*Environmental Engineering and Management Journal*

*July 2015, Vol.14, No. 7, 1713-1719*
*http://omicron.ch.tuiasi.ro/EEMJ/*

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# MYCELIAL GROWTH AND ENZYMATIC ACTIVITIES OF WHITE-ROT FUNGI ON ANAEROBIC DIGESTATES FROM INDUSTRIAL BIOGAS PLANTS

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## Abstract

Solid digestate (SD) represents an environmental problem due to the continuous increase of biogas production. In the present study four white-rot fungal strains were screened for their ability to grow on two different SD samples, obtained from industrial biogas plants, using wheat straw (WS) as a control medium. Results show that i) the feedstock used in the biogas plant influences the time required for the colonization of the corresponding SD and ii) different fungal strains have different capabilities to colonize the same digestate. In particular, *Pleurotus ostreatus* SMR 684 reached the maximum proliferation on corn silage digestate (CSD) in the shortest time (12 days). Subsequently, *P. ostreatus* was grown on WS and CSD for 42 days with measurement of lignocellulolytic activities and lignocellulose components (lignin, cellulose and hemicellulose). Enzymatic activities reached a maximum around the 24$^{th}$ day of incubation. Ligninolytic activities showed similar trends on CSD and on WS, while some differences were observed in the levels of cellulolytic and hemicellulolytic activities. Lignin was reduced by 12% on CSD, this suggesting that the fungal treatment can increase polysaccharides accessibility in view of a further utilization of SD for biorefinery purposes.

*Key words:* biogas, biorefinery, solid digestate, white-rot fungi

*Received: December, 2014; Revised final: June, 2015; Accepted: June, 2015*

## 1. Introduction

Anaerobic digestion (AD) for biogas production generates a residue, the anaerobic digestate, which is often still rich in nutrients. When AD takes place in a liquid phase, the solid digestate (SD) is mechanically separated from the liquid fraction, which can be recirculated into the biogas plant. The most widely used feedstocks for biogas production are energy crops, harvest residues, animal manure and food residues. The SD deriving from AD of such substrates is mainly composed by cellulose, hemicellulose and lignin, as these polymers undergo relatively little changes during conventional AD processes because of the esther and ether linkages occurring between the cell wall polymers (Yue et al., 2010). Due to its recalcitrant structure, SD is not generally considered suitable for a further conversion into other useful products (Tambone et al., 2009), and it is currently used by the agricultural industry for soil amendment or animal bedding (Makádi et al., 2012). However, the continuous increase of biogas production poses serious problems in terms of SD disposal, since the high content of N and P in such material might cause environmental pollution if excessive amounts of it should be spread over agricultural soils (Udayasimha and Vijayalakshmi, 2012).

Mushrooms may represent a valid alternative for recycling nutrients contained in SD, since they

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are usually cultivated on lignocellulosic substrates such as wheat straw. Moreover, the ability of some fungi to degrade lignin, which is extensively reported and reviewed as biological pretreatment for second generation bioethanol production (Tian et al., 2012), might help to increase the accessibility of structural polysaccharides in SD, thus making such material susceptible to a further digestion step, with the consequent increase in the final biogas yield.

Some studies report the cultivation of fungi on various substrates supplemented with SD (Banik and Nandi, 2000, 2004; Gangulli and Chanakya, 1994; Isikhuemhen et al., 2009; Udayasimha and Vijayalakshmi, 2012), but there is still lack of knowledge about the ability of ligninolytic fungi to grow on SD as a unique nutrient source.

In the present study four species of edible fungi were screened for their ability to grow on two digestates obtained from AD at commercial scale of corn silage (CSD) and of a mixture of substrates (MSD), using wheat straw (WS) as a control. The best performing strain was then incubated for a longer time (42 days) with sampling approximately twice per week for the determination of proteins, ligninolytic, cellulolytic and hemicellulolytic activities, as well as residual lignin, cellulose and hemicellulose in the substrates used, with the aim of evaluating if fungal growth is able to increase the accessibility of polysaccharides for a further digestion step.

