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Enzymatic activities and analysis of a mycelium-based composite formation using peach palm (*Bactris gasipaes*) residues on *Lentinula edodes*

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Abstract

By seeding fungus on top of industry residues, a mycelium can grow and form a compact network structure; however, it may not develop due to lack of optimal nutrients from the substrate. Consequently, peach-palm residues can be a potential alternative; so, to test this hypothesis, this work evaluates the effect of peach-palm residues as substrate for the growth of mycelium based on *Lentinula edodes*. They were also supplemented with cassava bran and various sources of nitrogen-ammonium sulphate, potassium nitrate, and soy flour—to analyse its effects on its physico-chemical, enzymatic activities, and thermal and mechanical properties of the final composite at 12 and 20 days of cultivation. This mycelium was able to grow at optimum source treatment conditions, which depends on the ratio of Carbon to Nitrogen, within only 12 days of inoculation. Furthermore, the enzyme activities directly correlate with the mycelium growth with optimum conditions of pH, water activity, and moisture for *L. edod*es to grow having lower enzyme activities for a well-developed composite; whereas higher activities were seen for a weakly developed material, and this material demonstrates mechanical and thermal properties similar to common mycelium-based composites. Therefore, this work demonstrates that peach-palm residues can be a potential alternative for mycelium-based composite.

Keywords: Filamentous fungi, Agro-industrial residues, Mycelium foams, Enzyme properties, Hydrolytic enzymes

Introduction

The peach-palm tree, *Bactris gasipaes,* is culturally and economically important in Latin America, and it has been used from the inhabitants of the Amazon forest for centuries, benefitting from all of its parts (Mora-Urpí et al. 1997). Three main layers composes this tree, the external, middle, and internal sheaths; the external and middle sheaths, which protects and surrounds this tree, are highly fibrous. However, the internal layer, known as heart-of-palm, is the edible part (Bolanho et al. 2014; Clement et al. 2016). Additionally, Brazil is the largest producer, and consumer, of heart-of-palm in the world (de Oliveira et al. 2019) with a planted area of 27,603 ha and US$ 43 million of value of marketed production, aiming mainly to meet the country's internal demand (IBGE 2019).

The heart-of palm is mainly sold as picked or canned, with minimal process to preserve its contents that presents significant amounts of minerals (Mora-Urpí et al. 1997). However, to extract this, food source also comes with its consequences—about 90% are considered as residue (Zenni et al. 2018); for instance, the median and internal sheaths of this tree have a low degradation rate

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(Seben et al. 2012). It is generally thrown into the soil as composting, but becomes an environmental liability in the case of peach-palm residues.

Peach palm residues (Bellettini et al. 2017) are described to have a nutrient competition, mainly nitrogen, between the soil, for its composting process, and the plant. When peach-palm residues act as compost for lettuce, it leads to a lack of nitrogen availability to the plant and reduces its normal growth. In addition, authors recommend a period of maturation of these residues before composting, but such allocated time, which is between 2 and 6 months (Chang 1987; Bellettini et al. 2017), could well be undermined with alternative usages, since they are very nutritious (Helm et al. 2013).

Wood degrading fungi are commonly used to degrade lignocellulosic materials, which are categorized into white-rot, brown-rot, and soft-rot decomposition (Blanchette et al. 1994). From within the white-rot fungi order, stands out the *Lentinula edodes* fungus, known as "Shiitake" mushroom of the Basidiomycetes class. This fungus degrades lignocellulosic material, by producing active enzymes in a solid-state fermentation process (Elisashvili et al. 2008) that breaks down the plant cell wall, which is then used as a source of carbon and energy for its life cycle (Kües and Liu 2000).

From within the fungi structure, the mycelium form hypha and can merge with others to produce a random fibre network structure that have a few microns in diameter (Islam et al. 2017). By seeding mycelium on organic material, it can consume upon it by secreting hydrolytic enzymes (Glass 2004), and embeds them in such hyphae network that contains binding properties, which grows as an interconnecting fibrous network so as to form a composite board (Jones et al. 2017).

Mycelium-based-materials or -composites, are reported to be useful in a variety of applications (Antinori et al. 2020); such as an alternative of polystyrene foam (Abhijith et al. 2018), a material that is neither biodegradable nor compostable. These materials possess interesting properties such as low density, low energy consumption during production, biodegradable, and can grow on a wide range of substrates (Stella et al. 2017). Besides, after usage, they can be reused as animal supplement, organic fertilizer, among others (Teixeira et al. 2018).

Overall, researchers have been using such residues into natural polymers as reinforcement (Singha and Thakur 2009), to produce renewable materials with good overall properties (Ates et al. 2020). However, mycelium-based materials could be a potential alternative, as it consists on a very simple methodology (Islam et al. 2018). The most studied fungi for the formation of this material are the division from Basidiomycota which are known for branching their hyphae into cavities (Attias et al. 2020). The growth of this structure is dependent on the fungi used (Haneef et al. 2017), directly changing the material density, thickness, and topography.

However, most of the papers presently in literature provide little in terms of fungal species, substrate composition, and additional steps of composite formation (Antunes et al. 2020). This could be related to the fact that more than half is published by co-authors affiliated with commercial companies, as stated by a previous report (Attias et al. 2020); besides, their growing process is time-consuming (Zeller and Zocher 2012).

The biochemical mechanism during fermentation and the overall properties of the composites are also needed to be studied due to the limited research focused (Antinori et al. 2020). Furthermore, since the substrate directly influences the overall properties of the composite, it is important to understand the colonization trends and a detailed description of the substrate.

So far, only one study reported the usage of shiitake as an effective mycelium-based material, using coconut powder with wheat bran as substrate, on the effect of fungi growth and mechanical properties (Matos et al. 2019). Therefore, this work investigates the effect of solid-state fermentation from *L. edodes* on peach-palm sheath fibres using three different nutrient growing feed source treatments and evaluates the amount of enzymes produced. In addition, to investigate the overall properties of the mycelium-based composite produced with the best formulation.

Materials and methods

Characterization of the substrate

The fungus *Lentinula edodes* (Berk.) Peglar, maintained on Castellani method (Castellani 1967), was obtained from the macro-fungi culture collection at Laboratory of Nontimber Products within the Brazilian Agricultural Research Corporation—EMBRAPA FLORESTAS (Colombo, PR, Brazil) (internal code EF 50) registered on AleloMicro database of EMBRAPA as [BRM 055640—BRM stand as Brazil Microorganism (EMBRAPA)]. The isolate was cultivated and kept in Petri dishes containing potato dextrose agar (PDA) medium for 7 days, in an environmental chamber at 25 °C in the absence of light, and after growth, was stored at 4 °C.

The *Bactris gasipaes*, peach-palm external inedible sheaths, used in the study were collected at the region within EMBRAPA Florestas in Colombo, Brazil. The external sheaths were crushed in a Disintegrator/Chopper/Grinder (DPM Júnior, Nogueira LLC, Brazil) reaching a final length of 2.5 cm.

The crushed sheaths were oven-dried at 60 °C for 24 h, followed by supplementation with cassava bran and three sources of nitrogen (ammonium sulphate, potassium

nitrate, and cooked soy flour) (Table 1), which were autoclaved at 121 °C (1 atmosphere pressure) for 15 min. An experimental design with three replications was applied at the central point, consisting of seven feed source treatments with equivalent nitrogen concentration.

After cooling, the sheathes were inoculated in two batches. The first batch was inoculated with 1/6 from mycelium plate of *L. edodes EF50* grown in PDA medium (solid inoculum), while the second batch was inoculated with 2/6 from mycelium plate of *L. edodes* that was previously crushed for 15 s in a modified Socarean solution (Couri and Farias 1995) (liquid inoculum). Cylindrical flasks (8.6 cm in diameter and 13.6 cm in height) were incubated using a BOD incubator (MA1415/450, Marconi Ltd, Brazil) at 25 °C, in the absence of light, for 12 and 20 days of culture for both types of inoculum.

Moisture content (%), water activity (w_a), and pH

The moisture content was determined by the gravimetric method described by (Hermann et al. 2013). Briefly, samples were dried at 60 °C until a constant weight was reached, and the content was calculated through the difference between the dried weight and the initial weight.

