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Pretreatment of lignocellulosic biogas substrates by filamentous fungi

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ABSTRACT

Decomposition of lignocellulosic plant biomass by four filamentous fungi was carried out to facilitate subsequent anaerobic degradation and biogas formation. Agricultural side products, wheat straw and corn stover and forestry energy plant willow chips were selected as plant biomass sources. The substrates were confronted by pure cultures of *Penicillium aurantiogriseum* (new isolate from rumen), *Trichoderma reesei* (DSM768), *Gilbertella persicaria* (SZMC11086) and *Rhizomucor miehei* (SZMC11005). In addition to total cellulolytic filter paper degradation activity, the production of endoglucanase, cellobiohydrolase, β-glucosidase enzymes were followed during the pretreatment period, which lasted for 10 days at 37 °C. The products of pretreatments were subsequently tested for mesophilic biogas production in batch reactors. All 4 strains effectively pretreated the lignocellulosic substrates albeit in varying degrees, which was related to the level of the tested hydrolytic enzyme activities. *Penicillium aurantiogriseum* showed outstanding hydrolytic enzyme production and highest biogas yield from the partially degraded substrates. Corn stover was the best substrate for biomass decomposition and biogas production. Scanning electron microscopy confirmed the deep penetration of fungal hyphae into the lignocellulosic substrate in all cases.

1. Introduction

Experts (https://www.ipcc.ch/report/ar6/wg1/) and politicians (https://unfccc.int/sites/default/files/resource/cop26_auv_2f_cover_decision.pdf) keep sending frequent alarming messages about the increasing global dangers related to climate changes, global warming and environmental disasters due to the elevation of greenhouse gases (GHGs) in the atmosphere as a result of extravagant human exploitation of our fossil energy resources.

A reasonable escape route from this global dead-end street is the increased utilization of renewable biomass, which accumulates continuously due to the conversion of the virtually inexhaustible solar energy into the chemical bonds of biomolecules. Agricultural products provide food and feed for the heterotrophic and mixotrophic living creatures. The by-products and leftovers of the human and animal nutritional activities, sometimes called as “waste”, is the fraction of the entrapped solar energy to be primarily used for renewable energy generation from biomass. The renewable resource utilization integrates biomass conversion processes to produce fuels, power, and chemicals, making agricultural operation economically more feasible and sustainable (Fermoso et al., 2018).

The bulk of plant biomass is composed of lignocellulosic materials, which has the largest potential for biofuel production, including gaseous and liquid biofuels (Saini et al., 2015). Its major constituents are cellulose, hemicellulose and lignin in varying ratios (Stamatelatou et al., 2012). Cellulose and hemicellulose are the main target substrates of bioconversion, whereas lignin, being actually a barrier for the efficient exploitation of lignocellulosic feedstocks, is hardly metabolized by most microbial species (Sawatdeenarunat et al., 2015). Pretreatment is necessary to disrupt the resilient lignocellulose matrix.

The common feature of the straws of wheat, barley, soybean, rice and corn stover is that they are by-products of human agricultural food production activity and have predominantly lignocellulose as main composite. Globally, these materials could contribute between 19 and 76 EJ (1 exajoule = 10^{18} Joule) to the worldwide primary energy use every year (Andersen et al., 2020). In other units, the annual global

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straw/stover production is in the range of 1500 Tg (1 teragram = 10^{12} gram) biomass (Fjortoft et al., 2019).

In spite of the enormous potential, this relatively inexpensive material is used to a very limited extent in biorefinery applications. A large number of the technologies, using straw in biorefinery applications, aim at ethanol production although the limited robustness of the necessary enzymes and their high cost jeopardizes the success of generalized large scale production. In contrast, conversion of straw to biogas is a relatively simple and robust process and can eminently close the nutrient cycle in agriculture. Nevertheless, the chemical structure and recalcitrance of lignocellulose hinder its degradability in the anaerobic digestion (AD) reactors.

Although corn stover is commonly used for production of biofuels and other chemicals, the raw substrate is subjected to chemical, biological or steam explosion pre-treatments (Wang et al., 2018).

Willow is a hardwood-type biomass, with holocellulose (cellulose and hemicellulose) and lignin contents ranging from 63.7 % to 64.5 % w/w and 24.5–28.8 % w/w on a dry mass basis, respectively (Lavoie et al., 2010). The AD of raw or thermochemically pretreated willow biomass has already been proposed, leading to promising methane yields (Kakuk et al., 2021; Szűcs et al., 2021; Dudits et al., 2022). The liquid fraction of steam-pretreated willow was investigated as carbon source for β-glucosidase production by different *Aspergillus* and *Penicillium* strains (Réczey et al., 1998). Attempts were also made to employ this fraction for cultivating *Trichoderma reesei* (Gyalai-Korpos et al., 2011; Kakuk et al., 2017).

The biodegradability of the lignocellulosic materials can be increased by pretreatments. Numerous approaches have been developed and proposed for lignocellulose pretreatments (e.g. Rajendran et al., 2018; Martinez-Gutiérrez, 2018; Fjortoft et al., 2019; Andersen et al., 2020; Olatunji et al., 2021). The pretreatment technologies are usually classified in three categories, i.e. physical, chemical and biological, and sometimes combinations of these methods are recommended for more efficient results. Several physical approaches have been developed with varying results (Kakuk et al., 2017; Kaldis et al., 2020). All share the disadvantages of needing specific instrumentation and extra energy input, i.e. additional investment and operational costs. The benefit may come from the increased lignocellulose degradation and accessible sugar yield. Chemical pretreatment methods are diverse and applied frequently due to their effectiveness and relatively simple operational conditions. Some of the used chemicals are simple and inexpensive, e.g. acids, bases, ozone, $FeCl_3$, H_2O_2. Others are costly, e.g. organic solvents, supercritical CO_2, ionic liquids, eutectic solvents (Olatunji et al., 2021). The removal of the chemical agents or salts generated during neutralization may present technical problems in large scale applications.

Bacteria and numerous fungi produce enzymes designed to decompose lignocellulose in Nature as part of their lifestyle. The fungal groups of interest for lignocellulose degradation are the wood decaying fungi, such as the white-, brown-rot and cellulose degraders (Feng et al., 2013). Cellulolytic enzymes of basidiomycetes were recently reviewed (Rytioja et al., 2014). The fungal degradation of cellulose is catalyzed by: (1) cellobiohydrolases (CBHs, EC 3.2.1.91) and (2) endoglucanases (EGs EC 3.2.1.4), of which CBHs cleave the polymeric cellulose from reducing or non-reducing ends, and EGs randomly endo-wise along the glucose chain. The cellulolytic system also contains (3) extra- or intracellular β-glucosidases (EC 3.2.1.21), which hydrolyze the resulting cellobiose or cello-oligosaccharides to glucose. The hydrolysis products can also be oxidized by cellobiose dehydrogenase (CDH). In addition, numerous other carbohydrate-active enzymes are produced by fungi. In the carbohydrate-active enzyme database CAZy (http://www.cazy.org) glycoside hydrolases are grouped in as many as 115 families (Cantarel et al., 2009). Lignin is a large macromolecular and heterogeneous polymer the degradation of which is difficult as it does not contain any hydrolysable linkages (Kirk and Cullen, 1998). The enzymes must be therefore oxidative (i.e., oxidoreductases), and due to heterogeneity of the polymer and several types of linkages (C–C, C–O), also nonspecific. Lignin is also stereo-irregular, differing from e.g. cellulose or hemicelluloses (Hatakka and Hammel, 2010).