## 2. Materials and methods

### *2.1. Substrates*

Anaerobic digestate samples were kindly supplied by two industrial biogas plants after removal of the liquid fraction. Corn silage digestate (CSD) was collected at "Fattoria Autonoma Tabacchi" (Città di Castello, PG, Italy); the plant, with nominal power capacity of 1MW per day, operated with 50 Mg of corn silage per day. Digestate from mixed substrates (MSD) was collected at "Azienda Agricola Palombini" (Nepi, VT, Italy); the plant, with nominal power capacity of 0.7 MW per day operated every day with 49.5 Mg of a heterogeneous feedstock composed by cow manure (50%), grass silage (10%), milk whey (21%), poultry litter (10%) and sugar beet-molasses (9%). Wheat straw (WS) was used as a control medium.

### *2.2. Mycelial growth on anaerobic digestates*

Pure fungal cultures (*Agrocybe aegerita* SMR 206, *Pleurotus ostreatus* SMR 684, *Pleurotus columbinus* SMR 688 and *Pleurotus eryngii* SMR 151) were stored in the IBAF International Bank of Edible Saprophytic Mushrooms. Fungal mycelia were grown in sterilized Petri dishes containing 3% malt extract and 1.5% agar. When fungal biomass was abundant, plugs of 9 mm diameter were cut and inoculated in the centre of Petri dishes (11 cm diameter), previously filled with 15 g of the different substrates (WS 100% (control); WS 50%- CSD 50%; WS 50%-MSD 50%; CSD 100%; MSD 100%) with moisture fixed at 75% and sterilized at 121 °C for 30 min. Fungal growth was evaluated by measuring the diameter of the area covered by the mycelium every 24 hours. Radial growth rate (*Kr*) was calculated as $Kr = (R_1 - R_0)/(t_1 - t_0)$ (Trinci, 1971), where $R_0$ and $R_1$ are the colony radius at time $t_0$ and $t_1$, respectively.

### *2.3. Enzymatic activities assays*

Another set of Petri dishes, filled with WS 100% and CSD 100 %, was made ready to study the enzymatic activities during 42 days of fungal growth. Twice per week the whole content of 3 Petri dishes for each substrate was weighted and extracted with 0.1 M potassium phosphate buffer pH 7.0 in a ratio 1:2 (w/v), filtered through a gauze and centrifuged at 9,000 x *g* for 20 min. In the supernatant, total peroxidases, Mn-independent peroxidase and laccase were assayed spectrophotometrically at 420 nm ($\varepsilon_{420}$=36,000/M cm) by oxidation of 2 mM ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, diammonium salt) in buffer solution (100 mM sodium acetate pH 4.5). Total peroxidases were assayed in the presence of 0.04 mM $H_2O_2$ and 0.1 mM $MnSO_4$. In Mn-independent peroxidase test 0.1 mM EDTA substituted $MnSO_4$. Enzyme activity was expressed in U/mg protein (one unit of enzyme activity was defined as the amount of enzyme that oxidized 1μmol of ABTS per min at pH 4.5 and 25°C). Peroxidase activities were obtained by subtracting the laccase activity. Mn-dependent peroxidase activity was obtained by subtracting Mn-independent peroxidase from total peroxidases activity.

In the same supernatants, endoglucanase, cellobiohydrolase and xylanase activities were measured in a buffer solution (sodium acetate 50 mM, pH5) using Avicel (0.5% w/v), carbossimethylcellulose (1% w/v) and xylan from beechwood (0.25% w/v) as substrates, respectively. Reactions were started by incubating the fungal extracts with the substrates for 30 min at 37 °C and subsequently stopped by incubating at 100°C for 3 min (Federici et al., 2012). After the reaction, the reducing sugars produced were determined spectrophotometrically (Miller, 1959). Data are expressed as U/mg protein (one unit of enzyme activity was defined as the amount of enzyme that produced 1μmol of glucose or xylose per min at pH5 and 37°C). Proteins were detected according to Bradford (1976) using bovine serum albumin for calibration.

