Effects of fungal pre-treatments on wheat straw, a co-substrate for anaerobic digestion of manure

Pourcher Anne-Marie [1,2], Peu Pascal [1,2] Madhkour Sonia [1,2], Vasseur Valérie [2,3], Le Bras Quéré Marie [2,3], Dabert Patrick [1,2]
(1) Irstea, UR GERE, 17 av. de Cucillé, CS 64427, F-35044 Rennes, FR
(2)Université Européenne de Bretagne, FR
(3) Université de Brest, EA3882 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, IFR148 ScInBioS, ESIAB, Technopôle de Brest Iroise, 29280 Plouzané, FR
*Corresponding author: anne-marie.pourcher@irstea.fr

Abstract
This study aimed to estimate the efficiency of fungal pretreatments applied to wheat straw on improving CH_4 production. Strains of *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Trametes versicolor* were incubated with sterilised straw at 25°C during 30 days. Cellulose, hemicellulose and lignin contents of the straw were respectively 40.1, 42.7 and 6.3%. For *P. chrysosporium* and *T. versicolor* assays, 30-35% of the volatile solids (VS) were lost whereas for *P. ostreatus* assays, 15 to 20% of the VS were not recovered. The BMP decreased between T0 and T20 days and then remained stable (*ie.*, 239 NL CH_4 /kg VS). The decrease between T0 and T10 days is correlated with an increase of the soluble fraction of the VS. These results suggest that inhibitory compounds or by-products of hemicellulose degradation may interfere with the anaerobic digestion process. Fungal pretreatments before anaerobic digestion seem not relevant to improve the biodegradability of substrates containing low level of lignin.

Introduction
Sustainable biogas production from crop residues could be an interesting option to meet the demands for renewable energy. Indeed, lignocellulosic wastes are considered a major potential substrate for methane production [1]. Among crops residues, straw may represent an interesting substrate because of the large volumes of production and of the facility for the farmers to access to these residues. The production of wheat straw residues in France is estimated at 24 million tonnes per year. The biochemical methane potential (BMP) of this plant ranges between 163 and 195 NL CH_4 /kg VS [2, 3]. However, due to its high content of lignocellulosic products (lignin, cellulose, hemicellulose), the anaerobic bioconversion efficiency of straw is still limited [4]. To improve the digestibility of lignocellulosic substrates, pretreatments such as size reduction, acids, alkalis, solvents or oxidants and stream explosion have been investigated [5]. Biological pretreatments can also be carried out using microorganisms or microbial enzymes [6]. Given their high ability to degrade lignocellulosic substrates, fungi such as *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Trametes versicolor* have often been used as a model for the biodegradation of lignin and chemical pollutants [7, 8]. The aim of this study was to estimate the effectiveness of these three species of fungi applied to wheat straw on increasing methane production.

Material and Methods
Material
Wheat straw was cut into pieces of about 2 cm and sterilized by γ-ray irradiation at a dose of 3 Gy (Ionisos, France). Three species of fungi (2 strains per species) were obtained from the "Banque de Ressources Fongiques de Marseille" (BRFM): *P. ostreatus* (strains BRFM 17, BRFM 565), *P. chrysosporium* (strains BRFM 387, BRFM 413), *T. versicolor* (strains BRFM 1320, BRFM 1452).
Experimental setup
Fifty grams of sterile straw were placed in air-permeable bags (SacO2, Microsac, Belgium). Each analysis was performed in triplicate. Nine samples of straw were inoculated with each strain of fungus previously grown on malt-agar and mixed with 150 mL of distilled water. Nine uninoculated samples of straw were used as control. All samples were vigorously shaken to achieve homogeneity of the mixture. The bags were incubated for 10, 20 and 30 days at 25°C.

Microbial and chemical analyzes

Fungal growth was followed visually with respect to mycelial density and by molecular technique (18S rDNA qPCR). At each sampling date, subsamples were taken from 21 bags (3×6 strains and 3 controls). The content of the bag was homogenized and 10g of the mixture straw-fungi were collected. Samples disruption was performed by cryogenic grinding. DNA was extracted from 300 mg of sub-sample using the FastDNA SPIN Kit for Soil (MPBio, USA). Fungal-specific PCR reactions were carried out according to the protocol of Borneman and Hartin [9].

Cellulose, hemicellulose and lignin contents were quantified using the Van Soest method. The BMP (NL CH_4/kg VS) of each straw-fungi mixture was determined according to Vedrenne *et al.* [10]. BMP was performed in triplicate with controls (inoculum only) until biogas production ceased. The inoculum used was obtained from an anaerobic pilot plant (100 L) acclimated to degrade pig slurry supplemented with horse feed as co-substrate. The VS inoculum: straw-fungi mixture ratio was 1:1.

