Fungal Pretreatment of Sugarcane Bagasse: A Green Pathway to Improve Saccharification and Ethanol Production

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Abstract

Biological pretreatment was investigated to increase ethanol production from lignocellulosic biomass, like sugarcane bagasse. Enzyme secretion, changes in substrate composition, enzymatic hydrolysis and ethanol yield after pretreatment by different basidiomycetes were evaluated. Analysis by Fourier transform infrared spectroscopy showed that *P. pulmonarius* PS2001 and *T. villosa* 82I6 promoted more extensive selective modifications in the lignin content. Glucose release during enzymatic hydrolysis of samples pretreated with *P. pulmonarius* PS2001 for 35, 42 and 49 days and with *T. villosa* 82I6 for 21, 28 and 49 days were higher than the control (48.5±2.38 mg/g), i.e. 68.4 ±0.7, 76.3 ±1.6 and 76.5±2.1 mg/g and 70.9±8.3, 77.8±5.8 and 77.6±4.2 mg/g, respectively. During the fermentation of hydrolysates of samples pretreated with *T. villosa* 82I6 for 28 and 49 days, a maximum ethanol yield of 19.1±2.8 and 20.2±0.5 mg/g, respectively, was achieved. A positive effect of biological pretreatment on hydrolysis and fermentation was demonstrated.

1 Introduction

The lignocellulosic materials has become increasingly attractive due to its potential for the production of sugars and fuels [1][2]. Cellulosic biomass, derived from non-food sources such as trees and grasses, is being exploited as a feedstock for cellulosic ethanol production. All lignocellulosic materials are, in principle, suitable for the production of ethanol or other biotechnological products [3], [4], [5], [6].

Brazil is the world's largest producer of sugarcane, and it is estimated that the production of sugarcane in the 2019/20 harvest will be 642.73 million tons [7]. Bagasse, a fibrous material remaining after the extraction of sugarcane juice, corresponds to one-third of the energy content of sugarcane [8]. Sugarcane bagasse is low cost since it does not find many applications. However, due to abundant quantities, it is important to find its applications [8], [9].

The base structure of all lignocellulosic biomass consists of three main components: cellulose, hemicellulose and lignin, as well as small amounts of extractives and minerals [10]. These constituents are linked together, forming a complex network resistant to microbial attacks [11], [12].

According to Balat et al. (2008) [13], biomass transformation to second-generation ethanol is composed of four main operation units: pretreatment, hydrolysis, fermentation and product separation. Pretreatment and the production of the enzymes used in the hydrolysis step are the factors that contribute most to the cost of processing second-generation ethanol [14] and have attracted much attention in recent years [15].

Pretreatment is among the great challenges associated with converting lignocellulosic biomass to ethanol and is one of the most crucial steps in the process [16], [17], [18]. The main objective is to remove or minimize the physical and chemical barriers caused by the close association of the main components of lignocellulosic biomass, increasing the accessibility of cellulose to enzyme [19], [20].

Since physical, chemical and physicochemical methods require higher energy demand, high reagent costs and the treatment of process residues, which are often highly polluting, in addition to forming compounds

that inhibit the hydrolysis and fermentation stages, biological pretreatment had emerged as an alternative technology [21].

Biological pretreatment consists of the disorganization of lignocellulosic biomass by enzymes produced by microorganisms, especially basidiomycetes fungi, making the sugars accessible to the hydrolysis and fermentation stages for ethanol production. In contrast to chemical and physical-chemical pretreatment methods, there is no need to use chemicals or high amounts of energy and there is no generation of toxic compounds which could compromise the subsequent steps of hydrolysis and fermentation [22], [23]. Fungi have two extracellular enzyme systems: the hydrolytic system, which produces hydrolases responsible for the degradation of polysaccharides, and the oxidative system, which degrades lignin and opens phenyl rings [24], [25].

Based on these considerations, in the present study, sugarcane bagasse was pretreated with seven basidiomycetes, namely *Pleurotus pulmonarius* PS2001, *Trametes villosa* 82I6, *Schizophyllum commune* VE07, *Pleurotus albidus* 78F-13, *Pycnoporus sanguineus* OU-04, *Pycnoporus sanguineus* PR-32 and *Marasmiellus palmivorus* VE111. The main purpose was select the species most appropriate for improving the subsequent enzymatic hydrolysis and fermentation.

2. Material And Methods

2.1. Microorganisms

The strains used as biological agents in the biomass pretreatment stage (belonging to the collection of the Laboratory of Enzymes and Biomasses of the Biotechnology Institute of the University of Caxias do Sul) are listed in Table 1, with the collection and access code of the library of the University of Caxias do Sul. The strains were grown on sawdust medium for up 15 days at 28°C, and then stored at 4°C. These strains were selected considering previous screening, searching for strains that produce enzyme-producing strains involved in lignin degradation.

For glucose fermentation, *Saccharomyces cerevisiae* strain CAT-1 was used. This strain was kindly provided by Dr. Luiz Humberto Gomes of Escola Superior de Agricultura Luiz de Queiroz (ESALQ), University of São Paulo, Piracicaba, SP. The strain was grown on yeast extract peptone dextrose (YPD) medium for up to 2 days at 28°C, and then stored at 4°C until use.

2.2 Substrate

The sugarcane bagasse was used dry and previously ground, kindly provided by Professor George Jackson de Moraes Rocha, from the National Laboratory of Science and Technology of Bioethanol, Campinas, SP, Brazil.

2.3 Biological pretreatment of sugarcane bagasse with basidiomycetes

The biological pretreatment (BPT) was performed using previously dried ground sugarcane bagasse as the major compound of the culture medium. Wheat bran 2.5% (w/v) and calcium carbonate ($CaCO_3$ 1% w/v)

were added to the biomass and the humidity was adjusted to around 66%, as described by Gambato et al. (2016) [26].

As inoculum, mycelial discs 1.5 cm in diameter were used, one disc in each bag. To obtain these disks, each strain of basidiomycete was propagated in Petri dishes containing sawdust medium, constituted of (w/v) 2% ground sawdust (*Pinus* sp.), 2% ground wheat bran, 2% of agar-agar and 0.2% $CaCO_3$, completed with distilled water, for 15 days at 28ºC, until total colonization of the medium surface. Then, in a sterile manner, discs were cut using a glass tube. Each bag containing sterile medium received a disk of mycelial mass as the inoculum. Each basidiomycete was considered as a pretreatment, performed in triplicate for each sampling time point, i.e. 7, 14, 21, 28, 35, 42 and 49 days. These samples were collected for measurements of cell growth, medium characterization and enzyme production.

2.4 Estimate of fungal growth

Fungal biomass was determined indirectly via the enzymatic hydrolysis of cell wall chitin. For the determination of the N-acetyl-D-glucosamine content resulting from the hydrolysis of chitin, the methodology described by Novello et al. (2014) [4].

2.5 Determination of enzymatic activities

Extraction of the enzymes was performed by suspending the sample in distilled water at 4°C in a solid/liquid ratio of 1:2, then shaking manually for 30 minutes. After, the samples were centrifuged (30 minutes, 2862×g, 4°C) and the supernatant was stored under refrigeration (4°C) for further analysis.

The activity of laccases was determined according to Schneider et al. (2018) [27] and the activity of total peroxidases was assessed according to Heinzkill et al. (1998) [28], by quantifying the oxidation product of the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) used as the substrate. For the determination of the activity of manganese peroxidases, phenol red was used as the substrate, according to the method proposed by Kuwahara et al. (1984) [29].

The determination of xylanase activity was performed according to Bailey et al. (1992) [30]. The analysis of enzymatic activity on filter paper (FPA) was determined according to Ghose (1987) [31], with modifications proposed by Camassola & Dillon (2012) [32]. The determination of endoglucanase activity was performed according to the methodology described by Ghose (1987) [31], with modifications. The determination of β-glucosidase activity was performed using the methodology adapted from Daroit et al. (2008) [33].