### *2.4. Determination of lignin, cellulose and hemicellulose*

Residual solids after buffer extraction (see 2.3) were ground (MF 10 miller IKA, Staufen, Germany) to pass a 0.5 mm grid. According to

Sluiter et al. (2008a), samples were deprived of their extractives for the determination of lignin, cellulose and hemicelluloses. The final extractives-free solid residue was hydrolyzed to determine i) acid-soluble lignin (ASL); ii) acid-insoluble lignin (AIL); iii) total monosaccharides content according to the method reported by Sluiter et al. (2008b). Monomeric sugars resulting from the acid hydrolysis of the extractives-free solid residues were analyzed by high-performance anion exchange chromatography, with pulsed amperometric detection (HPAEC-PAD) (Thermo Scientific™ Dionex™ ICS-5000, Sunnyvale, CA U.S.A.), consisting of an isocratic quaternary pump, a pulsed amperometric detector, an injection valve with a 5 µl injection loop and an analytical CarboPac SA10 column (4 mm x 250 mm) with the guard column.

The detection cell contained a gold working electrode (1.0 mm in diameter) and an Ag/AgCl reference electrode. Pulsed amperometric detection was carried out with the following waveform: $E_1$= +0.10 V ($t_1$=0.4 sec), $E_2$= -2.00 V ($t_1$=0.01 sec), $E_3$= +0.60 V (t=0.01 s), E4= -0.10 V (t=0.06 s). The electrical signal was integrated in ncoulomb (nC). Runs were carried out at 45°C. NaOH (1 mM) was used as mobile phase at a flow rate 1.0 ml/min with a post-column addition of concentrated NaOH (300mM) using a second pump, at a flow rate of 0.5 ml/min. Samples were filtered through 0.2 µm PPII syringe filters prior to injection. The instrumentation control, data acquisition, and processing was performed by the software Chromeleon Data System (CDS) version 6.8 (Thermo Scientific™, Dionex™).

Cellulose and hemicellulose contents were calculated from the amounts of monomeric sugars according to Ververis et al. (2007).

### *2.5. Statistical analysis*

Experiments were performed twice; each mixture set in triplicate. Data are shown as the means ± standard deviation (SD).

## 3. Results and discussion

### *3.1. Mycelial growth on anaerobic digestates*

Ligninolytic fungi are able to colonize, degrade and bioconvert lignocellulosic substrates thanks to the concerted and synergistic action of many lignocellulolytic enzymes.

At present, very little is known on the ability of such fungi to grow on SD resulting from biogas production, which is still rich in carbohydrates and might be further exploited through biorefinery.

Fig. 1 shows the trend of mycelial growth on the two digestates obtained from AD of CSD and MSD compared with that in WS, used as a control. It is noteworthy that most of the fungal strains grew faster on the mixture of WS and CSD than on the control medium, this suggesting that anaerobic digestate might improve fungal growth when added to a conventional growth medium.

Among the tested strains, *P. ostreatus* showed the fastest growth on WS 50%-CSD 50%, reaching the full colonization (11 cm) in 8 days, followed by *P. columbinus* and *P. eryngii* (11 cm in 12 and 13 days, respectively). *P. ostreatus* also reached the maximum proliferation on CSD 100% in the shortest time (12 days).

MSD was the harder substrate to colonize for all the tested fungi. In particular, the maximum growth on WS 50%-MSD 50% was reached after 12 days by *P. ostreatus*, and after about 17 days by the other strains.

None of the four fungal species was able to fully colonize the medium composed by MSD 100% in 17 days. These results are confirmed by the growth rate constant (Kr) calculated by linear regression of each colony radius versus time, the highest rate being detected on *P. ostreatus* grown on WS 50%-CSD 50% (Table 1).

These results show that the ability of a certain fungal strain to grow on a digestate can be severely influenced by the feedstock type used in the AD process. Among the tested fungi, *P. ostreatus* resulted to be the most suitable for cultivation on CSD. Pictures reported in Fig. 2 show the mycelial growth of *P. ostreatus* on the 5 tested substrates after 9 days of incubation.

