# Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme to increase flexibility in feedstocks supply management of biogas plants

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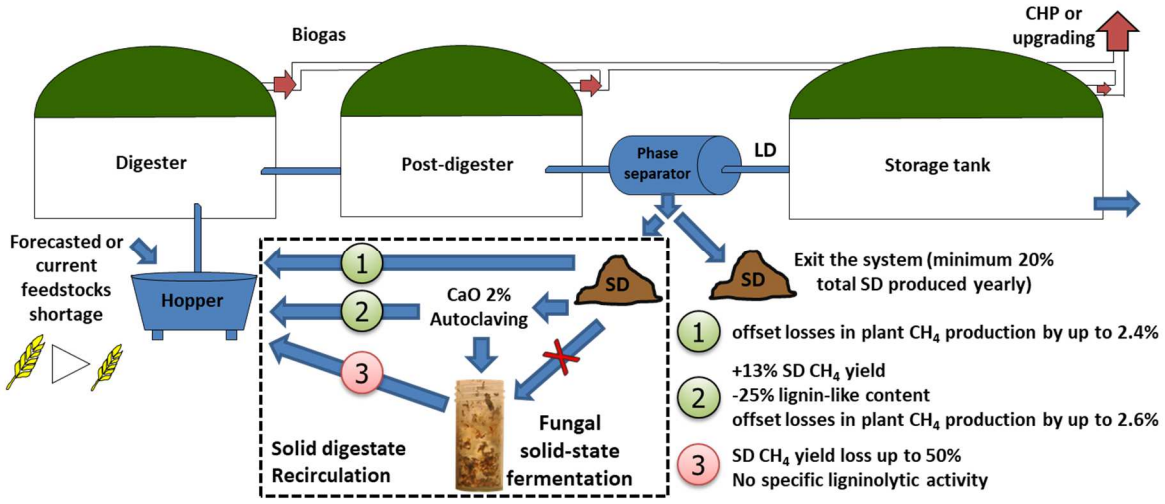
**Abstract**

Agricultural biogas plants can suffer occasional feedstock shortages (poor harvest, storage…) and recirculation of solid digestate (SD) into digester has been identified as a simple way to offset methane production loss from these situations. Calculations show that recirculation of SD could offset for losses in plant methane production by up to 2.4%. In that context, two post-treatments were evaluated to enhance residual potential methane of agricultural SD. Effect of fungal solid state fermentation (SSF) of SD on subsequent methane production has never been explored before. It was hypothesized that: (i) ligninolytic fungi would be able to specifically use the complex fraction of SD for their growth and (ii) energy generation from the subsequent anaerobic digestion of the colonized SD will be enhanced. However, experiments showed that thermo-alkaline treatment of SD (used as alkalinization and sterilization process) and a high spawn level (20% w/w) were necessary to perform fungal SSF. Besides, the observed fungal activities on SD did not target specifically the most complex fractions. This led to uncontrolled organic matter losses and subsequent decreases of biodegradability and methane yield of SD (up to 50%). Therefore, fungal SSF of SD before its recirculation into biogas plants appeared not to be a viable option.. Only thermo-alkaline treatment (CaO 2% w/w and 121°C 30 min) enhanced methane yield of SD by 13% and decreased its complex fraction by 25%. Further studies on optimization of this post-treatment may enhance efficiency of SD recirculation strategy to offset plant methane production losses.

## Graphical Abstract




## Highlights

- SD recirculation could compensate loss in plant methane yield by up to 2.4%.
- Necessary sterilization and high spawn level to allow fungal SSF of SD.
- Observation of losses in biodegradability and methane yield of SD after fungal SSF.
- No specific degradation of the lignin-like fraction of SD during fungal SSF.
- Thermo-alkaline treatment increases residual methane potential of SD (+13%).

**Keywords:**
Anaerobic digestion
Solid digestate recirculation
Fungal post-treatment
*Pleurotus ostreatus*
Thermo-alkaline post-treatment
Agricultural biogas plant

## Abbreviations

AD, anaerobic digestion; BMP, biochemical methane potential; COD, chemical oxygen demand; CSTR, continuous stirred-tank reactor; FM, fresh matter; HRT, hydraulic retention time; LD, liquid digestate; NEOM, Non extractable organic matter; OM, organic matter; PEOM, Poorly extractable organic matter; PO, *Pleurotus Ostreatus*; REOM, readily extractable organic matter; RMP, residual methane potential; SD, Solid digestate; SEOM, slowly extractable organic matter; SPOM, soluble extractable fraction from particular extractable organic matter; SRA, *Stropharia rugoso-annulata*; SSF, solid-state fermentation; TS, total solids; VFA, volatile fatty acids; VS, volatile solids.

## 1. Introduction

The global environmental crisis combined with fossil fuel depletion push governments to gradually shift their energy mix toward renewable solutions using incentive policies [1]. Among these solutions, anaerobic digestion (AD) was identified as a key technology as it treats waste to produce energy under the form of biogas and as it is relatively low cost compared to other existing technologies [2]. Concentrated mainly in Europe, with half of the global biogas production units in 2015, the number of industrial biogas plants continues to increase and energy production should meet the 2020 EU biogas energy targets [3]. Agricultural feedstocks are the main biomass deposit in Europe. So, agricultural biogas plants, mostly based on continuous stirred-tank reactor (CSTR) technology, are most widespread with 12,500 out of 17,660 existing installations in 2016 [4].

This growing industry is searching for innovations in order to improve its biogas production, conditioning and utilization. Concerning the former point, an optimization of the process is being sought, notably by a better conversion of organic matter (OM) into biogas via biomass pretreatment and a finer management of co-digestion [5–7]. Co-digestion, which is commonly practiced in agricultural plants, can lead to incomplete AD of biomass, especially of lignocellulosic biomass, due to too short hydraulic retention times (HRT). For instance, to reach 95% of methane recovery from manure, around 200 days HRT are needed while energy crops only need around 90 days [8]. Incomplete AD due to empirically selected HRT (for instance 100 days) can lead to methane emissions, in particular from the liquid fraction of digestate storage, hampering environmental benefit [9,10]. One option to improve methane recovery from biomass is to define good management practices of digestates from agricultural biogas plants before their land spreading.

