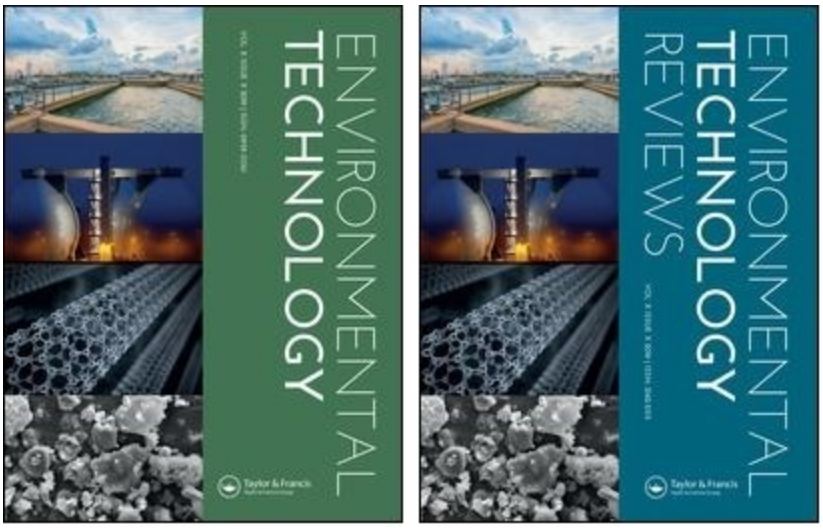

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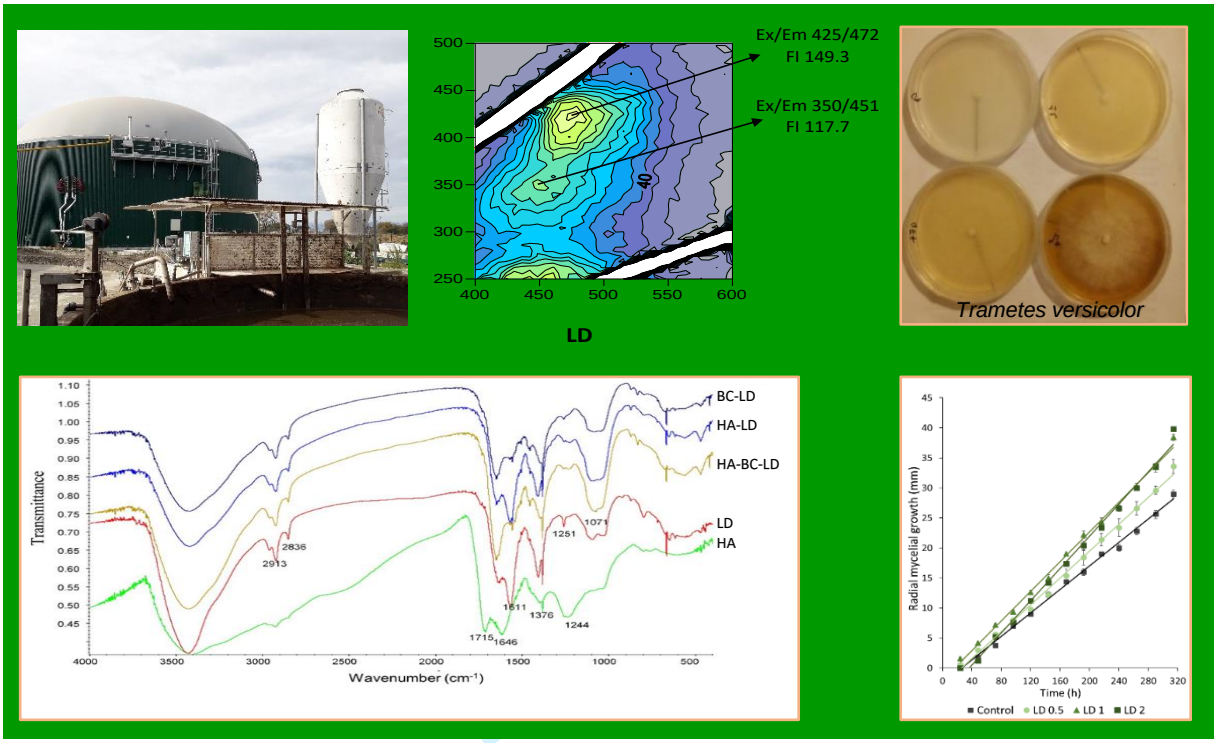

THE LIQUID BY-PRODUCT OF BIOGAS PRODUCTION: CHARACTERIZATION AND IMPACT ON SOIL FUNGI

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Abstract

In a laboratory study, a liquid digestate (LD) obtained from the anaerobic digestion of mixed organic waste was characterized and tested on three soil fungi, namely *Pleurotus eryngii*, *Irpex lacteus* and *Trametes versicolor*. Aqueous mixtures of LD at doses of 0.5, 1 and 2% (v/v) were tested directly or after interaction with 5% (w/v) biochar (BC-LD) and/or 100 mg L^{-1} soil humic acid (HA-BC-LD and HA-LD). Total luminescence (TL) spectroscopy and Fourier transform infrared (FTIR) spectroscopy were employed to investigate structural and functional properties of the organic fraction of the media. The TL spectrum of LD showed the presence of fluorophores typical of scarcely aromatic matter, while the FTIR spectrum evidenced absorption bands typical of labile and non-condensed material. Some spectroscopic variations of LD were observed after its interaction with the other materials. Differentiated effects on fungal growth were observed depending on the treatment, dosage and the fungus examined. In general, compared to the control (potato dextrose agar only), all LD treatments markedly promoted hyphal extension of *P. eryngii*, whereas significant stimulation of *I. lacteus* was observed only by LD previously interacted with BC and/or HA. The LD alone at each dose was irrelevant on the growth rate of *T. versicolor*, however, after interaction with both BC and HA, it exerted a clear stimulation of the fungus. The overall results obtained encourage the prudent addition of LD into the soil and, after further studies, a possible use of this material in the cultivation of edible mushrooms like *P. eryngii*.

Keywords: liquid digestate, ligninolytic fungi, mycelial growth, biochar, humic acid.

1. Introduction

A current emergency worldwide, especially in many European countries, is the need for an adequate energy supply. Given the opportunity to limit the use of fossil fuels that generate

climate-altering gas emission, technologies capable of providing renewable energy are becoming increasingly important (WBA 2021). Another current paradigm is the preservation of the environment endangered by the growing waste discharge which compromises the health of wildlife and the safety of the human and animal food chain. Therefore, sustainable processes for converting waste biomass into bioenergy are increasingly demanded. Besides generating biofuels, these technologies release large quantities of C-rich solid and liquid co-products and by-products suitable for various agricultural and environmental applications (Singh and Kalia 2017).

The anaerobic digestion (AD) process consists in the biological conversion of waste biomass by anaerobic bacterial and archaeal populations (Singh and Kalia 2017; Braguglia et al. 2018). Raw biomass used to feed the process originate from agricultural, industrial and domestic sectors (Tawfik et al. 2022). The main product of AD is biogas, which is a mixture of CH_4, CO_2 and small quantities of other gases. The biogas sector has been developing rapidly in recent years, with global biogas production tripled in the last decade (Karimi et al. 2022). The primary by-product of AD is a semisolid mixture with high moisture (about 90-95%) which, after a separation treatment, usually a centrifugation, produces a solid phase, commonly called 'solid digestate', and a clarified liquid known as 'liquid digestate' (LD) (Wang and Lee 2021).

During the AD process, the labile fraction of the organic matter is readily converted into biogas, while recalcitrant lignocellulosic components remain in the byproducts. Due to the degradation and homogenisation of the biomass during the AD, the bioavailability of the elements increases, and the material is partly sanitized (Nkoa 2014). The physicochemical properties of both the solid and the liquid digestates depend on the type of raw biomass and the operating conditions adopted in the AD process, such as retention time, working volume and temperature.

The LD is a mixture of undigested or poorly digested substances, anaerobic microorganisms (bacteria and archea), enzymes, metabolites, fatty acids, inorganic particulate and more stable organic molecules of humic-like type. It is characterized by low dry matter, high contents of P, N (mainly as NH_4^+-N) and K, and pH in the range 6.7 – 9.2 (Parra-Orobio et al. 2021). The direct discharge of LD into soil or natural waters raises concern for its very high N content and the possible ammonia emission and/or nitrate leaching. In addition, as cattle manure, municipal organic waste, agricultural residues and sewage sludge are very common feedstock for the AD technology, high levels of antibiotics and pesticides can be present in LD, which poses a serious threat to human health and to the protection of ecosystems (Wang and Lee 2021; Brueck et al. 2023). A further concern is the presence in LD of potentially toxic elements, such as Zn, Cu, Al and so on, and occasional plant and animal pathogens (Tawfik et al. 2022). For these reasons, LD has long been considered mainly waste to be managed with great caution (Nkoa 2014). However, the high phytonutrient content of this material induces farmers to apply it to the soil as a fertilizer, often through drip irrigation (Caruso et al. 2018; Peng and Pivato 2019), or to use it for hydroponic cultivation (Liu et al. 2011; Ronga et al. 2019). Only recently, the regulation of the European Commission authorized the use of digestates as soil fertilizers (European Commission 2019). Numerous scientific studies have demonstrated the benefits for plants of incorporating digestate into the soil (Cristina et al. 2020), while the activity exerted by this material on soil microbial populations has been poorly investigated. Differentiated and sometime controversial effects of LD on soil fungi have been reported (Tao et al. 2014).