### *3.2. Long incubation of P. ostreatus on CSD and WS: enzymatic activities and macromolecules content*

*P. ostreatus* was incubated for 42 days on CSD 100% and WS 100% (incubation conditions are described in 2.3) and samples were tested twice per week.

**Table 1.** $K_r$*s* of the fungi grown on different substrates

| ***Radial Growth Rate*** | ***$K_r$ (µm $h^{-1}$)*** | | | |
|---|---|---|---|---|
| ***Substrate*** | ***Agrocybe aegerita*** | ***Pleurotus columbinus*** | ***Pleurotus eryngii*** | ***Pleurotus ostreatus*** |
| WS 100% | 168 ± 2 | 209 ± 6 | 157 ± 5 | 278 ± 18 |
| WS 50%-CSD 50% | 175 ± 5 | 249 ± 12 | 187 ± 6 | 316 ± 25 |
| CSD 100% | 91 ± 4 | 199 ± 8 | 180 ± 4 | 256 ± 19 |
| WS 50%-MSD 50% | 136 ± 2 | 196 ± 6 | 167 ± 5 | 244 ± 21 |
| MSD 100% | 25 ± 1 | 27 ± 3 | 44 ± 4 | 93 ± 6 |

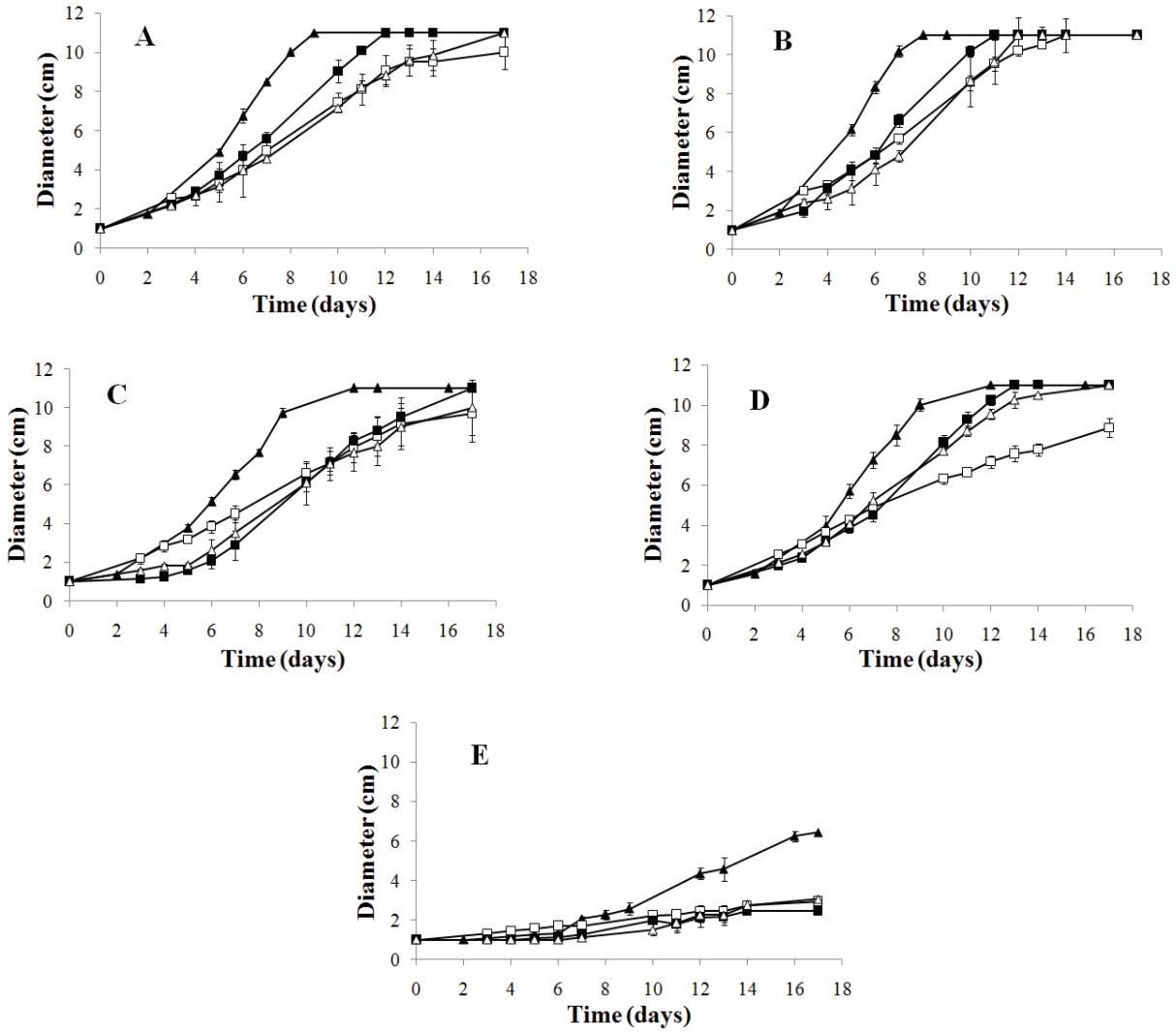


**Fig. 1.** Mycelial growth of *Agrocybe aegerita* (□), *Pleurotus columbinus* (■), *Pleurotus eryngii* (△) and *Pleurotus ostreatus* (▲) on the different substrates tested: WS 100% (A), WS 50%-CSD 50% (B), WS 50%-MSD 50% (C), CSD 100% (D); MSD 100% (E)

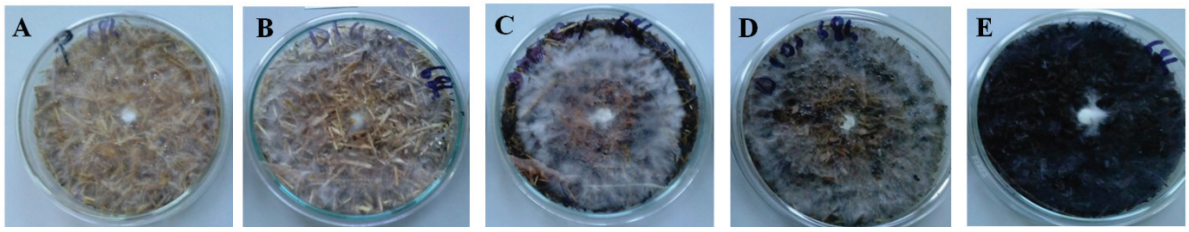