Concerning the liquid fraction of the digestate (LD), coverage and heating of the storage tank were identified as a good strategy to enhance both biogas plant yield (longer HRT for liquids) and its environmental footprint as methane is recovered [11,12]. For the solid fraction of the digestate or solid digestate (SD), strategies to apply are less obvious. It is generally stored in a composting pile until stabilization and application as soil amendment. However, it has been proven that SD coming from agricultural plants can contain residual methane potential (RMP) varying between 24-240 $Nm^3\ CH_4 \cdot ton^{-1}$ Volatile Solids (VS) as a function of the feedstocks and applied HRT [13]. SD recirculation into the plant can be an interesting strategy to enhance plant efficiency by recovering this RMP from SD and allow a more flexible management of plant feedstocks. Indeed, SD can be recirculated when: (i) there are some feedstock limitations (in terms of quantity/quality); (ii) feedstocks need to be economized (forecasted shortage), in that case SD can be integrated to the ration in replacement of feedstocks that are well stored (no methane loss over time such as well-prepared crop silage). Under these conditions, additional post-treatment can be performed to further degrade OM of SD (especially lignocellulosic compounds) and enhance methane production during recirculation. Until now, mechanical, thermochemical and enzymatic post-treatments have been tested on SD with various results; for instance, biological post-treatment using an enzyme cocktail on SD succeeded in increasing its methane potential by 13% [14]. In this recirculation scheme, the use of other types of biological treatments might be of interest due to their reasonable cost and environmental friendliness [15].

Fungal post-treatment of SD has been recently explored to valorize the remaining carbon into value-added products such as: (i) lignocellulolytic enzymes via submerged fungal fermentation of 21 different

strains [16] or via solid-state fermentation (SSF) using *Trichoderma reesei* [17]; (ii) volatile fatty acids (VFA) via SSF using *Pleurotus Sajor Caju* [18]; (iii) Edible mushrooms since *Pleurotus ostreatus* was successfully cultivated on up to 60% w/w solid digestate mixed with traditional substrates [19]. But none of these studies evaluated the impacts of fungal growth on SD methane yield in the case of a recirculation. Several fungal strains are known to be able to selectively degrade lignin and use it for their growth during SSF of straw. They can therefore potentially promote the conversion of lignin concentrated in SD into fungal biomass that will be accessible OM for AD microorganisms, which can subsequently produce methane after recirculation. Two strains were identified as potentially promising in that view, *Pleurotus Ostreatus* (*PO*) and *Stropharia rugoso-annulata* (*SRA*), as they are both robust, little demanding in cultivating techniques and known to efficiently degrade lignin when growing on wheat straw [20,21]. Besides, their cultivations are well known at industrial scales. Compost mushrooms such as *Agaricus bisporus* were not selected as they display lower ligninolytic activities and are generally more efficient in degrading cellulosic compounds [22].

The objective of this study is to evaluate an original process scheme where solid digestate coming from an agricultural CSTR biogas plant undergoes a post-treatment, which corresponds to a fungal SSF, before being recirculated back into the biogas plant. Growing conditions for fungal SSF were studied. The impact of fungi colonization over time on methane yield was analyzed as well as the evolution of OM composition, especially the lignin-like fraction. Impact of the sterilization, corresponding to a thermo-alkaline treatment alone on SD was also analyzed. Finally, feasibility at full scale of fungal SSF in a recirculation scheme in anticipation of a feedstock shortage or to overcome it is discussed.

## 2. Materials and methods

### 2.1. Solid digestate and fungal mycelium

SD was collected from a French agricultural CSTR plant, directly on-site after digestate mechanical separation. SD was then stored at -20 °C until the start of experiments in order to avoid any matter degradation. Biogas plant features are given in **Table 1**. This plant was selected for the following reasons: (i) a ration that is relatively rich in lignocellulosic biomass; (ii) the use of a screw press, a covered storage tank and an intermediate HRT which are representative of the process conditions applied in the agricultural biogas sector [23]. Besides, annual methane production ($V_{CH4}$ produced) was calculated by multiplying the injected biomethane flow rate (145 $Nm^3 \cdot h^{-1}$) by 8,200 hours annual functioning (taking into account technical maintenance and possible operational contingencies). A total yearly methane production of 1,189,000 $Nm^3$ was obtained. Methane yield (84.9 $Nm^3$ $CH_4 \cdot ton^{-1}$ feedstock) was obtained by dividing the yearly methane production by the total amount of feedstock used each year.

| Feedstocks composition | Amount of feedstock (tons/year) | Temperature | HRT (days) | Digestate separation technique | Quantity of SD produced (tons/year) |
|---|---|---|---|---|---|
| Catch crop (30%)<br>Bovine manure (18%)<br>Beet pulp (11%)<br>Cereal dust (8%)<br>Whey (35%) | 13,600 | Mesophilic | 100 | Screw press | 1,800 |
| **Type - reactors volume (m3)** | **%Total solids in digester** | **Type of valorization** | **Biomethane injected ($Nm^3$/hour)** | **$V_{CH4}$ produced ($Nm^3$/year)** | **$CH_4$ yield ($Nm^3$ $CH_4$/ton feedstock)** |
| Digester - 2000<br>Post-dig. - 2000<br>Storage tank - 6000 | 12 | Upgrading | 145 | 1,189,000 | 87.4 |

Table 1: Agricultural plant features

The two Basidiomycetes fungus strains were ordered from Mycelia bvba (Deinze, Belgium), a professional spawn and mycelium culture laboratory. Strain M2191 of *Pleurotus* Ostreatus, also called winter oyster mushroom, and strain M5012 of *Stropharia rugoso-annulata*, also called garden giant mushroom, were received under spawn form. For *PO*, spawn consisted in millet seeds colonized by mycelium while for *SRA*, spawn colonized wheat straw. Both were stored at 4 °C until experiments started.