2.6 Chemical analysis of the cellulosic substrates

The chemical composition of the samples was determined according to the methodology proposed by the National Renewable Energy Laboratory (NREL-TP-510-42618, NREL-TP-510-42619 and NREL-TP-510-42621) [34][35][36].

2.7Characterization of sugarcane bagasse before and after biological pretreatment

Scanning electron microscopy (SEM) (Shimadzu SSX-550 Superscan) was used to characterize the sugarcane before and after biological treatment. As part of the imaging procedure, the samples were sputter coated with gold layers.

Fourier transform infrared (FTIR) spectroscopy was performed according to ASTM E1252-98 (Reapproved 2013) or ASTM E573-01 (Reapproved 2013). Sample spectra were obtained in duplicate using an average of 128 scans in the range between 850 cm^{-1} and 2000 cm^{-1} with a spectral resolution of 2 cm^{-1}. Peak heights and areas of the Fourier transform infrared (FTIR) spectra were determined using Origin software version 6.0. The influence of the biological pretreatment was analyzed in terms of the percent diminution in the intensity of the lignin (1427 and 1515 cm^{-1}) and carbohydrate peaks (1395, 1098 and 898 cm^{-1}) [3],[37], [38].

2.8 Enzymatic hydrolysis of sugarcane bagasse

Enzymatic hydrolysis was carried out using the *Penicillium echinulatum* S1M29 enzyme complex, mainly composed of endoglucanases, cellobiohydrolases and β-glucosidases, produced in the Laboratory of Enzymes and Biomass (IB-UCS) [39], [40], [41]. The procedure was conducted in 50 mL Duran® flasks, containing 5% (w/v) of biomass biologically pretreated with basidiomycetes. This percentage of biomass was used to evaluate the reactivity of the fibers, before and after pretreatment. The enzyme loading was 20 FPU/g of dry biomass, suspended in 50 mmol/L citrate buffer, pH adjusted to 4.8, which was added until reaching a final volume of 50 mL. The flasks were kept at 50°C under reciprocal shaking at 150 rpm for 24 hours. Enzymatic hydrolysis occurred according to the sampling time of the culture of the basidiomycetes. As controls, the enzymatic hydrolysis of non-pretreated sugarcane bagasse was carried out under the same conditions. Samples of the hydrolyzate for the determination of reducing sugars and glucose were collected at 0, 6, 12 and 24 hours. A 1 mL volume was withdrawn at each sampling time, centrifuged (2862×g) and frozen (-4°C) for further analysis. The enzymatic hydrolysis of the biomass was carried out in triplicate.

2.9 Alcoholic fermentation

The alcoholic fermentation of sugars released from sugarcane bagasse was carried out using a *S. cerevisiae* CAT-1 at a concentration of 10^8 viable cells/mL for 24 h. Ethanol production was performed by a saccharification and fermentation process, carried out separately.

The experiments were performed in 2 mL tubes containing a suspension of sugarcane bagasse submitted to hydrolysis and supplemented to obtain a final concentration of 4 mg/mL of Prodex® (crude yeast extract) and 1 mg/mL of $(NH_4)_2SO_4$ at 28ºC under static conditions. Samples were taken to determine the consumption of glucose and xylose and ethanol production by high performance liquid chromatography (HPLC). Samples was analyzed using a Shimadzu chromatography system. The analysis was performed on an Aminex HPX-87H colum gn (Bio-Rad®) by the refractive index detector at 60°C, with 5 mmol/L H_2SO_4 (mobile phase) and a flow rate of 0.6 mL/min. The ethanol yield was calculated according to Menegol et al. (2016) [42].

2.10 Statistical tests

The results of fermentation were analyzed with GraphPad Prism 5.0, using analysis of variance and Tukey's post-test, with $p<0.05$.

3 Results And Discussion

3.1 Evaluation of the growth and production of enzymes in sugarcane bagasse

In Figure 1, the growth profiles of the different species in sugarcane bagasse medium are shown as a function of incubation time. It was found that all basidiomycetes species tested were able to colonize the medium.

It was observed that the species that apparently presented the greatest formation of mycelium were *S. commune* VE07, reaching about 0.25±0.004 g of mycelium per g of dry biomass (g/g), followed by *P. sanguineus* OU04, *M. palmivorus* VE111, *P. sanguineus* PR32 and *T. villosa* 82I6, which reached, respectively, 0.18±0.005, 0.16±0.001, 0.13±0.012 and 0.11±0.005 g/g. The two species that presented the lowest growth were *P. albidus* 88F-13 (0.07±0.006 g/g) and *P. pulmonarius* PS2001 (0.05±0.003 g/g).

When comparing the growth data of *P. albidus* 88F-13 in other lignocellulosic residues, Stoffel et al. [43] detected growth of 0.13 g/g of mycelium when grown in brewer spent grain and 0.03 g/g in grape bagasse. Although Stoffel et al. [43] used the determination of indirect growth through the dosage of ergoesterol, it is noticed that there is coherence between the growth data, indicating that the growth is variable according to the substrate used for the cultivation of the fungi.

The differences observed during growth may be due to several factors, among others firstly to the characteristics of the strain, the presence of macronutrients and micronutrients required for the species, the type of substrate and the pH of the medium [26], [44]. Biological pretreatment has been associated with modifications to the biomass via the action of enzymes produced during colonization. These enzymes act at specific substrate locations, and may degrade polyphenols in the lignin and also degrade the structure of the hemocellulose heteropolysaccharide, in addition to reaching cellulose fibers [45], [23], [18].

The enzymatic complex responsible for the degradation of lignin is composed of laccases and peroxidases [46]. The lignin peroxidase degrades non-phenolic units and manganese peroxidase acts on phenolic and non-phenolic lignin units [47]. Laccases are phenol-oxidases that act together with the peroxidases to oxidize phenolic components, leading to the complete degradation to CO_2 and H_2O [48], [49].

The hydrolysis of hemicellulose, a polysaccharide that forms a reticulated structural network and contributes to the integrity of the vegetal cell wall, is catalyzed by xylanases [47]. Endoglucanases act at randomly at various sites in the amorphous regions of the cellulose fiber, reducing the degree of polymerization and opening sites for the further action of cellobiohydrolases [50]. Endoglucanases and cellobiohydrolases act in synergy in cellulose hydrolysis [51] and β-glucosidases hydrolyze cellobiose and cellulose oligosaccharides to glucose. The presence of these enzymes during pretreatment may be

favorable to the process, provided that the microorganism does not use the sugars resulting from the action of this set of enzymes in its metabolism, since all sugars are important to the fermentation process. Therefore, it is necessary to evaluate the species of basidiomycetes in order to identify those that are able to promote the delignification of the biomass, but that have little or no metabolic activity for the use of cellulose and hemicellulose [52]. Thus, the production of different enzymes that act on these biomass components was evaluated.

Figure 2 shows the data on the production of laccases, manganese peroxidases, total peroxidases, β-glucosidases, endoglucanases, and xylanases, as well as filter paper activity (FPA) by basidiomycete species as a function of incubation time.

Regarding the production of laccases, it was observed that *M. palmivorus* VE111 stood out, showing increasing activity until day 42 of the process, with a peak of 1985±235 U/g. The species *P. pulmonarius* PS2001 and *P. sanguineus* PR32, followed by *P. albidus* 88F-13, achieved activities of 1256±65, 1185±89 and 785±72 U/g, respectively, at 35 days of culture (Figure 2A).

It was observed that the species that produced the highest amounts of manganese peroxidases were *P. albidus* 88F-13 (21.3±2.6 U/g), presenting increasing activities in up to 21 days of cultivation, and *P. pulmonarius* PS2001 (21±3.7 U/g) and *T. villosa* 82 I6 (17±1 U/g), which showed increasing activities in up to 14 days of cultivation (Figure 2 B).