The pH was determined using the potentiometric method (pH meter, Tecnal) (Lutz 2005). Briefly, 1 g of substrate was mixed in 10 ml of distilled water for 10 min and the pH was measured afterwards.

The water activity (w_a) was obtained by the relation between the vapor pressure of the culture medium (Pm) to that of the pure water (Pw), as described by the procedure from AOAC international (AOAC 2016). The w_a values were measured by a water activity meter (3TE Aqualab series 3B, Decagon Devices Inc., USA).

Enzymatic activities

The extraction of the enzymes complexes was performed by vacuum filtration. The extracts were centrifuged and maintained at 4 °C. Xylanase activity was determined by the decrease of reducing sugars carried out by xylan "birchwood", as described by (Bailey et al. 1992). The enzyme activity was initiated using 0.9 mL sample of 1% xylan with 0.1 mL of the enzyme extract, and the amount of sugar decrease was measured by the 3.5-dinitrosalicylic (DNS) method (Miller 1959).

The activities of endo-β-1,4-glucanase, or carboxymethylcellulase CMC, (EC 3.2.1.4) and exo-β-1,4-glucanase, or avicelase (EC 3.2.1.74) were determined according to the method described by (Tanaka et al. 1981). It consisted of conducting the hydrolysis of two solutions: the first was 0.44% carboxymethylcellulose solution in a buffer solution of 0.05 M sodium acetate (pH 5.0) for the EC 3.2.1.4 fraction activity; and, in the same buffer, 1.1% suspension of microcrystalline cellulose (Avicel) for the EC 3.2.1.74 fraction. The reaction was initiated by adding 0.9 mL of enzymatic extract in 0.9 mL of substrate, which was reacted for 60 min. The amount of reducing sugars was determined by the DNS method (Miller 1959).

β-Glucosidase activity (EC 3.2.1.21) was determined according to (Wood and Garcia-Campayo 1990). Briefly, 1 mL of 15 mM cellobiose solution (diluted in sodium acetate buffer pH 5.0) was added into 1 mL of enzymatic extract, and incubated at 50 °C for 30 min. The reaction was stopped by immersing the tubes in boiling water for 5 min. After transferring to a cold-water bath, the glucose produced was determined using a liquid enzyme glucose Kit (Doles Reagentes Inc., Brazil) based on the glucose oxidase–peroxidase reaction.

The statistical analysis of the experimental planning was performed using multivariate analysis. The model was simplified to exclude terms that were not considered statistically significant ($p > 0.05$) by analysis of variance (ANOVA). Analysis of the feed source treatments were initially performed as independent variables for the enzymatic activities, pH, moisture, and water activity, to assess which of the sources had a significant effect. A second-order polynomial fit was adjusted to the treatments on all variables. Pareto diagram was also obtained to investigate which of these treatments had significantly contributed to the work. All of these processes were performed using the STATISTICA software, version 8.0 (Stat Soft Inc., Tulsa, OK, USA).

Table 1 Feed source treatments used in this study, the acronym follows the feed source used followed by its composition ratio, as percentage

Sample name	Feed source (%)		
	Ammonium sulphate	Potassium nitrate	Soybean flour
A100	100.0	–	–
P100	–	100.0	–
S100	–	–	100.0
P50 S50	–	50.0	50.0
A50 P50	50.0	50.0	–
A50 S50	50.0	50.0	–
A33 P33 S34	33.0	33.0	34.0

Qualitative analysis of microbial growth

Through visual observation, the flasks containing fungus and cellulose fibres were determined according to the standard adapted from ASTM (American Society for Testing Materials) G21-90 (1990) (ASTM 1990), depicting the following growth ranges:

- (−) lack of growth.

• (+) little growth, with presence of small fragments from mycelium produced in the medium.
• (++) moderate growth with the appearance of a thin pellet on the surface of the medium.
• (+++) optimum growth of mycelium within half to full growth from the flask volume.

Pre-compression tests

The pre-composite formed from the best mycelial growth result (S100) was carefully removed from the flask with the help of a spatula, without breaking the structure. The compression test (ASTM 165-07) (ASTM 2017) was performed as a preliminary test, to analyse its behaviour for further studies using a universal testing machine model DL2000 and EMIC brand. After compression, these samples were oven-dried at 60 °C for 3 h. They were further used in the next studies naming it as composite cold-pressed (from S100 condition).

Composite formulation

To verify the composite integrity, the condition with the best mycelium growth was repeated using a larger mould, higher surface area, and smaller height ($350 \times 250 \times 11$ 5 mm = length × width × height), than the composites cold-pressed that were grown in flasks from glass. They were labelled as composite non-pressed (from S100 condition); after 12 days, samples were oven-dried at 60 °C for 3 h.

Carbon/nitrogen and ash analysis

The percentage of carbon (C), nitrogen (N), sulphur (S), and hydrogen (H) (CHNS) in the samples of pure soy flour, cellulose peach-palm sheath fibres, and the mycelium-based composite were determined using an elementary analyser equipment CHNS (CHNS Elementar, model Vario MACRO Cube, Langenselbold, Hesse, Germany).

Histological sections

Histological sections were performed with a microtome (Microm GmbH, Walldorf—Germany, Type HM325). Peach palm sheath fibres and composite (formed from S100 condition) were analysed. Samples previously chopped with a maximum size of 1.0 cm were included in paraffin and sectioned on a rotating microtome. They were double stained with 1% astra blue and 1% safranin which were mounted on permanent slides with synthetic resin, according to the conventional techniques (Kraus and Arduin 1997). The samples were observed and analysed in the Axio imager A2 microscope.

Scanning Electron Microscopy (SEM)

To observe and compare the formation of the mycelium from the fungus in the peach-palm sheath fibres, Scanning Electron Microscopy (SEM) was used. Small samples from the composites (formed from S100 condition) and pure peach-palm sheaths were covered using a gold sputtered equipment. For the mycelium-based composite, the equipment used was a Shimadzu—SSX-550 Superscan; whereas for the pure sheath fibres, a Hitachi TM-1000 was used.

Compression test

Three samples from the mycelium-based composite were cut to the following dimensions $60 \times 60 \times 20$ mm^3 and followed the ASTM 165-07 (ASTM 2017) standard. The compression tests were performed with a universal testing machine brand EMIC, model DL 2000. A load cell of 2 tons was used for the test.

Water absorption and swelling

Since the composites are hydrophilic, the water absorption capacity and the swelling of the composite were evaluated. The standard procedure used for water absorption was the ASTM D-570 98 (ASTM D570-98 2018) and the EM 317 for swelling (British Standards Institution BSI 1993).

Specimens of dimensions from $20 \times 20 \times 20$ mm^3 were cut from the mycelium-based composite. The material was weighed and measured before and after each period, to determine the percentage of water absorption and swelling at constant temperature 23 °C. The specimens were immersed in water for a period of 2, 24, and 48 h.

Thermal analysis (DTG and DSC)

The stability of peach-palm sheath fibres, and the composites, were investigated by thermogravimetric analyses (TG). The equipment DTG-60H—Shimadzu was used for TG/DTA and DSC-60A was used for DSC technique, using about 5 mg of each sample. The heating rate was 10 °C/min until reaching 600 °C, in a nitrogen atmosphere at 20 mL/min, and for the DSC, aluminium pans were used.

