indicated before, this behavior was attributed to enzymatic degradation by both fungi of hemicellulose mainly, but also of cellulose (Cohen and Persky 2002; Isroi *et al.* 2011). Thus, the amount of holocellulose decreased after a long colonization period (Table 1).

T. versicolor had a greater ability to degrade lignin in a short time (Table 1); however, this fungus has a high capacity for lignin and cellulose degradation (Cohen and Persky 2002).

Another important aspect to highlight about pulp from fungal colonization is that the changes in the amounts of the different organic compounds occurred on the fourth week. The amount of lignin decreased to a minimum value of 11.4% on week 4 and showed no statistical difference in subsequent weeks (Table 1). This result indicates that 4 weeks is the period that pineapple fibers must remain under colonization of *T. versicolor* (Table 1). In the case of *P. ostreatus*, however, there was a greater decrease in the amount of lignin, extractives, and cellulose at week 6. Thus, the colonization period necessary to attain the lowest lignin and extractives content was 6 weeks. The stabilizations in these weeks occurred by some components of the lignin are stable to further biodegradation of ligninolytic fungus (Martinez *et al.* 1994). At this point, the unstable component had been biodegraded and the remaining component was relatively stable.

As shown in Table 1, PALF had a higher amount of holocellulose (85%), which was higher than in hardwood (31% to 64%) and softwood (30% to 60%). Previous studies reported that PALF has higher cellulose content than oil palm fronds, coconut, and banana stem fibers (Razak *et al.* 2015). The higher holocellulose content in PALF is explained by its need to support the greater weight of the fruit and the fact that they are less perishable (Reddy and Yang 2005). Lignin is an undesirable polymer, as its removal during pulping requires high amounts of energy and chemicals (Nadirah *et al.* 2012). Although the percentage of lignin content in pineapple leaves found in these studies was low, it was still higher than those of previous studies (Nadirah *et al.* 2012).

Extractives obstruct the formation of microbes. In paper production, decreasing the extractives content can result in increased yield as well as decreased consumption of chemicals and material and the cooking time (Nadirah *et al.* 2012). However, in biopulping with both fungi, no considerable differences in the extractives appeared after colonization as the weeks passed. The only exception was the extractives content in NaOH solution, which was greater in *T. versicolor*, and their decrease was less in relation to the amount of extractives before fungal colonization (Table 1). This means that biopulping is possible with both fungi if levels of extractives contents are low, in comparison with other types of biomass used for pulp production.

Thermogravimetric Analysis

The DTG spectrum of the biopulp exposed to two white-rot fungi (*T. versicolor* and *P. ostreatus*) is represented in Fig. 5. The thermal behavior of biopulp produced in each colonization period was slightly different.