Fungi are very important organisms in terrestrial ecosystems because they give a fundamental contribution to the degradation of biowaste, especially plant debris. Ligninolytic fungi, also known as ‘white rot fungi’, belong to the Basidiomycota phylum and are soil-resident species playing a crucial role in the soil carbon cycle that ensures the continuous release

of plant nutrients (Lundell 2010). The extracellular enzymes of these fungi, namely lignin peroxidases, manganese peroxidases and laccase, have low substrate specificity which render them apt to degrade a wide range of phenolic and non-phenolic compounds (Yang et al. 2013). For this ability, ligninolytic fungi are used in remediation programs of wastewater and sediments from persistent organic pollutants (Loffredo et al. 2016). Moreover, white rot fungi are also involved in the important economic sector of edible and medicinal mushrooms (Brezáni et al. 2019). Fungal growth is very simple to evaluate, and the data collected can be processed and interpreted with various models (Dantigny et al. 2005; Tao et al. 2014). Very few studies are reported in the literature on the effects of digestate on soil fungi (Tao et al., 2014), and even less on ligninolytic ones (Santi et al. 2015; Brezáni et al. 2019).

The application of LD to the soil implies the occurrence of chemical interactions between LD components and native or anthropogenic organic fractions already present in soil. Biochar (BC) is the by-product of the thermochemical conversion of biomass. This carbonaceous material has a strong capacity to retain water, plant nutrients and inorganic and organic contaminants, and for its properties it is increasingly used for both agricultural (Igalavithana et al. 2016) and environmental applications (Loffredo 2022). In soil, the organic components of BC, especially the more polar ones, dissolve in pore water and interact with other dissolved compounds forming bonds of different strength (Rombolà et al. 2021). Furthermore, the native soil organic matter, especially the humic fraction, is notoriously very reactive towards other soil organic components. Humic acids (HA), which are ubiquitous in soil, can exert a remarkable influence on microbial growth by mitigating biotic and abiotic stress (Kulikova et al. 2005; Loffredo et al. 2012). Based on these considerations, it is reasonable to expect that the simultaneous presence of LD, HA and BC in the soil could modify the activity of LD only on ligninolytic fungi. Previous works report that fungal response to some bioactive compounds

could be modified or even reversed when these compounds were in the presence of humic substances (Loffredo and Traversa 2014).

The objectives of this work were: (i) to characterize a LD from mixed feedstock using both conventional and advanced spectroscopic techniques, and (ii) to evaluate *in vitro* the effects of various doses of LD, applied both directly and after interaction with a BC and/or a soil HA, on three ligninolytic fungi of agricultural and economic importance.

2. Materials and methods

2.1. Liquid digestate, biochar, humic acid and fungi

The LD sample was obtained from a local biogas-producing plant (F.lli Caione Azienda Agricola La Quercia Soc. Coop., Foggia, Italy) fed with a mixture of oat silage, manure, slurry, cereal byproducts, and two-phase olive pomace. The AD process was of the wet type, with a dry matter content < 10% and a retention time of about 45 days. After solid/liquid separation of the raw digestate by centrifugation, the sampled LD was stored in glass bottles at a temperature of 4 °C. Before starting the experiments, LD was autoclaved at 121 °C for 15 min. Total solids and ashes were determined at 105 °C and 550 °C, respectively. The electrical conductivity and pH were measured directly in the LD sample through conventional methods. Total N and total organic C were determined using the Kjeldahl distillation method and oxidation with $K_2Cr_2O_7$, respectively. Some properties of LD are shown in Table 1.

Biochar (BC), provided by Blucomb s.r.l., Udine, Italy, was obtained from grapevine pruning residues through a micro-gasification process with a thermal maximum of 550 °C, a residence time of 3 h and final dry cooling. Moisture, pH, ash, and EC of BC were, respectively, 4.5%, 9.9, 9.9% and 2.23 dS m^{-1}, while C, H and N contents (on dry- and ash-free basis) were, respectively, 755, 13 and 5.3 g kg^{-1} (Taskin et al. 2019).

The HA sample used in this work was the standard HA isolated from a prairie soil located near Joliet, Ill, USA, provided by the Standard and Reference Collection of Humic and Fulvic Acids of the International Humic Substances Society (IHSS 2022). Total acidity, COOH and phenolic OH contents of HA were, respectively, 10.2, 8.3 and 1.9 meq g^{-1}, while C, H and N contents (on dry- and ash-free basis) were, respectively, 581, 37 and 41 g kg^{-1} (IHSS 2022).

The *Pleurotus eryngii* (DC.) Qu el. (ITEM 13681) isolate was provided by the culture collection of the Institute of Sciences of Food Production (ITEM Collection, http://www.ispa.cnr.it/Collection/), Bari, Italy. Isolates of *Trametes versicolor* (L.:Fr.) Pilat (CBS 114372) and *Irpex lacteus* (Fr.:Fr.) (CBS 108555) were obtained from the Centraal Bureau voor Schimmelcultures (CBS-KNAW), Utrecht, The Netherlands. Fungal cultures were maintained on potato dextrose agar (PDA, Oxoid, 4% w/v) in Petri dishes in the dark at 20 ± 1 °C. Sample inoculation was performed in a laminar flow hood using a 2-mm PDA disk overgrown by the mycelium excised from the growing margin of young colonies.

2.2. Preparation of fungal substrates

The LD was diluted in double distilled water at dosages of 0.5, 1 and 2% (v/v) (LD 0.5, LD 1, LD 2). Then, diluted LD samples were interacted with BC and/or HA according to the following procedure. An aliquot of 5 g of BC was packed in a piece of tissue-non-tissue, closed with a cotton thread and dipped into 100 mL-volume of each diluted LD in a sterile glass pot. The pots were closed with appropriate caps and kept at room temperature (~ 20 ± 1 °C), in the dark, under magnetic stirring for 24 h. After that, the packed BC was removed from each pot and the interacted samples (BC-LD 0.5, BC-LD 1, BC-LD 2) were collected. In another set of media, aliquots of 10 mg of HA were added to 100-mL volumes of double distilled water (HA), LD 2 (HA-LD 2) and BC-LD 2 (HA-BC-LD 2), separately, then the samples were stirred for 24 h under the conditions described above. The pH and EC values of all media are shown in

Table 2. Approximately, the highest LD dose used in this study (2%) corresponds to a soil application of 14 t ha^{-1}, considering a soil depth of 5 cm and a soil bulk density of 1.4 g cm^{-3}. This concentration was lower than the recommended LD rates for field applications (30-60 t ha^{-1}) (Velechovský et al. 2021) because it was cautiously considered more appropriate in *in vitro* experiments.

2.3. Spectroscopic analysis

2.3.1. Total luminescence (TL) spectroscopy

An aqueous mixture of LD at a dose of 10% (v/v) was prepared and analyzed as such or after interaction with 5% (w/v) BC (BC-LD) and/or 100 mg L^{-1} HA (HA-BC-LD and HA-LD, respectively) according to the procedure described in section 2.2. The HA sample was dissolved in distilled water at a concentration of 100 mg L^{-1} and, after pH adjustment to pH ~ 8 with NaOH, was equilibrated overnight at room temperature (~ 20 ± 1 °C). The total luminescence (TL) spectra of all media were recorded in the form of excitation-emission matrices (EEMs, contour maps), over the emission wavelength range of 300-600 nm, increasing sequentially by 5 nm step the excitation wavelength from 250 to 500 nm. A scan speed of 1200 nm min^{-1} was selected for both monochromators. The luminescence spectrophotometer used was a Perkin-Elmer LS 55 equipped with the WinLab 4.00.02 software (Perkin-Elmer, Inc., 2001, Norwalk, CT) for data processing. The EEM plots were generated as contour maps from spectral data using the Surfer 8.0 software (Golden Software, Inc., 2002, Golden, CO).

2.3.2. Fourier Transform infrared (FTIR) analysis

The FTIR spectra were performed on the same media analyzed by TL spectroscopy (LD, BC-LD, HA and HA-BC-LD) described in section 2.3.1. Spectra were recorded on pellets obtained by pressing a mixture of about 2 mL of each sample and 400 mg of dried KBr,

spectrometry grade, under reduced pressure, after heating overnight the mixture at 30 °C. The pellet for recording the HA spectrum was obtained adding 1 mg of solid sample to 400 mg KBr and following the previously described procedure. The FTIR spectrophotometer used was a Thermo Nicolet Nexus, equipped with a Nicolet Omnic 6.0 software. The instrument settings were the following: range 4000–400 cm^{-1}, 2 cm^{-1} resolution and 64 scans min^{-1} for each acquisition.