The species that excelled in the production of total peroxidases were *M. palmivorus* VE111, which presented an enzymatic peak of 518±31 U/g after 28 days of the process, followed by *P. pulmonarius* PS2001 (472±48 U/g in 42 days) and *P. albidus* 88F-13 (423± 35U/g in 21 days). *P. pulmonarius* PS2001, *P. albidus* 88F-13 and *T. villosa* 82I6 showed low β-glucosidase activities throughout the entire culture period. The *P. sanguineus* strains PR32 and OU04 presented the maximum activity of β-glucosidase, i.e. 7.1±1.0 and 6.0±0.4 U/g, respectively, after 35 days. *S. commune* VE07 and *M. palmivorus* VE111 had an enzymatic peak of 3.9 ±0.4 and 3.9±0.9 U/g, respectively, after 21 days (Figure 2D).

The endoglucanase activities were low for all evaluated species; *M. palmivorus* VE111 was the species that presented the lowest activity of this enzyme, reaching only 0.09±0.005 U/g. *P. albidus* 88F-13 showed an enzymatic peak of 0.2±0.05 U/g after 7 days of culture. At 21 days, *S. commune* VE07 had maximum activity of 0.3±0.03 U/g. For the other species, the activities increased throughout the process, reaching values close to 0.3 U/g at the end of 49 days (Figure 2E).

The species that presented the highest FPA activity at the end of 49 days of cultivation was *T. villosa* 82I6 (2.3±0.1 U/g), followed by *P. sanguineus* OU04, which had a peak activity of 1.4±0.1 U/g after 35 days of incubation. In general, the tested species showed low values of this enzyme activity (Figure 2F).

In relation to xylanase production, *S. commune* VE07 showed the highest activity of 6.5±0.2 U/g after 21 days of culture, followed by *P. albidus* 88F-13, which presented an activity of 6.0±1.1 U/g at 35 days. *T. villosa* 82I6 presented an enzymatic peak of 5.5±1.2 U/g after 42 days of processing. After the maximum

activities were reached, these species sustained activities on the order of 4.5 U/g until the end of the process (Figure 2G).

It was evident that the studied species secreted different enzymatic complexes, which resulted in differences in the degradation of the components of the sugarcane bagasse. However, when cell growth was related to the production of enzymes by *P. pulmonarius* PS2001, *P. albidus* 88F-13 and *T. villosa* 82I6, these species showed lower mycelial formation and produced high amounts of laccases and peroxidases. However, according to Leisola et al. (2012) [53], the secretion of enzymes capable of degrading lignin by white rot fungi has some peculiarities. Among them is the fact that lignin is degraded only after nutrient depletion, which triggers the secondary metabolism of the microorganism.

3.2 Evaluation of basidiomycetes biodegradation patterns by FTIR spectroscopy

FTIR spectroscopy was performed focusing on the bands corresponding to cellulose, hemicelluloses and lignin (Table 2). Five bands were evaluated: 1515 cm^{-1} (aromatic skeletal vibrations in lignin), 1427 cm^{-1} (syringyl and guaiacyl condensed nuclei), 1375 cm^{-1} (cellulose and hemicelluloses), 1098 cm^{-1} (crystalline cellulose) and 898 cm^{-1} (amorphous cellulose). The analysis of these spectra in terms of percent modifications of each treated sample relative to the control sample (non-pretreated) and the weight losses after pretreatment is shown in Table 2.

It is possible to estimate the degree of degradation of the substrate through the weight loss of the lignocellulosic biomass after cultivation [18]. The species that promoted significantly higher weight loss than the other species was *P. albidus* 88F-13 (40.9%±0.7). The species *P. pulmonarius* PS2001, *T. villosa* 82I6, *P. sanguineus* OU04 and PR32, promoted statistically equivalent weight loss. *M. palmivorus* VE111 presented significantly lower weight loss (19.9%±2.3) than that observed in pretreatments with the other strains. Weight losses are obviously associated with the growth of each fungus and are the result of the transformation of plant biomass into fungal biomass and CO_2 generation [3].

Decreases were observed in all FTIR bands analyzed after pretreatment; however, the modifications in the different bands were different between the biological pretreatments. Only *S. commune* VE07, which also presented a minor degree of weight loss, had null reduction values for all bands. This species probably consumed small amounts of biomass components, which was not possible to be detect by FTIR (Table 2).

The 1515 cm^{-1} band, related to the aromatic skeletal vibrations in lignin, showed the most pronounced decrease, indicating that lignin degradation occurred in all pretreatments, except for *S. commune* VE07. *T. villosa* 82I6, *P. pulmonarius* PS2001, *P. albidus* 88F-13, *P. sanguineus* PR32 and OU04 presented the largest reductions in this band and were statistically the same (reduction around 30%). Reductions in the 1427 cm^{-1} band (syringyl and guaiacyl condensed nuclei) corroborate the suggestion that there was a reduction in the amount of lignin present in the biomass. *P. sanguineus* PR32 presented a stronger reduction in this band compared to the other tested species (Table 2).

Decreases in the bands at 898 cm^{-1} (amorphous cellulose) and 1098 cm^{-1} (crystalline cellulose) were clear indications of the degradation of cellulose. The reduction in these bands was especially pronounced in the pretreatment performed with *P. sanguineus* PR32, which presented values superior to all species tested, followed by *P. albidus* 88F-13. In the band at 1375 cm^{-1} (cellulose and hemicellulose), again *P. sanguineus* PR32 resulted in the greatest reduction. It should be highlighted that cellulose degradation is considered a disadvantage in the process, since the sugars required in the hydrolysis step are provided by this fraction of the biomass; thus, the consumption of cellulose will result in a decrease in productivity (Table 2).

It was also observed that the strains OU04 and PR32 of *P. sanguineus* presented differences in substrate degradation. While the PR32 strain promoted a high reduction in all bands analyzed by FTIR, the OUO4 strain promoted a considerable reduction in the lignin bands, a minor reduction in the hemicellulose band and null reduction in the cellulose bands (Table 2). This differential degradation can be attributed to the differences between the metabolisms of the strains, such as the secretion of different enzymatic complexes. This demonstrates the importance of selecting different isolates of the same species.

The degradation of lignin by basidiomycetes is associated with variable levels of sugar consumption, obtained from holocellulose hydrolysis for growth requirements. For this reason, it is important to achieve a state of balance between both pathways. When selecting a basidiomycete for biological pretreatment, both parameters should be considered, since they represent a significant effect on the economics of the process. The best results will be those in which the reduction of lignin is the highest with the lowest sugar consumption, in a shorter period of time [54].

It is worth mentioning that the species *T. villosa* 82I6 and *P. pulmonarius* PS2001 presented favorable proportions between the consumption of cellulose, hemicellulose and lignin. In other words, they presented high reductions in lignin bands and low reductions in holocellulose bands. According to Lee et al. (2007) [55], this demonstrates that the system of hydrolytic enzymes secreted by these microorganisms did not efficiently degrade the holocellulose present in the biomass. As discussed earlier in Figure 2, these species showed low FPA and low β-glucosidase, endoglucanase and xylanase activities.

3.3 Microscopic analysis

Scanning electron microscopy of the sugarcane bagasse was performed to verify the structural changes caused after 49 days of biological pretreatment (Figure 3). The control sample (untreated) showed more ordered structures and some pores on the order of 1 μm. After pretreatment, the structures were modified, and the biomass was covered with fungal mycelia, resulting in fiber detachment and the appearance of larger pores and cracks in some cases. In pretreatments with the species *P. sanguineus* PR32 and OU04 and *P. pulmonarius* PS2001 and *P. albidus* 88F-13, it was observed that the sugarcane bagasse was totally enveloped by the fungal mycelium. In the pretreatment with *T. villosa* 82I6, the biomass modification was more evident, in which, besides the presence of mycelia, disorganization of the structure of the biomass, cracks and an increase in the surface pore size (to about 6 μm).