Results and discussion

Moisture content (%), water activity (w_a), and pH

Physico-chemical analysis of the feed source treatments related to the growth of fungus (Fig. 1) presents different profiles and efficiencies. The time period used in this study had initial pH values ranged from 3.65 to 5.65 for all treatments (Fig. 1i, ii). The highest pH values occurred using pure soy flour, for both inoculum; also, significant differences occurred for all pure treatments. Nonetheless, treatments containing potassium nitrate and ammonium sulphate had lower pH values, which can be observed by their surface contour profiles (Fig. 1vii and Additional file 1: Fig. S1A), and is related to their higher dosage. For

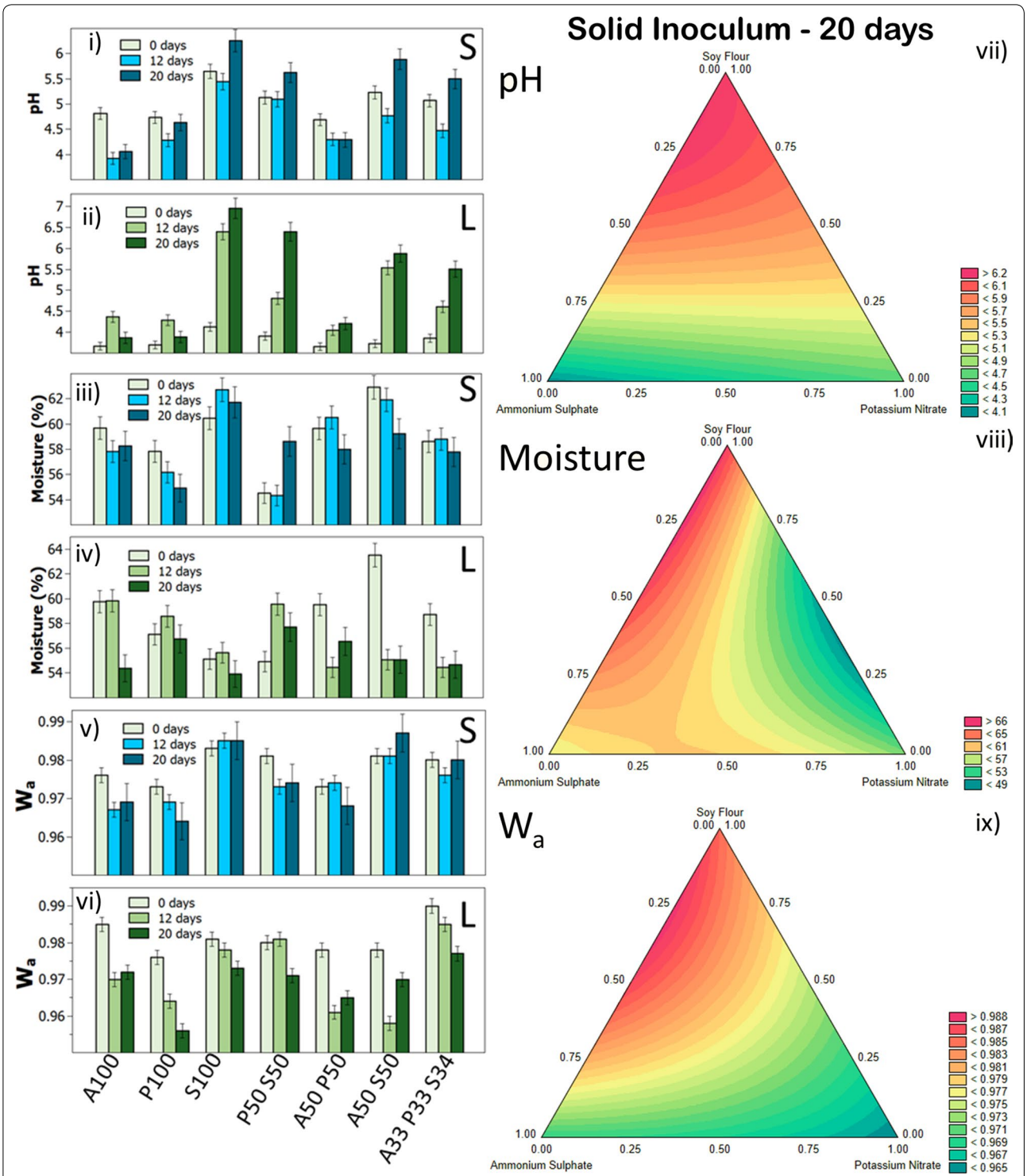

Fig. 1 Physico-chemical results in the studied time periods for pH, moisture, and water activity for solid (i, iii, v) and liquid (ii, iv, vi) inoculum, with their specific surface contour plot relating to a solid inoculum (vii–ix) at 20 days

the highest pH region, they were located near the corners of the treatments containing soy flour source, similar to a previous report (Hermann et al. 2013).

Some species of basidiomycetes have a self-regulating pH characteristic, with a tendency to stabilize at an optimum pH value for their growth, regardless of the initial

pH value (Mata et al. 2016; Chicatto et al. 2018); therefore, it might be the reason for the pH growth in feed source treatments containing soy flour. Nonetheless, treatments without soy flour had lower pH, and it further decreases with time compared to their initial values. This could be inferred to the ratio content of carbon nitrogen which may impart the mycelium growth; so, the pH of the medium does not self-regulate and the fungus cannot control the environment as it occurred at pH above 5.0 (Carvalho et al. 2018).

Samples moisture varied slightly depending on the treatment source used—ranging between 54 and 62% (Fig. 1iii, iv), similar to another report using *L. edodes* for solid-state fermentation (Bentolila de Aguiar et al. 2013), a condition favourable for the growth of the fungus. Consequently, the culture media must not have low relative moisture, since the water content is essential for the growth and metabolism of *L. edodes* (Antunes et al. 2020). Nonetheless, the results reported a decrease in moisture after the last cultivation time interval, for the majority of the samples, also detected in another work (Hermann et al. 2013); whereas the treatment containing only soy flour had the highest moisture content (58–60%). In a well-developed mycelium, it is possible to obtain precise values of moisture, but variations may occur depending on the fungus strain and treatments used.

The surface contour plot exhibits (Fig. 1viii) that treatments containing highest moisture was found for higher concentration of soy flour; though for the time period of 12 days and liquid inoculum (Additional file 1: Fig. S2A), the region with the highest values was found within a well-balanced source of soy flour and potassium nitrate.

For the water activity values (w_a) (Fig. 1v, vi), all feed source treatments exhibited activities greater than 0.955, and they were increased at the final studied time period for the majority of the treatments. These values are reported to be within a region for optimum fungus growth (higher than 0.950) (Pandey et al. 2000), which is also an indicative of large water available for the microorganism to develop.

The majority of the treatments presented to be significant in regards to their physico-chemical profiles. Increased values was perceived for the mixture of soy flour and ammonium sulphate for solid inoculum; while for liquid inoculum, optimum values were obtained for soy flour with potassium nitrate (Additional file 1: Fig. S3A–S6).

Moisture content (%), water activity (w_a), and pH of the mycelium-based composites

The physico-chemical characteristics of the mycelium-based composites exhibit some differences in the process performed (Table 2). The moisture exhibited to be positive for the growth of the fungus—as expected—and the low values are related to the drying methodology. Higher moisture from a more compacted material, cold-pressed, may be related to an increased difficulty of the water to be released compared to a non-pressed composite.

Nonetheless, the values of moisture content, w_a, and pH were similar to previous reports using *L. edodes* as solid-state fermentation biomass (Chicatto et al. 2014; Pedri et al. 2015).

Enzymatic activities

The enzymatic activities for avicelase, carboxymethylcellulose, β-glicosidase, and xylanase exhibited a similar behaviour, when comparing solid and liquid inoculum (Fig. 2). For the majority of the cases, lowest enzyme activities occurred when soy flour was used, which can also be seen with the surface contour plot (Fig. 3 and Additional file 1: Figs. S7A, S8). Contrarily, sources containing ammonium sulphate, followed by potassium nitrate, had the highest activities on all enzymes.

The composition of the substrate is an important factor for the growth and expression of various fungi, especially when the nutrients contains nitrogen and carbon. Therefore, a fungus may extract these elements more easily within a mixture of ammonium sulphate and/or potassium nitrate (Rughoonundun et al. 2012). Nonetheless, it has been reported that nitrogen is a key element in the growth of *L. edodes* (Lin et al. 2015). Therefore, an increase in organic matter content is expected where mycelium colonization is more advanced (Attias et al. 2020).