2.4. Assays on fungi

Each medium, prepared as described in section 2.2, and double distilled water (control) was added with PDA (4%, w/v) and autoclaved at a temperature of 121 °C for 15 min. An aliquot of 20 mL of each medium was poured into a 9 cm-diameter Petri dish and let cool and solidify to room temperature. Fungal inoculation was carried out placing a 2-mm radius mycelium inoculum in the centre of each plate. Then, the samples were placed in a thermostated chamber at a constant temperature of 20 ± 1 °C, in the dark. Radial growth (in mm) of the fungal colony was measured at intervals of 24 h for a time duration depending on the growth rate of the fungus tested, and until hyphal elongation reached the border of the plate in one replicate, i.e., 13, 9 and 7 d for *P. eryngii*, *I. lacteus* and *T. versicolor*, respectively. All experiments were replicated five times.

2.5. Data analysis

All fungal growth data were statistically analysed by one-way analysis of variance (ANOVA), and the means of the treatments were compared to the control by the least significant difference (LSD) test at 0.05*P*, 0.01*P*, and 0.001*P* levels.

To calculate the radial growth rate, μ (mm h^{-1}), the following linear model with breakpoint was adopted (Dantigny et al. 2005): $r\ (mm) = \mu\ (t - \lambda)$, where r is the mycelial radius, t is the

sampling time (h) and λ is the lag time (h). Although this model is very simple, it is very appropriate, showing generally regression coefficients greater than 0.99. Another advantage of using this model is that the parameters μ and λ can be obtained even when the Petri dish is not entirely covered with the mycelium (Dantigny et al. 2005). This model allows to calculate the lag period (λ) by extrapolation of the straight line. Under carefully controlled inoculum, the lag time can be considered the time required for the completion of the germination process of the fungus and usually it does not influence the radial growth rate (Dantigny et al. 2005).

The values of the diameters of the fungal colonies were used to calculate the absolute growth rate (AGR, mm d^{-1}) of the mycelium using the equation $AGR = \frac{D-2}{t}$ (Tao et al. 2014), where D is the diameter of the mycelium (mm), 2 is the inoculum diameter (mm), and t is the incubation time (h).

3. Results and discussion

3.1. Digestate properties

The physicochemical characteristics of LD are markedly influenced by the type of raw biomass used in the AD process and its retention time into the digester. Due to its composition, this liquid material can be considered as an organic fertilizer containing plant nutrients. The values of ~ 3% and 8% (on dry weight) measured, respectively, for total solids and total N content of the LD employed in this study (Table 1) were typical of the wet AD and comparable with those reported in the literature for LD from mixed feedstock, i.e., 0.8-4.0% for dry matter (Velechovský et al. 2021) and 7.7-9.2% for total N (Möller and Müller 2012). The pH value of 8.4 was also included in the range of 7.0-8.6 measured for other LD samples (Velechovský et al. 2021). Similarly, volatile solids content, TOC and EC values were in the range reported for this type of material (Ronga et al. 2019), while, as expected, NH_4^+-N content was lower than that found for a LD obtained from animal wastes only (Valentinuzzi et al. 2020).

3.2. Spectroscopic analysis

3.2.1. Total luminescence analysis

Fluorescence analysis revealed a lower complexity of the LD sample compared to soil native HA. The TL spectrum of LD (Fig. 1) showed the presence of two fluorophores at an excitation/emission wavelength pair (EEWP) of 350ex/451em and 425ex/472em. In contrast, the TL spectrum of the HA (Fig. 1) showed the presence of one fluorophore at an excitation/emission wavelength pair (EEWP) of 460ex/530em. The shorter wavelengths of fluorescence maxima and the higher fluorescence intensity (FI) values found for the LD sample, compared to HA, suggested the occurrence in LD of simple structural components, low degree of aromatic polycondensation, and low level of conjugated chromophores (D'Orazio et al. 2014), while HA was characterized by the presence of chromone derivatives, such as flavonoids, and extensively conjugated quinones and phenols with an elevated polycondensation degree (Senesi et al. 1991; Traversa et al. 2014). The spectrum of HA-LD resembled that of HA, with a single fluorophore at high wavelengths and a low FI, while the spectrum of BC-LD was similar to that of LD (Fig. 1). When the LD sample was treated with both HA and BC, the TL spectrum showed slight longer EEWP of the two fluorophores, compared to those of LD alone, reasonably ascribed to the addition of greater complex molecules of HA in the solution (Fig. 1). However, in the latter case, the FI of the two fluorophores greatly increased, suggesting possible chemical interactions between the three materials, which increased the number of electron-donating substituents such as hydroxyl, methoxyl, amino groups on low molecular weight molecules. In general, the fluorescence analysis showed a higher molecular complexity of HA, compared to the LD, and a greater contribution of HA, compared to BC, in determining the final conformation of the fluorescent molecules.

3.2.2. FTIR analysis

The FTIR spectra of HA and untreated and treated LD samples featured common bands, with some differences in their relative intensity (Fig. 2). Three peaks ranging from 2900 and 2800 cm^{-1} of LD samples are attributed to the C–H stretching of $-CH_3$, $-CH_2$, -CH and -CHO groups vibrations; a peak at 1715 cm^{-1}, present only in the HA spectrum, related mainly to the carboxylic groups but also to aryl- and unsaturated aldehydes and ketones; a peak at about 1650 cm^{-1}, usually attributed to the aromatic C=C vibration; a peak at about 1610 cm^{-1}, ascribed to the presence of COO^-, NH_2 and NH_3^+ groups, whose relative intensity was lower in BC-containing samples; two peaks at about 1400 and 1370 cm^{-1} attributable to the bending of phenolic O–H and alkyl P-O- groups vibrations; an absorption band at about 1250-1240 cm^{-1} in HA sample, whose relative intensity reduced all other samples, probably due to the O-H deformation of phenolic and aliphatic groups, C-O stretching of ethers and/or carboxyl groups; a band at about 1070 cm^{-1} that can be mainly ascribed to the C-O stretching of polysaccharide structures and to the vibrations of C=S and SO groups, whose relative intensity was lower in LD sample due to the possible presence of simpler saccharides.

The FTIR spectroscopy confirmed the lower molecular complexity and aromaticity of LD with respect to HA. All LD-based spectra showed bands and peaks related to the carboxylic groups with relative low intensity due to their salification with inorganic nutrients of the digestate. Finally, the presence of BC reduced the signal of carboxylate and N groups possibly for interaction with surface functional groups of BC.

3.3. Effects of LD on fungal growth

All treatments with LD, regardless of dosage, did not cause visual changes of the mycelium of any species, compared to the control. Different models have been adopted to study fungal

growth in *in vitro* conditions (Dantigny et al. 2005; Tao et al. 2014). The fitting of hyphal extension data in various development models depends on the type of fungus and the conditions adopted. A linear model with breakpoint was suggested by Dantigny et al. (2005) for filamentous fungi growing on PDA. This simple model can be very accurate in describing radial mycelial growth and has a very good fitting for a large number of fungal species, the regression coefficients being usually greater than 0.99. Plotting the radial mycelial growth as a function of time and applying the linear regression is possible to calculate the radial growth rate μ and the lag time λ, i.e., the initial period needed by the fungus to start hyphal elongation. Another valuable parameter expressing fungal growth is the absolute growth rate (AGR) of mycelium which is usually adopted to assess the occurrence of stimulating or inhibiting events on fungi when compared to a control sample (Tao et al. 2014).

3.3.1. Pleurotus eryngii

The radial growth of the fungal colony, measured at regular time periods, on all media is depicted in Figure 3, while μ and λ values along with the growth equations and the correlation coefficients, R, are referred in Table 3. At all samplings, the presence of LD only at each dose, especially at higher doses (1 and 2%), significantly ($P \leq 0.01$ in most cases) stimulated hyphal extension (Fig. 3A). Similar results were observed in all treatments with LD interacted with BC (Fig. 3B) and in the treatments HA-LD 2 and HA-BC-LD 2, whereas HA alone did not affect mycelian growth (Fig. 3C). Fitting the experimental data into the linear model, it resulted that LD 2, HA-LD 2 and HA-BC-LD 2 produced the maximum μ values which were about 38% higher than the control (Table 3). The same treatments also showed the longest delay times, indicating longer periods of fungus adaptation. These results suggest that in soil, where the humified fraction is ubiquitous or where BC may have been applied as an amendment, the stimulating activity of LD is preserved. Data of *P. eryngii* growth were very well described by

the linear model giving R values always equal or higher than 0.997. The AGR profiles recorded for the 13-day growth of this fungus are depicted in Figure 4. Compared to the control and except for the first sampling, the AGR of the fungus was always higher in all LD-based treatments, which confirmed the noticeable stimulating effects of this material both untreated and previously interacted with BC and/or HA. Very little information is available in the literature on the effects of digestate on ligninolytic fungi and mainly limited to solid digestate (Santi et al. 2015). Brezáni et al. (2019) tested some LD-treated substrates on edible and medicinal mushrooms, including *P. eryngii*, and concluded that the enrichment of fungal substrate with LD could be an economic and effective procedure for mushroom production. As far as we know, this is the first study where a LD was interacted with a BC or a HA or both and tested on ligninolytic fungi. The evident stimulating effects of the LD examined on *P. eryngii* are very encouraging. Differentiated effects on the *in vitro* growth of some ligninolytic fungi were observed by Taskin et al. (2019) using byproducts of bioenergy production, such as BC and hydrochar.