### 2.2. Cultivation method

An isolated wooden box was designed. Inside the box, a system using a vacuum pump KNF Laboport™ (Freiburg-im-Breisgau, Germany) bubbling air in vessels containing water ensured efficient air humidification. Temperature within the box was lowered and regulated using a Minichiller Huber (Offenburg, Germany) and piping system. Temperature and air relative humidity inside the box were measured via a Hygrasgard® RPFTF – 20 – Modbus sensor from S+S Regeltechnik (Nürnberg, Germany) and values were recorded every 10 minutes via a software developed internally. During experiments, relative humidity was maintained close to 75% while box temperature varied between 20-25°C as a function of the variation of the external temperature with day-night cycle. Finally, the box could be closed ensuring a dark environment. These conditions were inside the range for good substrate colonization according to both Mycelia bvba strains factsheets and previous studies [24,25].

### 2.3. Screening of solid-state fermentation conditions

Screening was performed to determine SSF conditions on SD of *Pleurotus ostreatus*. Two conditions were applied to SD before inoculation: (i) no treatment; (ii) quicklime (addition of calcium oxide, CaO) at 2% w/w and then autoclave for 30 minutes at 121 °C. Following these treatments, four spawn levels were tested: 5, 10, 15 and 20% w/w of SD. Fungal SSF was carried out by mixing 10 grams of SD, potentially treated, with the corresponding amount of *PO* spawn within Corning-Gosselin® 40mL polypropylene tubes (Borre, France). A control, where SD was replaced by moisturized and sterilized wheat straw, was added to be sure that *PO* spawn was active. Tubes were then placed inside the box in the dark. After 10 days, colonization inside the tubes, due to SSF, were visually assessed according

to the following scale: (0) no colonization at all; (+) some area of SD are colonized; (++) SD is totally colonized. Selected SSF conditions for *PO* were similarly applied to *SRA*.

### 2.4. Evaluation of optimal fungal solid state fermentation duration

Selected conditions for efficient SSF were applied, which consisted of 2% w/w CaO and 20 minutes autoclaving at 121 °C of SD followed by a 20% w/w spawn addition. Following inoculation, different SSF times under aerobic conditions were evaluated. For *PO,* five durations were tested: 5.5, 7.5, 10, 15 and 21 days. The first four ones reach the incubation/colonization phase, while the latter is in the fructifying phase. In that case, the wooden box was slightly opened after day 15 to ensure half-light and trigger fruiting. For *SRA*, three durations were tested: 5.5, 7.5 and 15 days. Two controls were also added, consisting in: (i) untreated SD, *PO* spawn and *SRA* spawn left under aerobic conditions inside the wood box for 5.5 and 15 days (ii) SD quicklimed at 2% w/w limed and autoclaved for 20 minutes, left under aerobic conditions inside the wooden box for 5.5 and 15 days. A summary of the experimental set-up is given in **Table 2**. For every 16 conditions, Corning-Gosselin® 40 mL tubes containing 2 grams of VS of corresponding substrates were prepared and placed for the given duration inside the wooden box that was subsequently closed.

| Aeration duration / Tested substrates | 5.5 days | 7.5 days | 10 days | 15 days | 21 days |
|---|---|---|---|---|---|
| Untreated SD | x3 | | | x3 | |
| CaO + autoclaved SD | x3 | | | x3 | |
| *PO* spawn untreated | x3 | | | x3 | |
| *SRA* spawn untreated | x3 | | | x3 | |
| *PO* spawn + treated SD | x3 | x3 | x3 | x3 | x3 |
| *SRA* spawn + treated SD | x3 | x3 | | x3 | |

Table 2: Experiments performed to evaluate optimal fungal SSF duration

### 2.5. Physico-chemical and microbial analysis performed on solid digestate and mycelia

To characterize the different substrates several methods were used. Total Solids (TS) and VS contents were obtained following the standard methods of the American Public Health Association [26]. OM corresponds to VS, therefore for the rest of the article VS term will be used to describe OM. An AutoKjehdahl Unit K-370, BUCHI (Flawil, Switzerland) was used to determine the total Kjeldahl nitrogen. Total carbon content was measured using a Shimadzu TOC-VCSN Analyzer (Kyoto, Japan) coupled to a Shimadzu solid sample module SSM-5000A. The pH was measured using a Mettler Toledo Seven S2-meter with an InLab® Expert Pro-ISM sensor (Colombus, US). The chemical oxygen demand (COD) of solid samples was performed using an Aqualytic 420721 COD Vario Tube Test MR 0–1500 $mg \cdot L^{-1}$ (Dortmund, Germany). Samples were first freeze-dried and then ground using a Retsch mixer mill MM 200 (Haan, Germany) and associated stainless steel grinding jar. Vibrational frequency was fixed at 25 $Hz \cdot min^{-1}.g^{-1}$ of material in the jar in order to obtain a homogenous powder between samples. 0.25 grams of sample powder were poured into 10 mL of 98% w/w $H_2SO_4$ and set under strong agitation overnight to solubilize solid particles. Dilution with MilliQ water up to 250 mL allowed pipetting. 2 mL of sample adequately diluted were then pipetted into each tube. Oxidation reactions in tubes were performed in a HACH COD reactor at 150 °C for 2 hours (Loveland, US). COD concentrations were measured using an Aqualytic MultiDirect spectrophotometer. To determine

microbial richness of untreated solid digestate, a bacterial quantitative Polymerase Chain Reaction (qPCR) was carried out following Braun et al., (2011) procedure.