Table 2 Physico-chemical, compressive, and sorption kinetic properties of the mycelium-based composites studied

Sample	Physico-chemical						Compressive		Sorption kinetics (dH_2O)	
	M_0 (%)	M_f (%)	w_{a0}	w_{af}	pH_0	pH_f	Modulus (kPa)	Strength (kPa)	Weight increase (%)	Thickness expansion (%)
Cold-pressed	60.4	14.1	0.98	0.50	5.6	5.8	–	–	245 ± 3	21.5 ± 0.5
Non-pressed	59.7	8.05	0.99	0.52	5.8	6.0	238 ± 16	223 ± 10	351 ± 4	18 ± 1

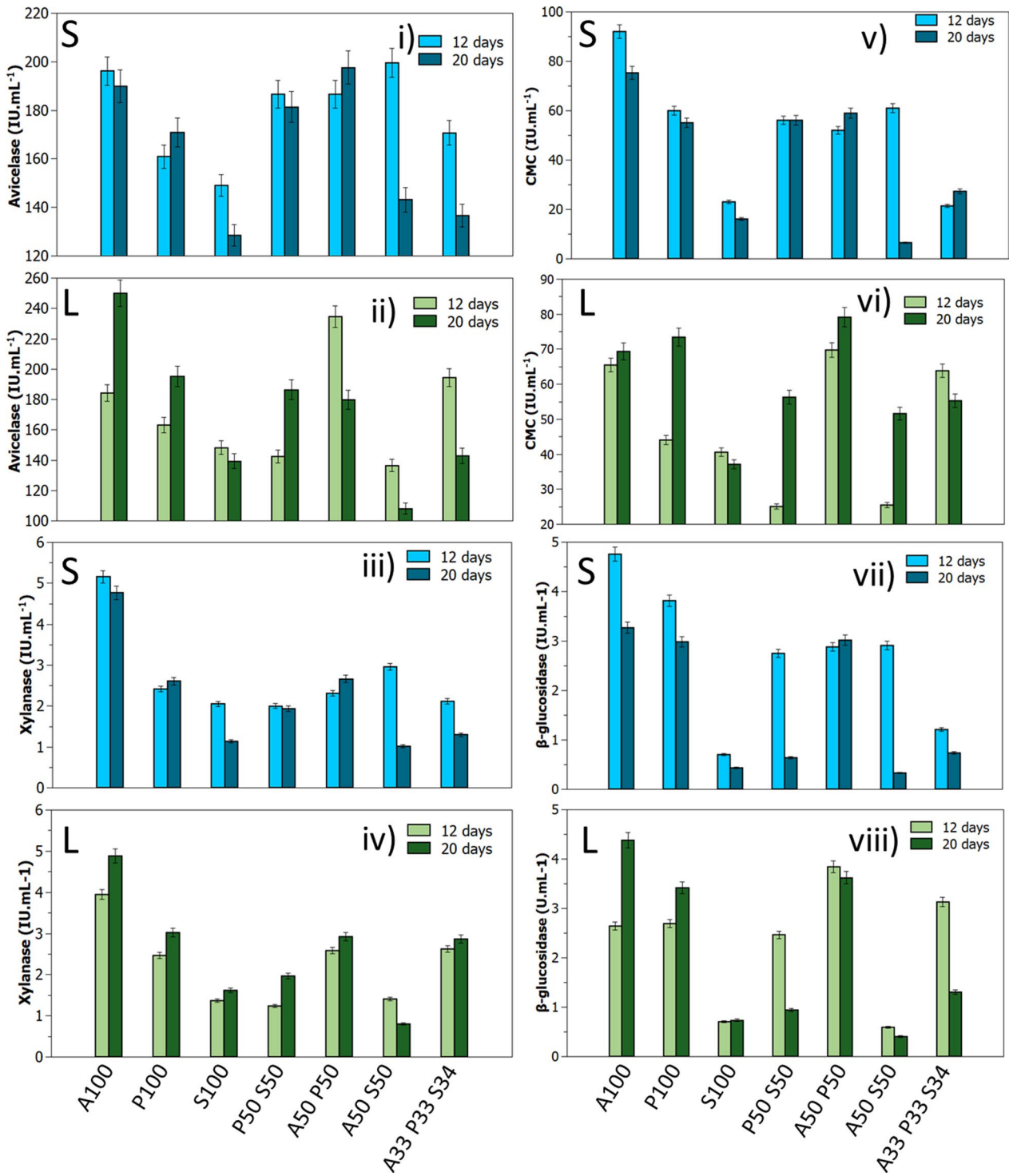

Fig. 2 Enzymatic activities of avicelase (i–ii), xylanase (iii–iv), CMC (v–vi), and β-glucosidase (vii–viii) using a solid or liquid inoculum at two studied time intervals (12 and 20 days)

The activities of the various feed source treatments using *L. edodes* evidenced it to be a good degrader of the peach-palm residue because of its increased enzyme activities. In addition to its nutritious for fungus growth, it is possible that, within 12 days, the majority of the growth from the mycelium might

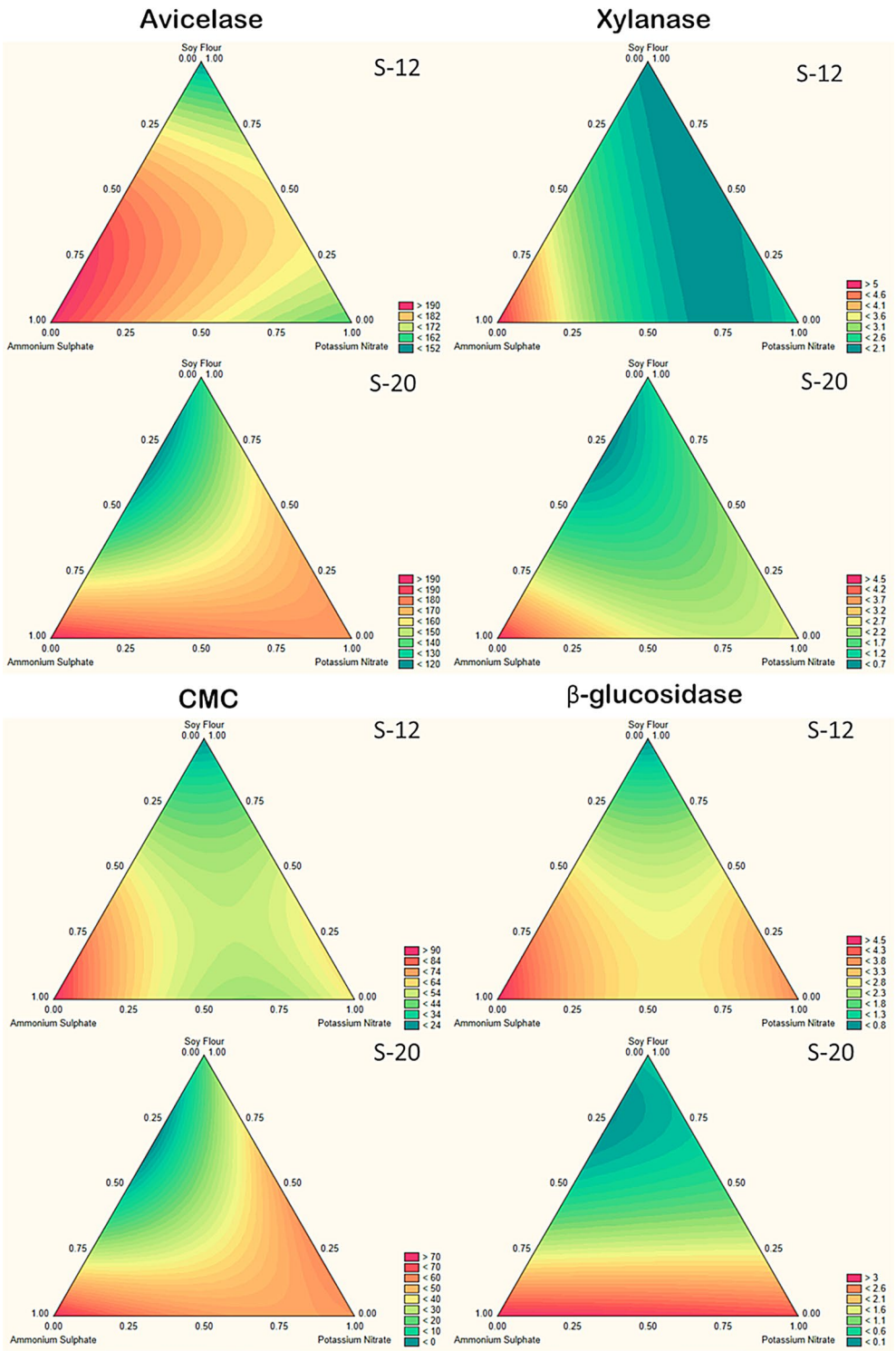

Fig. 3 Surface contour plot of avicelase, xylanase, CMC, and β-glucosidase activities under different feed source treatments using the solid (S) inoculum at time periods of 12 and 20 days

already occurred, and the trend saw within these results could be the last digestive enzymes cycles, which could be quite the opposite for the other treatments. Nonetheless, the majority of the enzyme activities presented a similar trend from a previous work that used *L. edodes* with solid-state fermentation (Philippoussis et al. 2011).