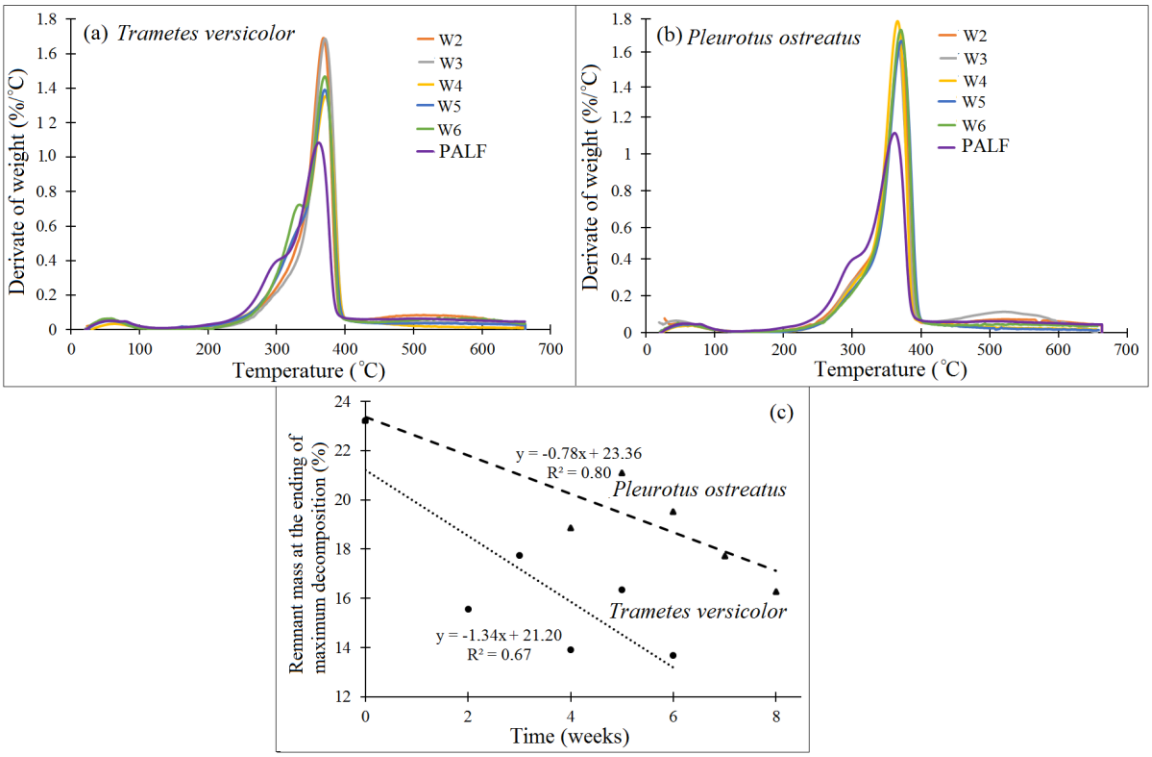

Fig. 5. Derivative weight of biopulp exposed to (a) *T. versicolor;* (b) *P. ostreatus* fungi; and (c) variation of remnant mass at the ending of maximum decomposition. Time of colonization: W2, two weeks; W3, three weeks; W4, four weeks; W5, five weeks; W6, six weeks

The DTG curves showed three important inflexion points in both pulps; however, temperatures evaluated in decomposition varied depending on the colonization period. In pineapple fibers without fungal colonization (Figs. 5a, b), the first inflexion was produced at a temperature of 297 °C, while the second inflexion occurred at 361 °C, corresponding to maximum degradation. No other inflexions were observed after this temperature.

The first inflexion, which occurred in fibers lacking fungal colonization, did not appear for biopulp produced by colonization with *T. versicolor* throughout the various weeks. The inflexion for maximum decomposition, on the other hand, appeared between 368 °C and 372 °C (Fig. 5a), showing slightly higher temperature values (from 7 °C to 11 °C) and decomposition rates (0.2 to 0.7% per °C) relative to natural or non-colonized fibers. In contrast to fibers with no fungal colonization, at week 4 there appeared a small inflexion close to 330 °C, which was more pronounced at week 6 (green line in Fig. 5a). Another difference observed in the DTG curve for colonized and non-colonized fibers was the apparition of a deflection between 448 °C and 630 °C, which was greater on the second week (orange line in Fig. 5a) and less pronounced in later weeks.

Regarding the biopulp produced through various colonization periods with *P. ostreatus* (Fig. 2b), the first inflexion was observed approximately at 304 °C of the DTG, a temperature slightly higher (by 4 °C) than in non-colonized fibers. In the second inflexion, again, an increase in temperature from 4 °C to 9 °C occurred, as well as increase in the decomposition rate (approximately 6% per °C) in comparison with natural fibers. Finally, only in pulp with a 5-week colonization period was observed a slight inflexion between 431 °C and 597 °C (grey line in Fig. 5b).

For both types of fungi, analysis of the differences at the point of maximum decomposition between natural fibers and colonized fibers, in addition to showing a greater decomposition velocity (Figs. 5a, b), revealed a decrease in the remnant mass at the end of maximum decomposition as a function of time (Fig. 5c).

In accordance with previous DTG results, after colonization with both fungi an evident change in the thermal behavior of fibers was observed from the absence of a first inflexion, the slightly higher temperature in the second degradation, and the higher decomposition rates (Figs. 5a, b). These variations in the thermal behavior may be explained by the exposition of the three basic chemical constituents of pineapple fibers (cellulose, hemicellulose, and lignin). Lignin is the component with greatest resistance to thermal degradation, followed by cellulose and, finally, by hemicellulose, which is the least thermally stable (George *et al* 2014). Various studies agree that the temperature range in which hemicellulose is degraded spans from 150 °C to 350 °C; cellulose presents its maximum decomposition between 275 °C and 350 °C; and the range corresponding to lignin degradation is from 250 °C to 500 °C (Neto *et al.* 2015).