### 2.6. Respiration assessment

VS loss due to respiration of fungi or endogenous SD microorganisms inside the wooden box was evaluated using TS/VS measurement. Initial VS in each tube was known. At the end of each post-treatment, 3 tubes were first placed at 105 °C to determine TS. Dry samples were then entirely transferred into ceramic crucibles and placed at 550 °C to determine remaining VS contents. Difference between the initial VS and the remaining VS content corresponds to VS loss from respiration.

### 2.7. Determination of methane yield and biodegradability

Biomethane Potential (BMP) tests were performed to simulate recirculation of SD within the biogas plant after post-treatment. For each condition of **Table 2**, BMP tests were carried out in triplicate and prepared similarly to Monlau et al., (2012). Briefly, for each tube containing tested substrates, the matter was mixed with a macroelement solution, an oligoelement solution, a bicarbonate buffer as well as an anaerobic sludge at 5 $g_{VS}{\cdot}L^{-1}$, before being incubated under agitation at 35 °C. Biogas production was monitored twice a week at the beginning and less frequently as production slowed down. Biogas volume was measured using a Keller LEO 2 digital manometer (Winthethur, Switzerland) and biogas composition was determined using a PerkinElmer® Clarus 580 gas chromatograph (Waltham, US) equipped with two Restek columns (Bellefonte, US): the first (RT®-Q-Bond) was used to separate $CO_2$ from other gases, the second (RT®-Msieve 5A) was used to separate $O_2$, $N_2$, $CH_4$, and $H_2$. The carrier gas used was argon delivered at 350 kPa and 35 $mL.min^{-1}$ into the column. Oven, injector and detector temperatures were set respectively at 60 °C, 250 °C and 150°C. A thermal conductivity detector was used for gas detection. According to standardized practices, for each condition, endogenous methane production was considered by subtracting the gas generated in the controls (inoculum only) and BMP tests were stopped when daily methane production during three consecutive days was <1% of the accumulated volume of methane [29].

Methane yield (in mL $CH_4{\cdot}g^{-1}$ VS initial) is defined as the accumulated amount of methane produced as a function of the initial VS content of the sample before post-treatment (≈2 g of VS), while biodegradability (in mL $CH_4{\cdot}g^{-1}$ VS final) is defined as the methane produced by the remaining matter. Biodegradability was expressed as a function of the final VS content of the sample after the post-treatment and respiration (less than 2 g of VS). BMP values obtained were compared to two types of control: (i) direct codigestion: untreated SD and spawn are directly anaerobically digested without contact time and (ii) treated codigestion: controls where treated SD (lime and autoclaving) and spawn are separately stored under aerobic conditions for 5.5 or 15 days before being anaerobically digested without contact time. In both cases, proportions between SD and spawn corresponded to identical VS ratios obtained in SSF trials with 20% w/w spawn levels. Overall BMPs for the controls were obtained as follows:

$$BMP_{direct\_codigestion} = BMP_{Spawn} * \%g\ VS_{Spawn} + BMP_{untreated\_dig} * \%g\ VS_{dig} \quad \textbf{(1)}$$

$$BMP_{\mathrm{treated\_codigestion\_Xdays}} = \mathrm{BMP_{aerated\_spawn\_Xdays}} * \%\mathrm{g\ VS_{spawn}} + \mathrm{BMP_{treated\_dig\_Xday}} * \%\mathrm{g\ VS_{dig}} \quad \textbf{(2)}$$

### 2.8. Volatile solids characterization

VS was characterized via sequential chemical extractions according to Jimenez et al., (2015) protocol. Firstly, selected samples were freeze-dried and ground to obtain fine and homogenous powders. Subsequent sequential extractions were performed on 0.5 g of sample. Four VS fractions of decreasing accessibility were then obtained by applying increasingly strong chemical solutions: (i) soluble extractable OM (SPOM) using $CaCl_2$ (10 mM), (ii) readily extractable OM (REOM) using NaOH (10 mM) + NaCl (10mM), (iii) slowly extractable OM (SEOM) using NaOH (0.1M) and (iv) poorly extractable OM (PEOM) using $H_2SO_4$ 72% w/w. At each step, the solubilized VS was recovered in the supernatant by centrifugation (18,750 g for 20 min at 4 °C) and filtered at 0.45 µm. VS of each fraction was then characterized via COD measurement. Finally, the non-extractable OM (NEOM) was calculated by subtracting the four fractions of VS extracted from the sample from the total VS. This total VS being obtained by measuring COD on the initial powder sample using the previously described COD method for solids (see 2.5.). Here, it can be specified that SPOM corresponds to soluble proteins and sugars, REOM and SEOM to recalcitrant proteins, lipids and some humic acids, PEOM to holocelluloses and finally NEOM to lignin-like molecules and complex humic acids. This characterization method was preferred to other existing methods of lignin quantification [31,32], as SD, contrary to crops or woody biomass, not only contains lignin but also complex humic acids that are not measured using these methods.

On some samples, the fluorescence spectra of liquid extracts were recorded on a Perkin Elmer LS55 and a complexity ratio was calculated according to Jimenez et al., (2015). This index is defined as the ratio of the sum of the fluorescence volumes of the most complex molecules (lignin, humic acid…) over the sum of the fluorescence volumes of the protein-like molecules.

Finally, correlations between the different VS fractions, methane yields and biodegradability were analyzed via a principal component analysis (PCA). PCA was performed using SIMCA software from UMETRICS (Umeå, Sweden).

