Biomass and Bioenergy 144 (2021) 105934

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: http://www.elsevier.com/locate/biombioe

Fungal pretreatment of non-sterile maize silage and solid digestate with a *Cephalotrichum stemonitis* strain selected from agricultural biogas plants to enhance anaerobic digestion

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ARTICLE INFO

Keywords:
Biogas
Methane
Digestate
Fungal pretreatment
Lignocellulose

ABSTRACT

The enhancement of the yields of the anaerobic digestion process was the primary target of the present study, thanks to the development of an innovative fungal pretreatment. The fungal community of agrozootechnical biogas plant feedstocks and by-products (i.e., maize silage and stover, zootechnical slurry and manure, wheat and rice straw, and solid digestate) were studied, isolating and identifying 172 strains belonging to 89 taxa. The mycobiota was dependent on the biomass: maize and zootechnical samples showed a higher biodiversity than straw and solid digestate. During the preliminary screening, *Cephalotrichum stemonitis* extensively grew on both non-sterile maize silage and solid digestate and it was then selected for their pretreatment. The fungus did change the biomasses features: the total solids decreased (approximately 20%), while the volatile solids slightly changed (<1%). Lignin, cellulose and hemicellulose degradation rate in maize silage was higher (55.2%, 25.0% and 24.5%, respectively) than in solid digestate (8.7%, 0.6% and 10.9%, respectively), highlighting their different accessibility and recalcitrance. During the anaerobic digestion, fungal-pretreated maize silage produced lower cumulative biogas and methane than the control, probably due to an excessive degradation of easily accessible fibers. On the contrary, the pretreated solid digestate was less recalcitrant to the microbial transformation in the anaerobic digestion: as a result, cumulative biogas and methane yields were significantly enhanced (approximately 70%). By favoring the (re)use of digestate as a feedstock, the investigated fungal pretreatment could help the economic and environmental sustainability of the anaerobic digestion.

1. Introduction

Anaerobic digestion (AD) is a multistep bioprocess that converts organic matter into biogas, a renewable source of energy [1]. The heterogeneous microbial consortium involved in AD produces digestate as by-product, which mainly contains water, inorganic compounds and undigested organic matter [2]. The yields of biogas production and the features of the digestate strongly depend on the quality of the feedstocks that enter the biogas plant and on the AD operative conditions [1,3]. Agricultural biogas plants (ABP) often operate in co-digestion, with a mixture of zootechnical effluents and lignocellulosic biomass at different ratios [4]. In Europe, energy crops, especially maize silage (MS), are among the most exploited lignocellulosic feedstocks [5]. Unfortunately, the use of energy crops for biogas production shows some drawbacks as: i) the use of cereal and other starch-rich crops competing with land exploitation for food production [6]; ii) the economic feasibility of the AD process is a challenge as a consequence of the rising price of energy crops and of the future ban of financial incentives [7]. To overcome these issues, the enhancement of the process efficiency and the use of alternative feedstocks can improve the overall sustainability and competitiveness of the AD [7,8]. In the recent years, European Union is encouraging the use of alternative, economic and sustainable, lignocellulosic wastes and by-products [9]. Previous studies considered the possibility of exploiting the residual undigested organic matter retained in the solid fraction of digestate (SFD) as a feedstock for ABP [2,3]. Although the recirculation of SFD within the main tank digester could be the simplest option [2,3], a cost-benefits analysis should be performed; the use of a dedicated tank could be a valuable alternative. This new valorisation route could potentially reduce greenhouse gas emissions

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https://doi.org/10.1016/j.biombioe.2020.105934
Received 1 July 2020; Received in revised form 2 December 2020; Accepted 8 December 2020
Available online 16 December 2020

Abbreviations

AD	anaerobic digestion
ABP	agricultural biogas plants
BMP	biochemical methane potential
CS	cow slurry
FYM	farmyard manure
MS	maize silage
MST	maize stover
PCWP	plant cell wall polymers
RS	rice straw
SFD	solid fraction of digestate
WS	wheat straw

that represent an actual issue during SFD storage. Besides, this strategy might help recovering an economically attractive amounts of biogas, positively affecting the environmental and economic sustainability of ABP [3,10].

The use of SFD and other lignocellulose-rich biomass for biogas production challenges the microbial community involved in AD process that should deal with recalcitrant components [11]. In fact, cellulose crystallinity and lignin content decrease the digestibility and limit the theoretical biogas yields [12]. These problems can be addressed and solved with biological pretreatments: microorganisms (whole cell systems) or their enzymes can perform an aerobic pre-hydrolysis targeted to the most refractory components. This process can ultimately enhance the performances of the microbial community of AD against the now-transformed lignocellulosic biomass [13]. In comparison to the physicochemical processes, biological pretreatments are usually cheaper, easy-handling and environmental-friendly, due to low energetic input, mild reaction conditions, no use of chemicals and no formation of inhibitory compounds [14]. The use of fungi to improve the conversion of lignocellulosic biomass is extensively reported in literature [15–17]. By inducing mechanical and biochemical modifications of lignocellulose, fungi can make structural polysaccharides more accessible to the microorganisms involved in AD process, with a consequent improvement in energetic yields [18]. On the other hand, some studies reported a decrease in bioenergy production after fungal pretreatment due to excessive degradation of organic matter [19,20]. In order to develop an efficient bioprocess, it would be necessary to select the best biological resources, fungal/biomass combination and process conditions [21]. Autochthonous fungi isolated from ABP feedstocks and other by-products could offer interesting microbial resources: being adapted to these ecological niches, they could efficiently colonize the matrix and transform the recalcitrant components. Despite this general background, little is known on the ABP mycobiota and its involvement in the biogas production chain [7].