Members of the genus *Penicillium* are well-known as one of the most common fungi occurring in a diverse range of habitats including soil, air or vegetation (Visagie et al., 2014; Yadav et al., 2018; Jiao et al., 2021). They enjoy worldwide distribution and exert large economic impact on human life, particularly since the discovery of penicillin, a „superstar" antibiotic in the 20th century (Vaishnav et al., 2018; Jiao et al., 2021). Research in the last decades uncovered that *Penicillium* strains are also important sources of cellulase enzymes. *Penicillium* species may also cause deleterious effects on cultivated crops, e.g. devastating rots on food crops (Frisvad and Samson, 2004; Pitt and Hocking, 2009; Samson et al., 2010), and producing a diverse range of highly toxic mycotoxins (Frisvad et al., 2004; Visagie et al., 2014). Some *Penicillium* species produce large array of enzymes capable of lignocellulose degradation, similarly to *Trichoderma* (Gusakov and Sinitsyn, 2012). *Penicillium* strains compete with *Trichoderma* species in the decomposition of lignocellulosic biomass to gain substrates fueling their metabolism (Fujian et al., 2002; Adsul et al., 2004; Dutta et al., 2008; Scholl et al., 2015; Gusakov, 2011). *P. brasilianum* (Jørgensen et al., 2003), *P. citrinum* (Dutta et al., 2008), *P. chrysogenum* (Hou et al., 2007), *P. crustosum*, *P. decumbens* (Liu et al., 2013a; Liu et al., 2013b; Liu et al., 2013c), *P. echinulatum* (Dillon et al., 2006; Reis et al., 2013), *P. funiculosum* (de Carvalho et al., 2014), *P. janthinellum* (Adsul et al., 2007; Singhania et al., 2014), *P. occitanis* (Chaabouni et al., 1994), *P. pinophilum* (Brown et al., 1987), *P. purpurogenum* (Suto et al., 1991), and *P. verruculosum* (Soloveva et al., 2005) were among the strains of highest cellulase production.

2. Materials and methods

2.1. Organisms and materials

2.1.1. Rumen content

Rumen samples were collected from beef steers farms in Csongrád and Bács-Kiskun County, Hungary. These areas have temperate continental climate and are located in South-East Hungary. Rumen contents were collected from 6 healthy adult steers directly from the rumen sac after slaughtering. The samples were transported in an anaerobic container and stored at 37 °C.

2.1.2. TS-oTS

Each substrate was soaked and sterilized at 121 °C in distilled water, then total solid (TS) and organic total solid (oTS) content were determined as follows The total solid (TS) content was measured after drying the biomass at 105 °C overnight. The organic total solid (oTS) value was calculated after all the organic mass of the sample was oxidized by heating the biomass to 550 °C for 1 h as recommended by the VDI 4630 protocol (VDI, 4630, 2016) (Table 1.).

2.1.3. Fungi

Penicillium aurantiogriseum was isolated and identified from rumen samples (see also Section 2.2).

Trichoderma reesei (DSM768) came from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Culture (Braunschweig, Germany).

Gilbertella persicaria (SZMC11086) and *Rhizomucor miehei*

Table 1
Characteristics of the substrates. TS: total solid, oTS: organic total solid, ash content and C/N ratio: carbon/nitrogen ratio.

Sterile substrate	TS (%)	oTS (%)	Ash content (%)	C/N ratio
wheat straw	9.5 ± 1.2	96.1 ± 1.4	3.9 ± 0.4	68.1 ± 17.5
willow chips	15.4 ± 0.7	97.3 ± 2.7	2.7 ± 0.2	48.3 ± 6.4
corn stover	10.1 ± 0.7	94.4 ± 4.4	5.6 ± 0.5	38.7 ± 1.9

(SZMC11005) was obtained from Hungarian strain collection. The fungal strains were provided by the Szeged Microbiology Collection (SZMC, Szeged, Hungary; http://szmc.hu/) (Takó et al., 2015).

2.1.4. Isolation and identification

In order to isolate cellulose-degrading fungi, 20 μL of the individual rumen samples were plated on carboxymethyl cellulose (CMC) agar, which contained 10 g CMC and 15 g agar in 1000 mL of distilled water. All media contained ampicillin, kanamycin, streptomycin (100 μg/mL). Plates were incubated at 37 °C.

Pure cultures were obtained by serial dilution and plating on Czapek-Dox medium (modified following DSMZ's 130. medium – Czapek-Dox). Czapek-Dox medium with 2% agar composition: in 1000 mL of distilled water: 1.988 g sodium nitrate, 0.994 g dipotassium phosphate, 0.52 g magnesium sulphate heptahydrate, 0.52 g potassium chloride, 20 g agar.

The Czapek-Dox medium was sterilized and contained ampicillin (100 μg/mL), kanamycin (100 μg/mL), streptomycin (100 μg/mL). All plates were incubated at 37 °C.

The fungi were identified with polymerase chain reaction (PCR) using DreamTaq (ThermoFisher Scientific) polymerase. The conditions were 95 °C for 2 min followed by 32 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 25 s followed by a final extension step of 72 °C for 20 s

For the identification calmodulin (CaM) and the RNA polymerase II second largest subunit (RPB2) genes as two marker options were used. RPB2 has the added advantage of lacking introns in the amplicon, allowing robust and easy alignments when used for phylogenies. The following primers were used in the case of RPB2 gene: RPB2 5F_Eur 5′-GAYGAYCGKGAYCAYTTCGG-3′ and RPB2 7CR_Eur 5′-CCCATRGCYTGYTTRCCCAT-3′ (Visagie et al., 2014).

Calmodulin gene amplification was performed using the set of primers Cmd5 5′-CCGAGTACAAGGAGGCCTTC-3′ and Cmd6 5′-CCGATAGAGGTCATAACGTGG-3′ (Hong et al., 2006).

After the PCR reaction, the purity of amplicons was checked on agarose gel, the sequences of the amplicons were determined by capillary electroforesis (3500 Series Genetic Analyzer, Life Technologies). The sequences were aligned using Genomic Workbench (CLC Bio) software. Identifications based on cultural features were confirmed by sequence analysis of the isolate. Basic Logical Alignment Search Tool (BLAST) results of RPB2 and calmodulin gene sequences of this study in National Center for Biotechnology Information (NCBI) provided relationships and similarities with reference sequences in GenBank. Our fungus showed 100% sequence similarity to *P. aurantiogriseum* CBS 324.89 (typestrain) for the calmodulin gene. For the RPB2 gene, the sequence of our isolate was 99.51% similar to *P. aurantiogriseum* CBS 324.89 (typestrain), and 100% similarity to *P. aurantiogriseum* CBS 112023.

