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 – in order 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, that 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 demonstrate 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

1. Introduction

The peach palm tree possesses in its structure a very important food source which is culturally and economically important in Latin America, commonly named as palmito – the heart-of-palm (Clement et al. 2016). However, to extract this food source also comes with its consequences – about 90% are considered a residue (Zenni et al. 2018); for instance, the median and internal sheaths of this tree are fibrous in nature and have a low degradation rate (Seben et al. 2012). They are generally thrown in the soil becoming an environmental liability; however, these residues could be used as low-cost substrate in various applications since they are also 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). The white-rot fungi can potentially be able degrade all components of the wood, mainly lignin, while brown mainly polysaccharides (Kirk and Cullen 1998). These fungi require cellulose - the main component of the plant cell wall - to grown because it is a source of energy (Ander and Eriksson 1977).

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 from its enzymatic system by breaking down the plant cell wall, to use as a source of carbon and energy for its life cycle (Kües and Liu 2000). Nonetheless, this mushroom is the second most cultivated in the world (Royse 2014) because of its medicinal, flavour, and nutritional properties. Furthermore, the degradation from *L. edodes* occurs via the hypha (Gow et al. 2017) elongation, tube-like fibres, producing active enzymes by a process called solid state fermentation (Elisashvili et al. 2008). They can convert cellulose into metabolizable sugars and completely digest lignin; these aspects enable this fungus to covert residues into renewable materials.

From within the fungi structure, the mycelium, vegetative part, form hypha and can merge with others to form a random fibre network structure that have the order of a few microns in length (Islam et al. 2017). By seeding mycelium on organic material it can consumes upon it by secreting hydrolytic enzymes (Glass 2004) and embeds them in such hyphae network containing binding properties that grows as an interconnecting fibrous network 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).

The most studied fungi for the formation of this material is 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, topography and the hyphae branching.

However, most of the papers presently in literature provides 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 have reported the usage of shiitake as a 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 source treatments and evaluates the number of enzymes produced and their properties when a mycelium-based composite is produced with the best formulation.

2. Materials And Methods

2.1 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) and 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 seven days in an environmental chamber at 25 ° C in the absence of light, and after growth stored at 4 ° C.

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

The crushed sheaths were oven-dried at 60 ºC 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 further submitted for autoclave, 121 ºC, 1 atm for 15 minutes. An experimental design with three replications was applied at the central point consisting of seven treatments of equal nitrogen concentration.

Table 1
Treatments used in this study containing different feed sources.

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

After cooling, the sheathes were inoculated in two batches. The first was inoculated with 1/6 of mycelium plate from *L. edodes EF50* grown in PDA medium (solid inoculum) and the second batch was inoculated with 2/6 of plate of *L. edodes* that was previously crushed for 15 seconds in a modified Socarean solution (Couri and Farias 1995). The flasks were incubated in a BOD incubator at 25 ºC, for 12 and 20 days of culture from both types of inoculum.

2.2 Moisture content (%), water activity (aw) and pH

The moisture content was determined by the gravimetric method described by (Hermann et al. 2013). Briefly, samples were dried at 60 ºC 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 in the supernatant 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) at the same temperature (Chemists 2005). The w_a values were measured by a water activity meter (3TE Aqualab series 3B, Decagon Devices Inc. WA, USA).

2.3 Enzymatic activities

The extraction of the enzyme complex 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 another work (Bailey et al. 1992). The enzyme activity was maintained for 5 minutes, using a 0.9 mL sample of 1% xylan with 0.1 mL of the enzyme extract, and the amount of sugar decrease was measured by 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). Which consisted of conducting the hydrolysis of a 0.44% carboxymethylcellulose solution in 0.05 M sodium acetate pH 5.0 buffer for the EC 3.2.1.4 fraction activity and a 1.1% suspension in the same buffer of microcrystalline cellulose (Avicel) for the EC 3.2.1.74 fraction. The reaction was initiated by adding the enzymatic extract to 0.9 ml of the substrates and proceeding the reaction for 60 minutes. 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), where 1 mL of 15 mM from 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 minutes. The reaction was stopped by immersing the tubes in boiling water for 5 minutes. After transferring to a cold-water bath, the glucose produced was determined using a kit 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 treatments were initially performed as independent variables for all enzymatic activities and pH, moisture and water activity, to assess which of the sources had a significant effect on these variables. 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).

2.4 Qualitative analysis of microbial growth

Through visual observation, the flasks containing the fungus and cellulose fibres were determined according to the standard adapted from ASTM (American Society for Testing Materials), Standard Methods 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.

2.5 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, in order 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 S100.

2.6 Composite formulation

In order to verify the composite integrity, the condition with the best mycelium growth was repeated using a large mould – composite non-pressed S100; though after 12 days, samples were also oven dried at 60 °C for 3 h.

2.7 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).

2.8 Histological sections

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

2.9 Scanning Electron Microscopy (SEM)

In order 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 composite S100 and pure peach palm sheaths were taken and 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.

2.10 Compression Test

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

2.11 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 × 20 × 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. The specimens remained at a temperature of 23 ºC, and were immersed in water for a period of 2, 24, and 48 h.

2.12 Differential Thermal Analysis (DTG and DSC)

The stability of peach palm sheath fibres and the composite were investigated by thermogravimetric analyses. The equipment DTG-60H - Shimadzu was used for DTG and DSC-60A was used for DSC, using about five mg of each sample. The heating rate was 10ºC/min until reaching 600ºC, in a nitrogen atmosphere at 20 ml/min and for the DSC, aluminium pans were used.