Castoldi et al. (2014) [3] observed the formation of pores on the surface of eucalyptus sawdust pretreated with *P. pneumarius, Trametes* sp. and *Ganoderma lucidum.* These structural changes in pretreated samples

may favor cellulose exposure [56]. The appearance of pores results in a greater available surface area and is generally considered an indication of increases in the accessibility of cellulose to enzymatic attack [57].

3.4 Enzymatic hydrolysis of pretreated sugarcane bagasse

The sugarcane bagasse pretreated with macromycetes was used in enzymatic hydrolysis assays in order to evaluate possible alterations in the release of sugars. In biomass hydrolyses pretreated with *P. sanguineus* OU04 and PR32 strains, glucose yields were null. For the pretreatments performed with the other species, after 24 hours of hydrolysis, the digestibility of the cellulose present in the pretreated sugarcane bagasse for 49 days was shown (Figure 3). Digestibility could represent the conversion yield of the raw material into fermentable sugars [18]. The digestibility of cellulose is the percentage of glucose released during enzymatic hydrolysis in relation to the theoretical maximum present in the biomass [54]. The chemical composition of sugarcane bagasse used in these experiments was 1.7±0.6% extractable components, 33.9±1.2% cellulose, 15.2±1.4% hemicellulose, 7.9±0.6% soluble lignin, 21.1±0.5% insoluble lignin and 5.9±1.8% ash.

It was observed that *M. palmivorus* VE111 treatment caused a decrease in digestibility (3.5 ± 0.7%) in relation to the control (12.9±2.4%). *P. pulmonarius* PS2001 presented similar digestibility to the control (12.8±8.0%), while *P. albidus* 88F-13, *S. commune* VE07 and *T. villosa* 82I6 species increased this parameter to 15.7±1.8%, 15.5±2.0% and 22.8±1.2%, respectively. It is worth mentioning that the species that promoted the greatest increase in digestibility, *T. villosa* 82I6, was the one that caused the greatest reduction in the percentage of total lignin present in the biomass (Figure 4).

In Figure 5, sugar and glucose release graphs are shown over time during the enzymatic hydrolysis of pretreated samples at different time points (7 to 49 days). The graphs refer to the pretreatments performed with the species that presented digestibility equal to or greater than the control. With regard to the hydrolysis of sugarcane bagasse pretreated with *S. commune* VE07, it was observed that both the release of reducing sugars and glucose formation were lower than the control.

In the hydrolysis of sugarcane bagasse pretreated with *P. albidus* 88-F13 and *P. pulmonarius* PS2001, in the first 21 and 14 days of pretreatment, respectively, a reduction in the release of reducing sugars was observed in relation to the control (Figures 6E and 6C). The same behavior was observed for glucose release in samples treated for up to, respectively, 35 and 21 days (Figures 6F and 6D). Only with longer periods of pretreatment was there an increase in the release of reducing sugars, including glucose, when compared to the control. It is likely that, in the first days of biological pretreatment, these strains consumed the accessible polysaccharides of the biomass, without the liberation of biomass deconstruction enzymes.

It is *interesting that,* in the pretreatment with *T. villosa* 82I6, an increase in the release of reducing sugars (Figure 5G) and glucose (Figure 5H) was observed in relation to the control, from the first days of biological pretreatment.

It is noteworthy that, for all pretreatments, the time of the enzymatic deconstruction process could be decreased from 24 hours to 12 hours, since after the initial period there was no increase in sugar release. In

addition, it was observed that the amount of glucose released during the enzymatic hydrolysis process was practically half of the reducing sugars released during the same period of time.

Figure 6 shows the analysis of glucose release in 24 hours of enzymatic hydrolysis from samples pretreated biologically for different periods of time.

In the enzymatic hydrolysis carried out with sugarcane bagasse pretreated with *P. albidus* 88F-13, it was observed that, in the samples pretreated for 42 and 49 days, glucose release was statistically equal to the control (48.5±2.38 mg/g), i.e. 45.4±2.6 and 56.7±1.7 mg/g, respectively. However, there was a trend towards increased glucose uptake with increasing pretreatment time, which may indicate the need for longer periods of pretreatment time (Figure 6A) or the use of a higher inocula concentration. Samples pretreated with *P. pulmonarius* PS2001 obtained statistically higher amounts of glucose were obtained in samples with pretreatment times of 35, 42 and 49 days (68.4±0.7, 76.3±1.6 and 76.5±2.1 mg/g) (Figure 6B). In the pretreatment with *T. villosa* 82I6, it was observed that samples collected at 21, 28 and 49 days released amounts of glucose statistically superior to the control (70.9±8.3, 77.8±5,8 and 77.6±4.2 mg/g) (Figure 6C).

It should be emphasized that decreasing the process time is one of the factors that interferes in the productivity and final cost of a product. Decreasing pretreatment time is the main challenge for this area. Thus, among the strains that proved to be efficient in the biological pretreatment, *T. villosa* 82I6 stood out among the tested species, since from day 21 of cultivation it promoted greater release of glucose and reducing sugars from sugarcane bagasse, in relation to the control. However, this time possibly could be shortened if more concentrated inocula were employed.

3.5 Fermentation

The evaluation of ethanol production is necessary to quantify the process final performance [58]. Since samples pretreated with *T. villosa* 82I6 presented better results in the hydrolysis stage, the hydrolysates of samples pretreated with this strain were fermented using the CAT-1 strain of *Saccharomyces cerevisiae*. Figure 7 shows the concentrations of sugars (glucose and xylose) and the concentration of ethanol over 24 hours of alcoholic fermentation of the sugarcane bagasse hydrolysates pretreated with *T. villosa* 82I6 at different times.

Evaluating the pretreated samples, it was observed that the initial amount of sugars as well as the concentration of ethanol reached in all pretreated samples were higher than in the control (Figure 7A). It was also found that, under all conditions, total glucose uptake occurred during the first 4 hours of fermentation. Decreases in the glucose concentration coincide with increases in the ethanol concentration. The xylose content remained constant throughout the fermentation process in all the samples, since the yeast used is not able to metabolize this sugar. Regarding the amount of available xylose, values around 1 mg/mL were observed in all conditions.

Table 3 shows the maximum ethanol yield from biomass. From the hydrolyzate of the control sample, a maximum yield of 10.1±0.8 mg/g was obtained. That is, for each gram of biomass used, 10.1±0.8 mg of

ethanol were obtained. The samples pretreated for 7, 14, 21, 35 and 42 days presented ethanol yields statistically equal to the control. Samples pretreated for 28 and 49 days showed significantly higher ethanol yields, corroborating the data shown in Figure 4C, in which it was observed that the glucose obtained in the hydrolysis of samples pretreated for 28 and 49 days was superior to the control. Based on this result, there was a positive influence of biological pretreatment on the subsequent stages of the process.

A disadvantage of the biological pre-treatments mentioned in the literature is the long time for this type of pre-treatment, but as the biomass is never used all at the same time, being stored, this pre-treatment can be carried out at this moment, therefore, even faster than other processes the same goal.

4. Conclusion

All strains tested were able to colonize the sugarcane bagasse and secreted different enzymatic complexes, which resulted in differences in the degradation of biomass components. Biological pretreatment of sugarcane bagasse by *T. villosa* 82I6 resulted in the selective degradation of lignin, evidenced by FTIR and microscopic analyses, facilitating the enzymatic hydrolysis of cellulose. The amount of reducing sugars and glucose released from samples pretreated by this species upon incubation with *P. echinulatum* cellulase were higher than the control sample and the other pretreated samples. Additionally, ethanol production was significantly improved in the fermentation stage.

Declarations

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Ethics declarations

Statement of Informed Consent, and Human/Animal Rights

No conflicts, informed consent, and human or animal rights applicable.

Conflict of Interest

The authors declare no competing interests.

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Tables

Table 1. Strains used in biological pretreatments and respective collection and access codes of the UCS library.