2.2. Scanning electron microscopy

Samples were washed, resuspended in phosphate buffered saline (PBS) and fixed with 2.5% (*v/v*) glutaraldehyde and 0.05 M cacodylate buffer (pH 7.2) in PBS overnight at 4 °C. The samples were washed twice with PBS and dehydrated with a graded ethanol series (30%, 50%, 70%, 80%, 100% ethanol (v/v), for overnight each at 4 °C). The samples were dried with a critical point dryer, followed by 12 nm gold coating (Quorum Technologies, Laughton, East Sussex, UK) and observed under a JEOL JSM-7100 F/LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

2.3. Pretreatments and biogas production

2.3.1. Enzyme assays

Cellulases are a multi-component enzyme systems, which consist of three major groups of enzymes: endo-β-1,4-glucanases, exo-β-1,4-glucanase and β-glucosidases. Total cellulase activity represents the collective measurement of endoglucanase, exoglucanase and β-glucosidase activities.

Total cellulase activity was determined as filter paper (FPase) activity by using Whatman No. 1 filter paper as substrate (Yu et al., 2016). Endoglucanases randomly cleave β-1,4-glycosidic linkages on the amorphous part of cellulose away from chain ends and was determined by using carboxymethyl cellulose (CMC) as substrate. Exoglucanases produce cellobiose by attacking cellulose from reducing and non-reducing chain ends while β-glucosidase converts cellobiose into glucose (Pathak et al., 2014; Kumar et al., 2016).

2.3.1.1. Determination of filter paper decomposition activity. Filter paper degradation activities (FPase) were determined by standard methods (Ghose, 1987). At first, 1 mL of 0.05 M Na-citrate buffer, pH 4.8 and 1 × 6 cm filter paper were added to a test tube. Then, 0.5 mL of samples was added to the tube. The tubes were incubated at 50 °C for 60 min. At the end of the incubation, each tube was removed from the 50 °C bath and the cellulase reaction was stopped by immediately adding 3 mL of 3, 5-dinitrosalicylic acid (DNSA) reagent (Sigma-Aldrich, USA). All tubes were boiled for exactly 5 min in a vigorously boiling water bath. Finally, after the colored solution was diluted with 20 mL of H_2O, the absorbance at 540 nm was measured using a GENESYS UV-Visible Scanning Spectrophotometer (ThermoFisher Scientific). Measurements were made in triplicate. A calibration curve was prepared using DNSA. One unit of each activity was defined as the amount of enzyme that released 1 μM of glucose equivalent from the respective raw substrate per minute under the assay conditions.

2.3.1.2. Endoglucanase assay. For endoglucanase activity, reaction mixture of 0.5 mL of 2% (w/v) carboxymethyl cellulose of medium viscosity (Sigma Chemical Co. St Louis, MO, USA), prepared in 50 mM citrate buffer (pH 5.5), and 0.5 mL of appropriately diluted crude enzyme was incubated at 50 °C for 30 min. The subsequent steps in endoglucanase activity measurements were the same as in the FPase assay (Ghose, 1987). Samples were measured spectrophotometrically at 550 nm. One unit of endoglucanase activity was defined as the amount of enzyme that releases 1 μM of glucose under the specified conditions.

2.3.1.3. β-glucosidase activity assay. β-glucosidase activity was measured by using p-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma-Aldrich) as substrate. The reaction mixture contained 250 μL acetate buffer (pH 5.5), 125 μL pNPG and 125 μL enzyme solution. Before adding the enzyme solution to the mixture of acetate buffer and pNPG, the mixture was incubated at 37 °C for 5 min. After adding 125 μL solution the reaction mixtures were incubated for another 15 min at 37 °C. The reaction was stopped by adding 500 μL of sodium carbonate (0.1 M), and the released p-nitrophenol (pNP) was measured spectrophotometrically at 400 nm. The calibration curve was prepared using *para*-nitrophenol (pNP). One unit of β-glucosidase activity is defined as amount of enzyme that released 1 μM of *p*-nitro phenol per min per millilitre under assay conditions (Wood and Bhat, 1988).

2.3.2. Experimental set-up

2.3.2.1. Inoculum sludge. The inoculum sludge, i.e. the fermentation effluent was obtained from an industrial scale mesophilic biogas plant, fed with pig slurry and maize silage mix (Zöldforrás Biogas Plant, Szeged, Hungary). The inoculum was used after one week of incubation at 37 °C, during this time the residual biogas production ceased.

2.3.2.2. Substrates for biogas fermentation. Dry corn stover, wheat straw and willow wood chip (Express variety, 3 years old) were milled and sieved to a maximum particle size of either < 2 or < 10 mm, with an electric grinder (Retsch SM 100, Haan, Germany). Dry corn stover and dry wheat straw were stored at room temperature, while willow wood chips were stored at − 20 °C. During the preparation for the fungal

pretreatment, the desired amount of substrates and 20 mL distilled water were weighed into the pretreatment flask and sterilized at 120 °C for 30 min.

2.3.2.3. 60 mL batch fermentations. All pretreatments were carried out in triplicate biological parallel experiments.

The fungal pretreatments lasted for 10 days in 100 mL batch reactor vessels. Substrates, containing 1.46 g oTS were inoculated with 10^7 spores in 15 mL sterile distilled water for the pretreatments (day 1). 45 mL of inoculum sludge (TS: 5.1 %, oTS: 70.3 %, pH: 7.3) was added to each sample on day 11. The negative controls contained only sludge. The biological methane potential (BMP) of the inoculum was tested using the internal standard α-cellulose as positive control (1.46 g oTS) added to the inoculum sludge as positive control samples (VDI 2006). The theoretical maximum biogas yield of α-cellulose is 740 mL_N/g oTS and its CH_4 content is 50–70 % (VDI. 2006). In practice, the inoculum is applicable when at least 80 % of the theoretical yield is produced. Hence at least 592 mL biogas/g oTS should be obtained in the VDI BMP test, which equals to 296–414 mL_N/g oTS CH_4.

2.3.2.4. 300 mL batch fermentations. 4.38 g oTS of the substrates were inoculated with 3 * 10^7 spores and added in 75 mL sterile distilled water in 500 mL batch reactor bottles to start the pretreatment stage (day 1). 225 mL of inoculum sludge, having identical composition as in the 60 mL experiments, was added to the pretreated samples to start the AD phase on day 11.

All pretreatments and anaerobic digestion (AD) experiments were carried out under mesophilic conditions at 37 °C, with manual mixing 3 times daily.

2.4. Analytical measurements

2.4.1. HPLC

4 mL samples were taken from the liquid phase of the pretreatment reactors for HPLC and enzyme activity assays on days 0, 3, 6, and 9. The samples were stored at − 20 °C until further analyses. Samples for organic acid analysis were taken from the liquid phase of the reactors. The small insoluble aprticles and molecular aggregates were removed by centrifugation (13,000 rpm for 10 min,) and the supernatant was filtered through PES (polyethersulfone) centrifugal filter (PES 516–0228, VWR) at 13,000 rpm for 20 min. The concentrations of volatile organic acids were measured with HPLC (Hitachi Chromaster) equipped with refractive index detector Chromaster 5450. The separation was performed on an Agilent Hi-Plex H column. The temperature of the column and detector were 50 °C and 41 °C, respectively. The eluent was 0.02 M H_2SO_4 (0.6 mL/min).