3. Results And Discussion

3.1 Moisture Content (%), Water Activity (aw) and pH

Physico-chemical analysis of the treatments, related to concentrations of various sources on fungus growth (Fig. 1), presents different profiles and efficiency. The time period used in this study presented initial pH values ranged from 3.65 to 5.65 for all treatments (Fig. 1.i-ii). The highest pH values were observed 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. 1.vii and Supplemental information – Fig. A.1), and is related to their higher dosage concentration. For the highest pH region, they were located near the corners of the treatments containing soy flour source and such data obtained herein was 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 treatments containing soy flour. Nonetheless, treatments without soy flour had lower pH and it further decreases with time compared to their initial values – due to the necessity of nitrogen and carbon tolerable limits for mycelium growth, 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 54–62% (Fig. 1-iii-iv), similar to another report using *L. edodes* for solid-state fermentation (Bentolila de Aguiar et al. 2013) which is a condition favourable for the fungus to grow. 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 the moisture after the last cultivation time interval for the majority of the samples, also observed in another work (Hermann et al. 2013); with the treatment containing only soy flour partaking the highest percentage of water. In a well-developed mycelium, it is possible that specific values of moisture are obtained, but these variations depend on the fungus strain and treatments used; however, it is interesting to notice that soy flour contained moisture values between 58–60%.

The surface contour plot exhibits (Fig. 1-viii) that the treatments containing highest moisture was found for higher concentration of soy flour; tough for the time period of 12 days and liquid inoculum (Supplemental information – Fig. A.2), the higher value region was found within a well-balanced source of soy flour and potassium nitrate.

For the water activity values (w_a) of the present work (Fig. 1v-vi), all treatments exhibited activities greater than 0.955; while also 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 availability for microorganism development.

The majority of the treatments presented to be significant in regards to their physico-chemical profiles. They also presented higher values for the mixture of soy flour and ammonium sulphate for a solid inoculum; and for liquid inoculum, higher values were obtained for soy flour with potassium nitrate (Supplemental Information – Fig. A.3–6).

3.2 Enzymatic activity

The enzymatic activities for avicelase, carboxymethylcellulose, β-glicosidase and xylanase exhibited a similar behaviour, when compared to both solid and liquid inoculum (Fig. 2). For the majority of the cases, the lowest enzyme activities occurred when soy flour was used, which can also be seen with the surface contour plot (Fig. 3 and Supplemental information A.7–8). Contrarily, sources containing ammonium sulphate, followed by potassium nitrate, had the highest activity on all enzymes.

The composition of the substrate is an important factor for the growth and expression of fungi, especially when the nutrients contains nitrogen and carbon. Therefore, fungi may extract these elements more easily within a mixture of ammonium sulphate and/or potassium nitrate (Rughoonundun et al. 2012).

The activity of various treatments using *L. edodes* evidenced it as a good degrader of the peach palm residue because of its increased enzyme activity. Besides the carbon and nitrogen as well as its nutrients

values for fungus growth availability, it is possible that within 12 days the majority of the mycelium growth might already occurred and the trend saw within these results could be the last digestive enzymes cycles which could be on the contrary for the other treatments. Nonetheless, the majority of the enzymatic activities presented a similar trend from a previous work which used solid-state fermentation of *L. edodes* (Philippoussis et al. 2011).

The presence of soy flour that influenced a decrease in enzyme activity may be due to the nitrogen sources (Philippoussis et al. 2011; Chicatto et al. 2018), in which higher dosages decreases the hydrolytic system but induces an increase in oxidative enzymes. Nonetheless, treatments can also be converted into proteins by microorganisms, and, although the values seem to show a direct relation, their actual profile is also dependant 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 mixture containing potassium nitrate (Fig. 2 – i, 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. 2.i). 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 Supplemental File Fig.A.7) and most of the studied sources used were statistically significant in the enzymatic activity values (Fig. S.9–10).

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) - the 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 (Supplemental information Figure A.8), 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.

Lower 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 region was the least effective on enzyme activity. However, only ammonium sulphate and potassium nitrate were able to exhibit a significant difference in terms of enzymatic activity (Supplemental Information Figure A.9–12).

Endoglucanase, CMC, activity is reported to produce in moderate quantities for *L. edodes*, this is enhanced depending on the amount of hemicellulose (Philippoussis et al. 2011). Furthermore, variations on the cellulolytic activity has 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 3rd colonization week leading to a decrease in the enzyme activity because of the limitation of utilizable nutrients and soluble carbon sources (Philippoussis et al. 2011).

Ammonium sulphate, as nitrogen source, can have positive effects for CMC and proteins but can inhibited the production of avicelase; also, 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 presented substrate; which in turns depends on the fungus ability to utilize the majority of substrate components as nutritive elements (Elisashvili et al. 2008). Likewise, presence of certain compounds, such as phenolics, from the substrate could also inhibit fungus growth (Mata et al. 2016).

3.3 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 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 conditions had no mycelial growth with the liquid inoculum, for both endpoints studied herein, and this could have been due to the concentration of the elements (Helm et al. 2013). Furthermore, previous works reports that growth of many fungi species can be halted when the nitrogen concentration or C:N ratio surpass a certain value (Philippoussis et al. 2011). In addition to the fact that, within both time periods, the growth rates are directly related to their enzymatic activities; whereas poor growth had a moderate activity, a well-developed fungus had lower activity values (Fig. 1). 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).

Mixing soy flour with the other treatments had a moderate mycelial growth of *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, the 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* are 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 total volume, an effect that occurred for sources containing mainly ammonium sulphate. This effect can be reversed if the media is richer in compounds that is easily digested by the mycelium, such as D-glucose, leading to an increased material volume (Antinori et al. 2020). In the case of peach palm sheaths, they contain higher values of non- and reducing sugars (Helm et al. 2013) that other common residues such as sugarcane bagasse (Rabelo et al. 2015) and may have contributed to the further growth of this fungus.