Strain	Colletion code	Access code
Pleurotus pulmonarius	PS-2001	HUCS/MIUCS 1215
Pleurotus albidus	88F.13	HUCS/MIUCS 1586
Trametes villosa	82I.6	HUCS/MIUCS 2081
Pycnoporus sanguineus	PR_32	HUCS/MIUCS 1829
Schizophyllum commune	VE_07	HUCS/MIUCS 1922
Marasmiellus palmivorus	VE_111	HUCS/MIUCS 2025
Pycnoporus sanguineus	OU_04	HUCS/MIUCS 2055

Table 2. Average weight losses and diminutions in specific bands in the FTIR spectra of sugarcane bagasse caused by biological pretreatment.

		FTIR analysis percent diminution in specific bands				
Biological pretreatment (PTB)*	**Weight losses (%)**	898 cm^{-1} (amorphous cellulose)	1098 cm^{-1} (crystalline cellulose)	1375 cm^{-1} (cellulose and hemicellulose)	1427 cm^{-1} (syringyl and guaiacyl condensed nuclei)	1515 cm^{-1} (aromatic skeletal vibrations in lignin)
P. pulmonarius PS2001	31.7 ± 3.7^{ab}	2.1 ± 2.3^{bc}	4.0 ± 3.6^{c}	0 ± 0^{b}	4.6 ± 2.8^{ab}	29.8 ± 0.9^{ab}
T. villosa 82I6	23.9 ± 4^{bc}	4.3 ± 2.9^{bc}	4.3 ± 1.2^{c}	3.0 ± 4.3^{b}	11.1 ± 4.9^{ab}	34.4 ± 7.9^{a}
S. commune VE07	21.3 ± 0.8^{c}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}
P. sanguineus OU04	24.8 ± 5.9^{bc}	0 ± 0^{c}	0 ± 0^{c}	4.3 ± 5.7^{b}	11.6 ± 3.4^{ab}	29.8 ± 1.9^{ab}
P. albidus 88F-13	40.9 ± 0.7^{bc}	13.9 ± 3.9^{b}	11.2 ± 1.2^{b}	5.9 ± 0.2^{b}	8.2 ± 7.8^{ab}	29.6 ± 0.6^{ab}
P. sanguineus PR32	22.3 ± 3.7^{bc}	32.2 ± 5.7^{a}	39.4 ± 0.8^{a}	20.7 ± 2.7^{a}	21.1 ± 3.2^{a}	27.3 ± 2.1^{ab}
M. palmivorus VE111	19.9 ± 2.3^{c}	3.2 ± 1.0^{bc}	1.5 ± 0.8^{c}	0 ± 0^{b}	5.9 ± 3.8^{ab}	11.9 ± 10.2^{c}

*Samples pretreated biologically for 49 days.

Table 3. Evaluation maximum ethanol yields obtained from fermentation of the glucose released via the hydrolysis of sugarcane bagasse pretreated with *T. villosa* 82I6 for diferent periods, using the yeast *Saccharomyces cerevisiae* CAT-1.

Sample	Time (h)	Ethanol Yield (mg/g)*
Control	8	10.1 ± 0.8^{b}
Pretreated for 7 days	4	13.8 ± 1.6^{ab}
Pretreated for 14 days	4	17.8 ± 1.9^{ab}
Pretreated for 21 days	4	15.6 ± 3.7^{a}
Pretreated for 28 days	4	19.1 ± 2.8^{ab}
Pretreated for 35 days	12	14.4 ± 3.2^{ab}
Pretreated for 42 days	24	16.0 ± 0.6^{ab}
Pretreated for 49 days	12	20.2 ± 0.5^{a}

Figures

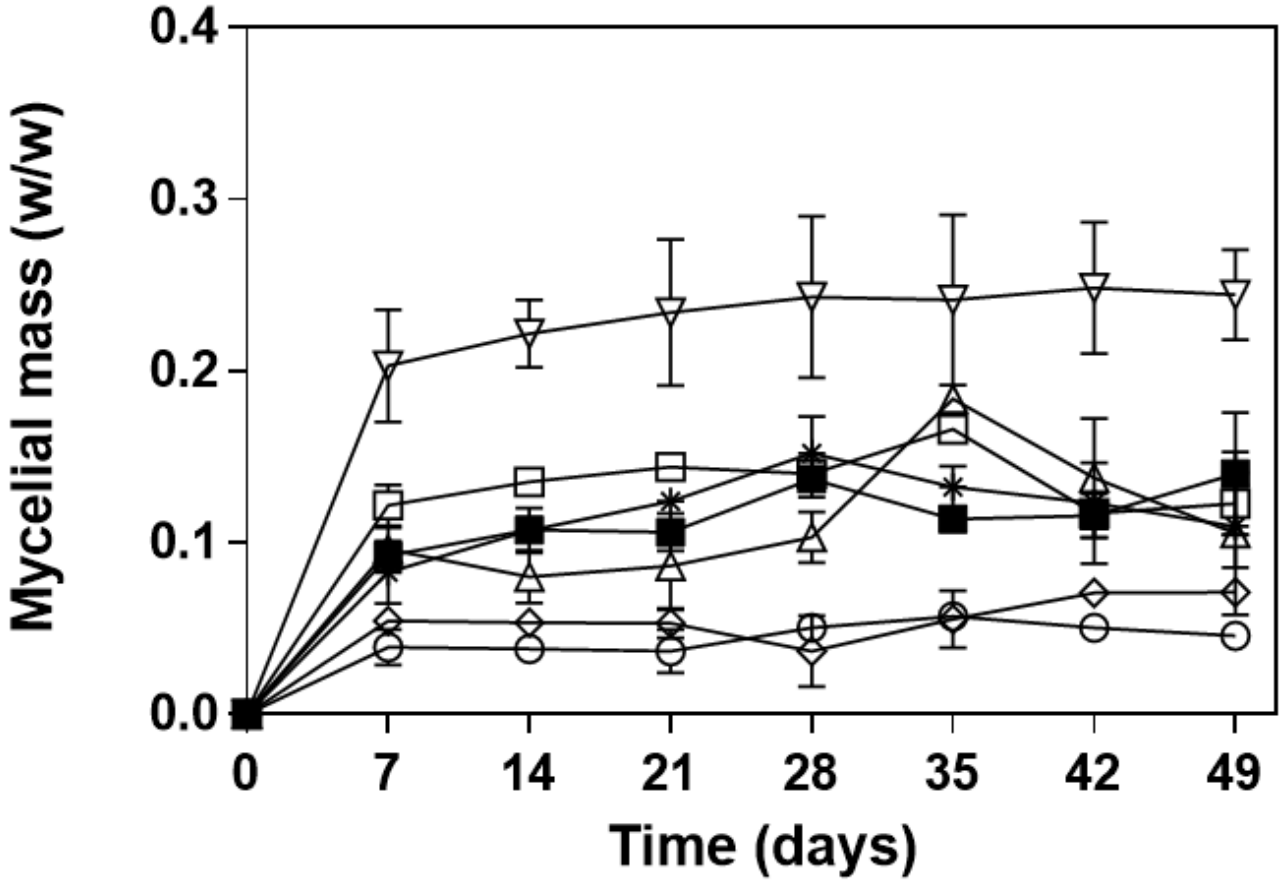

Figure 1

Growth variation of different basidiomycetes, shown as mycelial mass by dry biomass mass, as a function of the time of cultivation in sugarcane bagasse medium. Legend: (●) Pleurotus pulmonarius PS2001; (■) Trametes villosa 82I6; (Δ) Pycnoporus sanguineus OU04; (∇) Schizophyllum commune VE07; (◊) Pleurotus albidus 88F-13, (∗) Pycnoporus sanguineus PR32 and (□) Marasmiellus palmivorus VE111.