2.4.2. Gas chromatography

Bio-methane concentrations in the headspace were measured on a daily basis with an Agilent 7880 Gas-chromatograph (GC), on a HP Molesieve column, with a length of 30 m and an inner diameter of 0.53 mm, equipped with a Thermal Conductivity Detector (TCD). The carrier gas was Ar, oven (column) temperature was 37 °C, flow velocity was 1.2 mL/min and detector temperature was 160 °C. 250 μL Hamilton syringes were used to inject 50 μL of gas sample.

2.5. Statistical analysis

All experiments were performed in triplicates. Standard deviations of mean values were calculated using Microsoft Office Excel 2007 function and the Sigma Plot (USA) software was used for statistical significance analyses.

3. Results

3.1. Isolation and screening of cellulolytic fungi

The rumen samples were spread on CMC plates at 37 °C for 3 days. Cellulase producing fungi were selected after congo red test: plates were flooded with 0.1% w/v congo red, followed by destaining with 0.1 M NaCl. Thirteen colonies that showed largest halo were selected for further analysis.

3.2. Optimization of fermentation temperature in submerged fermentation (SmF)

During the first round, carboxymethyl cellulose (CMC), α-cellulose and microcrystalline cellulose were screened, CMC gave the highest endoglucanase activity when fermented with *Penicillium aurantiogriseum* under SmF.

Incubation temperature plays a key role in the assay. During the experiments 4 different temperatures (20 °C, 24 °C, 30 °C and 37 °C) were tested to reach the maximum enzyme activity on CMC. 37 °C was selected as optimum temperature.

3.3. Submerged fermentation (SmF) and inoculum concentration

Microscopic observations of enrichment cultures revealed cellulose fibers, which presumably were released from corn or wheat straw disintegrated by cellulolytic enzymes, surrounded by fungal hyphae.

It was established that an optimal concentration of 15–20 w/w% substrate increased significantly the cellulase production in SmF. Under these conditions rapid growth of fungi was observed, which was accompanied with elevated enzyme activity. Higher or lower substrate ratios resulted in a significant decrease in endoglucanase production. In contrast, β-glucosidase activity showed constant increase between the lowest and the highest concentrations. Among the tested substrates, the cultivation of the fungus *P. aurantiogriseum* on wheat straw and corn stover provided the highest endoglucanase and β-glucosidase production.

3.4. Hydrolyzing enzyme activities

3.4.1. β-glucosidase activity

The activity of the β-glucosidase enzyme of *R. miehei* and *P. aurantiogriseum* showed a decreasing tendency over time (Fig. 1 A, 1B). The substantial decrease is explained by the fact that the supernatant was removed every 3 days during the pretreatment phase. The order of the β-glucosidase activities in the crude extracts of willow fermentations after 9 days was *P. aurantiogriseum* > *R. miehei* > *G. persicaria* > *T. reesei*, while it was *P. aurantiogriseum* > *G. persicaria* > *R. miehei* > *T. reesei* in the corn system.

3.4.2. Total cellulolytic filter paper activity

In every case, the highest concentrations of reducing sugars were detected on day 3 (Fig. 2). The activity markedly decreased by day 6, except in the *P. aurantiogriseum* pre-treated straw residue samples (Fig. 2B). FPase activity had similar rate for both *T. reesei* and *G. persicaria* during the whole fermentation period. Interestingly, these fungi had the lowest activity with every substrate.

3.4.3. Cellobiohydrolase activity

Fig. 3. shows the time course profiles of CBH activities during the pre-treatment. It is noteworthy that the *p*NPC hydrolysis activities in the samples pre-treated with *P. aurantiogriseum* was at least 35% higher than those detected by the other strains. CBH activity decreased in the case of all fungal strains in time when willow or corn substrates were tested. Interestingly, on wheat straw substrate the opposite tendency was detected for all fungal strains.

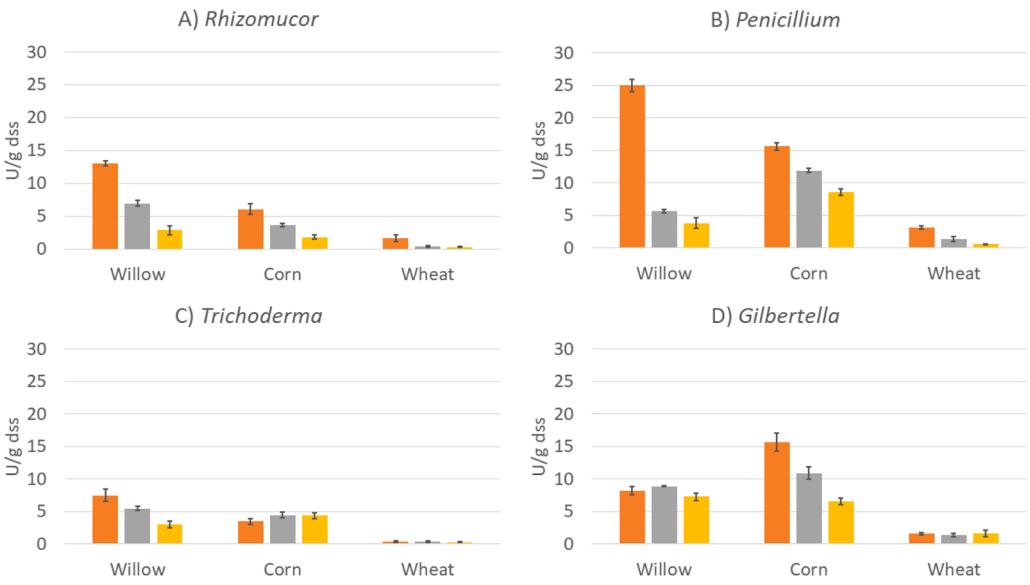

Fig. 1. β-glucosidase activities of the fungi on willow wood chip, dry corn stover and wheat straw substrates: *R. miehei* (A); *P. aurantiogriseum* (B); *T. reesei* (C); *G. persicaria* (D). Orange: on day 3; grey: on day 6; yellow: on day 9.

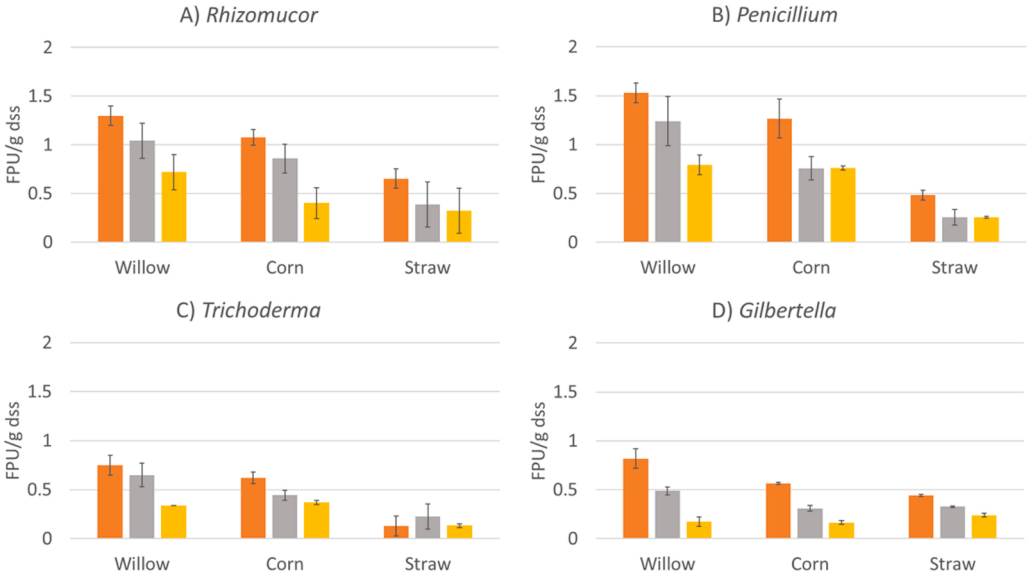

Fig. 2. FPase activities on willow wood chip, dry corn stover and wheat straw substrates by the fungi: *R. miehei* (A); *P. aurantiogriseum* (B); *T. reesei* (C); *G. persicaria* (D). Orange: day 3; grey: day 6; yellow: day 9.