Therefore, it is possible that 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, and 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 source containing pure soy flour, features a compact mycelium block on the peach palm sheath fibres (Fig i-iv), and this source preference by the fungus may be related to the presence of amino acids (Leonowicz et al. 1991). The growth and nutrient parameters of the residues exhibited mycelium composite formation, a significant achievement by the usage of this fungus compared to substrates containing higher values of hemicellulose and nitrogen (Philippoussis et al. 2011). These are also related to be the main promoter of growth in mycelia stage (Gaitán-Hernández et al. 2011); nonetheless, the content of cellulose and hemicellulose on peach palm sheaths are lower than other common residues such as bamboo and sugarcane (Franco et al. 2019) lower hemicellulose is a good indicative for mycelium development (Gaitán-Hernández et al. 2011).

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

This treatment, S100, and time, 12 days, with solid inoculum were chosen, due to the higher mycelia growth rate within few days of cultivation, facilitating the process of manufacturing this material. 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 (Figure S.13). The force was applied until the rupture of the material close to 4 cm deformation and it was labelled as cold-pressed.

However, in order 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. 4.iii-iv); therefore, it is possible to tailor its volume based on the media and source used for the fungus to grow. Besides, the material formed within 12 days were very similar to the one formed at 20 days and it was also the reason this time period was selected.

Nonetheless, this material had some characteristics similar to a Styrofoam plate presenting a rigid structure of polysaccharides (matrix) over fibres that are biodegradable and with characteristics of fibre / matrix association

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

The physico-chemical characteristics of the mycelium-based composites exhibits 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.

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 (%)	$w_{a\,0}$	$w_{a\,f}$	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

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).

3.5 Carbon / Nitrogen and Ash Analysis

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 mycelial growth. It is already known that the addition of supplements increases the levels of nitrogen and available carbohydrates (Pedri et al. 2015).

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

Material (*)	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
(*) For all components, the results were calculated from the average.					

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 30–40 to favour the mycelial growth of L. edodes (Song et al. 1987), which indicates the relation between growth rate and availability of nitrogen readily usable. Therefore, since peach palm sheaths already possess a relation of C:N within a favourable growth, it is also possible that soy flour – containing lower amount of nitrogen than other sources –may have contributed to the growth of this material.

3.6 Histological sections

The microscopic images of the lateral (Fig. 5.i) and transversal (Fig. 5.ii) sections of the plant cell from peach palm sheaths exhibits, 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. 5-iii and iv). The same region examined for the pure sheaths exhibits that, after formation of the mycelium-composite, they were further degraded and a disorder of small fragments stands out around the fibres (arrows in (iii-iv) and marked area in (iv) from Fig. 5) which are directly linked to *L. edodes*; though, no fungus hyphae were found in the images.

The biodegradation of lignocellulosic materials by fungi primarily occurs in an extracellular form, since they must initially be depolymerized to smaller compounds in order 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 produce a great diversity of extracellular metabolites, which then act by degrading the plant cell wall.

3.7 Scanning Electron Microscopy (SEM) Analysis

The SEM images of the mycelium based-material was analysed on the cold-press composite, non-pressed also presented a similar structure – not shown, and their morphology exhibits the interconnected network,

formed from *L. edodes* mycelium (Fig. 6-i), and also their hyphae (Fig. 8.ii-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. 6 ii-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 growth throughout the whole composite limits the bonding between the hyphae and the substrate, and are reported to be responsible for the limited mechanical performance (Islam et al. 2018; Liu et al. 2020).

Furthermore, the binder network from the mycelium also affects its mechanical properties and tensile resistance of mycelium-based composites are more influenced by failure of the binder than the substrate itself (A. R. Ziegler, S. G. Bajwa, G. A. Holt, G. McIntyre 2016; Jones et al. 2018b).

3.8 Compression test

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

Under compression, mycelium-composites behave as an elastic regime at small strains followed by a localization strain regime – multiple bands of localized strain that connects the weakest points - and finally, at larger strains, a densification regime occurs whereas large number of fibre contact induces rapid stiffening (Islam et al. 2018). These stress-strain regimes were also observed in the studied composites (Figure S.12) using peach palm sheaths as residues, and similar to other works (A. R. Ziegler, S. G. Bajwa, G. A. Holt, G. McIntyre 2016; Jones et al. 2017).

Mycelium-composites under compression presents an elastic modulus on the order or 0.14–0.19 MPa and at lower strain rates, it seems that the matrix (peach palm residue) is the one that controls the response (Haneef et al. 2017; Islam et al. 2017).

Nonetheless, it is important to state that the compressive values obtained herein (Table 2) are within the region of EPS (0.23 compared to 0.15–0.7 MPa) and are characterized as rigid foam material (Xie et al. 2018). This can be related to the chitin, that is presented in the cell wall, and is also able to reduce the occurrence of cracks when subjected to compressive forces (Yang et al. 2017). 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 nutrient substrate, the bonding and extent of their hyphae network is increased, consequently, increasing the material bonding 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 are only suitable for foam like structures.

3.9 Water absorption and swelling

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

One of the problems on mycelium-based materials, compared to EPS, is their water absorption and their need to reduce its density. The water is absorbed very fast in these materials, and 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 and proteins that are able to bind to others and form its network and these components are known to have high swelling values.

3.10 Thermal properties

The DSC curves for the raw peach palm sheaths and the mycelium-composite exhibits (Fig. 7.i) an increase in heat flow close to 350ºC, and it can be attributed to the exothermic event of cellulose decomposition. Compounds that are degraded at a temperature above 400 °C, such as lignin, and chitosan at 300ºC that are present in the composite have exothermic profile. Because of the degradation that occurred with the mycelium, it contributed to the temperature stabilization before 500 ºC and the temperature profile are close to the degradation values of each individual fibre and matrix component of cellulose-based materials (Averous and Boquillon 2004).