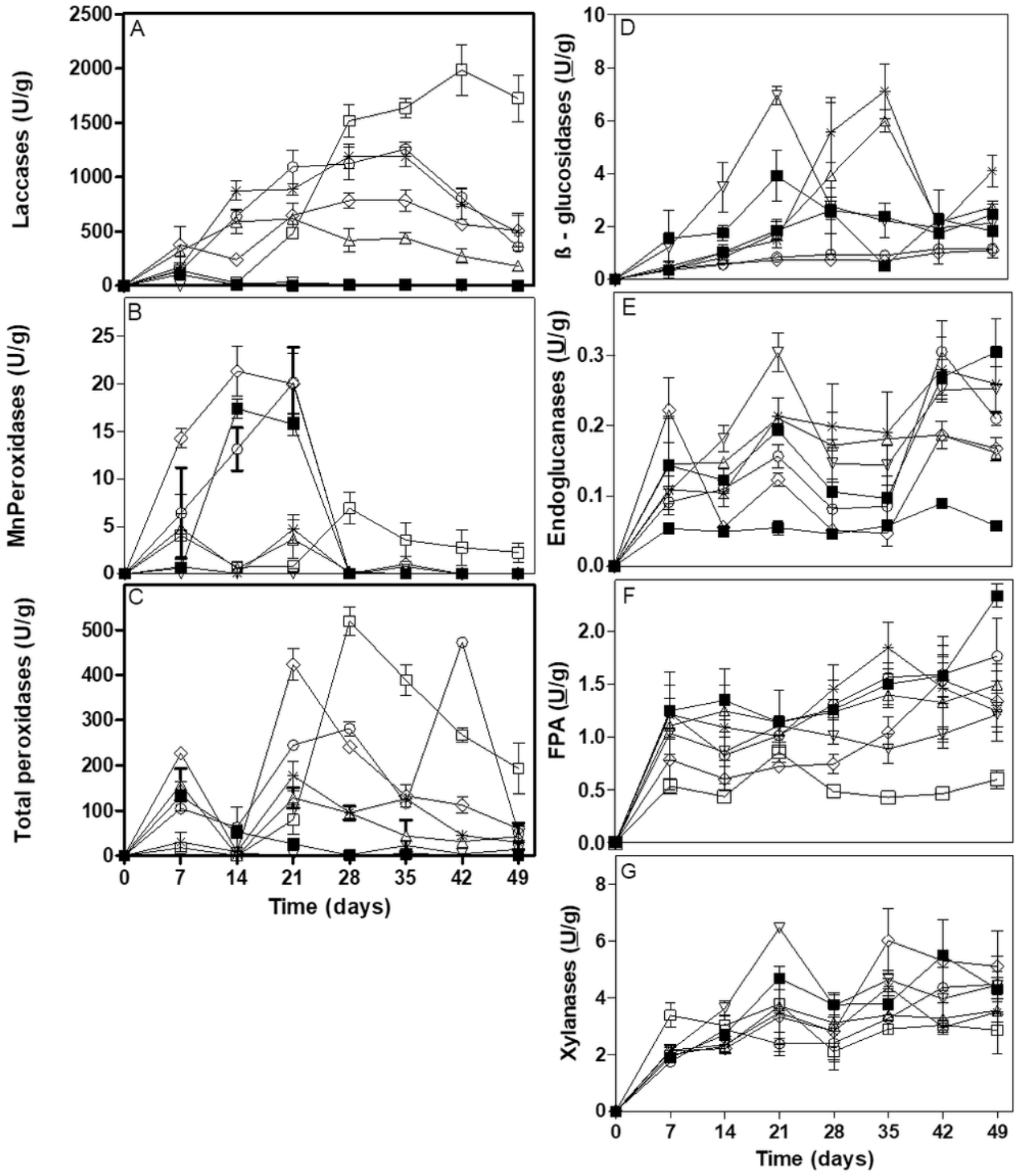

Figure 2

Variation in laccase [A], manganese peroxidase [B], total peroxidase [C], β-glucosidase [D], endoglucanase [E], FPA [F] and xylanase [G] activities as a function of the time of incubation of different basidiomycetes. Legend: (●) Pleurotus pulmonarius PS2001; (■) Trametes villosa 82I6; (Δ) Pycnoporus sanguineus OU04; (∇) Schizophyllum commune VE07; (◊) Pleurotus albidus 88F-13, (*) Pycnoporus sanguineus PR32 and (□)Marasmiellus palmivorus VE111.

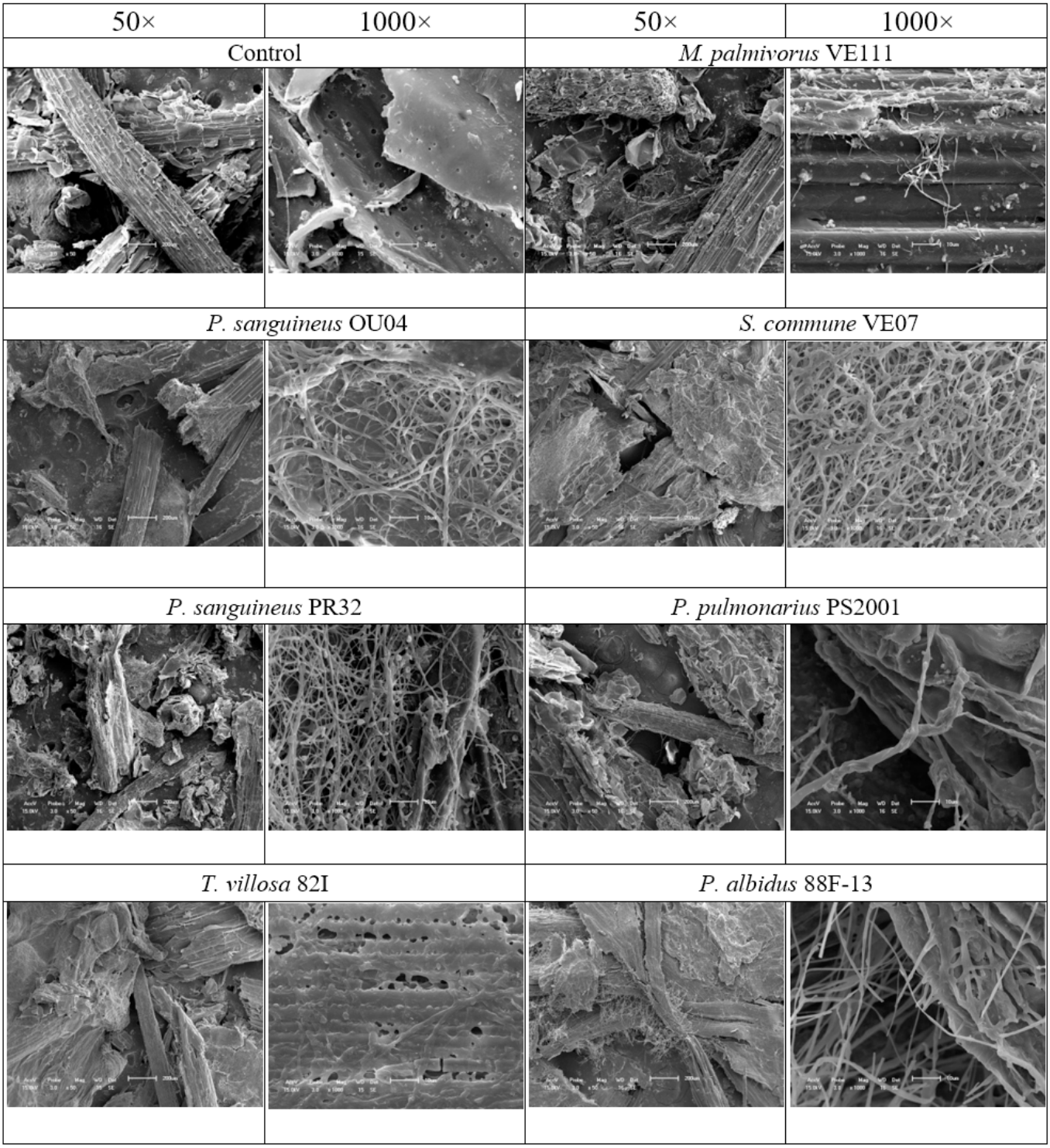

Figure 3

Scanning electron microscopy of sugarcane bagasse was performed to verify the structural changes caused after 49 days of biological pretreatment.