The presence of soy flour, which influenced a decrease in enzyme activity, may be related to the nitrogen sources (Philippoussis et al. 2011; Chicatto et al. 2018); in which higher content decreases the hydrolytic system, but induces an increase in oxidative enzymes. Nonetheless, ammonium sulphate can also be converted into proteins by microorganisms, and, although these values seems to show a direct relation, their actual profile is also dependent of the substrate used—peach-palm residues.

From within each enzyme activity, avicelase and xylanase lowest activities occurred with treatment containing soy flour, or with a mixture containing potassium nitrate (Fig. 2i, ii and iii,iv); however, it actually exhibited the highest activity for avicelase (199.46 UI mL^{-1}) using solid inoculum within the time period of 12 days (Fig. 2i). Apart from that, avicelase highest activities in all groups were produced in treatments with ammonium sulphate, either as pure source or with the addition of potassium nitrate (234.6 and 249.9 UI mL^{-1}, for 12 and 20 days, respectively).

For xylanase, the highest activity was found for pure sources of ammonium sulphate (5.15 UI mL^{-1} and 4.87 UI mL^{-1} within time period of 12 days, using solid inoculum, and 20 days using liquid inoculum respectively). Overall, the enzymatic activities' profile of avicelase and xylanase exhibits a similar behaviour when comparing the time period and the inoculum used (Fig. 3 and Additional file 1: Fig. 7a), and most of the studied sources used were statistically significant in the enzymatic activity values (Additional file 1: Fig. S9, 10).

Since peach-palm sheaths are reported to contain 19.5% of lignin on a dry basis, and are lower than other common organic residues such as soy, sugarcane bagasse, rice, and corn (Franco et al. 2019), avicelase activities produced in this study were higher than other residues using another white-rot fungi *Agaricus brasiliensis*, same Agaricales order of fungi from *L. edodes* (de Siqueira et al. 2010). However, low values of xylanase were found herein compared to the aforementioned residues.

For CMC and β-glucosidase enzymes (Fig. 2v–viii), the highest activities were found for treatments containing pure ammonium sulphate, or with a combination of soy flour (91 IU mL^{-1} and 4.7 IU mL^{-1} at 12 days using solid inoculum; also, 79 IU mL-1 and 4.4 IU mL^{-1} at 20 days using solid inoculum for CMC and β-glucosidase respectively).

Their enzymatic profile also exhibits that, for solid inoculum (Fig. 3), ammonium sulphate region had increased activity on all time periods, and for liquid inoculum (Additional file 1: Figure S8A), potassium nitrate had a major part on CMC enzyme. For β-glucosidase enzyme activity, ammonium sulphate had a major role; though on a shorten time period, 12 days, the mixture of ammonium sulphate and potassium nitrate region had higher activity.

Lowest enzyme activity region was found within the mixture of soy flour and ammonium sulphate for the time period of 20 days, using low concentrations of ammonium sulphate (Fig. 3—CMC and β-glucosidase); while for a shorten time period, 12 days, pure soy was the least effective on enzyme activity. However, only ammonium sulphate and potassium nitrate were able to exhibit a significant difference for CMC and β-glucosidase (Additional file 1: Figure S9–12).

Endoglucanase, CMC, activity is reported to be produced in moderate quantities for *L. edodes*, this could be further enhanced depending on the amount of hemicellulose (Philippoussis et al. 2011). Furthermore, variations on the cellulolytic activity have been reported for *L. edodes* which depends on the fungus growth stage (Chicatto et al. 2014). It has been reported that a deceleration in mycelium growth rates could occur after the third colonization week, leading to a decrease in the enzyme activity because of the limitation of utilizable nutrients and carbon sources (Philippoussis et al. 2011).

Ammonium sulphate, as nitrogen source for the fungus, can have positive effects for CMC and proteins, but can inhibit the production of avicelase. The *L. edodes* strain can also result in variation of the enzyme activity (de Siqueira et al. 2010). Therefore, due to differences in chemical composition of substrates for cultivation, it is important to select genotypes with suitable characteristics for growth in the existing substrate; which in turn depends on the fungus ability to utilize the majority of the substrate components as nutritive elements (Elisashvili et al. 2008). Likewise, the presence of certain compounds, such as phenolics, from the substrate could also inhibit fungus growth (Mata et al. 2016).

Qualitative analysis of mycelium growth and composite formation

The growth of *L. edodes* over time (Fig. 4) exhibits that the lowest microbial growth was observed in feed source treatments containing either pure ammonium sulphate, or potassium nitrate; also, a mixture of those two had a small formation of *L. edodes* mycelium in the medium.

The treatment with a mixture of all supplements, A33 P33 S34, had no mycelial growth with the liquid inoculum, for both endpoints studied herein, and this could

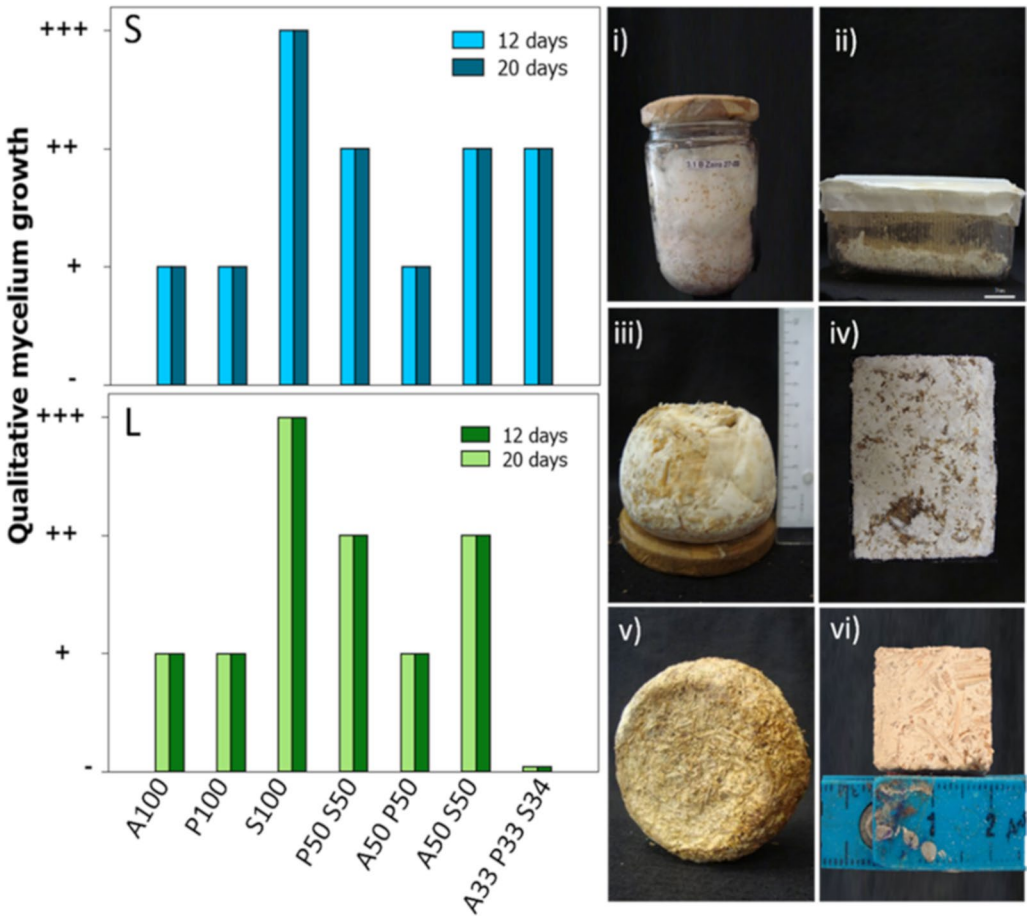

Fig. 4 Qualitative mycelium growth from the studied feed –-source treatments, varying from lack to optimum at time points of 12 and 20 days using a solid (S) or liquid (L) inoculum. Also, macroscopic images of the S100 treatment (i–ii) before and (iii–iv) after mycelium-composite removal from the flask, (v) is after compression and drying (cold-pressed); (vi) oven-dried, grounded, and final piece (non-pressed)