### 2.9. Evaluation of the impact of post-treatments and solid digestate recirculation on plant methane yield compensation

To determine the extent to which SD recirculation can compensate a feedstock shortage or be integrated in the ration ($\gamma_{CH4\_yield\_compensation}$), we used the following equation:

$$\gamma_{CH4\_yield\_compensation} = \frac{Corrected\_BMP_{SD} * \%VS_{SD} * M_{SD\ recirculated}}{V_{CH4}\ produced\ yearly} \quad (3)$$

*Corrected_BMP$_{SD}$* (in $Nm^3\ CH_4 \cdot ton^{-1}$ VS) aimed to simulate full-scale methane production value. It was obtained by applying a 0.8 correction factor to obtain BMP values according to Holliger et al. study [33]. *%VS$_{SD}$* corresponds to the ratio of volatile solids measured at lab scale over fresh matter of SD. $M_{SD\_recirculated}$ (tons/year) corresponded to 20%, 50% and 80% of the total SD produced yearly (1800 tons/year). We assume that it is not possible to recirculate 100% of the SD as some minerals and recalcitrant organic matter need to exit the system notably to avoid AD inhibitions and be used for land fertilization. For fungal post-treatment calculations, 20%w inoculum was added to $M_{SD\_recirculated}$ and *%VS$_{SD}$* of the mix was calculated based on initial VS content of SD as well as inoculum (21% for

PO+SD and 17% for SRA+SD). Besides, methane yields taking into account VS losses were used. At last, the volume of methane produced yearly as previously calculated corresponded to 1,189,000 $Nm^3$.

To determine a number of tons of feedstocks ($\Delta_{feedstock}$) that would be replaced by such strategy, we applied equation (4), where $CH_4$ yield corresponds to the average methane production per ton of feedstock (84.9 $Nm^3$ $CH_4$·$ton^{-1}$ feedstock):

$$\Delta_{feedstock} = \frac{Corrected\_BMP_{SD} * \%VS_{SD} * M_{SD\ recirculated}}{CH_4\ \text{yield}} \quad (4)$$

Finally, to estimate the impact of recirculating SD on digester TS ($Digester\_TS_{recirculation\_SD}$), we used the equation herein below:

$$Digester\_TS_{recirculation\_SD} = \frac{(M_{feedstock} - \Delta_{feedstock}) * \%TS_{digester} + M_{SD\ recirculated} * \%TS_{SD}}{M_{feedstock} - \Delta_{feedstock} + M_{SD\ recirculated}} \quad (5)$$

In this equation, the amount of feedstock ($M_{feedstock}$) corresponds to the initial ration of the digester (14,000 tons/year) and initial TS content of the digester was 12% ($\%TS_{digester}$). $Digester\_TS_{recirculation\_SD}$ can be defined as the new TS content of the digester, following implementation of the SD recirculation strategy.

## 3. Results and discussion

### 3.1. Substrates features

Several substrate factors are known to affect mushroom growth such as C/N ratio, moisture, pH, microbial load and spawn level [34]. SD was characterized in order to determine if it was a suitable substrate for fungus growth (**Table 3)**. Moisture and pH were slightly higher than what is usually stated as optimal for growth. In the case of *Pleurotus* species: (i) moisture contents between 50-75% are often targeted as higher levels can favor fungus diseases and microbial competition and (ii) an initial pH of 6.5-8.7 is preferred [34,35]. However, high nitrogen content (C/N ratio below 40 in the case of synthetic medium) was identified as a potential way to enhance ligninolytic activities of *Pleurotus* species, which was relevant for the tested post-treatment [36]. It can be noticed that the pH of spawn, also characterized in **Table 3**, were around 5.5 due to the fact that mycelium colonization led to an acidification of the bulk growth substrate. Initial trials at different spawn levels (5-20% w/w) for *PO* did not lead to any significant colonization of untreated SD. This can be seen in **Fig. 1**. This is not due to a problem in *PO* spawn activity, as wheat straw control was partly colonized. Treatment of the SD is thus necessary to allow its colonization by *PO*.

| Substrate | %TS | %VS | %C·$g^{-1}$ TS | %N·$g^{-1}$ TS | C/N | pH |
|---|---|---|---|---|---|---|
| Solid Digestate (SD) | 20.5 ±0.3 | 15.5 ±0.1 | 33.2 ±0.2 | 1.7 ±0.1 | 19.3 | 9.1 |
| SD quicklimed & autoclaved | 22.9 ±0.2 | 15.2 ±0.1 | 32.4 ±0.2 | 1.4 ±0.1 | 23.6 | 11.4 |
| *PO* spawn | 48.9 ±0.1 | 43.1 ±1.5 | 39.7 ±0.3 | 2 ±0.1 | 19.6 | 5.7 |
| *SRA* spawn | 26.7 ±0.5 | 24.3 ±0.4 | 31.2 ±0.3 | 1.4 ±0.1 | 22.9 | 5.4 |

Table 3: Substrates features

### 3.2. Determination of fungal SSF conditions

Sterilization appears to be the most important parameter to work on as the microbial load of SD was high and *PO* is reported to be sensitive to bacteria competition during its development [37]. Results of

the qPCR on untreated SD displayed an absolute abundance of $7.3 \cdot 10^{11}$ ($\pm 3.9 \cdot 10^{11}$) numbers of 16S gene copies per gram of fresh matter (FM). This corresponds to an average of $1.8 \cdot 10^{11}$ bacterial cells$\cdot g^{-1}$ FM, which is in the range of values found in literature for similar digestates coming from AD plants (Braun et al., 2011). However, this amount is high in comparison to other growth media for fungi such as bulk soil ($10^8$ cells$\cdot g^{-1}$) or even unsterilized straw ($10^9$ cells$\cdot g^{-1}$) [38,39].