Due to the substrate and the characteristics of the fungi, the thermal degradation is similar to most cellulosic materials and the lignin-based substrate are responsible for the fire-resistant properties of these composites (Jones et al. 2018a). 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 - (Fig. 7 ii-iii) the first stage from 25–200 °C indicates evaporation of free and bonded water (~ 5%). The second stage have higher degradation within 200–375 °C are due to organic constituents such as protein and polysaccharides (70%), and the final stage having residual char which degrades forming carbonaceous char 450–600 °C (Jones et al. 2018b). Similar profiles for TGA has 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).

Chitin and chitosan present in hyphae of the fungus when heated to higher temperatures undergo degradation and the composite thermograms exhibits exothermic events (Fig. 9-iii) at 329ºC, 471ºC and 480ºC, in agreement to another work (Peniche-Covas et al. 1988). 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.

4. Conclusions

Mycelium-based composites can be a potential alternative for packages or similar applications by their similar properties of polystyrene. However, the properties of this composite are dependent on the fungi strain used, material used as a food source and its time growth period. The usage of peach palm sheaths can be a good source for mycelium growth of *Lentinula edodes*, and the composite shows an interconnected hyphae network resulting in the expected properties obtained for this type of material within a reasonable formation time of 12 days.

Declarations

Conflicts of Interest

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

Availability of data and material

Data will be made available upon request

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing Interests

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

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Authors's contributions

de Lima, G. G. wrote the manuscript and analysis of the data. Pedri, Z. C. carried out the experiments, Magalhães, W.L.E. Tavares, L.B.B. and Helm, C. V. supervised and reviewed the manuscript.