Therefore, the two inflexions occurring in non-colonized fibers were due to thermal degradation of hemicellulose and cellulose (at 297 °C and 361 °C, respectively), as well as the little amount of lignin. The small inflexion observed after 400 °C was attributed to lignin, which was not as evident in non-colonized fibers, probably due to the low lignin content contained in this type of lignocellulosic material (less than 15%) (Table 1). For cellulosic fibers, lignin degradation started at around 200 °C, while other polysaccharides, mainly cellulose, were oxidized and degraded at higher temperatures (Nadirah *et al.* 2012). The second stage of degradation occurred at a temperature ranging from 200 to 500 °C, where weight loss corresponded to the formation of volatile products that arose from random chain scission and intermolecular transfer involving tertiary hydrogen abstractions from the hemicelluloses, cellulose, and lignin (Neto *et al.* 2015).

The greater stability obtained in fibers under fungal colonization is explained by the increase in cellulose content and the decrease in lignin content (Table 1). According to Kim and Eom (2001) and Rachini *et al.* (2009), as cellulose is thermally more stable than lignin and hemicellulose, it is expected that the initial degradation temperature of pineapple fibers with white-rot fungal treatment should increase by reducing their lignin and hemicelluloses contents and increasing their cellulose content. The major source of stability in cellulose is hydrogen bonding, which allows thermal energy to be distributed over many bonds (Nadirah *et al.* 2012).

Pulps generated by both fungi showed different thermal behaviors (Figs. 5a, b). The first inflexion, which occurred at 297 °C, decreased as fungal colonization time increased; this indicates that a big portion of hemicelluloses was degraded before week 2 for *T. versicolor* and before week 4 for *P. ostreatus*. In the latter, hemicellulose degradation was slower, and a small inflexion was seen at week 8 (Fig. 5b).

Regarding the second inflexion, the pulp produced by colonization with *T. versicolor* showed a decrease in the percentage of mass at the peak for maximum decomposition from week 4 onwards (Fig. 5a); therefore, the range of thermal degradation of cellulose (George *et al.* 2014) was affected starting at this stage, probably due to the decrease in the amount of cellulose as colonization time progressed (Table 1). *T. versicolor*, from a certain point in the colonization period, aggressively degraded not only lignin and hemicellulose but cellulose as well, as its action was not selective upon lignin, thus decreasing the cellulose content and rendering the material less thermally stable (George *et al.* 2014; Bari *et al.* 2015). This behavior was nevertheless not so noticeable in pulp

generated by *P. ostreatus*, for which variation of the mass at maximum decomposition was almost constant (Fig. 5b). Hence, in pulp from this fungus, thermal degradation was not very affected by the colonization time and thus the thermal stability of the pulp was unaffected (Chen *et al.* 2012).

At higher temperatures, an inflexion was observed around 330 °C in week 4 in pulp generated by *T. versicolor*, which may correspond to a persisting presence of lignin. The amount of lignin was still high in relation to non-colonized fibers (Table 1) and the ranges of temperature of thermal degradation rose up to 500 °C (Neto *et al.* 2015). This behavior differed from that of pulp exposed to *P. ostreatus*, as no inflexion was seen around 330 °C in the thermogram (Fig. 5b) other than the one that would correspond mostly to cellulose, and the amount of lignin contained in the pulp was low (Table 1).

Infrared Analysis

Fourier transform infrared spectra corresponding to each biopulp showed behavior similar to that described by Neto *et al.* (2015) and Singhal *et al.* (2015) for lignocellulosic material (Fig. 6). The three main compounds observed in this material were cellulose, hemicellulose, and lignin. The presence of these compounds was evidenced by signals between 2850 cm^{-1} and 2900 cm^{-1}, which was due to the asymmetric stretching of -CH and $-CH_2$ (Morán *et al.* 2008; Neto *et al.* 2015).

In non-colonized pineapple fibers and in the pulp generated by both types of fungus, an absorbance peak was observed at 3340 cm^{-1} (Fig. 6a), which corresponds to the hydrogen-bonded -OH stretching vibrations associated to the intermolecular and intramolecular hydrogen bonds of the free -OH in cellulose (Razak *et al.* 2015). Maximum intensity of this absorbance in biopulp generated by *T. versicolor* was achieved in week 5, but in week 6 this intensity was lower than in previous weeks and was inferior to the signal from non-colonized fibers. Meanwhile, the pulp generated by *P. ostreatus* again showed maximum intensity of vibration at 3340 cm^{-1} in week 6. Later, the intensity of this signal decreased and became stable towards week 8 (Fig. 6c).