The sterilization strategy was based on the combination of an alkaline and thermal treatment to reduce microbial load and activity and results are displayed in **Fig. 1**. Indeed, alkaline conditions appear to reduce activity of competing microorganisms, while mycelium development of *PO* is not greatly affected [35,40]. Therefore, a 2% w/w quicklime powder was used to alkalinize SD as it is a cheap chemical, generally used on farms for agricultural practice, it is more concentrated than slacked lime previously successfully used by Hernandez et al., (2003) and it has an additional delignification effect (Ramos-Suárez et al., 2017). Autoclaving was subsequently performed to reduce microbial load before inoculation. Following this treatment, moisture was close to 75%, C/N ratio increased slightly, as nitrogen probably volatilized under ammonia form during autoclaving, and pH was alkaline (see **Table 3.**). Only a high 20% w/w of *PO* spawn succeeded in colonizing efficiently SD after 10 days of SSF, a white coating was formed around the SD. According to these results, selected growth conditions for *PO* and *SRA* consisted in addition of quicklime (2% w/w), autoclaving and a 20% w/w spawn level. Full-scale feasibility of such a strategy will be discussed thereafter.

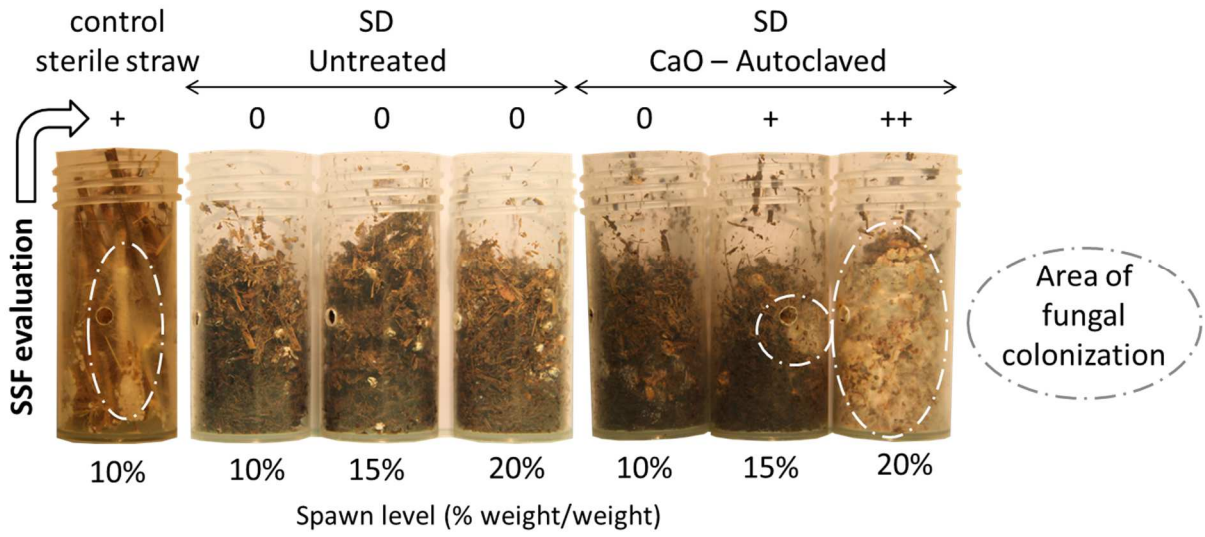



**Figure 1: Screening of colonization conditions during SSF of *Pleurotus ostreatus* on solid digestate and visual assessment according to predefined evaluation scale (5% spawn level is not shown as no colonization occurred).**

### 3.3. Impact of fungal SSF duration on volatile solids loss

Selected growth conditions led to colonization of SD by *PO* during SSF. For each SSF duration, from 5.5 to 21 days, all tubes had successful colonization with a mycelium that tightens and forms an increasingly strong coating around the SD in time. Some tubes at 21 days even showed some fructification starting with small carpophores growth at the top of SD. For SSF of *SRA*, growing and SD colonization was less obvious than for *PO*. At 5.5 and 7.5 days colonization was not significant at sight, and at 15 days some white mycelium had developed but it was not comparable with *PO* colonization. To illustrate these observations, some tubes are shown in **Fig. 2**.

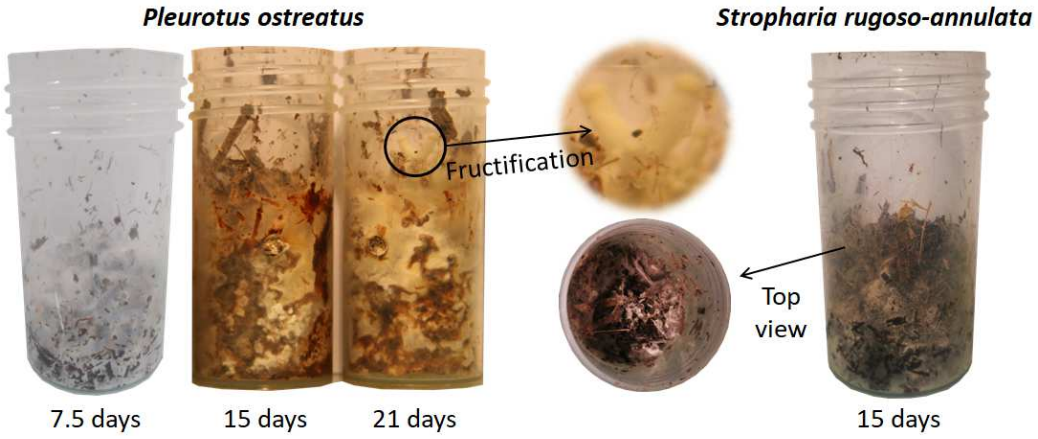



**Figure 2: Photographs of solid digestate colonized by *PO* and *SRA* after SSF at different times**

The trend of VS loss for each condition was plotted in time in **Fig. 3**. VS is lost in time for every condition except at 5.5 days in the case of quicklimed and autoclaved SD (thermo-alkaline post-treatment). This is probably due to an efficient sterilization and alkaline conditions that suppressed microbial aerobic activity for a few days. However, at 15 days, thermo-alkaline post-treatment led to a VS loss higher than the SD only aerated (19% of VS loss against 12.8%). This is probably due to the fact that this treatment enhanced the amount of easily accessible VS that can be subsequently respired. Aerated spawn controls show VS losses between 13.3-23.5% after 15 days, which can be explained by further colonization and respiration by *PO* and *SRA*, respectively. For *SRA* colonizing SD, VS loss at 5.5 days was higher than the control, probably due to some colonization of SD by *SRA*. Nevertheless, after 15 days, VS loss was similar to the control showing that there were no specifically strong interactions between *SD* and *SRA* over time. For *PO,* VS loss was always higher than the control reflecting the colonization of the SD by *PO* and a higher activity. VS loss reaches around 20% after 15 days. Similar VS loss was observed in studies using fungi to pretreat biomass before AD [42–44]. VS loss directly impacts methane yield since the VS consumed for respiration is no longer available to be converted to methane.