3.4.4. *Endoglucanase activity*

CMCase activity of the isolates using the three different substrates is shown in Fig. 4. Each fungus showed the highest enzyme activity on day 3 of the cultivation on willow. After 72-h incubation, the trend of CMCase activities in the crude extracts was *P. aurantiogriseum* > *G. persicaria* > *T. reesei* > *R. miehei*. On corn substrate, the relative CMCase activities followed the trend *P. aurantiogriseum* > *R. miehei* > *G. persicaria* > *T. reesei*.

3.5. *HPLC*

Samples were collected from the supernatants of the pre-treatment experiments every third day for the determination of hydrolysis products by HPLC. The predominant hydrolysis product was cellobiose (Fig. 5.). The results correlate with the total cellulolytic activity. As the pretreatment progressed, the amount of degradation by-products in the liquid solution decreased, the rate of degradation slowed down. This is also true for glucose and cellobiose as well as acetate and ethanol.

3.6. *Scanning electron microscopy*

The biodegradation of the three substrates was morphologically visualized by SEM before and after fungal cultivation using *P. aurantiogriseum*, as the most effective degrader of the four tested strains. Fig. 6. shows the SEM surfaces and visible structural degradation of the various substrates, i.e. willow chips (A), corn stover (B) and wheat straw (C). In each picture set the untreated biomass is shown on the left side and the fungal pre-treated substrate on the right side. The untreated substrates exhibited a rough, continuous and compact surface. Compared to them the surface morphology changed considerably in the pre-treated samples, channeling, fractures, and the irregular textures were observed in addition to the fungal hyphae. It is also apparent that

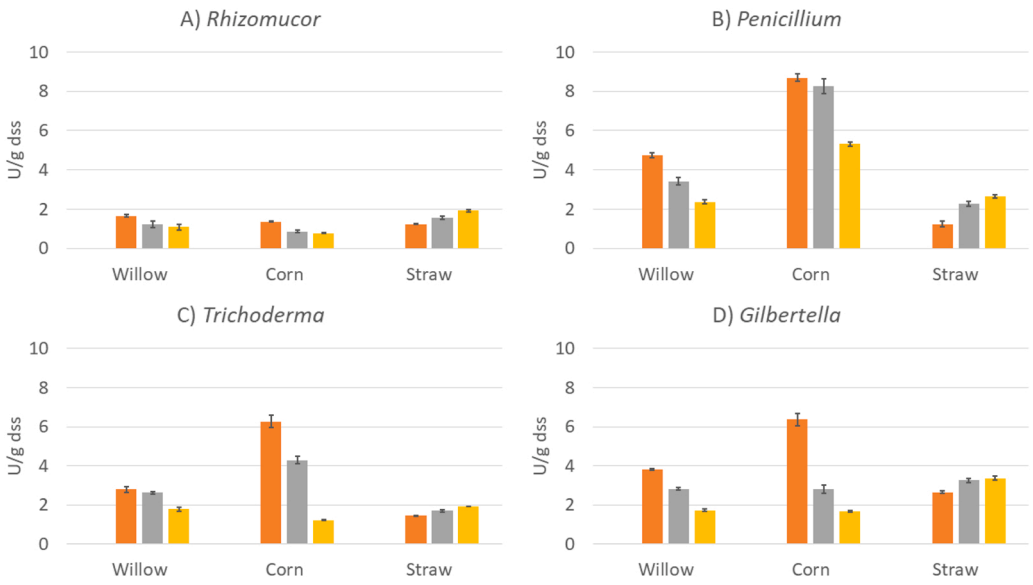

Fig. 3. CBH activities of the various fungal strains on willow wood chip, dry corn stover and wheat straw substrates: *R. miehei* (A); *P. aurantiogriseum* (B); *T. reesei* (C); *G. persicaria* (D). Orange: day 3; grey: day 6; yellow: day 9.

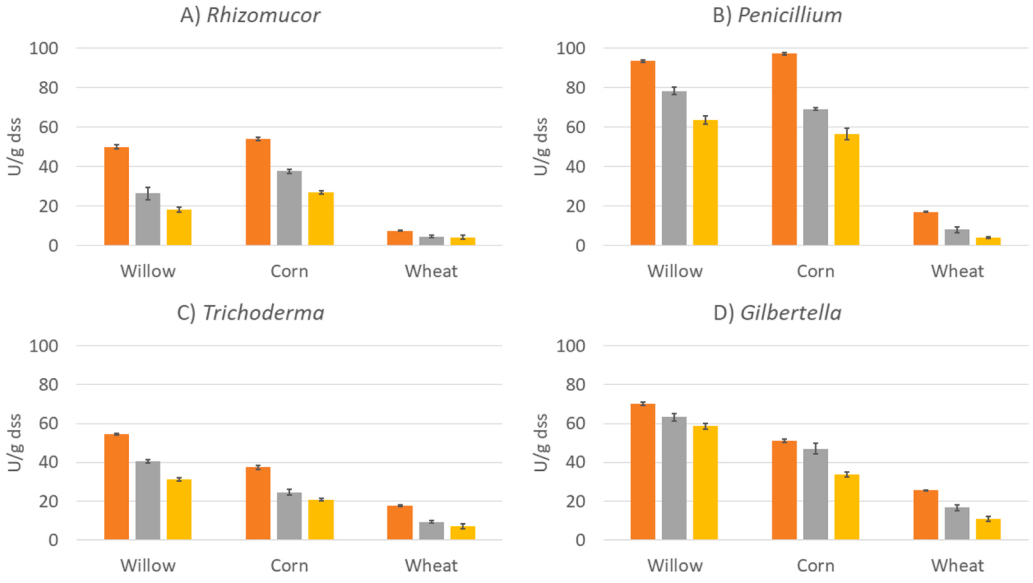

Fig. 4. Endoglucanase activities of the fungi on willow wood chip, dry corn stover and wheat straw substrates: *R. miehei* (A); *P. aurantiogriseum* (B); *T. reesei* (C); *G. persicaria* (D). Orange: day 3; grey: day 6; yellow: day 9.

the fungal hyphae penetrate deeply into the lignocellulosic substrate in all cases, which facilitates the subsequent hydrolysis process.