Furthermore, in non-colonized fibers and in pulp generated by colonization from both fungi, a peak was observed between 2900 and 2950 cm^{-1}, which was attributed to the presence of –OH from hemicellulose (Razak *et al.* 2012). The maximum absorbance associated with hemicellulose also occurred between 2900 and 2950 cm^{-1} similar to that of cellulose absorbance (3340 cm^{-1}), after 5 weeks of colonization with both fungi. After this period, the peak intensity tended to decrease until the end of the colonization period, at 6 weeks for *T. versicolor* (Fig. 6a) and at 8 weeks for *P. ostreatus* (Fig. 6c).

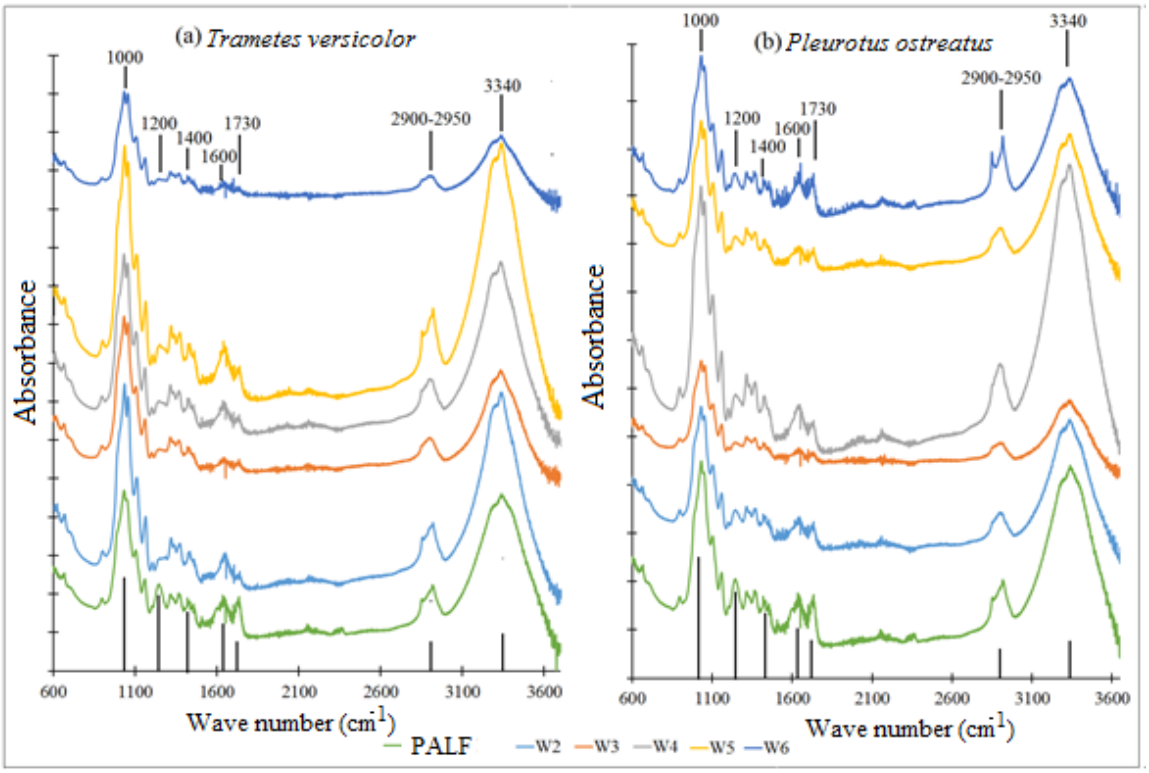

Fig. 6. Fourier-transform infrared (FTIR) spectra of pineapple leaves treated with (a) *Trametes versicolor* and (b) *Pleurotus ostreatus*. Time of colonization: W2, two weeks; W3, three weeks; W4, four weeks; W5, five weeks; W6, six weeks

In accordance to previous changes in peak intensity, the increase of absorbance between 3100 and 3600 cm^{-1} and the increase in intensity of hemicellulose peaks (intensity between 2900 and 2950 cm^{-1}) observed in the fibers up until 5 or 6 weeks, after it had been colonized by both fungi, suggests that this increase in hydroxyl groups (-OH) was associated with greater colonization due to lignin degradation (Nayan *et al.* 2014). Meanwhile, the decline occurring in hemicellulose absorbance after week 5 or 6 was attributed to cellulose and hemicellulose degradation occurring after the lignin decreased (Cohen and Persky 2002; Isroj *et al.* 2011).