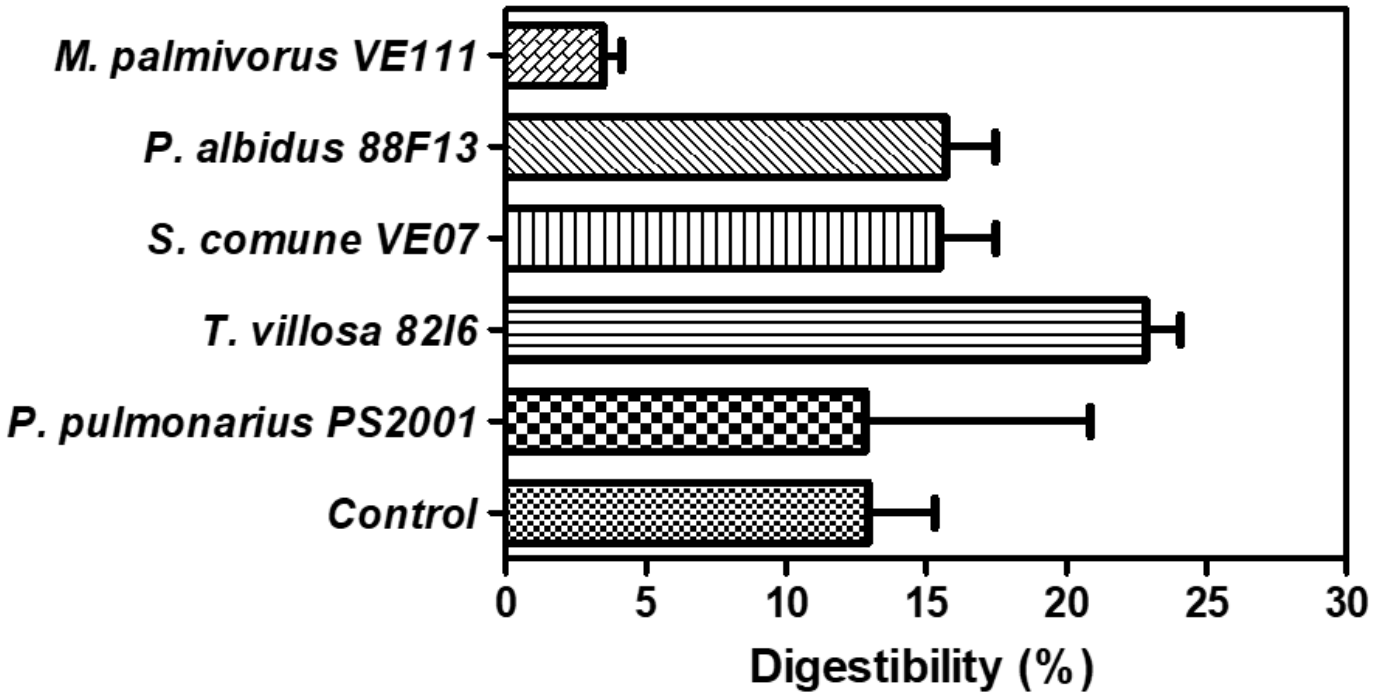

Figure 4

Digestibility of sugarcane bagasse cellulose pretreated with different species of basidiomycetes for 49 days, after 24 hours of enzymatic hydrolysis.

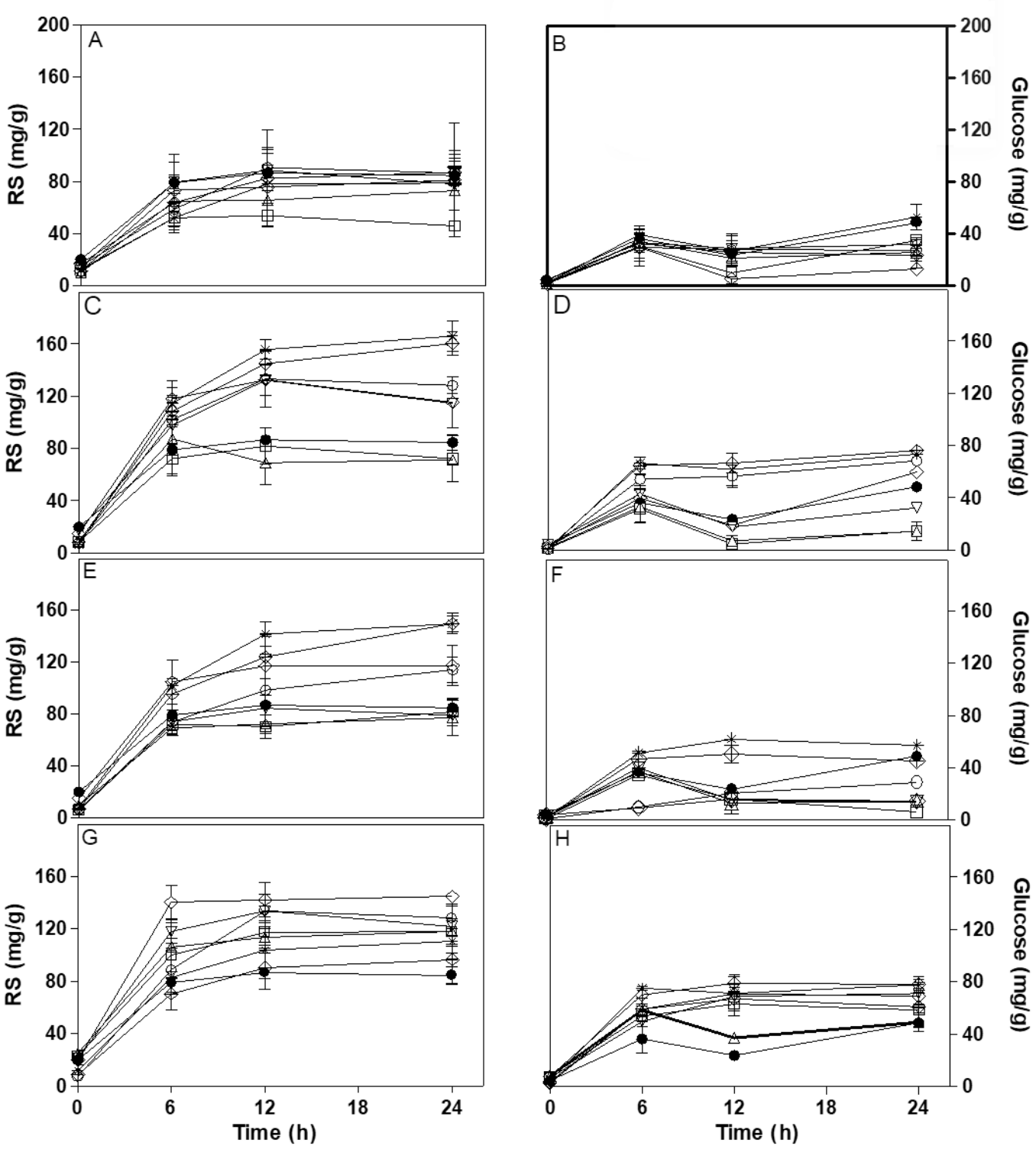

Figure 5

Please see the Manuscript file for the complete figure caption.