3.3.2. Irpex lacteus

Data of radial mycelial growth of *I. lacteus* and their plot in the linear model are reported in Figure 5. Treatments with diluted LD generally did not affect the growth of this fungus, except for a slight inhibition at the first sampling and a slight stimulation by LD 1 at 96 h after inoculation (Fig. 5A). Conversely, treatments with BC-LD showed a clear growth stimulation when used at the dose of 1% and some inhibition at the highest dose (Fig. 5B), compared to the control. In this set of trials, the observed biostimulant action of BC-LD on the fungus may probably depend on soluble components released by BC during its interaction phase with LD. In fact, in a previous study, a BC water extract was able to promote the growth of some ligninolytic fungi (Loffredo and Taskin 2018). The treatments HA, HA-LD 2 and HA-BC-LD

2 caused a significant improvement in hyphal elongation in almost all samplings (Fig. 5C). This activity could reasonably be attributed to the biostimulating effects of HA on the fungus, an activity already observed on other ligninolytic fungi (Kulikova et al. 2005), in particular on *Bjerkandera adusta*, *T. versicolor* and *Pleurotus ostreatus* (Loffredo and Taskin 2018).

A very good linearity ($R \geq 0.997$) was obtained fitting the experimental data in the linear equation (Table 4). Based on the growth parameters, only BC-LD 2 reduced ($P \leq 0.05$) the μ value, compared to the control, while all treatments with HA highly significantly stimulated ($P \leq 0.01$) hyphal elongation (Table 4). As already observed for *P. eryngii*, the lag times of this fungus were rather high for all treatments and the control, with the lowest value measured for HA only (Table 4). Profiles of AGR are shown in Figure 6. At the end of the experiments, the maximum and the minimum AGR of *I. lacteus* were recorded for HA and BC-LD 2 treatments, respectively.

3.3.3. Trametes versicolor

At each sampling time, a significant reduction of the mycelial growth was measured in LD and, especially, BC-LD treatments, with the only exception of LD 0.5 which, starting from 72 h after inoculation, resulted ineffective (Fig. 7A and B). Different results were observed using LD 2 and BC-LD 2 preliminary interacted with HA (Fig. 7C). In general, all treatments with HA did not show any inhibition on *T. versicolor*, being the growth of the fungus not different from the control (Fig. 7C). Therefore, a clear antitoxic activity was exerted by HA, possibly due to the adsorption of some toxic components released by LD and BC in the medium. The strong adsorption capacity of HA towards both hydrophilic and, especially, hydrophobic compounds has been widely recognized (Senesi et al. 2001; Chianese et al. 2020).

When the linear model was used to describe the experimental data, R values of all treatments were ≥ 0.99. The μ values obtained indicated that only BC-LD treatments at each dose

significantly reduced the mycelial growth, compared to the control, whereas LD treatments did not really alter the radial extension of the fungus at any dose (Table 5). This finding suggests that the presence of LD in the medium was not suppressive on the fungus but only caused a noticeable delay in growth initiation after inoculation, which is confirmed by the high λ values of LD 1 and LD 2 treatments (Table 5). The inhibitory effects observed with BC-LD treatments were possibly due to same toxic components released by BC during its interaction with LD. These results agree with the findings of Ascough et al. (2010) who reported that a dose of 0.5% of two BCs obtained at 300 and 400 °C from Scots pine reduced the hyphal extension of *T. versicolor*, being the effects more evident for the BC obtained at the higher pyrolysis temperature. The enzyme Mn peroxidase released by white rot basidiomycetes can effectively decompose brown coal, which would suggest the ability of these fungi to degrade BC components and use them as the sole C source (Hofrichter et al. 1999). However, it is known that not all ligninolytic fungi have the complete enzymatic equipment suitable for degrading any lignocellulosic material (Yang et al. 2013). Gibson et al. (2016) found that a pinewood BC produced at a temperature of 450 °C mildly inhibited the respiration rate of *T. versicolor*. These results would suggest particular caution in choosing the type and dosage of BC to be incorporated into soil in order to avoid or limit inhibition of this fungus. However, results showed that when BC-LD was previously interacted with HA (HA-BC-LD 2), the activity changed from inhibition to slight stimulation, with the μ value reaching the maximum value recorded in this set of experiments (Table 5). Hence, it was evident that the interaction of BC-LD with HA annulled the initial chemical stress on the fungus which showed a mycelium expansion even greater than the control. It can be hypothesized that this activity reversal is due to the retention and/or inactivation of some toxic compounds of BC by HA during the interaction phase. The HAs are ubiquitous in soil and their activity on these fungi has already been ascertained (Kulikova et al. 2005). Furthermore, in soil, HA can bind both natural and

xenobiotic organic compounds through various physical and chemical mechanisms, thus also regulating their bioavailability and bioactivity (Pukalchik et al. 2019). The numerous and different functional groups of HA allow a very high sorption efficiency towards organic molecules, especially the most hydrophobic ones. However, further studies are needed to better clarify this aspect. A recent study has shown the stimulating action of BC and hydrochar on various ligninolytic fungi, including *T. versicolor* (Taskin et al., 2019).

Among the fungi examined in our study, *T. versicolor* showed the fastest growth (higher μ values) and the shortest lag times (lower λ values) (Table 5). The AGR of this fungus seemed reduced by all LD and BC-LD treatments, compared to the control, but this effect clearly decreased along the experiments (Fig. 8). Differently, compared to the control, all treatments with HA did not change the AGR of the fungus (Fig. 8).

3.4. Relationship between substrate properties and fungal growth

To evaluate the possible relationships between the properties of the various media and their activity on the fungi, the correlation coefficients between the pH and EC values of the media (Table 2) and the μ and λ values of the fungi (Tables 3-5) were calculated. Significant positive correlations were obtained only between pH ($P \leq 0.03$) or EC ($P \leq 0.04$) and radial growth rate (μ) of *P. eryngii*, while non-significant negative correlations were found in all other cases. The unexpected positive correlations obtained only for *P. eryngii* suggest a greater tolerance of this fungus to the alkalinity and salinity of the medium, compared to the other fungi. Based on these results, we can hypothesize that the fungal response to the different treatments is regulated by a multitude of factors, including the fungal species. Unfortunately, there is no information in the literature on this matter and therefore it is not possible to compare our findings with those of other authors.

Trying to relate the spectroscopic characteristics of the media and their activity on the fungi is quite difficult and perhaps too speculative. However, when the FI values obtained from luminescence analysis for the various media (the higher FI for LD, BC-LD and HA-BC-LD) were correlated to the corresponding μ values (at the highest dose) of each fungus, a low *P* value ($P = 0.08$) was observed only for *P. eryngii*. For this fungus, the higher molecular complexity of the medium seemed to favour its growth. Unfortunately, from the total luminescence spectra it is not possible to deduce other useful information to understand the structure–activity relationship of these media on the fungi. Comparison of the FTIR spectra of the various media also did not provide further information in this respect. As far as we know, there is no information in the literature on this matter that could contribute to a discussion.

4. Conclusions

As biogas production increases, the amount of LD released as by-product increases. In compliance with the principles of the circular economy which recommend the conversion of waste into a resource, it appears important to investigate the best applications of this material. In the present pioneering study, we aimed to characterize a LD from mixed biomass and test it on three fungal species that are not only involved in the soil C-cycle but also in the important food (*P. eryngii*) and medicinal (*T. versicolor*) sectors. Various fungal growth media were prepared using only diluted LD or LD previously treated with a BC and/or a HA. The examined media exerted a differentiated activity on the tested fungi. The LD showed a surprising stimulation of *P. eryngii* that persisted also in the presence of BC and HA. Negligible effects were exerted by LD alone on *I. lacteus* who, on the other hand, was significantly improved by the same LD after interaction with BC and HA. The ineffectiveness of LD alone and the inhibition of BC-treated LD on *T. versicolor* turned into an evident stimulation when the two media were interacted with HA which allowed the same fungus to achieve the highest growth

rate. The different response of the fungi to the examined media suggests the involvement of specific compounds or some medium characteristics that are not easily identifiable. The information obtained from the spectroscopic characterization of the media, such as total luminescence analysis and FTIR spectroscopy, did not allow to understand the mechanisms underlying the fungal growth response to the various media. Further studies are needed to clarify this aspect. However, the overall results obtained in this study appear to encourage the use of LD to promote the growth of these fungi. In particular, an interesting economic exploitation could be the use of LD in the cultivation of the edible mushroom *P. eryngii*.