**Fig. 2.** Mycelial growth of *Pleurotus ostreatus* on the different substrates tested: WS 100% (A), WS 50%-CSD 50% (B), WS 50%-MSD 50% (C), CSD 100% (D); MSD 100% (E) after 9 days of incubation

The mycelium in CSD grew as fast as in the control. Fig. 3 reports ligninolytic activities (laccase, Mn-independent and dependent peroxidase) in CSD and WS during the 42 days of incubation with *P. ostreatus*. The enzymatic activities started to increase with the mycelial growth and continued to increase after the full colonization of the Petri dish (9 days) reaching a maximum around the 24$^{th}$ day of incubation. This trend is characteristic for *P. ostreatus* grown in wheat straw in Petri dishes (Galli et al., 2008). The ligninolytic activities in the plates containing CSD followed the same trend than those in the control medium (WS). Therefore the anaerobic digestate results to be a good material for mycelial growth and it is also able to stimulate the enzymatic activities.

Fig. 4 reports cellulolytic and hemicellulolytic activities (endoglucanase, cellobiohydrolase and xylanase), which were produced simultaneously with the ligninolytic activities during the 42 days incubation with *P. ostreatus*. Endoglucanase and xylanase activities on CSD were higher than on WS in the first period of mycelium growth, reaching the maximum at the 17$^{th}$ day. Cellobiohydrolase activity

was higher on WS, with a maximum of activity at 24th day. After 30 days all the activities decreased with similar values in both of the substrates.