have been due to the concentration of the elements (Helm et al. 2013). In addition to the fact that, within both time periods, the growth rates are directly related to their enzymatic activities, as discussed in previous "Moisture content (%), water activity (w_a), and pH of the mycelium-based composites." section.

Mixing soy flour with the other treatments had a moderate mycelial growth for *L. edodes*, corresponding to half of the substrate covered by a mycelium network, for both time periods. Additionally, the treatment with the highest mycelial growth was found for pure soy flour source, in both inocula, in which the fungus reached the surface of the bottle.

The enzymes released from the mycelia hyphae contributes to the mycelium-composite formation, by degrading the substrate and increasing its mycelia density (Tacer-Caba et al. 2020). It is important to remind that the yield of materials based on mycelium depends on the strain, medium, conditions of growth, and growth cycle (Philippoussis et al. 2011; Elisashvili et al. 2015; Attias et al. 2019). Nonetheless, mycelium growth of *L. edodes* is affected by substrate cellulose, hemicellulose, and lignin proportions, along with nitrogen content (Philippoussis et al. 2011).

It has been suggested that if a media is too difficult to digest, it can induce the mycelium to secrete more enzymes—having a wide and fast surface but reduced volume, an effect that occurred for sources containing mainly ammonium sulphate. This effect can be reversed if the media is rich in compounds that are easily digested by the mycelium, such as D-glucose, leading to an increase in the material volume (Antinori et al. 2020). In the case of peach-palm sheaths, they contain higher values of non-reducing and reducing sugars (Helm et al. 2013) than other common residues, such as sugarcane bagasse (Rabelo et al. 2015), and may have contributed to the further growth of this fungus.

Therefore, due to the optimum condition for *L. edodes* to grow using pure soy flour, mycelium grew more than the other conditions, and what is seen by their enzymatic profile is at the end of the composite formation. This suggests that the majority of the enzyme activity, in digesting and consuming the residue, already occurred prior to the first time period observed in this study; alternatively, other sources shown higher values due to the difficulty in digesting, relating to a poor growth of the mycelium. This trend was already reported in a previous work of our group, with the usage of residues from *Eucalyptus benthamii* using the same fungus (Pedri et al. 2015).

The visual mycelial density, for a supplement containing pure soy flour, features a compact mycelium block on the peach-palm sheath fibres (Fig. 4i–iv), and this source preference by the fungus may be related to the presence of amino acids (Leonowicz et al. 1991). The nutritive parameters of this residue were able to produce a mycelium composite, a significant achievement by the usage of this fungus compared to substrates containing higher values of hemicellulose and nitrogen (Philippoussis et al. 2011). Nonetheless, the contents of cellulose and hemicellulose on peach-palm sheaths are lower than other common residues, such as bamboo and sugarcane (Franco et al. 2019), and lower hemicellulose content is a good indicative for mycelium development (Gaitán-Hernández et al. 2011).

Because of the limited space found for the fungus to grow on the flask, they reached to its top, and a volume from about half the size of the flask was formed. Therefore, they were compressed to remove excess of water, and evaluate how this material would behave for future analysis.

This treatment and period, S100 and 12 days, using a solid inoculum was chosen, due to the higher mycelia growth rate within few days of cultivation. However, the liquid inoculum did not show the same characteristics of mycelial growth, as its interior was not completely colonized by the fungus, containing empty spaces, and it led it to be impossible to continue the mechanical test.

The interaction of the hyphae with the fibres formed a compact material after compression. According to the curves obtained in the test, points were estimated to determine the force and deformation (Additional file 1: Figure S13). The force was applied until the rupture of the material, which was close to 4 cm of deformation, and it was labelled as cold-pressed.

However, to compare this composite, a new one was produced using the same treatment of cold-pressed with a bigger size mould or flask in length. The composite—labelled as non-pressed—was able to grow evenly and detached over the days from the mould on its own (Fig. 4iii, iv); therefore, it is possible to tailor its volume based on the media and source used for the fungus to grow. In addition, the material formed within 12 days was very similar to the one formed at 20 days, and it was also the reason this time period was selected.

This material had some characteristics similar to a polystyrene presenting a rigid structure of polysaccharides (matrix) over fibres that are biodegradable and with characteristics of fibre/matrix association.

Elemental analysis by CHNS

A relation of carbon and nitrogen was analysed in the peach-palm sheaths, soy flour, and the mycelium-composite (Table 3), exhibiting that the presence of soy in the substrate had positive effects on the time and in the mycelial growth. It is already known that the addition of supplements increases the levels of nitrogen and available carbohydrates (Pedri et al. 2015).

Supplementation with flour has shown that, for the cultivation of *L. edodes*, it is necessary a source of nitrogen within the substrate (Queiroz et al. 2004). It has been reported that the C:N ratio should be between 30 and 40 to favour the mycelial growth of *L. edodes* (Song et al. 1987), which indicates the relation between growth rate and availability of nitrogen. Therefore, since peach-palm sheaths already possess a relation of C:N within a favourable growth, it is also possible that soy flour—containing the lowest amount of nitrogen than other supplements—may have contributed to the growth of this material.

Histological sections

The microscopic images of the lateral (Fig. 5i) and transversal (Fig. 5ii) sections of the plant cell from peach-palm sheaths exhibit, on the transversal section, larger circumferences related to the sap transport duct (arrow). Like others lignocellulosic materials, peach-palm sheaths are basically composed of cellulose and lignin.

Peach palm sheaths that underwent solid-state fermentation using *L. edodes* fungi presented a similar aspect of the raw sheaths, but with traces of the soy and cassava flour (Fig. 5iii and iv). The same region examined for the pure sheaths shows that, after formation of the mycelium composite, they were further degraded, and a disorder of small fragments stands out around the fibres [arrows

Table 3 Elemental analysis of the studied materials, obtained from the CHNS equipment

Material[a]	Carbon	Nitrogen	C:N	Sulphur	Hydrogen
Peach palm sheaths	40.87	1.14	42:1	0.164	7.33
Soy flour	54.56	7.59	8:1	0.236	10.41
Non-pressed composite	42.61	4.51	11:1	0.273	7.51