The aims of the present study were the following: i) to fill a knowledge gap concerning ABP mycobiota and exploit this biological resource to develop a fungal pretreatment aimed to improve the performance and sustainability of the AD process; ii) to select fungi that can be used as inoculants for pretreatment of non-sterile MS and SFD, in the future perspective of (re)use them as AD feedstocks; iii) to evaluate the effects on both biomasses of a fungal pretreatment performed with a selected strain on plant cell wall polymers (PCWP - cellulose, hemicellulose and lignin) and subsequent anaerobic production of biogas and methane.

2. Materials and methods

2.1. Biomass sampling and storage

Agrozootechnical biomass used for fungal isolation were collected from storage facilities of two mesophilic full-scale ABP operating in the Piedmont region (Italy) and fed with a mixture of zootechnical effluents, MS and crop residues. Samples of SFD, MS, maize stover (MST), cow slurry (CS) and farmyard manure (FYM) were taken from ABP-1. Samples of MS, MST, CS, FYM and wheat straw (WS) were taken from ABP-2. In addition, a sample of rice straw (RS) was taken from a local farm. The collected biomasses were stored individually at room temperature until use (within 1 month).

Fresh samples of MS and SFD used for the fungal pretreatment at different scale and biochemical methane potential (BMP) tests were collected periodically at a third selected biogas plant (ABP-3) operating in the Piedmont region. ABP-3 is a completely stirred tank reactor with 1 MWel of installed power. It is usually fed with MS (75%), triticale silage (13%) and other cereals (12%). The organic loading rate is 2.25 kg volatile solids per m^3 digester day^{-1}. Hydraulic retention time is approximately 60 days. The resulting digested slurry (approximately 70 t day^{-1}) is processed through a screw-press (CRIMAN® mod. SM260) to separate SFD (approximately 5 t day^{-1}) from the liquid fraction (approximately 65 t day^{-1}). Samples were stored under vacuum at 5 °C for maximum 1 month before use.

2.2. Fungal isolation and characterization

2.2.1. Isolation

Isolation was carried out with selective media containing the agro-zootechnical biomass as sole source of nourishment, to maximize the isolation of samples-specific fungi, capable of growing on them. The methods were adapted according to the characteristics of the biomass. For zootechnical samples (CS and FYM), moist chambers were prepared [22]. As for lignocellulosic samples (SFD, MS, MST, WS and RS), biomass extracts were prepared by autoclaving them with deionized water (1:9 fresh weight/volume ratio) and used as agarized (18 g L^{-1} agar) culture media (modified methods reported by Kim et al. [23] and Lee et al. [24]). All culture media were supplemented with a mix of antibiotics (gentamicin sulfate 40 mg L^{-1}, piperacillin plus tazobactam 11 mg L^{-1}) to limit bacterial growth. Ten replicates for each biomass were performed and incubated in the dark at 15–25–37 °C. Plates were inspected every 3 days for 30 days. The growing fungal colonies were picked up with a sterilized needle and sub-cultured onto solid (18 g L^{-1} agar) Malt Extract Media (MEM: 20 g L^{-1} malt extract, 20 g L^{-1} glucose, 2 g L^{-1} peptone) to obtain pure cultures. Isolated fungi are preserved at the *Mycotheca Universitatis Taurinensis* (MUT- www.mut.unito.it) of the Department of Life Sciences and Systems Biology, University of Turin (Italy).

2.2.2. Identification

Fungal isolates were identified by means of a polyphasic approach. Morphological observations were useful to group isolates into similar morphotypes and to guide the choice of specific primers for molecular identification. Genomic DNA extraction and PCR amplification were performed as described in Bovio et al. [25]. Details on the loci amplified, primers and PCR programs used can be found in Supplementary Material (Table S1). PCR products were purified and sequenced at Macrogen, Inc. Europe Lab (Madrid, Spain). Consensus sequences were obtained by using the software Sequencer 5.0 (Gene Code Corporation). Taxonomic assignments were based on high percentage homologies (similarity values equal or higher than 98%; e-value $> e{-}100$) between the newly generated sequences and those available in public nucleotide databases (GenBank - NCBI database and CBS-KNAW Collection, Westerdijk Fungal Biodiversity Institute). Newly generated sequences were deposited in GenBank with the following accession numbers: MT151627 - MT151658; MT159813 - MT159827; MT180133 - MT180148; MT216344; MT219960; MT271821 - MT271828; MT274498 - MT274504.

For safety reasons, 22 out of 172 strains belonging to species classified as potentially harmful for human and animals (e.g. H2 and keratinolytic species) were excluded from further analyses.

2.3. Preliminary screening

Preliminary analyses led to the exclusion of the following fungi: i) yeasts and yeast-like organisms, because inadequate for the solid-state fermentation pretreatment; ii) slow growing fungi, being inadequate for the inoculum production.