However, the peak at 1730 cm^{-1} is the characteristic band for carbonyl (C=O) stretching that represents the ester linkage of lignin and acetyl group of hemicelluloses (Nayan *et al.* 2014). This peak was noticeable in non-colonized fibers (Figs. 6a, b), but its intensity was decreased as colonization progressed (Figs. 6a, b). This decreased intensity suggests that a substantial portion of uronic acid, a constituent of hemicellulose xylan, was removed (Nayan *et al.* 2014).

In non-colonized fibers, the major signals in the wavelength-region of 1000 and 1200 cm^{-1} (Figs. 6a, b), were associated with C-O stretching present in hemicellulose and cellulose (Singhal *et al.* 2015). However, the intensity of this band increased with colonization time for both types of fungus. According to Chen *et al.* (2011) and Singhal *et al.* (2015), the presence of lignin and hemicellulose disguises the signal corresponding to cellulose at 1000 cm^{-1} and 1200 cm^{-1}, thus weakening the signal in non-colonized fibers. However, this signal intensity increased as colonization time progressed, which may indicate further exposure of cellulose due to fungal degradation of lignin and part of the hemicellulose (Isroi *et al.* 2011).

Lignin shows characteristic signals between 1420 and 1600 cm^{-1}, which corresponded to C-H vibrations of the aromatic rings present in its chemical structure (Chen *et al.* 2012; Neto *et al.* 2015; Singhal *et al.* 2015b). These peaks were evident in non-colonized fibers (Figs. 6a, b). Whereas in the biopulp generated by colonization, the intensity of the bands decreased with time, which may be due to the scarce amount of lignin present in pulp produced through fungal colonization.

Finally, the change in previously studied band intensities (1000, 1200, 1400, 1600, 1830, 2900 to 2950, and 3340 cm^{-1}), starting from week 5 in *T. versicolor* (Fig. 6a) and week 7 in *P. ostreatus* (Fig. 6b), may reflect that the fungus digested the majority of lignin during this period, and, as it is not selective, it began digesting cellulose and hemicellulose that were exposed (Cohen and Persky 2002; Isroj *et al.* 2011). This indicates that this was the optimal period of colonization of the pineapple fibers by the fungus, as the amount of cellulose was higher and the lignin was present in the lowest amount.