[a] For all components, the results were calculated from the average

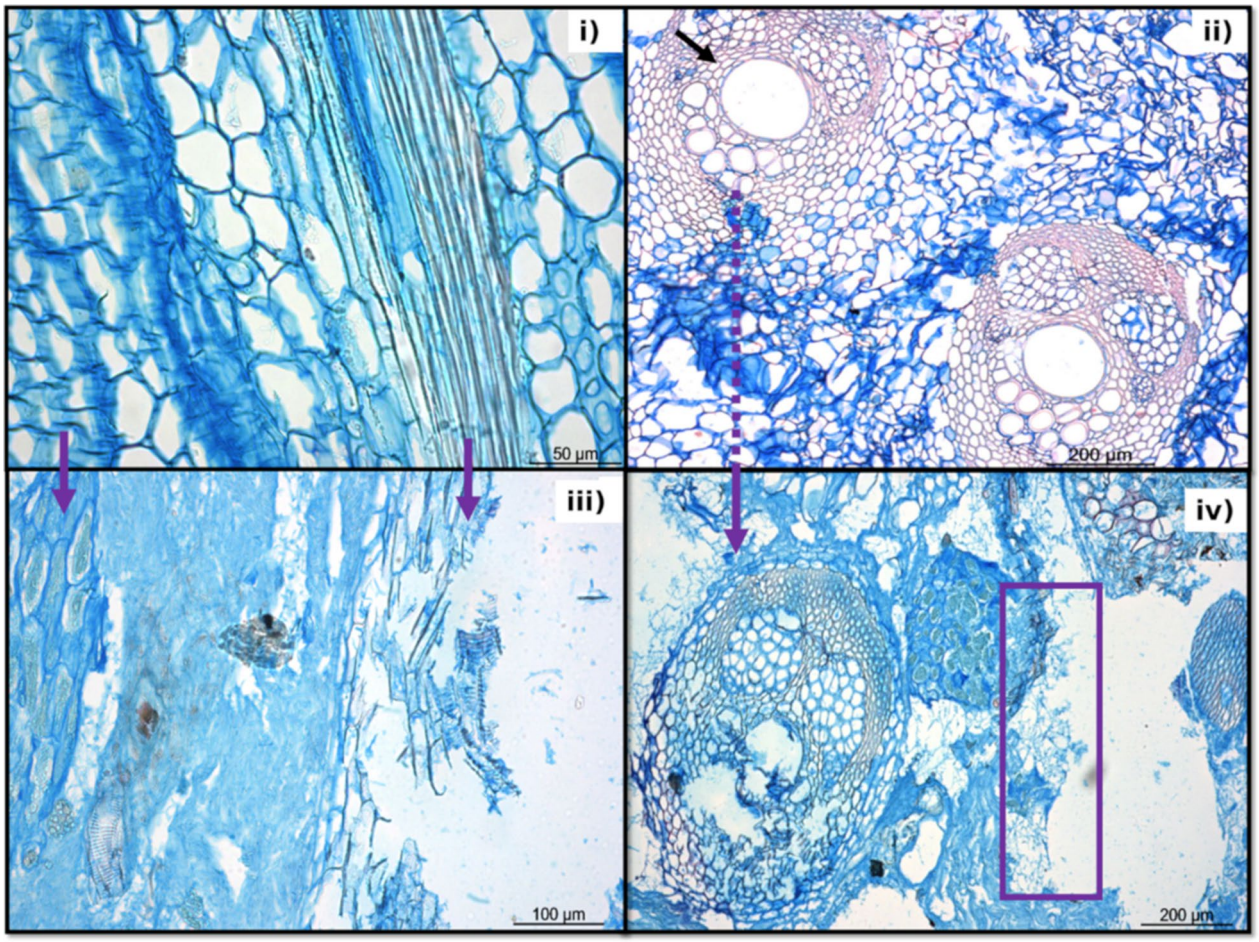

Fig. 5 Histological sections of peach palm sheaths (i) side ×400 and (ii) cross-section ×100. Cold-pressed mycelium-based composite side (iii) ×200 and (iv) cross-section ×100. The arrows indicate the degradation within the same region after the composite formation

in (iii–iv) and marked area in (iv) from Fig. 5], which are directly linked to *L. edodes* action; though, no fungus hyphae were found in the images.

The biodegradation of lignocellulosic materials by a fungus primarily occurs in an extracellular form, since they must initially be depolymerized to smaller compounds to be susceptible to be transported by the cell wall and intracellular fungi metabolism. Moreover, fungi-degrading action occurs through penetration of their hyphae in the lumen of the plant cells (Rodríguez et al. 1997); afterwards, their hyphae produces a great diversity of extracellular metabolites, which then act by degrading the plant cell wall.

Scanning electron microscopy (SEM) analysis

The SEM images of the mycelium-based material were analysed on the cold-press composite, non-pressed also presented a similar structure—not shown—and their morphology exhibits an interconnected network, formed from *L. edodes* mycelium (Fig. 6i), and also presents their hyphae (Fig. 6ii, iii) which corresponds to the composite matrix.

The substrate particles are shown to be deeply hidden by the mycelium, containing a hypha with a diameter on the order of 1–5 µm (Fig. 6ii, iii); they are either loosen due to degradation, or physically twisted with the mycelium, a morphology previously reported for mycelium-based composites (Liu et al. 2020). The fact that insufficient fungal had grown throughout the whole composite limits the bonding between the hyphae and the substrate, and is reported to be responsible for the limited mechanical performance (Islam et al. 2018; Liu et al. 2020).

Furthermore, the bind due to the network from the mycelium also affects its mechanical properties. The tensile resistance of mycelium-based composites is more influenced by failure of the binder than the substrate itself (Ziegler et al. 2016; Jones et al. 2018b).

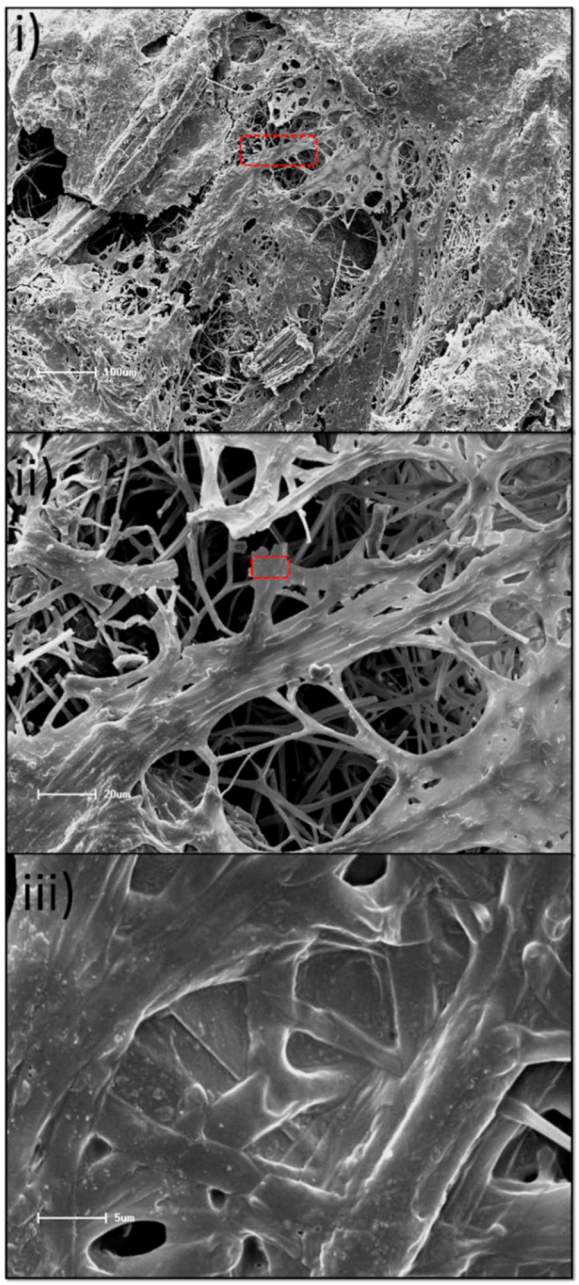

Fig. 6 Scanning electron microscope images of the mycelium-based composite cold-pressed at different resolutions at the same region, the dotted rectangles represent where the zoom was performed and are shown in ii and iii

Compression test

Compression tests revealed no rupture of the composite until the end of the test, with little deformation and greater resistance compared to polystyrene, and a commercial mycelium-based composite (Zeller and Zocher 2012).

The compressive stress–strain curve of the mycelium-based material presents a common behaviour, where at small compressive strains, the dominated response is related to be from the mycelium matrix, which presented elastic modulus on the order of 0.14–0.19 MPa. At larger strains, large number of fibre contact induces rapid stiffening (Islam et al. 2018) and the regime is dominated by the organic substrate particles (Haneef et al. 2017; Islam et al. 2017). These profiles were also similar to other works (Ziegler et al. 2016; Jones et al. 2017).