The remaining 124 strains, belonging to 67 species (Supplementary Material, Table S2), were screened for their ability to grow on non-sterile MS and SFD, being colonization of feedstocks fundamental for the establishment of a whole cell pretreatment. Fungi were pre-grown in submerged fermentation in 500 mL Erlenmeyer flasks containing 350 mL of diluted (1:10) MEM (without agar) with 10 g L^{-1} of MS. Flasks were incubated at 25 °C (37 °C for thermophilic species) in agitation at 120 rpm. After 7 days, the mycelium was filtered and inoculated into plates with 10 g of non-sterile MS and SFD to obtain a fungal inoculum/biomass ratio of 1:20 w/w. According to literature [26] and preliminary trials (data not shown), this inoculum load would lead to an extensive and fast fungal colonization. Sterile deionized water was added when necessary to achieve a final moisture content of about 65%. Three replicates were set up. Negative controls consisted of not-inoculated biomass. Plates were incubated in the dark at 25 °C (37 °C for thermophilic species). The grow rate, percentage of plates colonization and predominance of inoculated fungi over indigenous microorganisms were visually evaluated every 3 days up to 21 days (Fig. 1). Fungi with the best growth performance and competitive ability under non-sterile conditions were selected for further investigations.

2.4. Fungal pretreatment and biochemical methane potential tests

2.4.1. Fungal pretreatment

Cephalotrichum stemonitis MUT 6326 was selected to perform the whole cell pretreatment on both MS and SFD. The inoculation was conducted as reported in Section 2.3, scaling up the system to 300 g. The content of total solids (TS) and volatile solids (VS) of the mycelial biomass used as inoculum was determined according to Dinuccio et al. [2]; the fungal inoculum had a TS content of 4.3%, of which 98.2% were VS. Fungal pretreatment was performed at 25 °C under solid-state fermentation and non-sterile condition for 10 days. According to literature [27] and the screening data, this pretreatment period should ensure extensive colonization of the biomass. After the aerobic pretreatment, fungal-pretreated biomass and untreated controls were processed to determine the changes induced by the process and used as feedstocks in BMP tests.

2.4.2. Biomass characterization

Analyses on untreated and fungal-pretreated MS and SFD were conducted on homogenous composite samples obtained by mixing different replicates. In order to estimate the autochthonous microbial load of the non-sterile and untreated samples, microbial cell density was measured using a light-microscope (no staining needed). In detail, 1 g of each biomass was suspended in 6 mL sterile water and incubated at 25 °C and 150 rpm for 1 h in order to release all the microbial propagules in the supernatant: the microbial cells (i.e., bacteria, yeasts and fungal conidia or spores) was determined in a Bürker chamber [28,29], calculating the concentration of cells per gram of biomass (cells g^{-1}).

pH, TS and VS contents were analysed according to the Association of Official Analytical Chemists (AOAC) [30] and Dinuccio et al. [2,31]. The total fiber composition was estimated as neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) using the detergent Van Soest method [32]. Celluloses and hemicelluloses content were calculated as the difference between ADF and ADL, and NDF and ADF, respectively [31]. TS data were expressed as percentage on the raw wet biomass. Instead, VS and PCWP data are expressed as percentage on the TS content of the dry biomass, avoiding any bias due to samples with different water content [2,31]. Loss of TS, VS and degradation of PCWP induced by the pretreatment were calculated using the equations reported by Zhao et al. [26]. These parameters are expressed as variation percentage comparing data obtained after the pretreatment with those of the untreated samples, namely control.

2.4.3. Biochemical methane potential tests

BMP tests were performed in batch trials, according to Dinuccio et al. [31] and VDI 4630 [33]. The batch reactors were set up by filling 2 L capacity digesters with a mixture of feedstock, inoculum and deionized water to obtain a final feedstock to inoculum ratio of 1:2 based on VS content. The inoculum used consisted of the separated liquid fraction of digested slurry produced by ABP-3. Batches containing untreated MS and SFD were used as control. They were used to evaluate the performance of the batches run with biomass pretreated with *C. stemonitis* MUT 6326: the two trial conditions (control and pretreated) were then compared, and the results are expressed as variation percentage. Blank batches trials with inoculum only were also carried out to determine its