Macromolecules content in WS and CSD during mycelial growth is shown in Fig. 5. Insoluble lignin was reduced by 15% in WS and 12% in CSD after 42 days with reference to the initial amount. These results confirmed the expectations based on the enzymatic activities detected during fungal growth and are very interesting if compared to those reported by Isikhuemhen et al. (2009). In their study *A. aegerita* did not degrade lignin when cultivated on anaerobic digestate from broiler litter as unique feedstock. In the present study, part of the lignin was used as organic matter for fungal growth, while a small amount was converted into soluble lignin that was increased from 0.88 to 1.83% (108%) on CSD, and from 0.91 to 1.2% (32%) on WS.

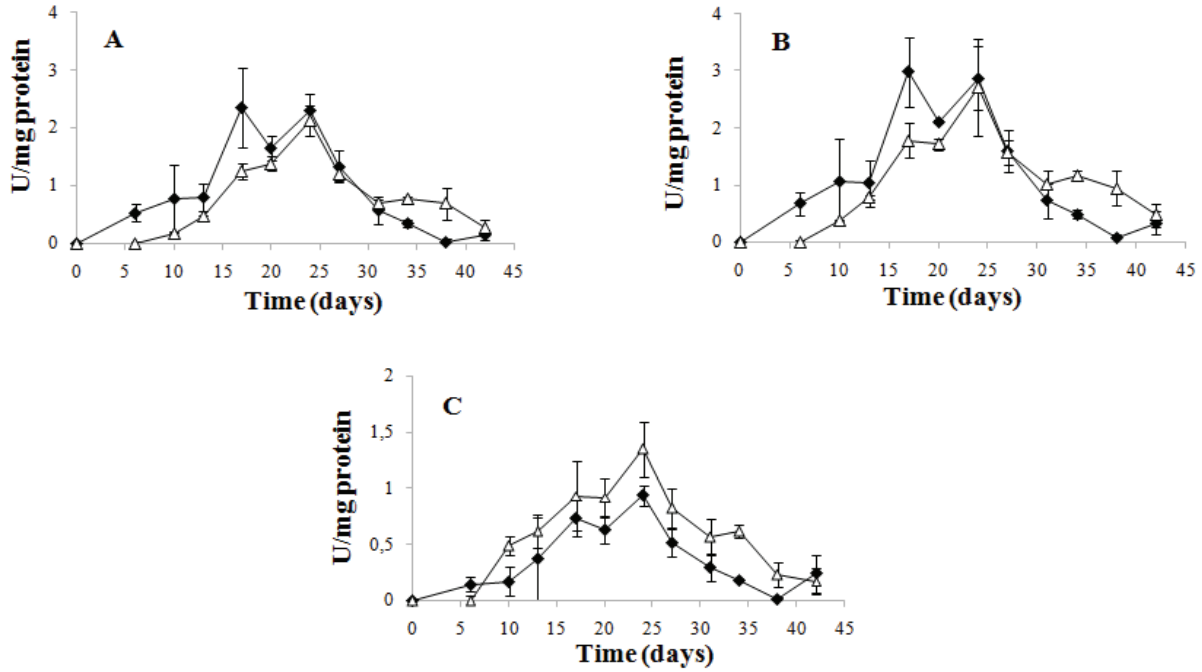


**Fig. 3.** Ligninolytic activities of *Pleurotus ostreatus* grown on WS (◆) and CSD (△): laccase (A), Mn-independent peroxidase (B), Mn-dependent peroxidase (C)