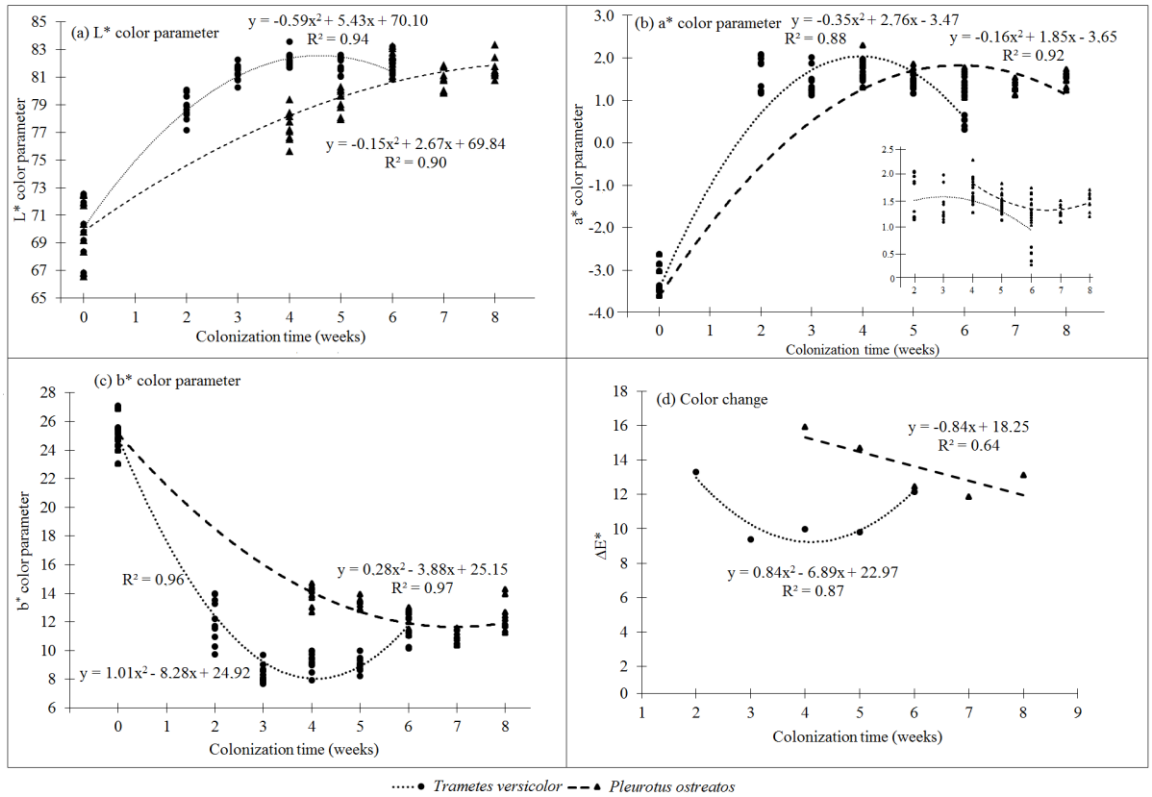

Fig. 7. Variation of color parameters (a, b, c) and color change (d) in relation to time for two different biopulps

Color

Color parameter variations (L^*, a^*, and b^*) produced through biopulping of pineapple leaves, as well as those of fibers in dry condition, are detailed in Fig. 7. The value for parameter L^* of both fungi increased in relation to non-colonized fibers, and it tended to grow with colonization time, going from a value of 69 to 83. Parameter a^* also increased in pulp generated by fungal colonization in relation to non-colonized fibers (Fig. 8b). The tendency of parameter a^* in relation to time varied for both types of fungus. It decreased in pulp produced by *P. ostreatus* as fungal colonization progressed each week. The value tended to stabilize between week 7 and 8. It remained unchanged during the first

4 weeks in pulp produced by *T. versicolor* and, at the end, decreased (Fig. 7b). In contrast, the color parameter *b** (Fig. 8c) decreased in biopulp in comparison with non-colonized fibers. Parameter *b** decreased up to 4 weeks of fungal colonization by *T. versicolor* and 7 weeks by *P. ostreatus*, after which it increased (Fig. 7c).

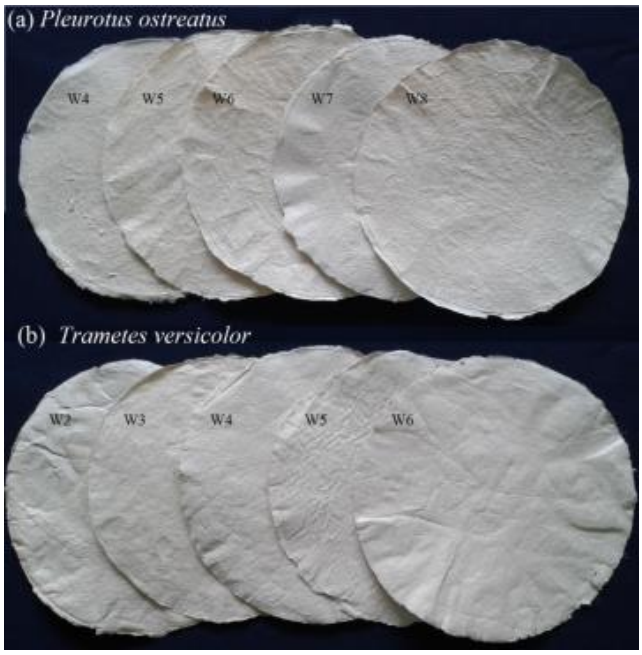

Fig. 8. Paper sheets produced from pineapple biopulp exposed to fungi (a) *P. ostreatus* and (b) *T. versicolor*. Time of colonization: W2, two weeks; W3, three weeks; W4, four weeks; W5, five weeks; W6, six weeks

Lightness is measured by color parameter *L**, and it indicates how light (values close to 100) or dark (values close to 0) the color is. Parameter *a** denoted the variation in redness (positive values) and greenness (negative values). Lastly, the parameter *b** indicated variation in hues of yellow (positive values) and blue (negative values) (Perng *et al.* 2015). Pulp from both fungi was lighter than from non-colonized fibers, becoming even lighter as colonization time progressed (Fig. 7a). Biopulp showed a red hue in contrast to normal fibers (Fig. 7b). However, when comparing the pulps produced by (Fig. 7b) *T. versicolor* and *P. ostreatus*, redness tended to disappear on week 6 (value close to 0). Finally, the yellowness was less in biopulp than it was in non-colonized fibers, due to a decrease in parameter *b** (Fig. 7c). The lowest yellow hue appeared between weeks 3 and 4 for *T. versicolor* and in week 7 in *P. ostreatus* (Fig. 7c), as parameter *b** values tended to pass from high to low values in the first weeks.