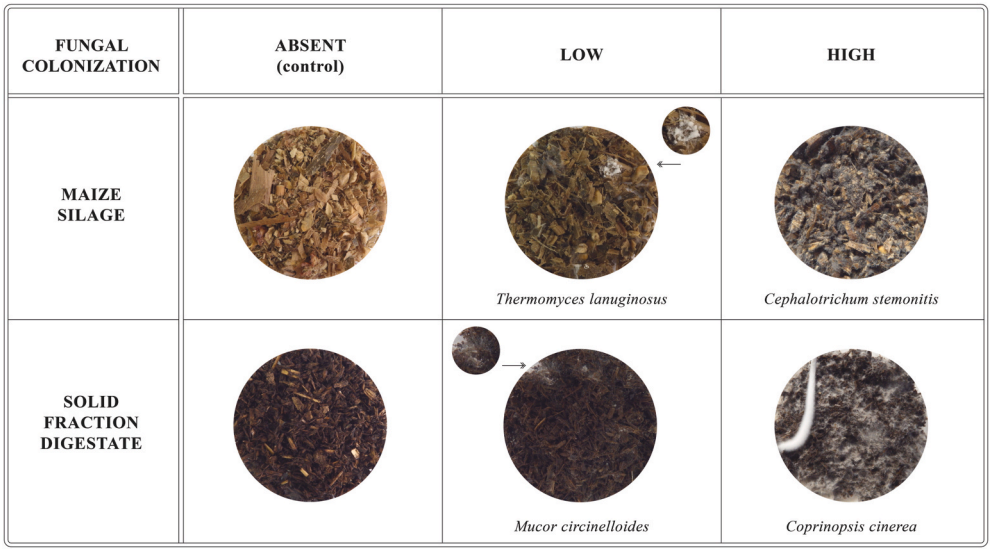

Fig. 1. Visual evaluation of the fungal biomass development and colonization on non-sterile maize silage (MS) and solid fraction of digestate (SFD). Fungal colonization: absent = negative control; low = no or very little plates colonization (0–25%), with dominance of indigenous microorganisms; high = total plates colonization (75–100%), with absence of visible indigenous microorganisms.

residual productivity and to correct the biogas yield from the tested biomass. For each trial condition four replicates reactors were run. Each batch reactor was sealed with glass taps connected, by means of tygon tubing, to a Tedlar bag (3 L capacity) for biogas collection. Trials were performed under mesophilic conditions (40 ± 2 °C) in a temperature-controlled incubator, for 50 days. The biogas volume and composition were monitored every 3 days for the first 2 weeks, and then weekly until the end of the trials. The volume of biogas produced was measured by means of a Ritter Drum-type Gas volume meter (TG05/5, Ritter Apparatebau GmbH & Co. KG, Bochum, Germany). The methane concentration in the biogas was determined with a gas analyser equipped with infrared sensors (model XAM 7000, Drägerwerk AG & Co. KgaA, Lübeck, Germany). The recorded data were normalized at standard temperature and pressure (0 °C and 1013 hPa) according to VDI 4630 [33]. The daily yields of biogas and methane were expressed as normal litres (L_N) per kg of VS fed into the digester ($L_N * kg\ VS^{-1}\ d^{-1}$). Finally, the cumulative biogas and methane production were calculated according to the procedure described by Dinuccio et al. [31] and the measured yields have been corrected for the VS losses during pretreatment, and expressed as L_N per kg of untreated biomass (wet basis) ($L_N * kg\ untreated\ biomass^{-1}$).

2.5. Statistical analysis

Data collected during isolation procedures were analysed using the statistical package PRIMER v. 7.0 (Plymouth Routines In Multivariate Ecological Research). Differences in the fungal community among different sampling sites and biomasses were evaluated by applying a Permutational Multivariate Analysis of Variance (PERMANOVA; pseudo-F index; $p < 0.05$). Principal Coordinate Ordination (PCO) was performed to visualize the data. SIMilarity PERcentage (SIMPER) analysis was carried out to determine the contribution of individual species (expressed in percentage) to the diversity observed.

Data obtained from BMP tests were analysed with the software RStudio Version 3.4.3. Data of biogas and methane yields were analysed by one-way analysis of variance (ANOVA) followed by Tukey's means grouping tests.

3. Results and discussion

3.1. Isolated mycobiota

One hundred and seventy-two (172) fungal strains, belonging to 89 taxa, were isolated (Supplementary Material, Table S2). The isolated mycobiota were affected by both the area of collection and the biomass. The two mesophilic ABPs (ABP-1 and ABP-2) showed significant differences in their fungal communities (PERMANOVA, $p < 0.05$; SIMPER, 75% average intergroup dissimilarity). The qualitative and quantitative structure of the mycobiota showed significant differences among the agrozootechnical biomasses (PERMANOVA, $p < 0.05$; Fig. 2). The average intergroup dissimilarity ranged between 71% and 93% (SIMPER), suggesting that maize samples (MS and MST), WS, RS, CS, FYM and SFD are distinct ecological niches, each inhabited by a unique fungal mycobiota (Fig. 2). The highest number of taxa was retrieved from biomasses rich in nutrients easily accessible for fungi, such as maize samples (41 taxa from MS and MST) and zootechnical samples (40 taxa from CS and FYM). Lower fungal loads and biodiversity were found on more recalcitrant biomass like straw (34 taxa, from WS and RS) and SFD (12 taxa). The lack of easily degradable carbon sources may have led to the reduction of biodiversity, fostering the development of species that can grow in limiting conditions [34]. Interestingly, the fungal community of SFD showed the highest similarity with CS one (SIMPER; 27% average intergroup similarity). This finding could be due to the fact that SFD and CS are by-products of a similar digestion process; the peculiar anaerobic conditions occurring in both the digestive tract of ruminant animals and ABP [35] may have favored the selection of a similar mycobiota.