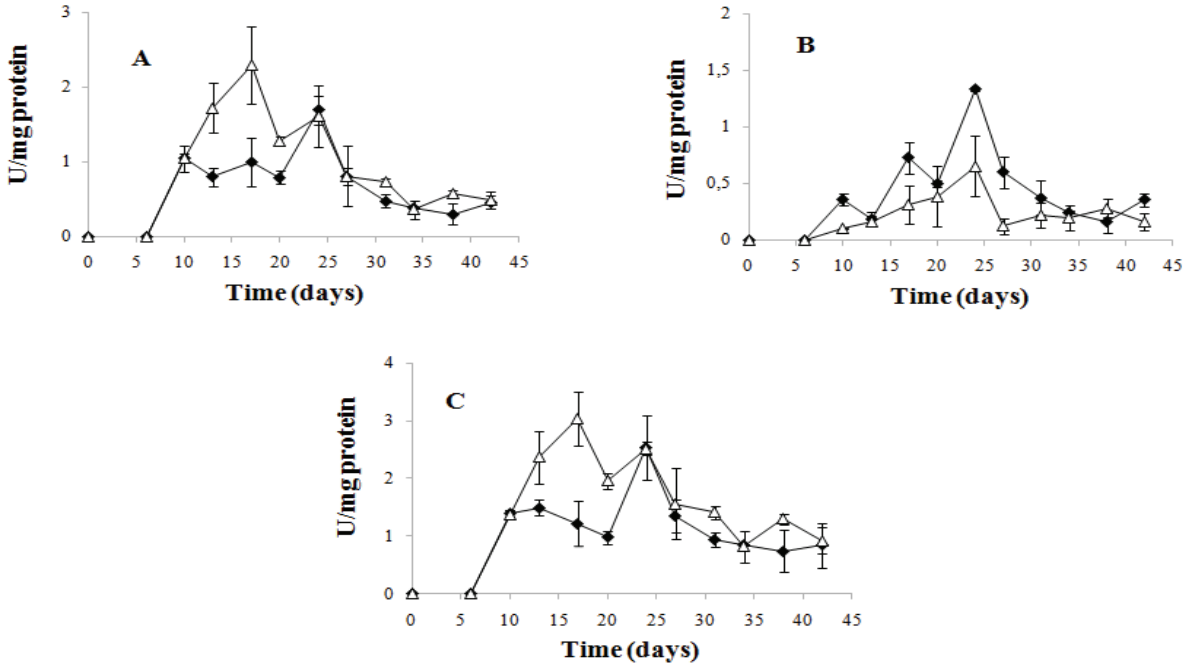


**Fig. 4.** Cellulolytic and hemicellulolytic activities of *Pleurotus ostreatus* grown on WS (◆) and CSD (△): endoglucanase (A), cellobiohydrolase (B), xylanase (C)