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Figures

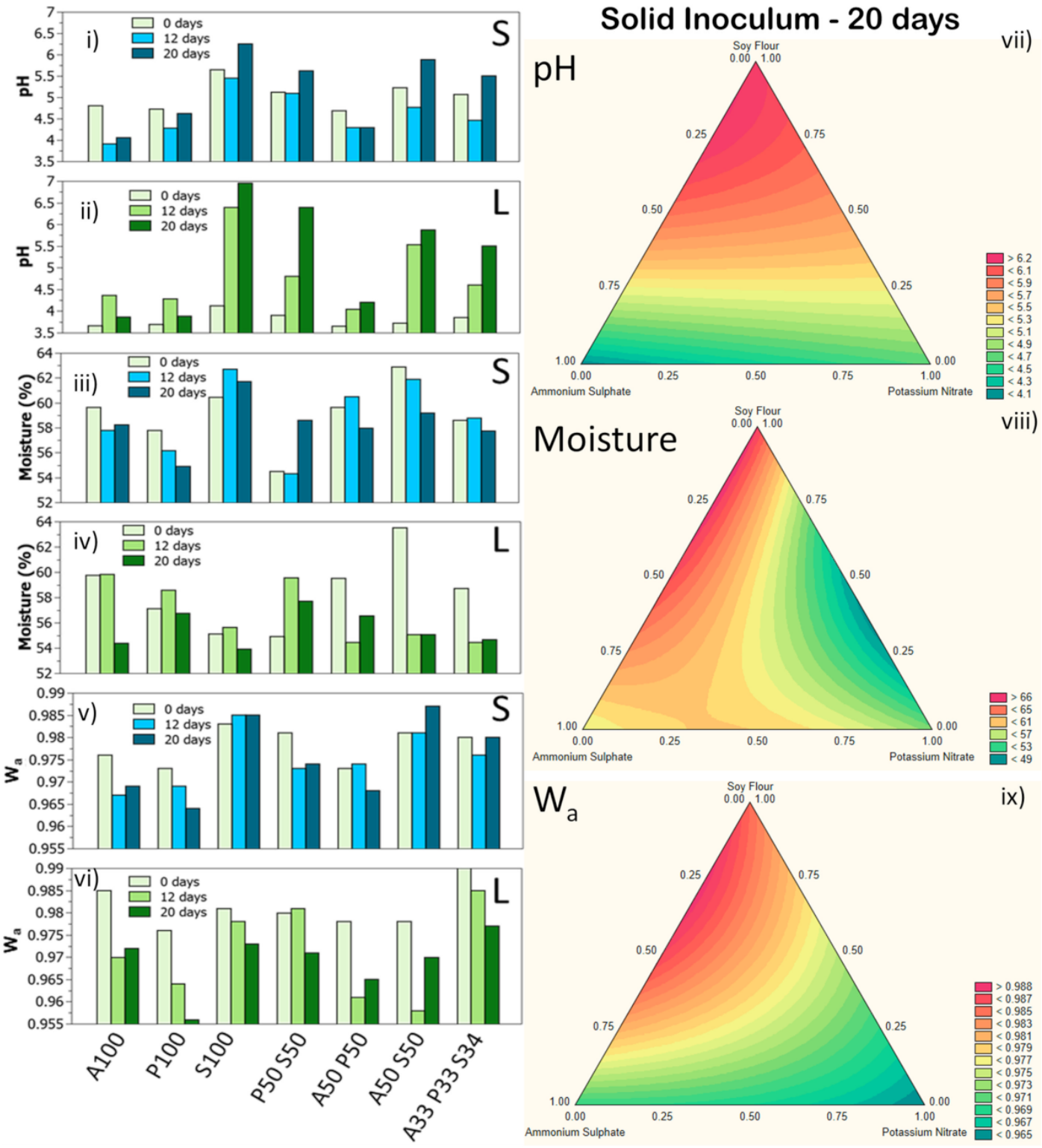

Figure 1

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

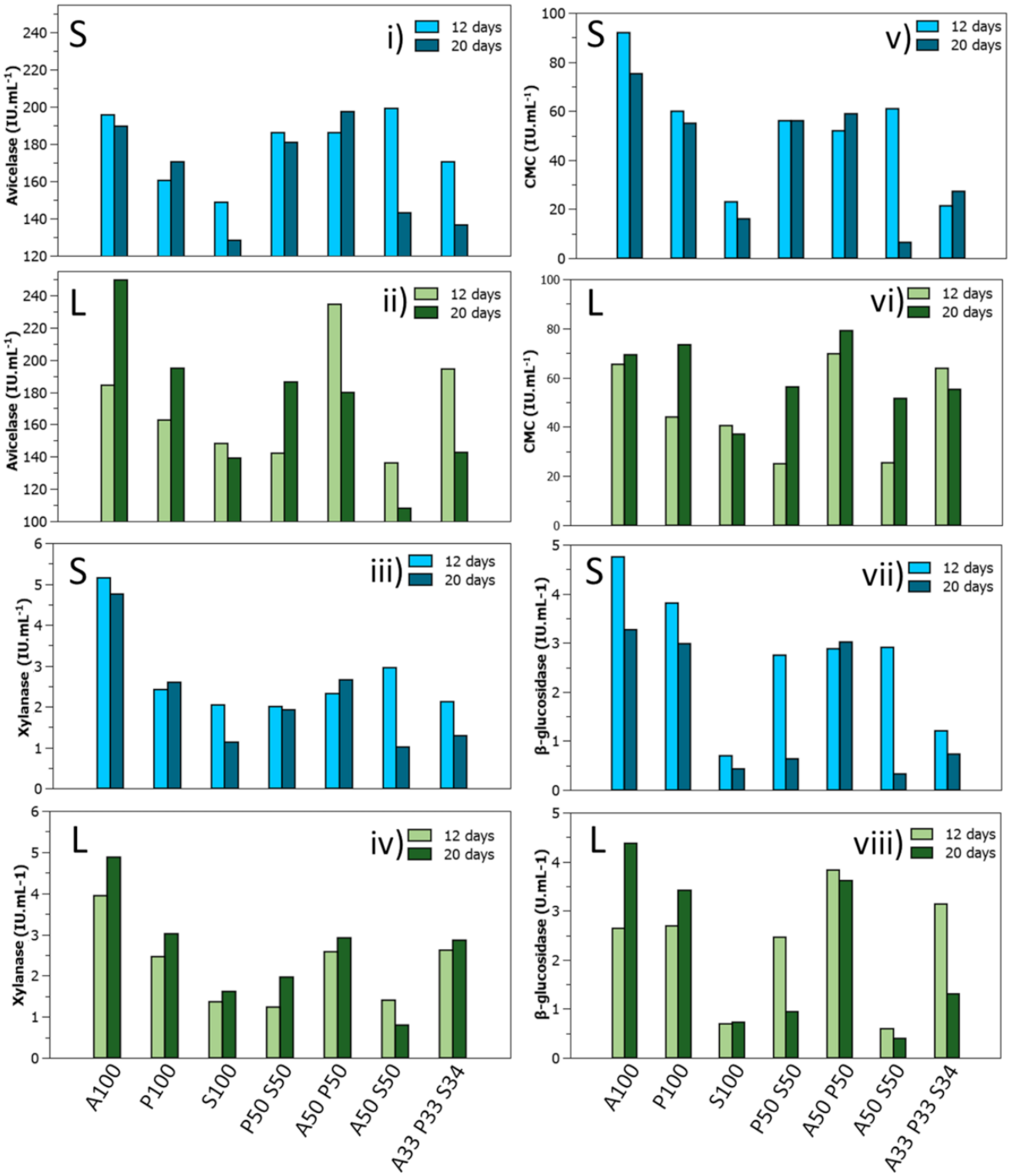

Figure 2

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