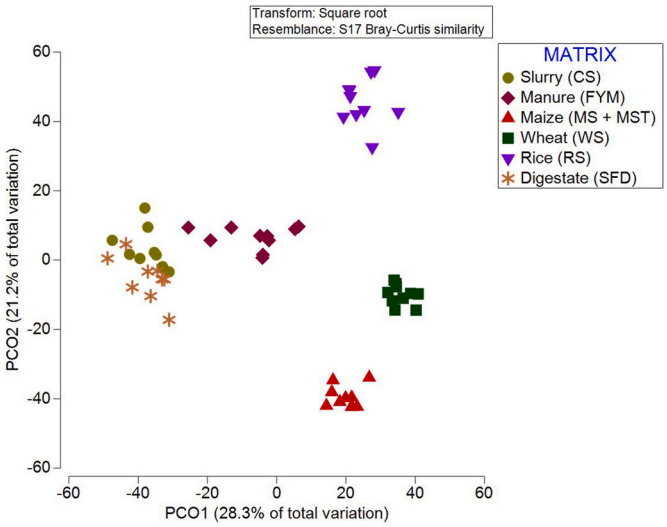

Fig. 2. PCO plot showing how different fungal communities are associated with the biomass investigated. CS: cow slurry; FYM: farmyard manure; MS + MST: maize silage and stover; WS: wheat straw; RS: rice straw; SFD: solid fraction of digestate. The first axis explains 28.3% of the total variance and the second axis 21.2%.

The isolated mycobiota were dominated by Ascomycota (153 strains, 81 species, 43 genera), followed by Mucoromycota (13 strains, 6 species, 5 genera), and, at a less extent, by Basidiomycota (6 strains ascribable to 2 species, *Coprinopsis cinerea* and *Trichosporon asahii*). The most frequently isolated genera were *Aspergillus* (13 species), *Fusarium* (10 species) and *Penicillium* (6 species). *Aspergillus* was the only genus found in all sampling sites and biomasses, while the occurrence of *Fusarium* and *Penicillium* was almost restricted to lignocellulosic feedstocks. These findings agree with literature data, where *Aspergillus*, *Fusarium* and *Penicillium* are reported to be dominant in agricultural and zootechnical residues [7,36–38].

Many isolated fungi are known for their ability to produce lignocellulolytic enzymes, a skill that can be crucial for improving the bioconversion of lignocellulosic feedstocks. For instance, the oxidative and hydrolytic enzymes of *C. cinerea, Aspergillus niger* and *Trichoderma* spp. are reported as effective in lignocellulose biodegradation [16,39,40].

As demonstrated also by Anastasi et al. [41], the use of different incubation temperatures allowed the isolation of rare and less competitive species, with different physiological features. Noteworthy thermophilic and thermotolerant species were isolated including *Thermomyces lanuginosus, Thermoascus crustaceus*, *Scytalidium thermophilum*, *Thermothelomyces thermophilus*, *Remersonia thermophila*. These fungi gained particular attention for their capability of producing thermostable extracellular lignocellulolytic enzymes that can find several biotechnological applications [42].

Unfortunately, members of the mycobiota were also emerging opportunistic human and animal pathogens (e.g. *Aspergillus fumigatus* and *Scedosporium apiospermum*), phytopathogens and potentially mycotoxin producers (e.g. *Alternaria alternata* and *Aspergillus flavus*), which therefore raise safety issues for the operators of ABP and for the potential final uses of the digestate [43].

3.2. Preliminary screening

The selection of fungi that can efficiently grow in the presence of non-sterile biomass is a prerequisite for future industrial application. Significant energy input may be reduced by developing a pretreatment technology that does not require sterilization of feedstocks [26]. The

choice of the most effective fungus is a critical step. In this study, a wide fungal biodiversity (124 strains - 67 species) was investigated. The screening strategy used was a powerful method to select a restricted number of fungi suitable for the pretreatment of non-sterile MS and SFD.

As regard MS, 35% of the strains (20 species – 9 genera) were massively growing on the non-sterile biomass (Table 1). The fastest fungi belonged to the genera *Lichtheimia*, *Mucor* and *Trichoderma,* that completely colonized MS in 5 days, while the other fungi needed more time, e.g. 7–9 days. SFD was more recalcitrant than MS to fungal growth: only *C. cinerea* and *C. stemonitis* efficiently grew on it, reaching the full colonization in 5–7 days. The differences observed between MS and SFD could be associated to the lack of easily degradable carbon source and to the presence of inhibitory factors (e.g., higher lignin content, pH level and ammonia concentrations) that may limit the fungal growth in the presence of SFD [44]. The high recalcitrance of SFD was also confirmed by the presence of a lower indigenous microbial load than MS (about 1.5 $\times$ 10^7 cell g^{-1} in SFD and 2.7 $\times$ 10^7 cell g^{-1} in MS).

The most promising strains for the development of a pretreatment on both ABP feedstock (MS) and by-product (SFD) belonged to *C. stemonitis* species, being the only ones capable of colonizing both non-sterile biomasses. Interestingly, *C. stemonitis* strains were isolated only from FYM and CS, indicating their coprophilous behavior. Indeed, fungi that inhabit herbivore feces are known to secrete enzymes capable of degrading recalcitrant PCWP still present after the digestion process [45]. Noteworthy, very scattered information are available on the skills of *C. stemonitis* in lignocellulosic biomass transformation. Peterson et al. [45] analysed the secretome of *C. stemonitis,* revealing its ability to produce several enzymes involved in the degradation of PCWP (e.g. cellobiohydrolases endoglucanases, glucosidases, xylanases, xylosidases, oxidoreductases). Therefore, C. *stemonitis* MUT 6326 was selected for the next experimental phase.

3.3. Fungal pretreatment and biochemical methane potential tests

3.3.1. Characteristics of untreated and fungal-pretreated biomass

Untreated MS and SFD showed pH, TS, VS and fibers composition (Table 2) comparable with literature data [11,15]. About half of the TS of untreated MS was composed by fibers and the prevailing components were cellulose and hemicellulose. Fiber content in the untreated SFD was higher than in MS. Lignin and cellulose were the main components of the SFD, confirming its higher recalcitrance respect to MS [11].