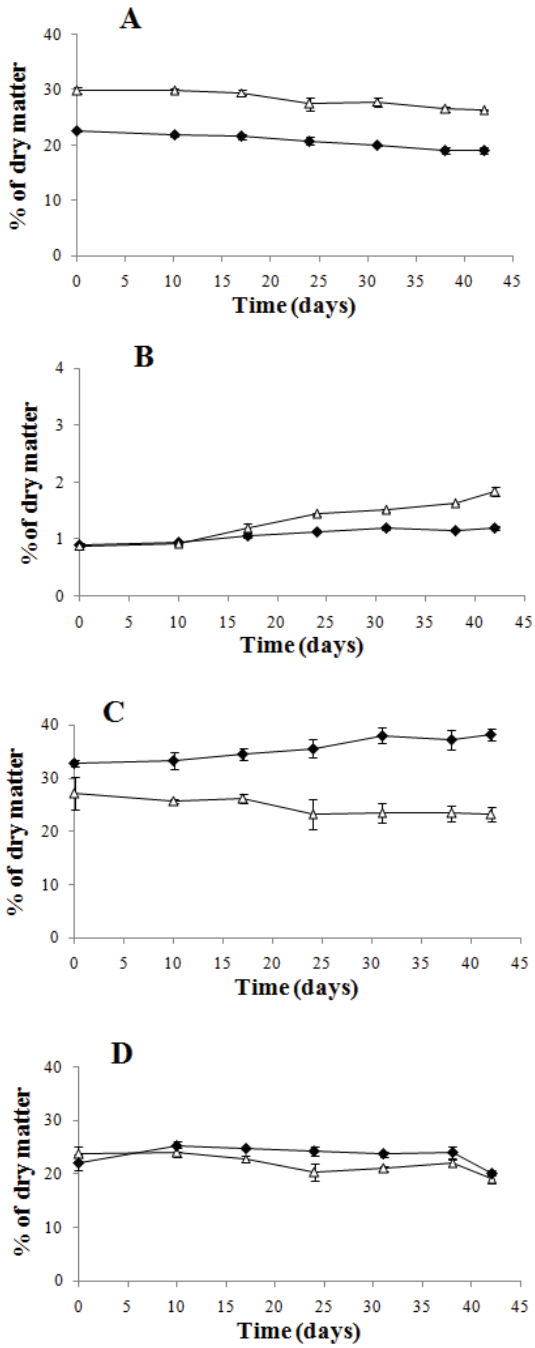


**Fig. 5.** Acid insoluble lignin (A), acid soluble lignin (B), cellulose (C) and hemicellulose (D) percentages during the growth of *Pleurotus ostreatus* on WS (◆) and CSD (△)

Lignin depletion during fungal growth has been reported to improve biogas production potential. In particular, Müller and Trösch (1986) demonstrated that a partial lignin removal (19% of the initial content) leads to a remarkable increase of biogas yield (30%). Muthangya et al. (2009) report an increase of methane production (about 50%) even after a smaller reduction of lignin (7%). Moreover, the same fungal biomass can also represent a feedstock for biogas production, as reported by Jasko et al. (2012). Thus, reintroducing SD in an anaerobic digester after cultivation of fungi might significantly increase biogas yield.

As for structural polysaccharides, cellulose was reduced by 15% in CSD while, unexpectedly, an increase of cellulose was detected in WS, in spite of the presence of cellulolytic activities during the fungal growth on such substrate. This might be due to the fact that, although a small amount of cellulose was consumed, as suggested by the presence of cellulolytic activities, the fungus found other nutrient sources, the consumption of which influenced the final mass balance more extensively than the utilization of cellulose in WS.

Cellulose increase after fungal growth on WS, in spite of the detection of cellulolytic activities, is also reported in another study (Dias et al., 2010). Moreover, it must be taken into account that endoglucanase activity was higher on CSD than on WS, while the highest level of cellobiohydrolase was measured on WS, but this value was quite low, corresponding to 1.3 U/mg protein. Hemicellulose depletion in CSD (19%) was higher than in WS (8%), this confirming the expectation based on the higher xylanase activity detected on CSD. Cellulose degradation on CSD was much lower than the value (27.7%) reported by Isikhuemhen et al. (2009), while hemicellulose consumption was more extensive in the present study.

In view of a further bioconversion of SD after fungal treatment, *e.g.* through the production of second generation bioethanol, it would be desirable to improve carbohydrate accessibility through the maximization of lignin breakdown. In this sense, the consumption of structural polysaccharides by the fungus might represent a disadvantage, especially with regard to cellulose, representing the main source of fermentable sugars.

Hemicellulose consumption by the fungus, instead, does not reduce the value of SD for bioethanol production, since such polysaccharide is mainly composed by pentose sugars which might pose problems of exploitation during ethanol fermentation, and are often removed through pretreatments (Santi et al., 2014).

## 4. Conclusions

The present study demonstrates that the feedstock used in anaerobic digestion can affect mycelial growth on the resulting SD, and that different fungal strains show large variation on their ability to grow on SD.

For the first time an anaerobic digestate was used as a unique nutrient source for the growth of *P. ostreatus*, with positive results comparable to the control. Therefore *P. ostreatus* resulted to be suitable for the conversion of CSD into valuable biomass like edible mushrooms. In this sense, further studies might be aimed at producing fruiting bodies.

Moreover, *P. ostreatus* was able to reduce the insoluble lignin contained in CSD by 12%, and

partially convert it into soluble lignin. Given the improvement of biogas yield in the presence of low quantity of lignin, the ability of this fungal strain could be exploited as a treatment in order to increase the accessibility of structural polysaccharides in CSD prior to its reintroduction into a digester or, alternatively, for further biorefinery applications such as bioethanol production.

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