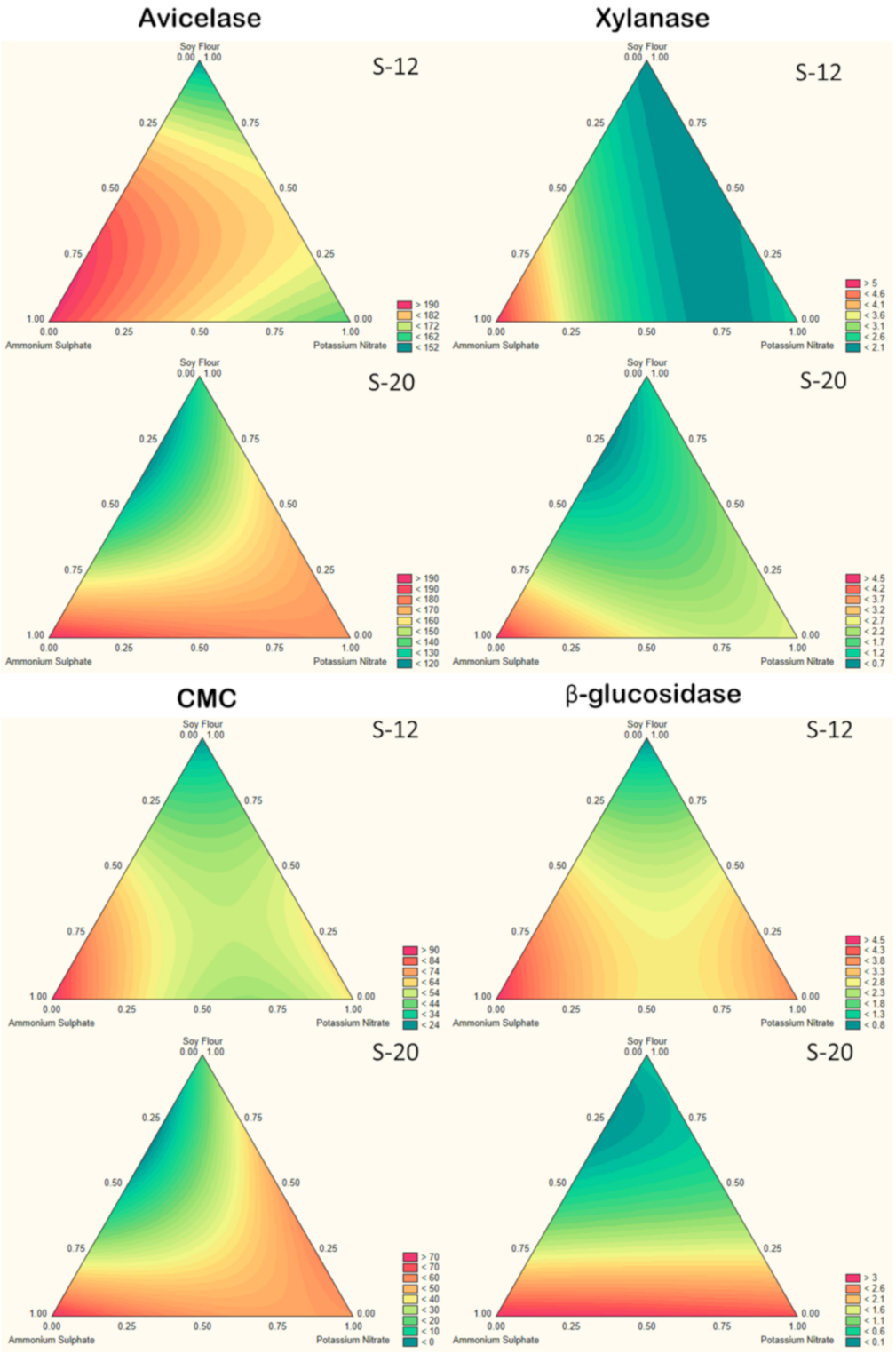

Figure 3

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

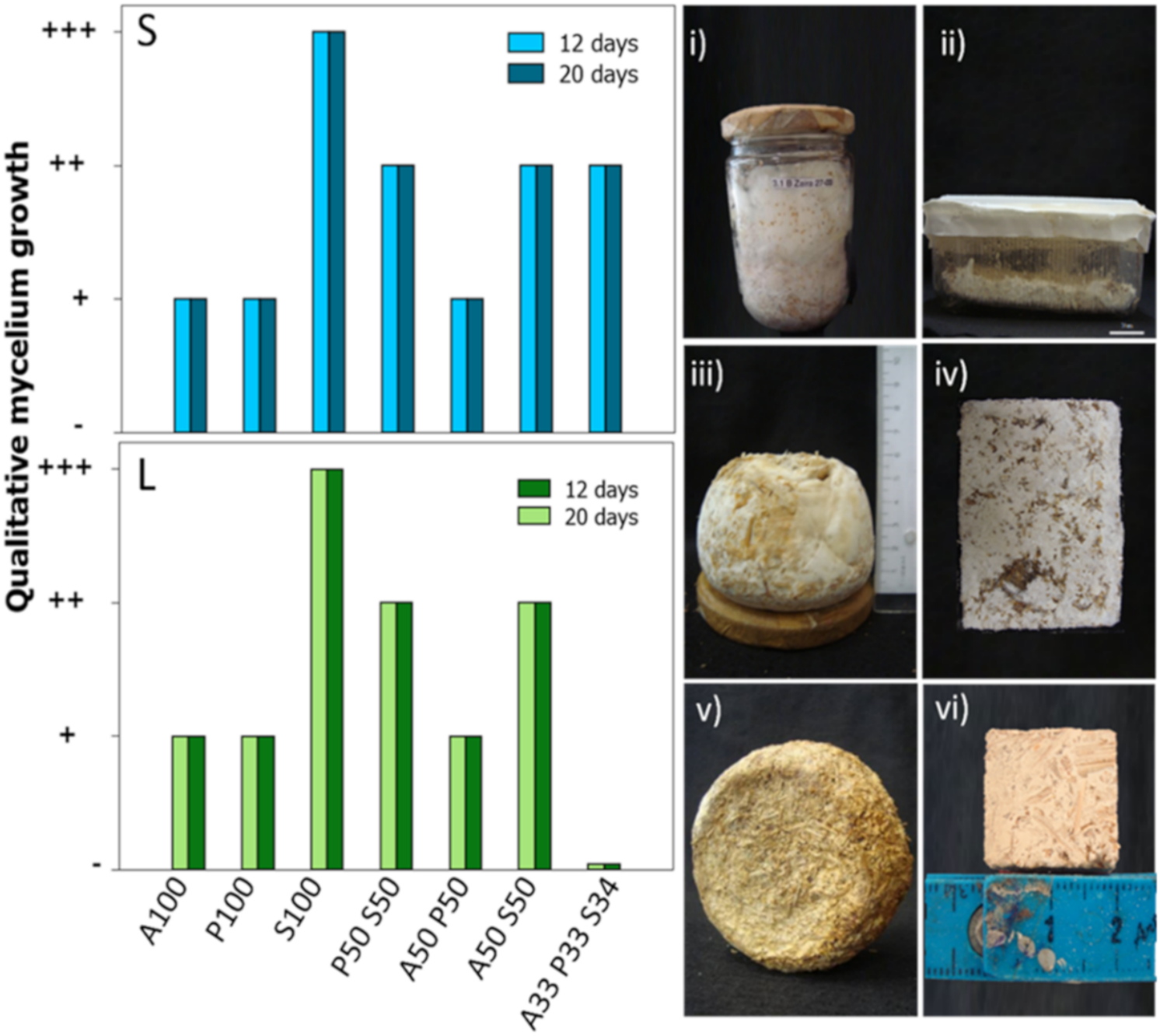

Figure 4

Qualitative mycelium growth from the studied 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).