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Disclosure statement

The authors report there are no competing interests to declare.

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Figure Legends

Figure 1. Total luminescence spectra of the media.

Figure 2. FTIR spectra of the media.

Figure 3. Effects of LD untreated (A) and interacted with BC (B) and/or HA (C) at different dosages on the radial mycelial growth of *P. eryngii*. The linear plot of data is shown on the right side. The vertical line on each bar indicates the standard error (n = 5). The mean of any treatment was compared to the control at any sampling by the LSD test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 4. Absolute growth rate of *P. eryngii*.

Figure 5. Effects of LD untreated (A) and interacted with BC (B) and/or HA (C) at different dosages on the radial mycelial growth of *I. lacteus*. The linear plot of data is reported on the right side. The vertical line on each bar indicates the standard error (n = 5). The mean of any treatment was compared to the control at any sampling by the LSD test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 6. Absolute growth rate of *I. lacteus*.

Figure 7. Effects of LD untreated (A) and interacted with BC (B) and/or HA (C) at different dosages on the radial mycelial growth of *T. versicolor*. The linear plot of data is shown on the right side. The vertical line on each bar indicates the standard error (n = 5). The mean of any treatment was compared to the control at any sampling by the LSD test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 8. Absolute growth rate of *T. versicolor*.

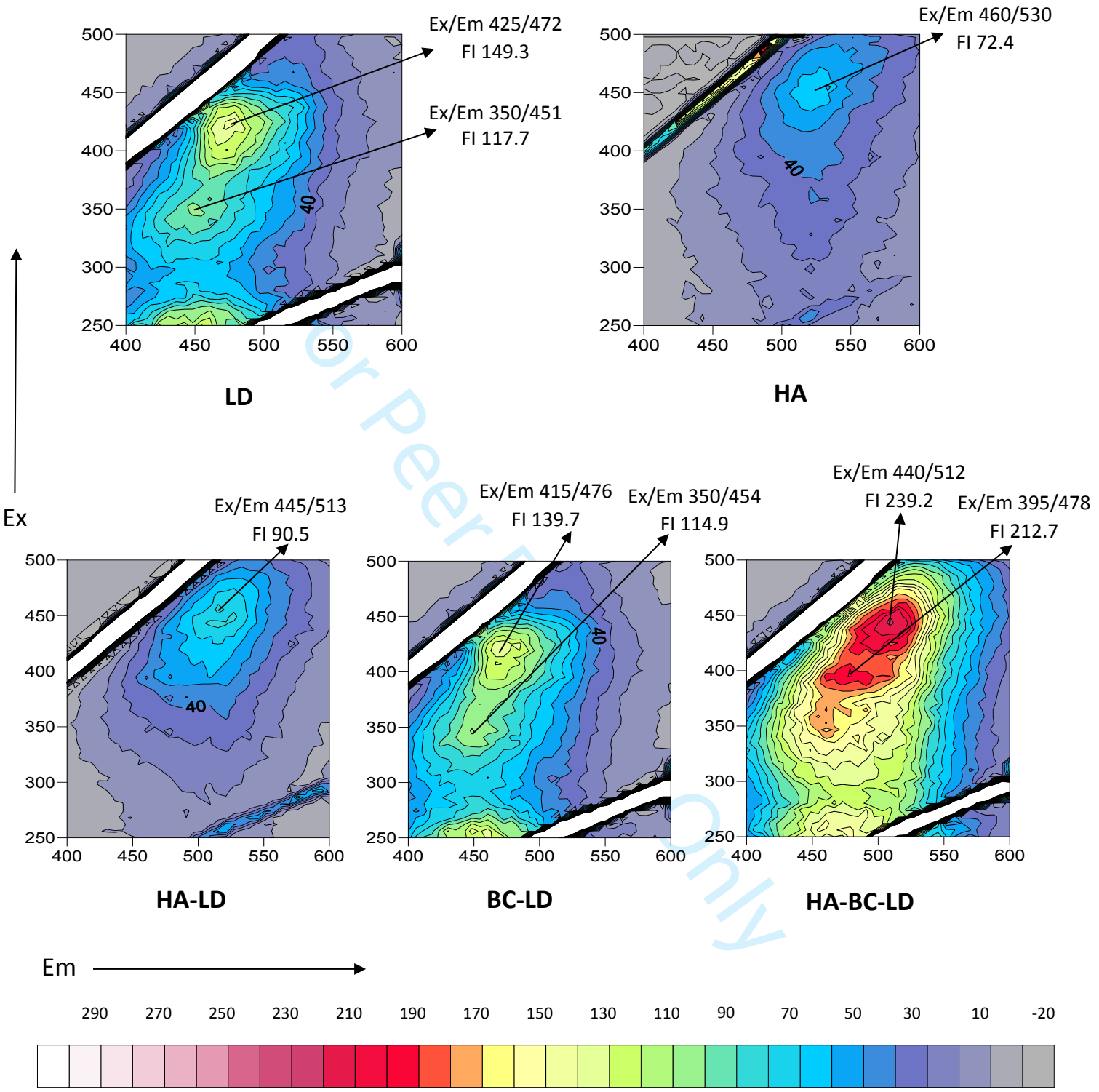

Figure 1

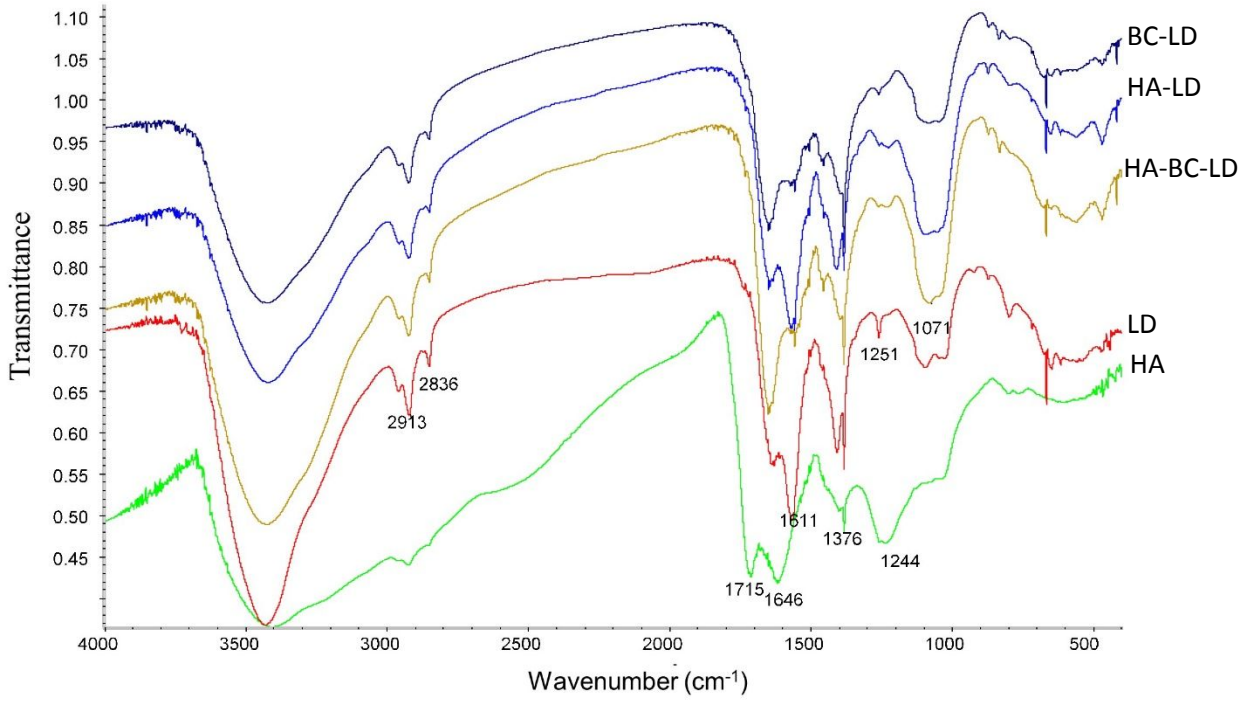

Figure 2

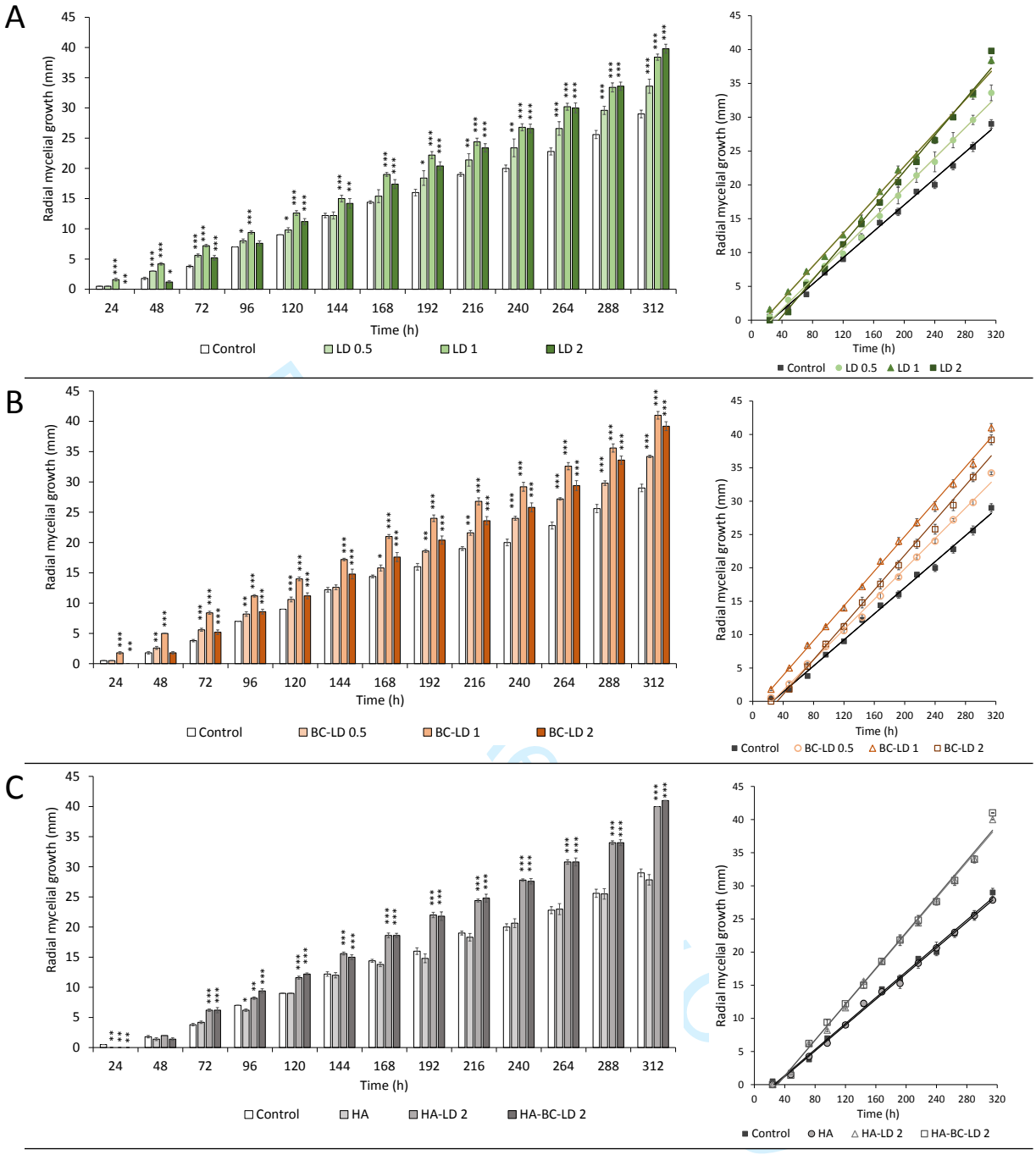
A
Radial mycelial growth (mm)
Time (h)
Control
LD 0.5
LD 1
LD 2
B
Radial mycelial growth (mm)
Time (h)
Control
BC-LD 0.5
BC-LD 1
BC-LD 2
C
Radial mycelial growth (mm)
Time (h)
Control
HA
HA-LD 2
HA-BC-LD 2

Figure 3

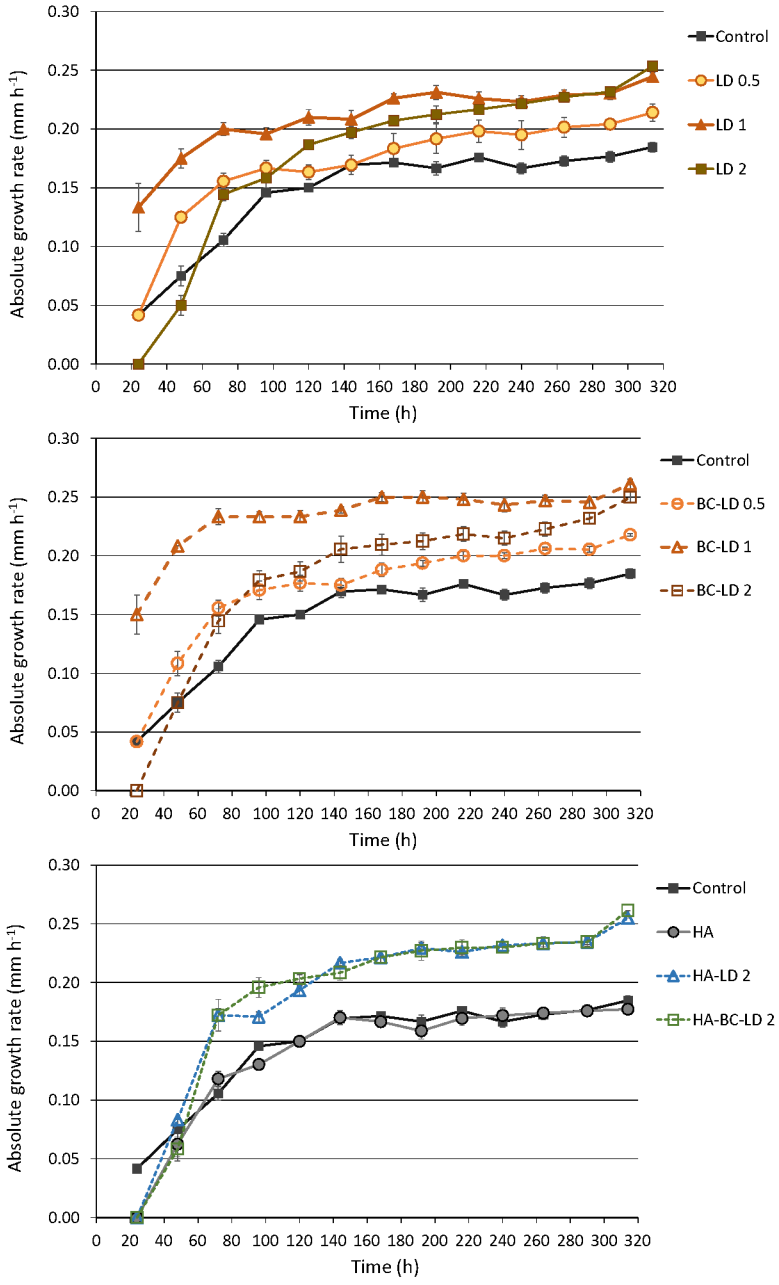
Absolute growth rate (mm h-1)
Time (h)
Control
LD 0.5
LD 1
LD 2
BC-LD 0.5
BC-LD 1
BC-LD 2
HA
HA-LD 2
HA-BC-LD 2