3.7. The effect of pretreatment on bio-methane production

3.7.1. 60 mL batch fermentations

The results obtained with the three substrates are presented in Fig. 7. A significant portion of the produced methane (95%) evolved within the first 20 days.

The relative amounts of methane evolved in the reactors treated with various fungi and containing various substrates were compared with the α-cellulose containing samples used as the positive control (Fig. 7). Corn stover fed reactors had the highest methane yield. The *P. aurantiogriseum* pretreated reactors yielded the highest average methane yield (281 mL_N/g oTS), which was 81% relative to the control α-cellulose (348 mL_N CH_4/g oTS), an easily degradable polysaccharide. The difference between the untreated corn stover and *P. aurantiogriseum* pretreated one, which is about 16% in case of corn stover, indicates the net gain in CH_4 production as the result of fungal pretreatment. It is noteworthy that corn stover substrate was preferred by all tested fungi for methane productivity. In addition, the methane yield from corn stover alone, i.e. without fungal pre-treatment, was higher than in the other two substrates. The reactors receiving willow wood chips gave the lowest results indicating that this was the least degradable substrate. Nevertheless, the pretreatment efficacy by *P. aurantiogriseum* was the highest in the case of willow substrate. This observation suggest that the fungal pretreatment is more efficient on more recalcitrant substrates. The *R. miehei* pre-treated reactors generated similar methane yields as the non-pretreated reactor (27 %). Reactors supplied with *G. persicaria* and *T. reesei* as pretreatment agents performed better, reaching 32 % and 35 % excess methane productivity relative to the positive control (Fig. 7).

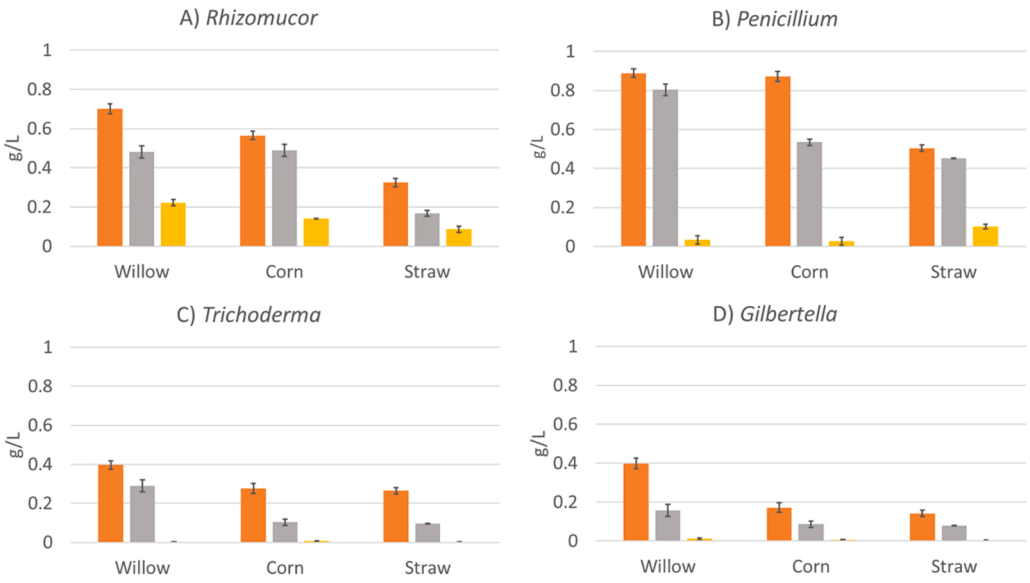

Fig. 5. Cellobiose concentrations (g/L) during the 9-day long pre-treatment on willow wood chip, dry corn stover and wheat straw using *R. miehei*, (A), *P. aurantiogriseum* (B), *T. reesei* (C) and *G. persicaria* (D). Orange: day 3; grey: day 6; yellow: day 9.

Using wheat straw substrate, *P. aurantiogriseum* proved to be the most effective, i.e., 48 % methane yield increase after pretreatment. Further methane yields were as follows: *R. miehei* (41 %), *T. reesei* (43 %), *G. persicaria* (44 %) relative to the untreated α-cellulose controls.

3.7.2. 300 mL batch fermentations

In the next step, the effect of a 5-fold increase in volumetric scaling-up was investigated. It should be noted first that the maximum difference between the results of the two reactor sizes was only 13.3 % (in case of *P. aurantiogriseum* reactors) indicating that the overall process was relatively insensitive to scaling-up. A similar behavior was observed in the cases of all substrates pretreated with *T. reesei, R. miehei* and *G. persicaria* as well.

The relative amounts of degraded organic total solid (oTS) material were measured in order to examine a potential correlation between the methane production and degraded oTS. Here again the value determined for α-cellulose was considered as 100%, the results of the other samples were compared to this positive control (Fig. 8).

Samples containing only the raw lignocellulosic substrates decomposed less oTS and produced lower biogas yields than their pretreated counterparts. Without fungal pretreatment the degraded oTS was 24 %, 35 %, 48 % in the case of willow, wheat straw and corn straw, respectively.

Bars represent SD values of replicates. Columns with various letters indicate a significant difference at $P < 0.05$, $n = 3$ according to the Duncan's multiple range tests.

4. Discussion

Microorganisms that degrade plant cell wall are both abundant and ubiquitous in nature. They include fungi, bacteria and actinomycetes, aerobes and anaerobes, mesophiles and thermophiles. Aerobic and anaerobic fungi are among the most effective plant biomass degraders known and have high potential to increase the efficiency of lignocellulosic biomass utilization, such as for biogas generation.

In a partial list of cellulolytic fungi, Ljungdahl and Eriksson (1985) named 60 different species. Nevertheless, it should be emphasized that while many fungi can grow on cellulose, or produce enzymes that degrade amorphous cellulose, relatively few can synthesize the complete extracellular cellulase systems that degrade crystalline cellulose extensively in vitro (Mandels, 1975; Fogarty and Kelly, 1990). Aerobic fungi (AeF), as main decomposers of plant biomass in nature, and anaerobic fungi (AnF), as key fiber degraders in the ruminants' digestive tract (Silva et al., 2017), have great potential to facilitate the deconstruction of lignocellulose-rich biomass due to their mechanical fiber penetration and lignocellulolytic capabilities (Dashtban et al., 2009, 2018). Lignocellulolytic fungi are phylogenetically widespread but mainly found in the phyla Ascomycota, like the soft-rot *Trichoderma sp.* (Schuster and Schmoll, 2010), Basidiomycota, including many brown- and white-rot fungi (Mester et al., 2004) and the anaerobic Neocallimastigomycota (Dashtban et al., 2009).

In a preliminary experiment a similar pretreatment strategy has been examined using filamentous fungi (Szűcs et at al, 2021). *Aspergillus nidulans*, a close relative of *P. aurantiogriseum* showed the best performance in pretreatment of the various lignocellulosic biomass sources. In the current study, the additional oTS degradation efficacy, HPLC and scanning electron microscopy results corroborated the advantageousness of additional filamentous fungi in this function.