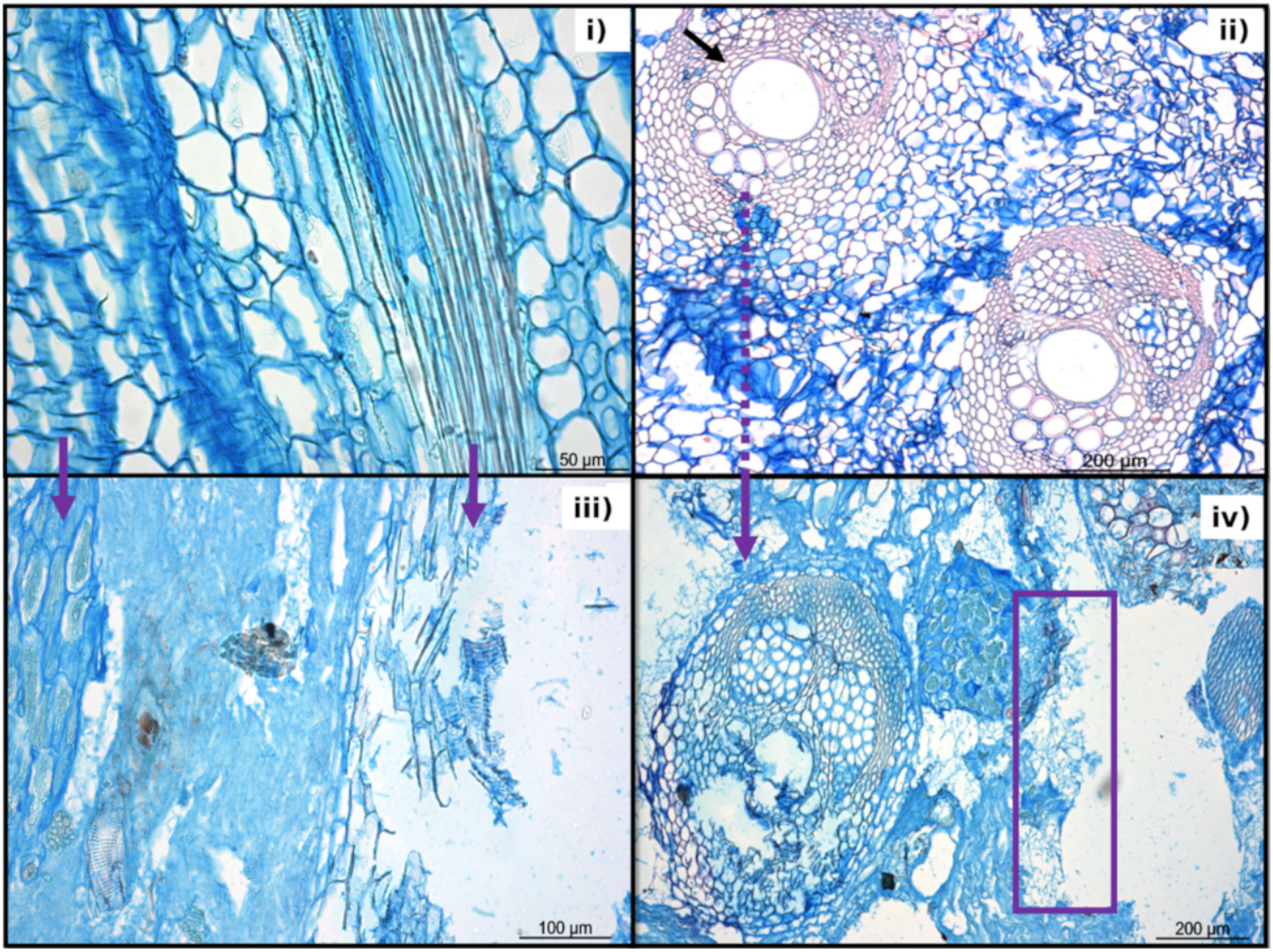

Figure 5

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

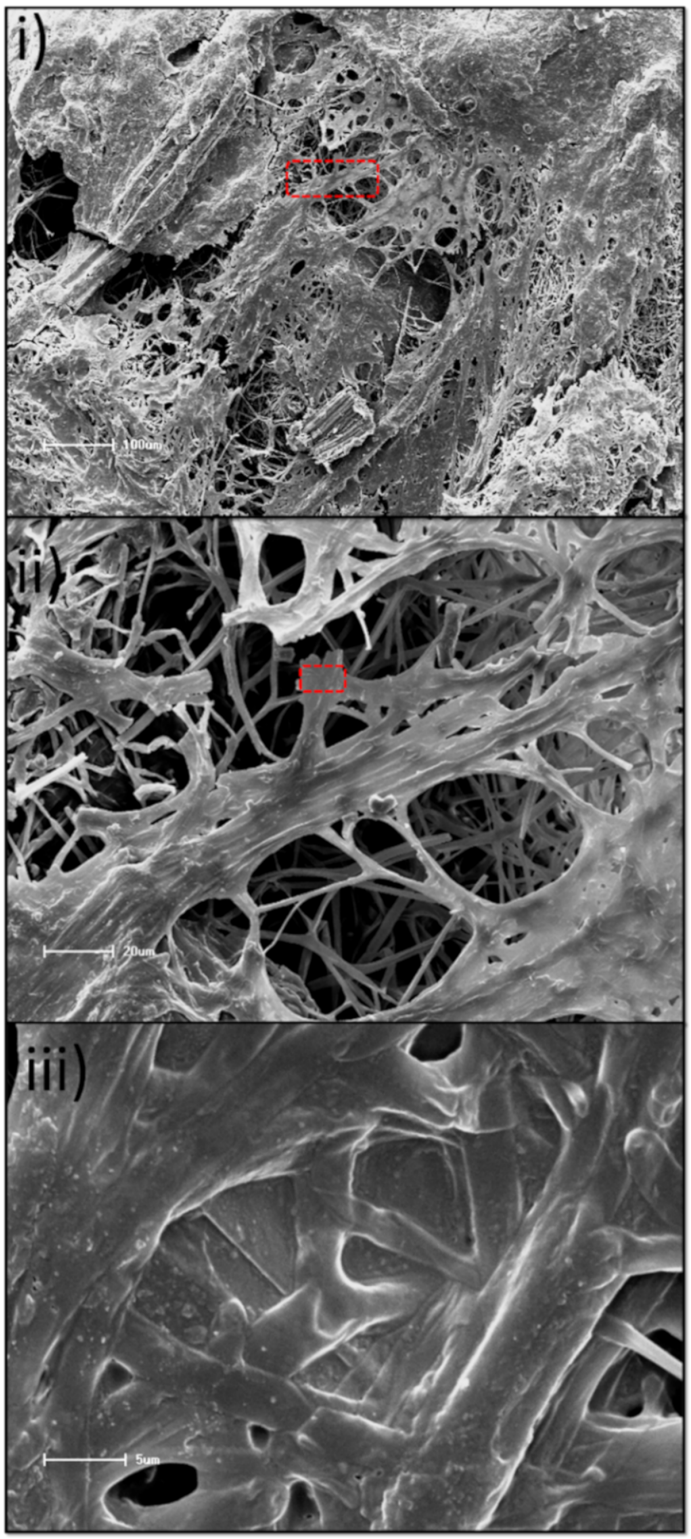

Figure 6

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