Results

Straw was composed of 91.5% of dry matter, comprising 89.2% of VS and 2.3% of ash. The γ-ray treatment did not impact the straw composition which did not significantly change after irradiation. The cellulose, hemicellulose and lignin contents of the straw were 40.1, 42.7 and 6.3%, respectively. The level of the lignin was lower than expected according to the literature. However, it is noteworthy that the composition of straw differs according to the authors. Thus, cellulose and hemicellulose contents ranged between 34.1 and 43.2% and between 23.1 and 34.1% respectively, whereas lignin content ranged between 7.6 and 17.7% [11, 12]. The BMP measured on untreated straw after 50 days was about 350 NL CH_4/kg VS, which is higher than the values reported by Moller *et al.*, [3] and Moletta [2] who observed BMP ranging between 163 and 195 NL CH_4/kg VS. The fungal growth observed visually throughout the incubation period is reported in table 1. No growth of fungus was observed in the control bags. Highest mycelia density was observed for *T. versicolor* and to a lesser extent, for *P. ostreatus* and *P. chrysosporium*. The growth of the fungi led to pH ranging between 4.5 and 5.5 (Table 1).

Table 1. Growth of the 6 strains and final pH observed in the bags

	P. ostreatus		*P. chrysosporium*		*T. versicolor*		control
	BRFM 17	BRFM 565	BRFM 387	BRFM 413	BRFM 1320	BRFM 1452	
Mycelial density	++[a]	++	+ +	++	+++	+++	-[b]
pH	5.3	5.5	5.3	4.5	4.5	4.5	6.3

[a] the number of "+" is proportional to the mycelium development; [b] no growth.

The relative abundance of fungi determined by qPCR is reported on figure 1.

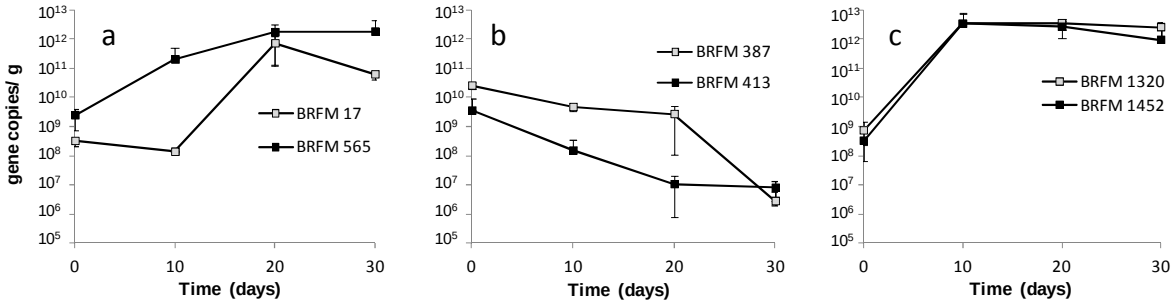

Figure 1. Growth kinetics of strains of *P. ostreatus* (a), *P. chrysosporium* (b) and *T. versicolor* (c). Bars indicate standard deviation.

The 18S rDNA gene copies of *T. versicolor* and *P. ostreatus* increased from 10^9 to 10^{12} copies per gram of sample throughout the incubation period whereas the copy gene number of *P. chrysosporium* decreased. Indeed, the qPCR method underestimates the growth of this species compared to visual

estimation. One possible explanation of this underestimation may be a bias introduced by DNA extraction. The kinetics of VS loss are presented on figure 2. Although irradiation has resulted in a reduction of 4 log units of the number of bacteria, a weak degradation of the VS (8% of loss) was still observed in the control bags at days 10 and 20 (data not shown). In presence of *P. chrysosporium* and *T. Versicolor*, 30 to 35% of the VS were lost within 30 days, respectively whereas in *P. ostreatus* assays, VS losses were limited to 15 to 20%. It is noteworthy that the organic matter content measured by VS determination is composed of both fungi and straw and does not only reflect the fungal consumption. As a result we can assume that the straw degradation after 30 days of incubation is higher than 30% and 15% for P *chrysosporium and P. ostreatus*, respectively.

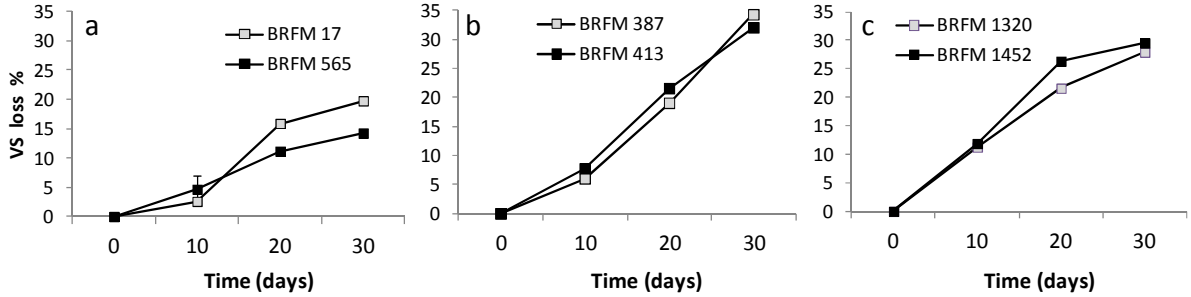

Figure 2. VS loss profiles (expressed in %) of straw in presence of *P. ostreatus* (a), *P. chrysosporium* (b) and *T. versicolor* (c) Each value is an average of 3 replicates. Bars indicate standard deviation. If no error bars are shown, sd is smaller than symbol.