Trichoderma and *Phanerochaete* species are the best studied plant cell wall degraders (Ljungdahl and Eriksson, 1985) although bacteria, such as *Clostridium thermocellum* (Béguin et al., 1987; Tailliez, a, b et al., 1989), and the anaerobic rumen fungus, *Neocallimastix frontalis* (Wood and McCrae, 1986), could be similarly promising for commercial exploitation (Mandels, 1985). Much effort and ingenuity has been devoted to the isolation of hypercellulolytic mutants and to optimizing fermentation conditions. Productivities of 200–500 filter paper units (FPU) per litre per hour by mutants of *T. reesei* have been achieved using fed-batch techniques and soluble substrates (Watson et al., 1984; Pourquié et al., 1988; Fogarty and Kelly, 1990). Eveleigh (1987) considered that productivities of as much as 1000 FPU per litre per hour could be achievable.

Fungi produce lignocellulolytic enzymes and contribute synergistically to the decay of lignocellulosic residues in nature. If the lignin complex is removed, cellulose and other carbohydrates can be released for use in industrial processes and to meet the nutritional needs of ruminants (Abrão et al., 2017). Fungi also produce enzymes that facilitate polysaccharide degradation in ruminants, and cultures of *Aspergillus oryzae* and their extracts have been used as supplements in ruminant diets to improve productivity (Wang and McAllister, 2002). In contrast, little is known about the population of aerobic fungi that naturally occur in the bovine rumen. An earlier study of the cellulolytic activity of fungi in the digestive tract of dairy cattle revealed *Aspergillus* and *Paecilomyces*

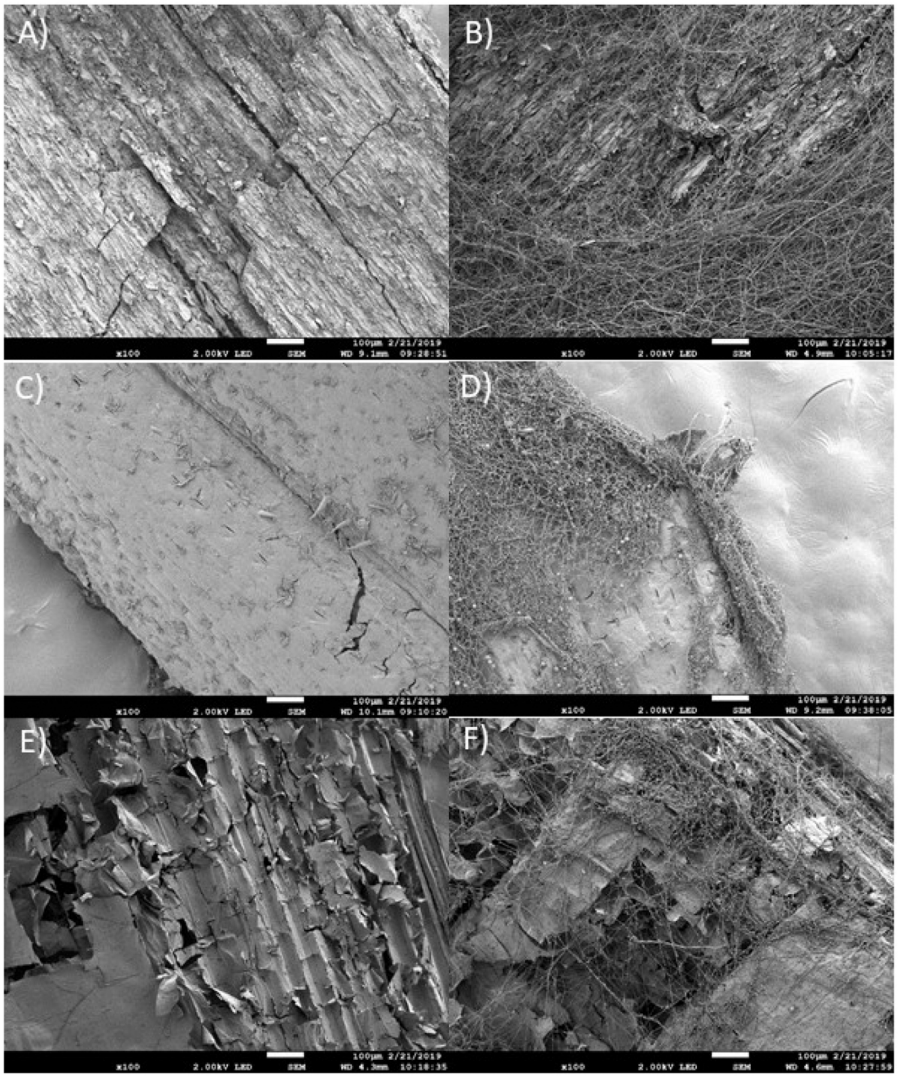

Fig. 6. SEM pictures of the surfaces of willow chip (A, B), corn stover (C, D) and wheat straw (E, F) before (left) and after (right) pre-treatment with *P. aurantiogriseum* for 15 days.

isolates that degrade microcrystalline cellulose (Almeida et al., 2014).

Trichoderma reesei is a mesophilic soft-rot fungus that is extensively used as a source of cellulases and hemicellulases for various applications. It has also long been a model system for the degradation of plant cell wall polysaccharides. The concept of how fungi degrade cellulose (and hemicelluloses) is almost totally based on the studies of the enzyme system of *T. reesei*, while only rather incomplete data are available on other ascomycetes.

Cellulolytic, lipolytic and proteolytic enzyme production of zygomycetes *Mucor corticolus*, *Rhizomucor miehei*, *Gilbertella persicaria* and *Rhizopus niveus* were investigated using agro-industrial wastes as substrates. Rapid production of endo-glucanase (CMCase) was observed with maximal activity reaching after about 48-h fermentation, while cellobiohydrolase (CBH) and β-glucosidase enzymes generally had their peak after 72-h incubation. (Takó et al., 2015). In most cases the observed enzyme acitivites decreased in time in the experiments presented here. Taking into account that the supernatants were replaced after every 3-days incubation period, this may indicate an inducible hydrolytic enzyme production. Interestingly, the cellobiohydrolase enzyme production behaved distinctly on wheat straw substrate.

Some of the isolates selected for this study proved to be good extracellular β-glucosidase producers in previous experiments (Takó et al., 2015). In this study, *P. aurantiogriseum, R. miehei*, *G. persicaria* and *T. reesei* effectively assisted the deconstruction of all tested plant biomass, i.e. wheat straw, corn stover and willow chips. Each fungal strain showed intensive growth under the applied conditions, although they revealed noticeable differences in the production of the tested hydrolases.

It should be noted that biological methane potential (BMP) tests must be carefully executed to make the results from distinct laboratories comparable. A sensitive element of the BMP tests, i.e. the quality of the inoculum is often disregarded and appropriate controls are not included. We have found that α-cellulose is an excellent material to relate to the biogas/biomethane production from complex lignocellulosic substrates (VDI, 2006). α-cellulose as internal standard can serve reliably to test the quality of the inoculum in the BMP tests and offer an easy way to normalize the data obtained in various laboratories employing inocula of various biodegradation capacity (VDI, 2006). The presented results indicate that fungal pre-treatment could be a useful strategy in industrial scale biogas fermentation to avoid the accumulation of undigested biomass and gain a higher biogas potential from lignocellulosic materials. Production of the fungal hydrolytic enzymes facilitated the breakdown of cell wall structure. The fungi increased the surface area of exposed lignocellulose, hence the pretreatment promoted contact for other microbes and their enzymes.