Compression test values presented small deviation on compression resistance tests, which can indicate that the mycelium developed homogeneously with a good bonding structure. Furthermore, it is important to mention that the compressive value obtained herein (Table 2) is within the region of polystyrene (0.23 compared to 0.15–0.7 MPa) and is characterized as rigid foam material (Xie et al. 2018). The time period of cultivation is also reported to affect the mechanical properties, whereas longer periods can increase it due to hyphae aggregation. By comparison, the cultivation period also influences the volume loss due to the drying of the substrate and hyphae collapsing (Haneef et al. 2017), though hyphae can colonize these vacancies left by water removal.

With a more nutritious substrate, the bonding and extent of their hyphae network is increased, while also increasing the material density, and is one of the main factors when failure occurs in these materials (Jones et al. 2017). Therefore, mycelium grown on substrate from residues to form mycelium-based materials is only suitable for foam like structures.

Researchers have been trying to conceive, with the overall properties of mycelium-based composites, industrial applications with computer-aided and design tools (Attias et al. 2019), possible applications suggested may be an insulating water container (Attias et al. 2020), insoles (Ziegler et al. 2016a, b), and indoor decoration (Zeller and Zocher 2012), among many others' complex geometries.

Water absorption and swelling

Water is an important criterion for many practical applications of mycelium-based products, thus, determining the performance under adverse conditions. In such cases, the water absorption values for the composite absorbed large quantities of water (245.1%) (Table 2) with a thickness expansion of 21%. The cold-pressed material had lower water absorbed and expansion, due to its compacted and rigid structure.

A major drawback on mycelium-based materials, compared to polystyrene, is their high-and fast water absorption. It is reported that they increase in weight by 40–580 wt% in contact with water for 48–192 h (Jones et al.

2020). This is due to their cellulose fibres having various hydroxyl groups, as well as its mycelium binder which is hydrophilic (Jones et al. 2017). The gel formed by these composites prevents dehydration of their hyphae (Antunes et al. 2020), allowing adhesion to other cells or surfaces. Moreover, the hyphae of *L. edodes* fungus is composed of β-glucans, chitin (Peniche-Covas et al. 1988), and proteins that are able to bind to others and form its own network, and these components are known to have high swelling values. Therefore, differences in water uptake from various mycelium-based materials are related to the difference in their chemical structure (Tacer-Caba et al. 2020).

Thermal properties

The DSC curves for the raw peach-palm sheaths, and the mycelium composite, exhibit (Fig. 7i) an increase in heat flow at ~ 350 °C, attributed to the exothermic event of cellulose decomposition. Compounds that are degraded at a temperature above 400 °C, such as lignin, have exothermic profile, and because of the degradation that occurred within the mycelium, it contributed to the temperature stabilization before 500 °C. The temperature profile presents degradation values similar to each individual fibre and matrix component of cellulose-based materials (Averous and Boquillon 2004).

Due to the substrate and the characteristics of the fungus, the thermal degradation is similar to most cellulosic materials. Furthermore, it can be seen that the non-pressed composite presented no significant variations at temperatures higher than 500 °C compared to cold-pressed. This might be related to the conditions that were grown the cold-pressed material, and may had not degraded all components at this specific temperature.

The thermal stability of the composites exhibits a three-stage process in the thermal degradation, and follows the profile of the substrate—peach-palm sheaths—(Fig. 7ii, iii), the first stage from 35 to 100 °C indicates evaporation of free and bonded water. The second stage is where the combustion started with a fast degradation rate within 250–350 °C associated with devolatilization process of the organic constituents of the substrate (60%) (Jones et al. 2018a), and the final stage can be related to volatile matter that burned at such higher temperature, due to the air combustion, 450–600 °C. Because of the denser, and more compact network found in the cold-pressed composite, it may have contributed to the increased values of the exothermic event at the final stage of 480 °C.

The thermal profiles were similar to the peach-palm substrate, meaning that the fungus did not altered the thermal behaviour, and may be related to the substrate used as previously mentioned. Similar profiles for TG have been shown for *T. multicolor* fungus using rapeseed straw, and the variation profile, whether cold-pressed and non-pressed, resulted in a similar graph (Appels et al. 2019).

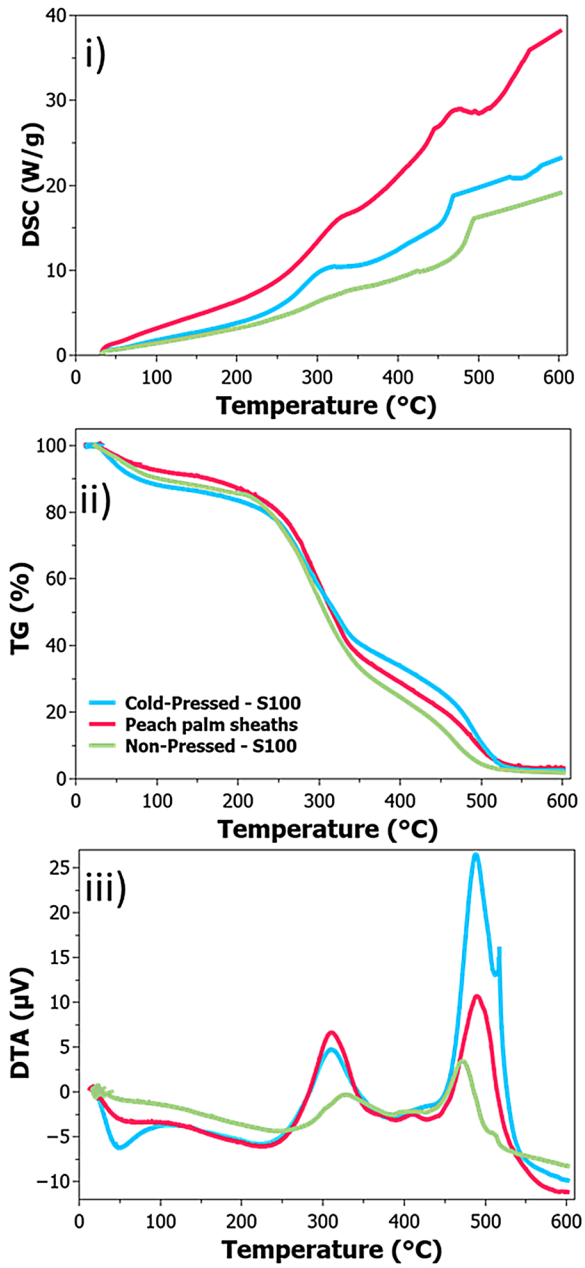

Fig. 7 Thermal analysis of the studied mycelium-based composites (i) DSC, (ii) TG, and (iii) DTA, within the comparisons are the pure raw peach-palm sheaths residue used to grow the fungus

Conclusions

The physico-chemical and enzymatic activities for the mycelial growth of *L. edodes* in the peach-palm sheaths presented that the best feed source for mycelium growth and density was justified by the ratio content of carbon/nitrogen for mycelium to grow, while also due to the large source of available amino acids from the soy flour. This condition formed a well-developed composite within 12 days, with no visual differences from 20 days. However, soy flour from both inoculum, presented low enzymatic activity attributed to the fact that the mycelium grew in optimum conditions of pH, moisture, and acceptable water activity; so, the enzymes were at the last digestive enzymes cycles. Contrarily, feed sources containing ammonium sulphate and potassium nitrate had a poor mycelial growth, with a decrease in pH due to the increased nitrogen source in the media; however, these treatments presented the highest enzymatic activities meaning that the mycelium had an improper condition to grow and was trying to establish a controllable environment to grow. The composite formed from the mycelium of *L. edodes* presented similar values of other mycelium-based composites on compressive strength and elastic modulus; however, even though the hyphae network completely filled the peach-palm residue from this mycelium, it did not fully degrade the residue and resulted in similar thermogravimetric results without differences in the overall thermal stability.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40643-020-00346-2.

Additional file 1. Additional figures.

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Authors' contributions
GG wrote the manuscript and analysis of the data; ZCPS carried out the experiments; WL, LB, and CV, supervised and reviewed the manuscript. All authors read and approved the final manuscript.

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The authors declare no conflicts of interest regarding the publication of this manuscript.

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