Van Soest method was performed to determine the fractions of VS (cellulose, hemicellulose, lignin and soluble fractions) after pretreatment. Figure 3 shows the impact of the fungal growth on the chemical composition of fibers after 20 and 30 days, compared to the initial composition of the irradiated straw (at T0).

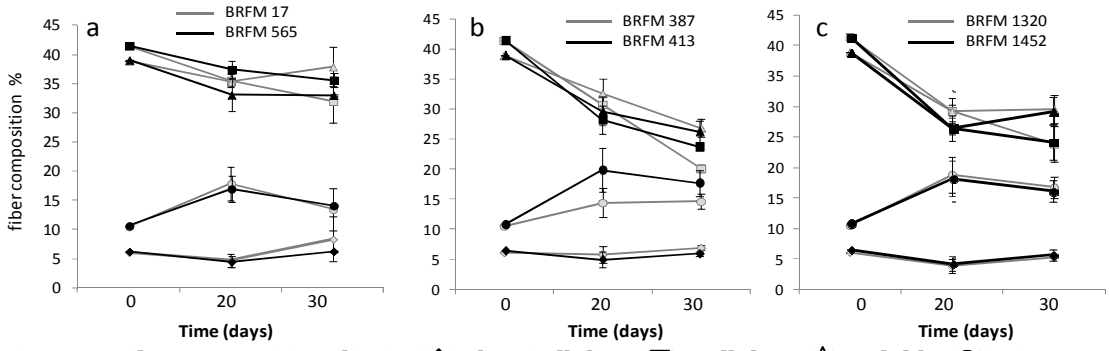

Figure 3. Fiber composition (lignin (◇), hemicellulose (□), cellulose (△), soluble (○)), in presence of *P. ostreatus* (a), *P. chrysosporium* (b) and *T. versicolor* (c). Bars indicates standard deviation.

Lignin fractions of wheat straw measured by Van Soest fractionation did not significantly change during incubation with a stable content close to 6% of the VS. However, holocellulosic fractions (hemicellulose and cellulose) significantly decreased due to the fungal growth and led to the formation of soluble compounds. *T. Versicolor*, *P. chrysosporium* and *P. ostreatus* degraded respectively 34, 40 and 14% of the holocellulosic compounds. A higher lignocelluloytic activity of *P. chrysosporium* compared to *Pleurotus* has also been observed by Dorado *et al.* [13] who have inoculated different genera of fungi in straw. We observed that the 3 species of fungi degraded preferentially holocellulosic fractions of the VS whereas Santoyo *et al.* [8] reported that *P. ostreatus* degraded the lignin fraction of the substrates and did not degrade most of the cellulose fraction. It is noteworthy that the straw used in our experiment contained low amount of lignin (6%) compared to the amount of hollocellulose (80% of the VS). This repartition of the organic fractions probably influenced the metabolism of the fungi. It may explain why the experiments carried out by Santoyo *et al.* [8] on substrates with 16-18% of lignin led to a consequent decrease of this fraction after fungal pretreatment. Indeed, Singh *et al.* [14] who studied straw containing 16% of lignin, observed that *P. chrysosporium* degraded within three weeks approximately 30% of the lignin. However, these

authors have autoclaved the straw for 1h at 121°C before inoculating the fungi, which could involve changes in the structure of the lignocellulosic substrate.
The biochemical methanogenic potentials obtained from pretreated straw after 10 and 30 days are presented in Table 2.

Table 2. BMP of the samples inoculated or not with the strains of fungi (in NL CH_4/kg VS)

Days of incubation	*P. ostreatus*		*P. chrysosporium*		*T. versicolor*		control
	BRFM 17	BRFM 565	BRFM 387	BRFM 413	BRFM 1320	BRFM 1452	
10	251	262	279	266	257	273	270
30	249	255	258	227	218	226	269

For the control incubation at T0, BMP was 270 NL CH_4/kg VS. The BMP of the control did not change after 10 and 30 days of incubation whereas the BMP of the samples inoculated with the fungi decreased to a final average value of 239 NL CH_4/kg VS. Globally, the decline of BMP between T0 and T10 was correlated with the increase of the soluble fraction of the VS. These results suggest that inhibitory compounds or by-products of the hemicellulose degradation may interfere on the anaerobic microbial community. Indeed, Larsson *et al.* [15] and Xu *et al.* [16] have shown that the hydrolysis of hemicellulose could produce inhibitors of methanogenic *archaea* such as uronic acids, 4-O-methyl-D-glucuronic acid or 5-hydroxymethylfurfural.

Conclusion and perspectives

The 6 strains of fungi selected for their ability to degrade lignin, preferentially degraded holocellulosic fractions of wheat straw. Thus, fungi pretreatments before anaerobic digestion seem not relevant to improve the biodegradability of substrates containing low level of lignin. The effect of fungi should be tested on other crops residues with higher lignin content, such as corn stover or rape straw.

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