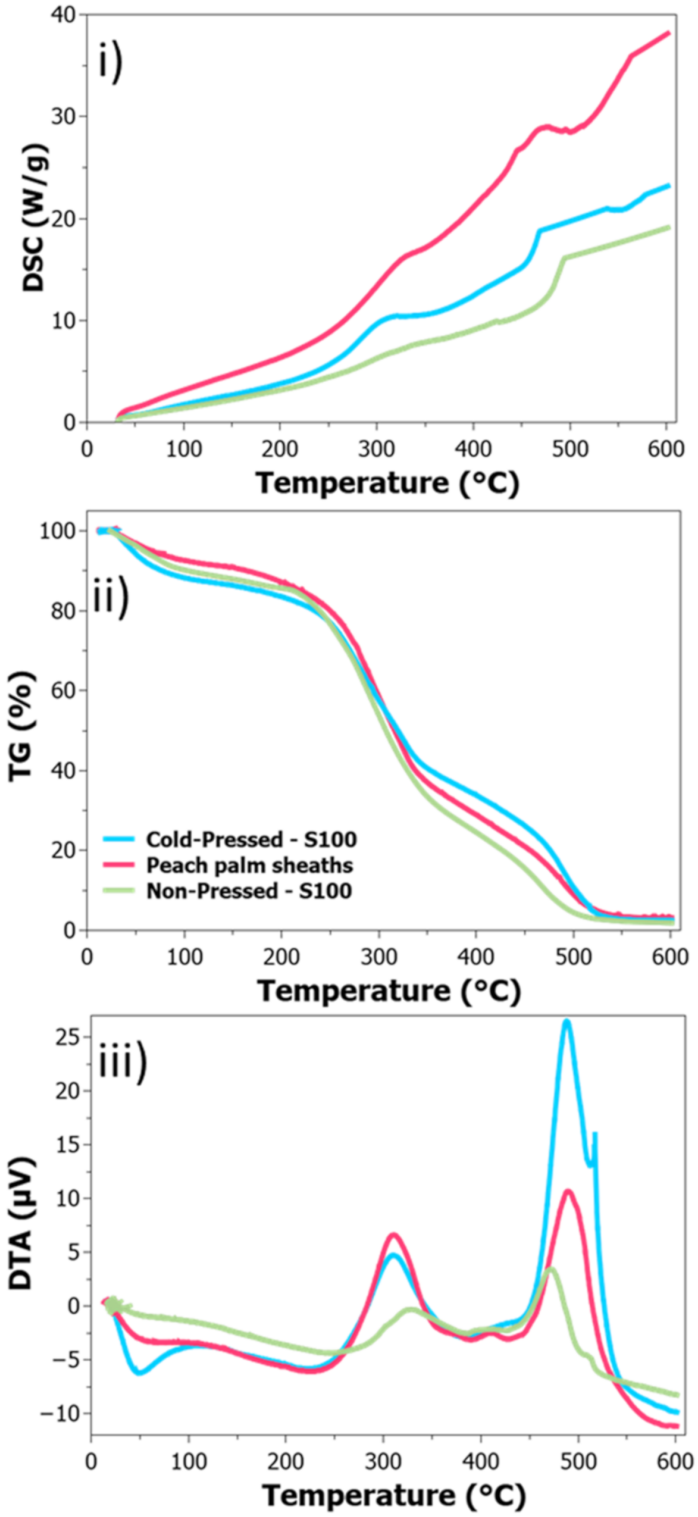

Figure 7

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

Supplementary Files

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- Graphicalabstractc.tiff
- Supplementalinformation.docx