Variation in color parameters may be affected by the different components of the pulp (cellulose, hemicellulose, and extractives). For example, in solid wood, Gierlinger *et al.* (2004) mentioned that redness (*a**) and lightness (*L**) indexes are more correlated to the extractives content of wood, while the yellowness index was primarily related to the photochemistry of the lignin. Moreover, Moya *et al.* (2012) found that parameter *L** decreased as the total extractives and phenolic contents increased. However, parameter *a** increased as the content of extractives and phenols increased, while parameter *b** was not affected by any chemical components. Although pineapple fibers were slightly different, there were certain similarities with previous findings. In biopulp colonized by *P. ostreatus* and *T. versicolor*, the parameter *L** was statistically affected only by the holocellulose

content, with its value increasing depending on the holocellulose content (Table 2). In biopulp produced with *T. versicolor*, parameter *L** was negatively affected by the content of extractives in ethanol-toluene and NaOH solution (Table 2), while redness (*a**) increased significantly with the amount of extractives in ethanol-toluene.

Table 2. Pearson Correlation between Color Parameters and Chemical Components of Biopulp Colonized by Two White-Rot Fungi

White-rot Fungus	Color Parameter	Ethanol-toluene	NaOH	Holocellulose	Lignin
Pleurotus ostreatus	*L**	-0.38	-0.47	0.63*	-0.15
	*a**	0.39	0.26	0.36	0.11
	*b**	0.43	0.53	0.34	0.23
Trametes versicolor	*L**	-0.63*	-0.61*	0.67*	-0.20
	*a**	0.72*	0.42	-0.34	-0.13
	*b**	-0.02	0.23	-0.33	-0.39

* Statistically significant at the 95% confidence level

In sum, the increase in lightness, decrease in yellow hues, and the neutrality between redness and greenness indicates that the pulp produced by fungal colonization was perceived as white (Perng *et al.* 2015), which was due to the increase in the proportion of holocellulose in the biopulp (Table 2). To illustrate this, the paper sheets prepared from biopulp are shown in Fig. 8, laid out according to colonization time, where the white hues of the sheets can be appreciated. The greatest whiteness was achieved on week 5 for *T. versicolor* (Fig. 8a) and week 7 for *P. ostreatus* (Fig. 8b). Furthermore, the color of this paper was compared to that of a commercial bond paper sheet with the following parameters: *L** = 84.06, *a** = -0.02, and *b** = 12.57. Figure 7d shows the tendency to color change (ΔE^*) with time. Biopulp produced by colonization with *P. ostreatus* showed a tendency to decrease its color change, very similar to that shown by biopulp exposed to *T. versicolor*. Nevertheless, the least difference in regards to commercial paper appeared during weeks 4 and 5.

CONCLUSIONS

1. Fibers extracted from pineapple leaves were subjected to colonization with *Pleurotus ostreatus* and *Trametes versicolor* for production of biopulp, with yields between 55% to 70% in periods from 4 to 8 weeks, and 35% to 50% in periods from 2 to 6 weeks, respectively. Then this lignocellulose material can be transformed in pulp with aproppiate equiment and process and to produce paper from PALF with unit operations at industrial scale.
2. A greater colonization period significantly increased the amount of holocellulose and considerably decreased the amount of lignin and extractives in ethanol-toluene and NaOH solutions. These changes were confirmed by thermogravimetric analysis (TGA) and the FTIR. Furthermore, these signals in temperatures and the peaks of vibration confirmed the change at 5 weeks of colonization in *P. ostreatus* and at 4 weeks in *T. versicolor*.

3. Colonization by *P. ostreatus* and *T. versicolor* caused the color of the pulp to rise in lightness (*L** parameter) and decrease in yellowness (*b** parameter), but little variation was observed in red and green hues (*a** parameter), indicating that the biopulp presented considerable whiteness.

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