The fungal pretreatment greatly affected the characteristics of both biomass (Table 2). *C. stemonitis* MUT 6326 transformed PCWP of both MS and SFD, potentially increasing their digestibility during the subsequent AD [46]. However, according to the results of Wan and Li [47], different degradation profiles were observed depending on the tested biomass. The differences observed between MS and SFD reflected their different physicochemical features. The recalcitrant nature and the alkaline conditions of SFD could have been detrimental for ligninolytic enzymatic production and activity, leading to lower PCWP degradation than the one observed in MS [7,44]. In comparison with the untreated sample, fungal-pretreated MS showed decrease in lignin content up to 55.2%. The degradation of cellulose and hemicellulose in MS was 25.0% and 24.5%, respectively, indicating that *C. stemonitis* preferred lignin rather than holocellulose. However, other than the recalcitrant fractions, also the easily accessible MS fibers had been substantially metabolized by the fungus. When grown on SFD, *C. stemonitis* caused a selective degradation of lignin and hemicellulose (8.7% and 10.9%, respectively) while cellulose loss was minimal (0.6%). This is an important process outcome. Indeed, degradation of lignin is one of the main factors for a successful fungal pretreatment, as it increases the accessibility of AD microorganisms to the more easily degradable structural carbohydrates, directly contributing to the enhancement of anaerobic digestibility [48]. Cellulose and hemicellulose degradation are also correlated with improved biomass digestibility, although an excessive loss during pretreatment could be undesirable, since they constitute the main sources of sugars for microorganisms involved in AD [48].

Table 2
Characteristics and fibers composition of untreated and fungal-pretreated maize silage (MS) and solid fraction of digestate (SFD).

	Untreated MS	Fungal-pretreated MS	Untreated SFD	Fungal-pretreated SFD
pH	3.7	6.9	8.4	7.9
TS %	34.4	26.8	34.2	27.2
VS (% TS)	96.4	95.5	87.3	87.0
Lignin (% TS)	6.1	2.8	28.8	26.2
Hemicellulose (% TS)	20.0	15.1	16.6	14.8
Cellulose (% TS)	23.3	17.5	25.3	25.1

The TS concentration decreased up to 22.1% and 20.5% in fungal-pretreated MS and SFD, respectively. Most likely, this result was a consequence of the dilution due to the water content in fungal inoculum (95.7%) and to the VS and PCWP degradation during the pretreatment [26]. However, small variations (<1.0%) in VS content were observed for both fungal-pretreated MS and SFD respect to the untreated groups. These data suggest that although during pretreatment PCWP were degraded by the fungus (Table 2), the inoculated fungal biomass (4.2% VS content) and the grown mycelia may have compensated the loss in VS. It is known that, in the presence of easily degradable biomass as MS, fungi could lead to considerable TS and VS losses due to consumption of PCWP, proteins, soluble sugars and volatile fatty acids. For instance, Liu et al. [15] reported that the fungal pretreatment of corn stover silage decreased the TS content up to 55.3%. On the contrary, as regards recalcitrant materials such as SFD or *Albizia moluccana* (albizia) biomass residues, the loss of TS and VS is generally lower and not so relevant for the economic efficiency of the downstream process [49].

3.3.2. Biogas and methane yields

The daily biogas yields and methane concentration recorded during BMP tests are illustrated in Fig. 2. During the first 7 days of AD, untreated MS showed higher daily biogas and methane production rates than fungal-pretreated samples (Fig. 3 a, b). From day 9, the average daily biogas production rates recorded from untreated and pretreated MS were comparable, although fungal-pretreated samples produced biogas with higher methane concentration (maximum peak of about 60% maintained up to the end of the trial) (Fig. 3 a, b). In detail, the fungal pretreatment on MS increased the daily methane concentration in biogas up to 10% points (Fig. 3 a, b). As a result, fungal-pretreated MS exhibited a significant ($p < 0.05$) decrease (- 11.4%) of the cumulative biogas yield compared to the untreated sample, while cumulative

Table 1
List of fungal species capable of growing on non-sterile maize silage (MS) and solid fraction of digestate (SFD).

MS				SFD
Aspergillus alabamensis	*Aspergillus tubingensis*	*Lichtheimia ramosa*	*Penicillium roqueforti*	*Cephalotrichum stemonitis*
Aspergillus cejpii	*Bauveria felina*	*Mucor circinelloides*	*Penicillium simplicissimum*	*Coprinopsis cinerea*
Aspergillus giganteus	*Byssochlamys nivea*	*Mucor fragilis*	*Trichoderma asperellum*	
Aspergillus niger	*Cephalotrichum stemonitis*	*Penicillium oxalicum*	*Trichoderma harzianum*	
Aspergillus terreus	*Fusarium verticillioides*	*Penicillium paneum*	*Trichoderma longibrachiatum*	