Figure 4

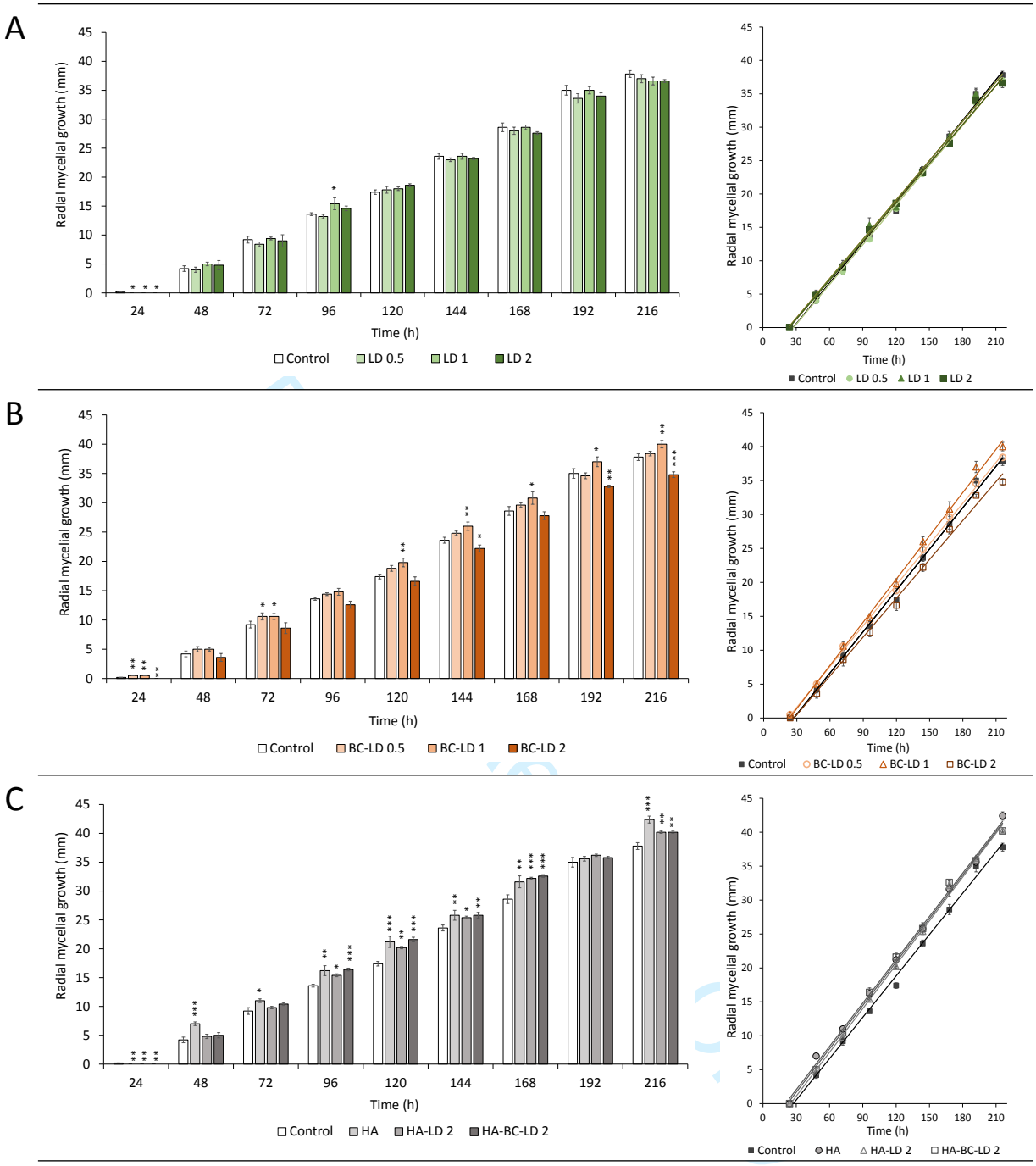
A
Radial mycelial growth (mm)
Time (h)
Control
LD 0.5
LD 1
LD 2
B
Radial mycelial growth (mm)
Time (h)
Control
BC-LD 0.5
BC-LD 1
BC-LD 2
C
Radial mycelial growth (mm)
Time (h)
Control
HA
HA-LD 2
HA-BC-LD 2

Figure 5

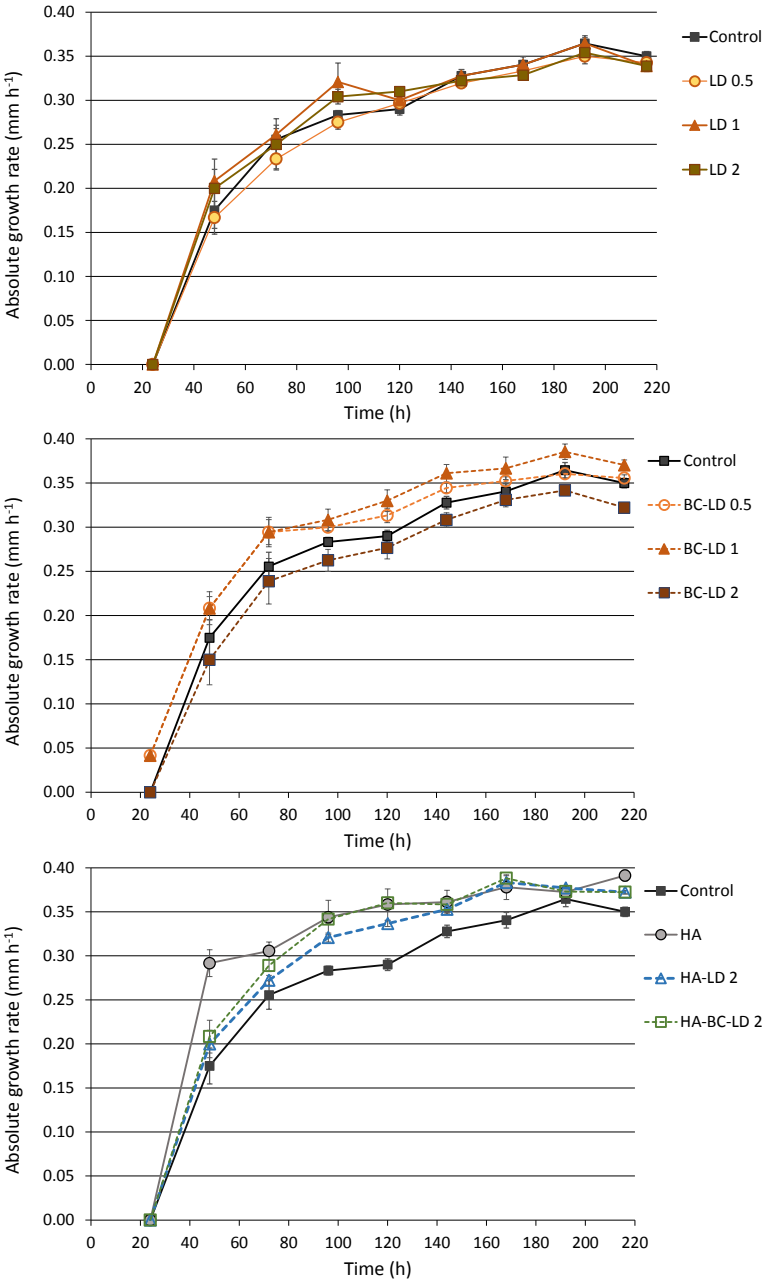
Absolute growth rate (mm h-1)
Time (h)
Control
LD 0.5
LD 1
LD 2
Absolute growth rate (mm h-1)
Time (h)
Control
BC-LD 0.5
BC-LD 1
BC-LD 2
Absolute growth rate (mm h-1)
Time (h)
Control
HA
HA-LD 2
HA-BC-LD 2

Figure 6

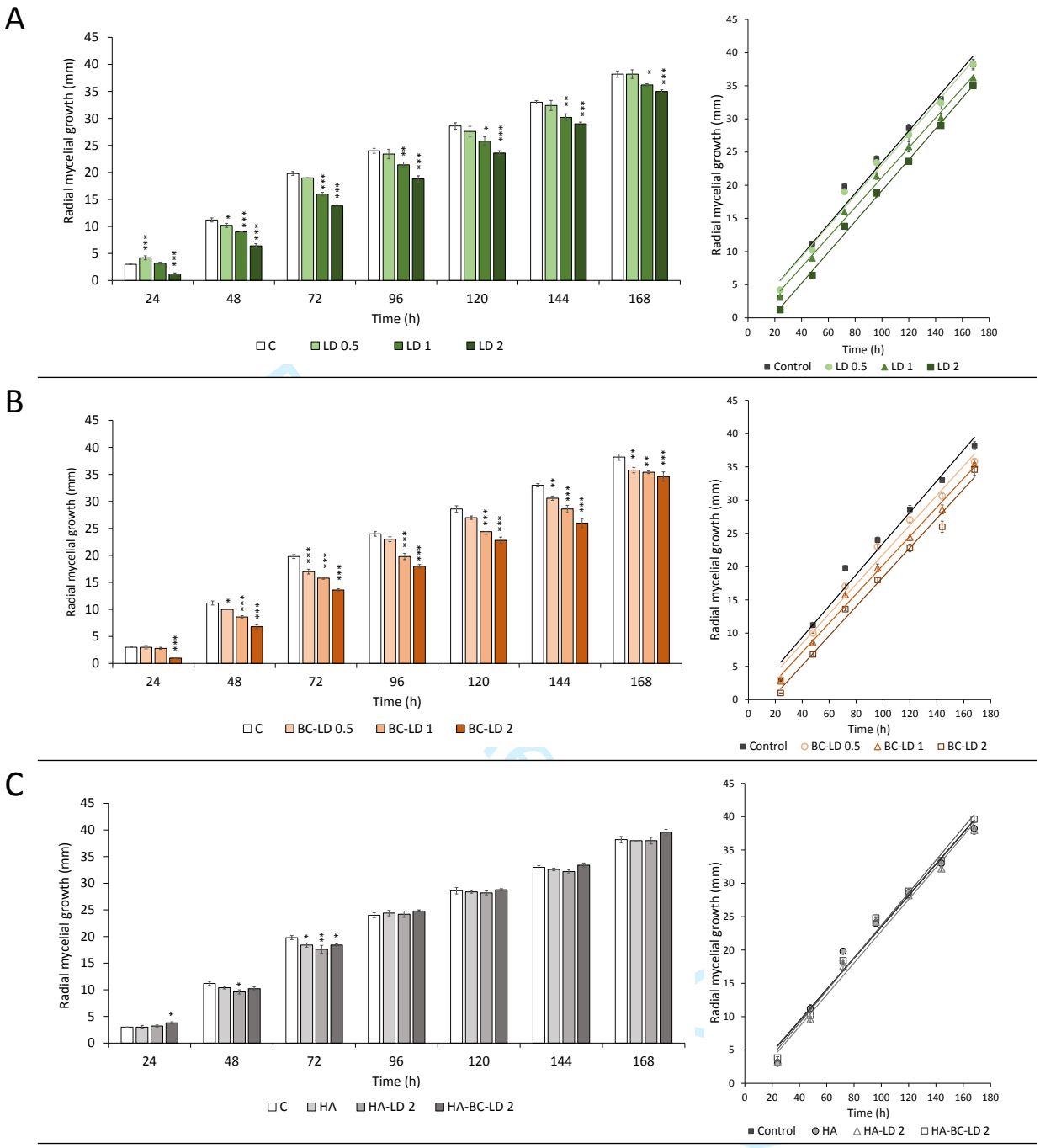
A
Radial mycelial growth (mm)
Time (h)
C
LD 0.5
LD 1
LD 2
Control
B
BC-LD 0.5
BC-LD 1
BC-LD 2
C
HA
HA-LD 2
HA-BC-LD 2