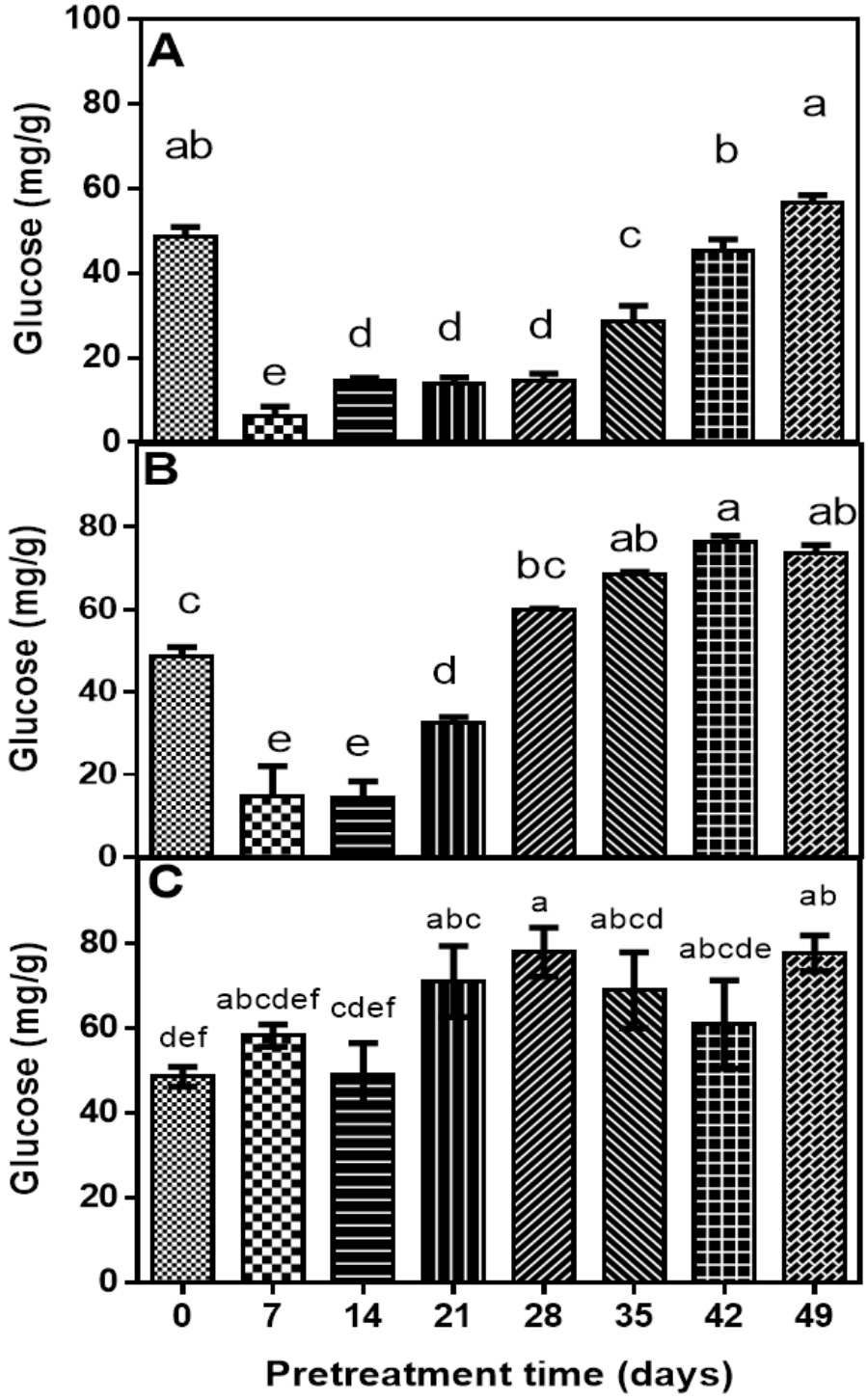

Figure 6

Glucose released after 24 hours of enzymatic hydrolysis of sugarcane bagasse after different periods of biological pretreatment with Pleurotus albidus 88F-13 [A]; Pleurotus pulmonarius PS2001 [B] and Trametes villosa 82I6 [C].

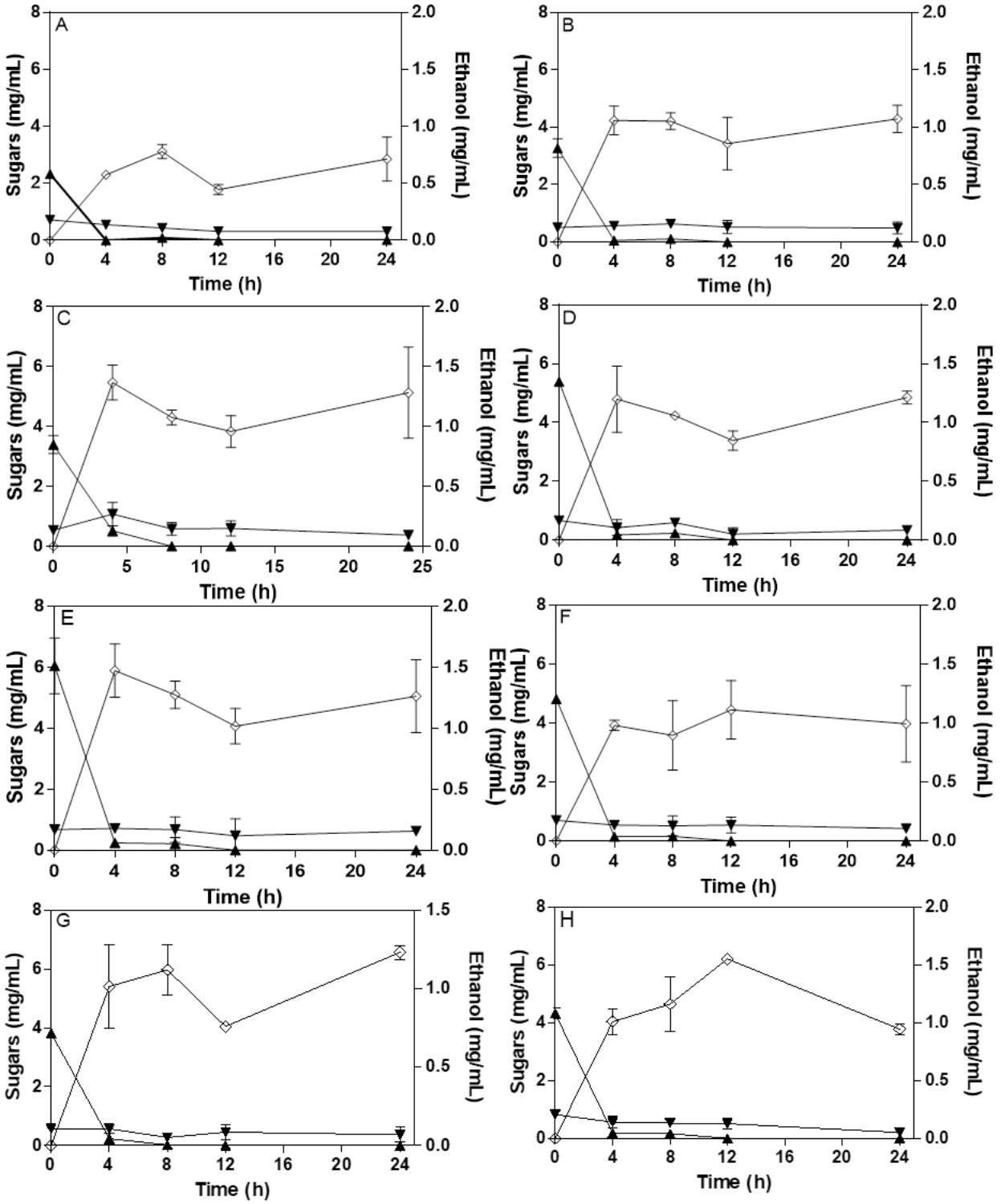

Figure 7

Concentration of glucose, xylose and ethanol (mg/mL) obtained from the fermentation of samples pretreated with Trametes villosa 82I6 for 0[A], 7[B], 14[C], 21[D], 28[E], 35[F], 42[G] and 49[H] days. Legend: (▲) glucose, (▼) xylose and (◊) ethanol.

Supplementary Files

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- Graphycalabstract.png