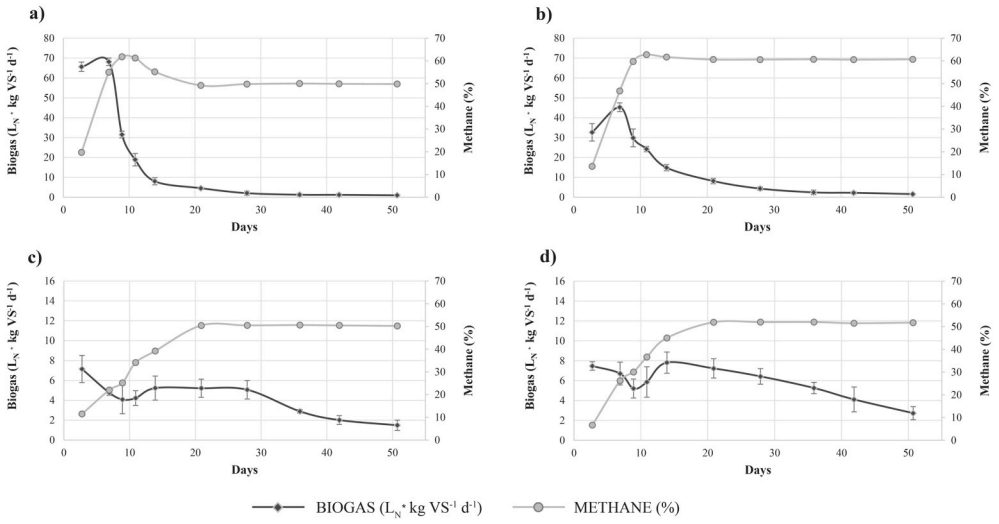

Fig. 3. Daily biogas yield, expressed as normal litres (L_N) per kg of VS fed into the digester (L_N * kg VS^{-1} d^{-1}), and respective methane concentration (%) in biogas of untreated and fungal-pretreated maize silage (MS) and solid fraction of digestate (SFD). 3a: Untreated MS; 3b: Fungal-pretreated MS; 3c: Untreated SFD; 3d: Fungal-pretreated SFD. Results are expressed as mean ± standard deviation from four replicates.

methane yield was comparable between control and fungal-pretreated MS (- 4.6%; $p > 0.05$) (Fig. 4). In detail, fungal-treated MS led to cumulative biogas and methane yields of 179 ± 10 L_N * kg untreated biomass^{-1} and 95 ± 6 L_N * kg untreated biomass^{-1}, respectively, while data with untreated MS were 202 ± 11 L_N * kg untreated biomass^{-1} and 99 ± 6 L_N * kg untreated biomass^{-1}, respectively. In agreement with these findings, other authors reported a decrease in AD productivity after fungal pretreatment on lignocellulosic biomass [19,20]. The lower yields obtained with fungal-pretreated MS compared to the untreated samples could be explained by the excessive consumption of cellulose and hemicellulose during pretreatment (Table 2) in comparison to the amounts of recalcitrant compounds became available for anaerobic bacteria [20]. On the other hand, the fungal pretreatment on MS improved the daily methane concentration in biogas (Fig. 3 a, b) and the cumulative biogas quality (Fig. 4). This finding has positive implications, as it is well known that the energy content of biogas is in direct proportion to the methane concentration [50]. We may hypothesize that the inoculated fungal biomass and the grown mycelium has increased the total fat and protein content in the feedstock, boosting AD microorganisms, and ultimately leading to the increase of methane level. However, based on technological progression, data of BMP test in 2 L batch cannot be exhaustive for the future scale up of the process. As regards the actual economic feasibility of the process, continuous 5 L trials would be necessary to clearly define the precise biogas and methane productivity [51].

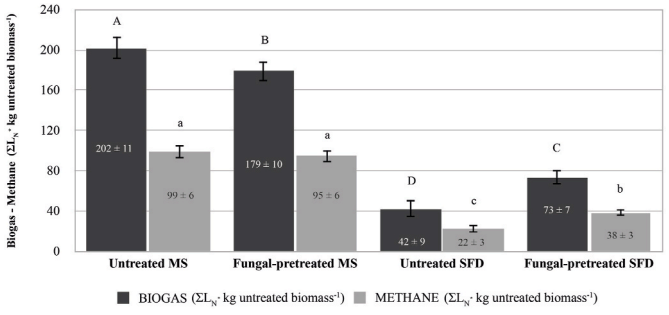

Fig. 4. Cumulative biogas and methane yields, corrected for the VS losses during pretreatment and expressed as L_N per kg of untreated biomass (wet basis) (ΣL_N * kg untreated biomass^{-1}), of untreated and fungal-pretreated maize silage (MS) and solid fraction of digestate (SFD). Results are expressed as mean ± standard deviation from four replicates. Different letters indicate significant differences ($p < 0.05$).

Concerning SFD, the batch trials results confirmed that it still contained residual biogas and methane potential (Fig. 3 c, d; Fig. 4) [52]. However, a large fraction of its organic matter is not readily biodegradable, as demonstrated by the poor biogas and methane yield obtained from untreated samples (about 42 ± 9 L_N * kg untreated biomass^{-1} and 22 ± 3 L_N * kg untreated biomass^{-1}, respectively) (Fig. 4). Fungal pretreatment affected the anaerobic digestibility of SFD, leading to a higher daily biogas and methane production rates from fungal-pretreated SFD than from the untreated control (Fig. 3 c, d). However, in contrast to MS, fungal pretreatment of SFD did not vary the methane concentration in daily produced biogas (Fig. 3). As a result, cumulative biogas and methane yields from fungal-pretreated SFD (about 73 ± 7 L_N * kg untreated biomass^{-1} and 38 ± 3 L_N * kg untreated biomass^{-1}, respectively) resulted approximately 74.7% and 71.5% higher ($p < 0.05$) than the control, respectively (Fig. 4). The fungal pretreatment seems a cost-effective and environmental-friendly option to increase the digestibility and yields of SFD. The improved performance can be associated to the reduced biomass recalcitrance due to selective lignin and hemicellulose removal (Table 2), as confirmed by other studies [18,53].