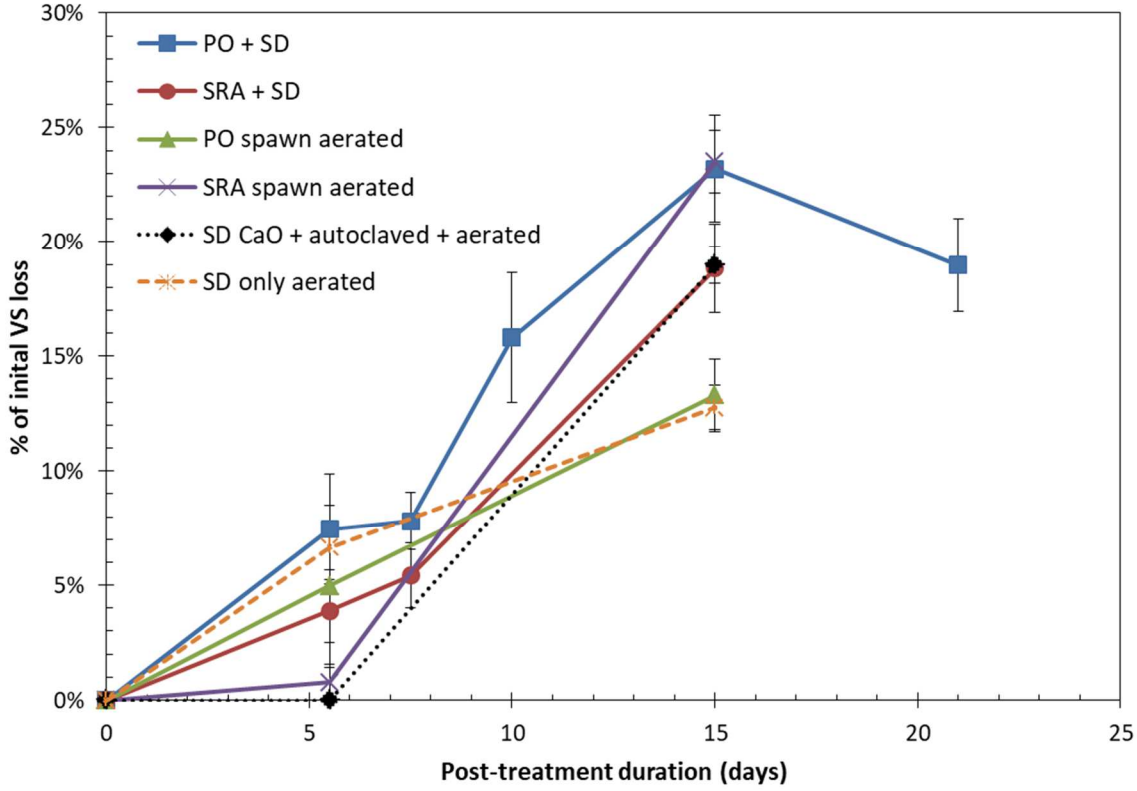



Figure 3: Volatile solids loss in time for the different conditions applied to SD

### 3.4. Impact of fungal SSF duration on methane yield and biodegradability

Results of *PO*/*SRA* SSF, thermo-alkaline post-treatment and simple aeration of spawn or SD on methane yield and biodegradability are gathered in **Table 4**. Several observations can be drawn from that: (i) Methane yield of SD, *SRA* spawn and *PO* spawn only aerated were respectively around 10%, 20% and 30% lower than their corresponding control (no aeration). VS loss was not compensated by a gain in biodegradability, which significantly decreased for *PO* (by 25%) at 5.5 and 15 days. This is also the case at 5.5 days for *SRA* (by 17%) and SD (by 7.6%), but biodegradability was not significantly different after 15 days. One hypothesis is that endogenous SD microorganisms or *PO* and *SRA* used VS which was easy to access for their growth/metabolism during this period which can explain the loss in biodegradability generally observed. Lignin-like molecules were not specifically degraded.

(ii) Thermo-alkaline post-treatment followed by a short 5.5-day aeration displayed an enhanced methane yield and biodegradability of 13% in comparison to untreated SD. It is likely that this kind of post-treatment acted on VS biodegradability and notably lignin as it was recently observed by Mustafa et al., (2018) on sugarcane bagasse. After 15 days, similarly to only aerated SD, methane yield was significantly lower (15%) and biodegradability was not significantly enhanced. It is likely that endogenous or/and exogenous microorganisms, after a lag time due to sterilization and increased pH, used the additional easy to degrade VS fraction that was released during post-treatment for their growth, explaining the biodegradability decline.