Scale-up experiments of corroborated that the beneficial effects of

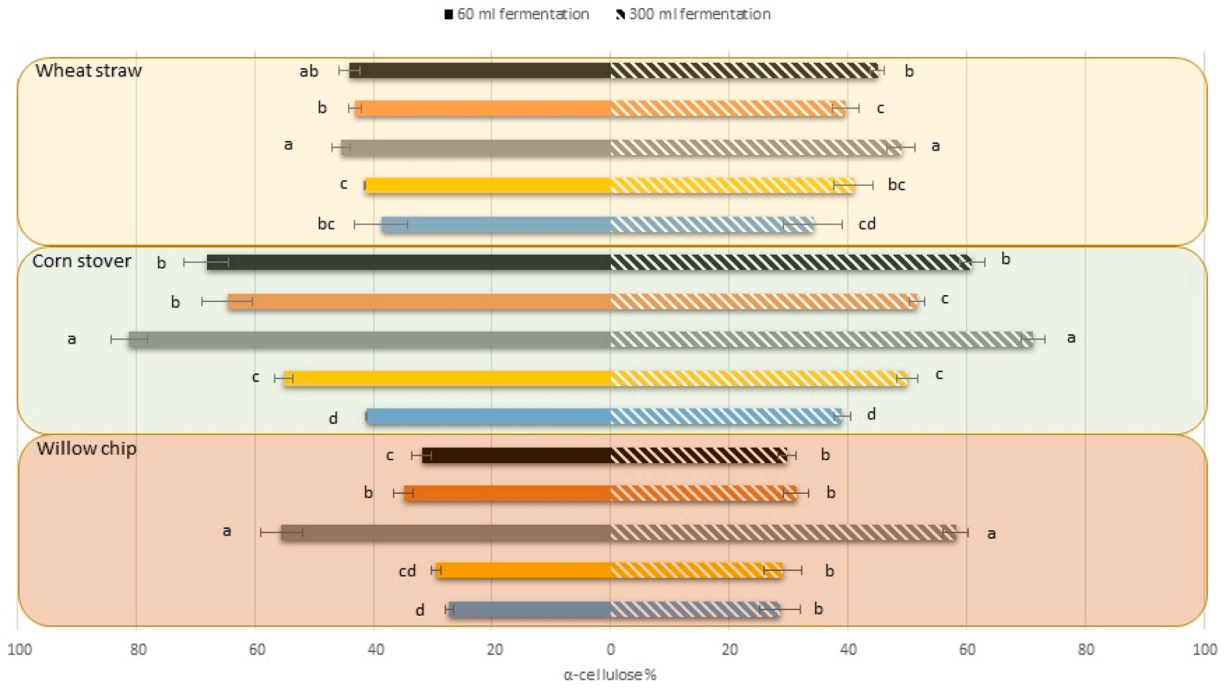

Fig. 7. Methane yields (% of α-cellulose) of untreated substrates (black), and pretreated with *R. miehei* (orange), *P. aurantiogriseum* (grey), *T. reesei* (yellow) and *G. persicaria* (blue) on wheat straw (yellow background), corn stover (green background) and willow chips (orange background) substrates in 60 mL (filled columns) and 300 mL (patterned columns) fermentations following 10 days of pretreatments (see Sections 2.3.2.3 and 2.3.2.4). Bars represent SD values of replicates. Columns with various letters indicate significant differences at $P < 0.05$, n = 3 according to the Duncan's multiple range tests (see for example Szűcs et al., 2021).

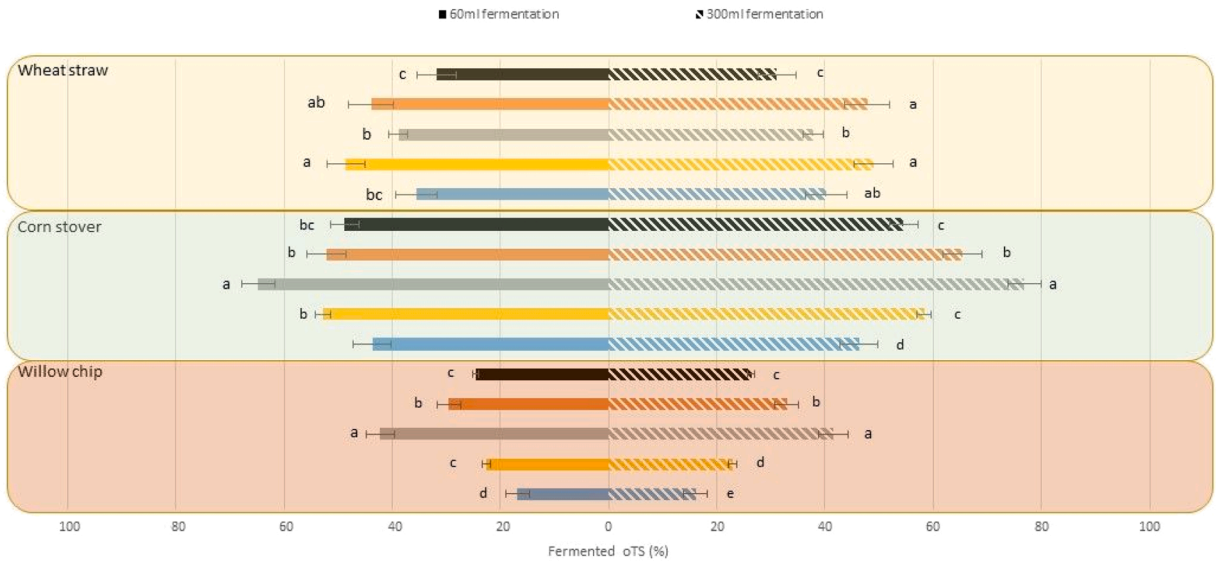

Fig. 8. Fermented organic dry mass (control% of α-cellulose) of *R. miehei*, *P. aurantiogriseum*, *T. reesei* and *G. persicaria* on willow, corn and straw substrates.

fungal pre-treatment is independent of reactor size.

P. aurantiogriseum was apparently an eminent filamentous fungus for the pretreatment of lignocellulosic agricultural by-product for methane fermentation. The cellobiohydrolase (CBH) activity of *P. aurantiogriseum* was at least 35% higher, carboxymethyl cellulose (CMCase) activity was 22% higher than those of the other tested filamentous fungi.

Fungal pre-treatment is attractive way to increase the methane yields as they efficiently degrade lignocellulosic materials through their outstanding enzyme-producing ability.

CRediT authorship contribution statement

Etelka Kovács: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Csilla Szűcs**: Methodology, Validation, Investigation. **Attila Farkas**: Methodology, Investigation, Visualization. **Márk Szuhaj**: Validation, Investigation. **Gergely Maróti**: Methodology, Investigation, Data curation. **Zoltán Bagi**: Investigation, Formal analysis, Data curation, Supervision. **Gábor Rákhely**: Project administration, Supervision. **Kornél L. Kovács**: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Resources.

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.

Data Availability

Data will be made available on request.

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