However, the fungal biomass added to the system may have also played a role on the final BMP value. In fact, the fungal biomass could be profitably used as a substrate in AD processes as it contains lipids, proteins and other organic molecules that may be exploited by anaerobic microorganisms. However, literature is scarce on this subject, thus a lack of knowledge must be filled with targeted investigations on the precise contribution of the fungal biomass to the AD. Hom-Diaz et al. [54] reported that the methane production of fungal biomass could vary from 281 to 595 L_N * kg VS^{-1}, depending on the inoculum (digested slurry) used to start the methanation. Considering the average methane productivity reported in literature per g of fungal VS (about 387.5 ± 123.8 L_N * kg VS^{-1}) [54] and the amount of fungal VS introduced inside each batch reactor (0.23 g VS), in this study, the fungal biomass could have contributed to approximately 0.9–2.5% to the total methane productivity.

Interestingly, previous studies [10,55] have investigated biological and/or physicochemical pretreatments to get a higher energy recovery from SFD, but they often obtained weak results compared to that here reported. For instance, two different Basidiomycota strains (*Pleurotus ostreatus* and *Stropharia rugoso-annulata*) on solid digestate led to uncontrolled organic matter losses and to subsequent decreases of methane

yield (up to 50%) [55]. Besides, Sambusiti et al. [10] revealed that thermal and alkaline treatments did not enhance methane potentials of SFD, while enzymatic treatment only slightly increased the methane yield (13%).

In conclusion, the fungal pretreatment with *C. stemonitis* could favor the (re)use of SFD as a feedstock for the anaerobic digester, contributing to the development of a next-generation by-products management strategy [3,11]. The reuse of fungal-pretreated SFD into the anaerobic digester has the potential to allow concurrent gaseous loss abatement and better energy recovery, leading to environmental and economic benefits that make even more attractive and effective the overall sustainability of AD technology [2,3,56]. Results of the BMP tests suggest that under the specific conditions of ABP-3 plant, fungal pretreatment of SFD could improve the daily methane production of the AD by 192.3 m^3_N day^{-1}. Considering the average hourly methane consumption (approximately 240 m^3_N h^{-1}) of the combined heat and power system of ABP-3 plant, the latter value allows the production of approximately 0.80 additional MWel per day (approximately 290 MWel per year). This extra electrical production could correspond to a significant economical income to ABP owners. Considering the low inputs required for pretreatment (e.g., use of diluted culture media for inoculum production, cultivation in non-sterile condition), the economical income could allow the coverage of the process costs. However, further studies are required to evaluate the actual techno-economic feasibility of the process in full-scale application.

4. Conclusions

The present study provides new insights on the mycobiota of common ABP feedstocks and by-products and reports a simple but powerful strategy to isolate and select promising fungi that can find different biotechnological applications, including the pretreatment of lignocellulosic feedstocks. The isolated fungi have demonstrated to adapt to and grow on maize silage and solid digestate as well as to compete with the indigenous microorganisms under non-sterile conditions. This result emphasizes the importance of investigating the fungal biodiversity of peculiar ecological niches in order to identify potential candidates for developing efficient bioprocess at industrial level.

The pretreatment with *C. stemonitis* MUT 6326 appears to be detrimental on the nutrient rich and easily biodegradable MS. Further studies are needed to optimize the experimental conditions in order to enhance the biogas and methane production from MS. The exploitation of other fungi may offer an alternative solution, as well as the use of crude enzyme cocktails, which can avoid the sugar loss that typically occurs with whole-cell processes.

On the contrary, the fungal pretreatment demonstrated to be a cost-effective and environmental-friendly tool to increase AD performances with the poorly digestible SFD. This finding primarily opens an alternative valorisation scenario of this by-product. The development of a next-generation by-product management strategy would improve the sustainability of AD process and could address some environmental issues that often mine the disposal of AD by-products.

Authors contributions

Andrea Zanellati: Conceptualization, Data curation, Formal analysis; Investigation, Methodology, Software, Visualization, Writing- Original draft preparation. Federica Spina and Anna Poli: Conceptualization, Data curation, Methodology, Software, Validation, Writing - review & editing for isolation, identification and screening of fungi. Luca Rollé: Methodology, Data curation, Validation for biomethanation tests. Elio Dinuccio and Giovanna Cristina Varese (Supervisors): Conceptualization, Funding acquisition, Project administration, Resources. Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the biogas plants owners for allowing the sampling of biomass. The technical and scientific support of MUT staff and JRU MIRRI-IT is greatly acknowledged.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2020.105934.

Supplementary material

E-supplementary data of this work can be found in online version of the paper.

Data availability

Datasets of nucleotide sequences related to this article can be found in Genbank https://www.ncbi.nlm.nih.gov/genbank/, hosted at the National Center for Biotechnology Information (NCBI) database

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