Figure 7

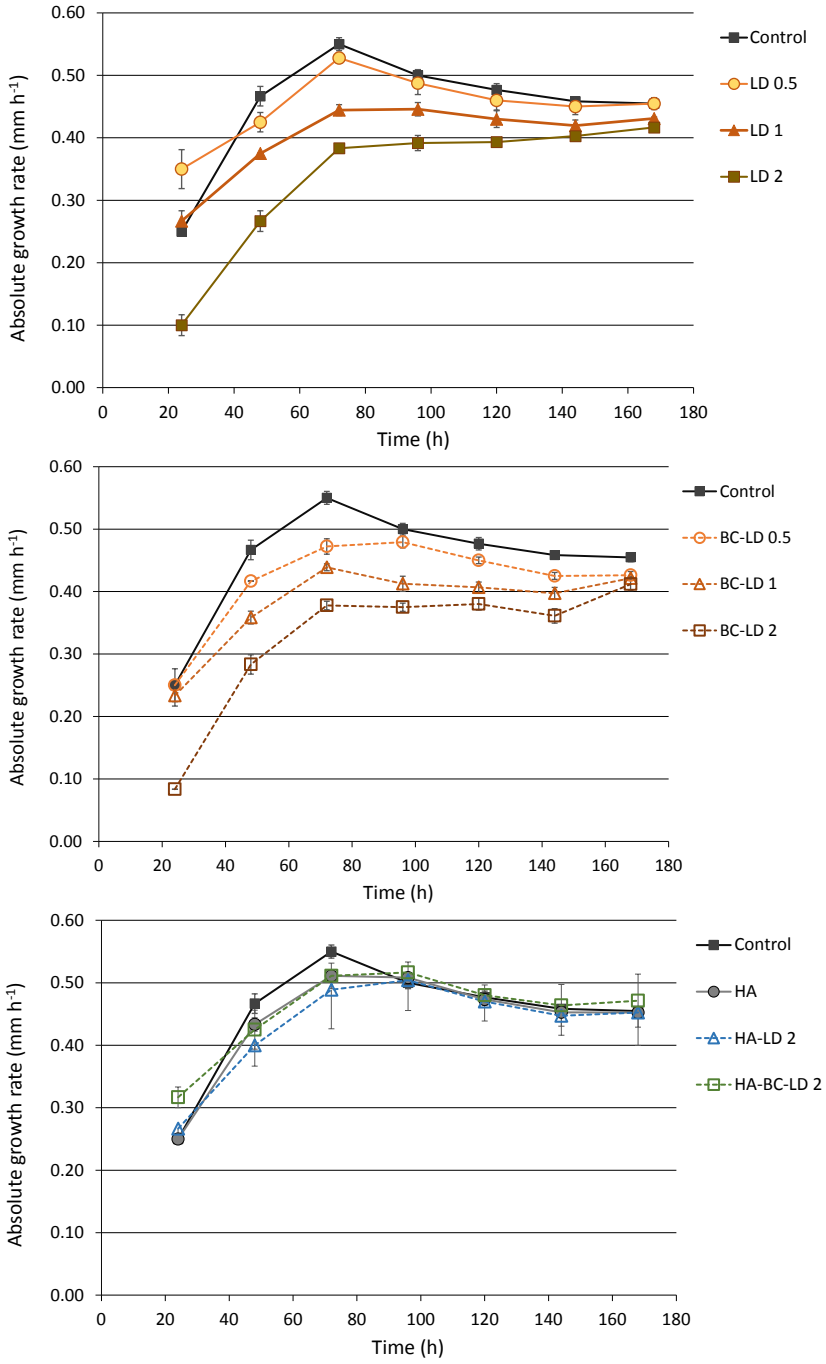
Absolute growth rate (mm h-1)
Time (h)
Control
LD 0.5
LD 1
LD 2
BC-LD 0.5
BC-LD 1
BC-LD 2
HA
HA-LD 2
HA-BC-LD 2

Figure 8

Table 1. Physicochemical properties of LD.

Parameter	Value
pH	8.45
Total solids (g L^{-1})	29.80
Total solids (% f.w.)	2.98
Volatile solids (g L^{-1})	21.79
Volatile solids (% t.s.)	73.12
TOC (mg L^{-1})	12640
TOC (% t.s.)	42.42
Total N (mg L^{-1})	2400
Total N (% t.s.)	8.05
NH_4^+-N (mg L^{-1})	1540
NH_4^+-N (% total N)	64.17
TOC/TN	5.3
COD (mg L^{-1})	19400
EC (dS m^{-1})	1.56
Ash (% d.w.)	26.88

f.w.: fresh weight; d.w.: dry weight; t.s.: total solids

Table 2. pH and EC values of the liquid media.

Medium	pH	EC (dS m^{-1})
LD 0.5	8.20	0.13
LD 1	8.26	0.28
LD 2	8.29	0.47
BC-LD 0.5	7.90	0.12
BC-LD 1	7.98	0.24
BC-LD 2	8.10	0.44
HA	7.59	0.37
HA-LD 2	7.94	0.42
HA-BC-LD 2	8.02	0.44

Table 3. Growth parameters of *P. eryngii* calculated from the linear model with breakpoint.

TREATMENT	μ (mm h^{-1})	λ (h)	R	Growth equation
Control	0.098	25.61	0.998	y = 0.098x – 2.51
LD 0.5	0.112**	25.63	0.998	y = 0.112x – 2.87
LD 1	0.124***	16.10	0.998	y = 0.124x – 1.98
LD 2	0.135***	35.82	0.997	y = 0.135x – 4.80
BC-LD 0.5	0.114***	25.61	0.999	y = 0.114x – 2.92
BC-LD 1	0.131***	10.08	0.999	y = 0.131x – 1.31
BC-LD 2	0.131***	31.98	0.997	y = 0.131x – 4.19
HA	0.097	27.63	0.999	y = 0.097x – 2.68
HA-LD 2	0.134***	30.15	0.998	y = 0.134x – 4.04
HA-BC-LD 2	0.135***	30.37	0.997	y = 0.135x – 4.10

μ: radial mycelial growth rate; λ: lag time.

Table 4. Growth parameters of *I. lacteus* calculated from the linear model with breakpoint.

TREATMENT	μ (mm h^{-1})	λ (h)	R	Growth equation
Control	0.203	27.30	0.998	y = 0.203x – 5.54
LD 0.5	0.198	27.62	0.999	y = 0.198x – 5.48
LD 1	0.197	22.99	0.997	y = 0.197x – 4.52
LD 2	0.194	23.70	0.999	y = 0.194x – 4.61
BC-LD 0.5	0.201	22.10	0.999	y = 0.201x – 4.43
BC-LD 1	0.212*	23.41	0.999	y = 0.212x – 4.97
BC-LD 2	0.191*	27.43	0.997	y = 0.191x – 5.23
HA	0.213**	20.30	0.999	y = 0.213x – 4.32
HA-LD 2	0.215**	24.87	0.999	y = 0.215x – 5.35
HA-BC-LD 2	0.213**	22.13	0.998	y = 0.213x – 4.72

μ: radial mycelial growth rate; λ: lag time.

Table 5. Growth parameters of *T. versicolor* calculated from the linear model with breakpoint.

TREATMENT	μ (mm h^{-1})	λ (h)	R	Growth equation
Control	0.235	0.13	0.989	y = 0.235x – 0.03
LD 0.5	0.231	0.00	0.994	y = 0.231x – 0.00
LD 1	0.225	5.96	0.997	y = 0.225x – 1.34
LD 2	0.233	17.55	0.999	y = 0.233x – 4.09
BC-LD 0.5	0.223*	2.06	0.992	y = 0.223x – 0.46
BC-LD 1	0.218**	7.20	0.996	y = 0.218x – 1.57
BC-LD 2	0.221*	16.56	0.995	y = 0.221x – 3.66
HA	0.237	0.12	0.990	y = 0.235x – 0.00
HA-LD 2	0.238	4.33	0.993	y = 0.238x – 1.03
HA-BC-LD 2	0.244*	3.03	0.994	y = 0.244x – 0.74

μ: radial mycelial growth rate; λ: lag time.