(iii) For *PO*, methane yield was always lower in comparison to direct codigestion (untreated SD and *PO* spawn). The longer the SSF time, the lower the methane yield, with up to 50% methane yield loss after 21 days SSF. Besides, codigestion of separately treated SD (CaO, autoclaving and 5.5 or 15 aeration

394 days) and *PO* spawn (5.5 or 15 aeration days) both at 5.5 and 15 days displayed higher methane yields.
395 This means that the interaction between SD and *PO* during colonization increased methane loss;
396 probably due to respiration of VS coming from SD by *PO*. Colonization by *PO* of SD does not appear
397 beneficial as biodegradability is decreased over time, probably reflecting no specific ligninolytic activity
398 of *PO* on SD. Similar results were observed by Rouches et al., (2015) for certain fungi strains. In their
399 study, methane yield of pretreated wheat straw was decreased due to carbohydrate consumption by
400 fungi.

401 (iv) For *SRA*, short term aeration (5.5 and 7.5 days) did not lead to any significant methane yield loss.
402 However, at 15 days methane yield was decreased by 16.8% as VS loss occurred without any significant
403 biodegradability increase. Again, *SRA* interaction with SD is not clearly shown as methane yield loss is
404 similar to the codigestion of *SRA* spawn and treated SD aerated 15 days separately. The lack of
405 colonization can be due to suboptimal conditions as *SRA* is generally growing on straw, so a pH around
406 6.5-7.5 and C/N ratio of 50-100 is preferred compared to 11.4 and 24 in our case, respectively. Besides,
407 growth rate is slower than *PO* and 15 days might not be long enough to see a clear colonization [47].

| | Sample | Aeration duration | VS loss (% initial VS) | Methane yield (mL $CH_4 \cdot g^{-1}$ initial VS) | % Methane yield variation | Biodegradability (mL $CH_4 \cdot g^{-1}$ final VS) | % Biodegradability variation |
|---|---|---|---|---|---|---|---|
| **Treated SD** | **SD control** | **0** | **0** | **157 (±3)** | **/** | **157 (±3)** | **/** |
| | SD Cao autoclaved aerated | 5.5 | 0 (±0.3) | 177 (±2) | 13 | 177 (±2) | +13 |
| | SD Cao autoclaved aerated | 15 | 19 (±0.8) | 133 (±4) | -15.2 | 158 (±5) | Not significant |
| | SD only aerated | 5.5 | 6.6 (±1) | 136 (±5) | -13.3 | 145 (±5) | -7.6 |
| | SD only aerated | 15 | 12.8 (±1) | 142 (±6) | -9.6 | 160 (±6) | Not significant |
| **Aerated spawn** | ***PO* spawn control** | **0** | **0** | **365 (±30)** | **/** | **365 (±30)** | **/** |
| | *PO* spawn aerated | 5.5 | 5 (±3.5) | 258 (±12) | -29.3 | 271 (±13) | -25.8 |
| | *PO* spawn aerated | 15 | 13.3 (±1.6) | 238 (±3) | -34.8 | 269 (±4) | -26.3 |
| | ***SRA* spawn control** | **0** | **0** | **203 (±6)** | **/** | **203 (±6)** | **/** |
| | *SRA* spawn aerated | 5.5 | 6.5 (±0.8) | 158 (±8) | -22.2 | 168 (±8) | -17.2 |
| | *SRA* spawn aerated | 15 | 23.5 (±1.4) | 160 (±5) | -21.2 | 197 (±6) | Not significant |
| **Treated SD + *PO*** | **Direct codigestion SD + *PO*** | **0** | **0** | **232 (±12)** | **/** | **232 (±12)** | **/** |
| | Codigestion treated separately | 5.5 | 2 (±1.4) | 210 (±6) | -9.5 | 215 (±6) | Not significant |
| | SD + *PO* | 5.5 | 7.5 (±2.4) | 174 (±8) | -25 | 187 (±9) | -19.4 |
| | SD + *PO* | 7.5 | 7.8 (±1.3) | 170 (±5) | -26.7 | 183 (±5) | -21.1 |
| | SD + *PO* | 10 | 15.8 (±2.8) | 143 (±14) | -38.4 | 166 (±16) | -28.4 |
| | Codigestion treated separately | 15 | 17.2 (±1) | 197 (±4) | -15.1 | 207 (±4) | -10.8 |
| | SD + *PO* | 15 | 23.2 (±2.3) | 149 (±13) | -35.8 | 184 (±16) | -20.7 |
| | SD + *PO* | 21 | 19 (±2) | 114 (±5) | -50.9 | 136 (±6) | -41.4 |
| **Treated SD + *SRA*** | **Direct codigestion SD + *SRA*** | **0** | **0** | **167 (±3)** | **/** | **167 (±3)** | **/** |
| | Codigestion treated separately | 5.5 | 1.5 (±0.2) | 173 (±3) | Not significant | 175 (±3) | +3.6 |
| | SD + *SRA* | 5.5 | 3.9 (±1.4) | 158 (±9) | Not significant | 164 (±10) | Not significant |
| | SD + *SRA* | 7.5 | 5.4 (±1.4) | 169 (±8) | Not significant | 178 (±9) | Not significant |
| | Codigestion treated separately | 15 | 19.9 (±0.9) | 139 (±4) | -16.8 | 166 (±5) | Not significant |
| | SD + *SRA* | 15 | 18.8 (±1.9) | 139 (±7) | -16.8 | 165 (±9) | Not significant |

408

**Table 1:** ***Volatile solids loss and BMPs results of solid digestate post-treatment***

### 3.5. Volatile solids fractions: evolution following post-treatments

To better understand the impact of these different treatments on VS, fractionation of 8 samples was carried out and results were gathered in **Fig. 4**. Impact of fungal treatment on VS was evaluated at 15 days as methane yield was lower both for *PO* and *SRA*. Controls A and C, which correspond to direct codigestion of untreated SD and spawn, allow to have an insight into VS evolution after thermo-alkaline treatment, aeration and fungal colonization. Controls B and D correspond to codigestion of thermo-alkaline post-treated SD and untreated spawn. They enable to distinguish the effect of the thermo-alkaline treatment from aeration and fungal colonization.

Firstly, untreated SD is in range with previously analyzed agricultural SD using the same method, notably with a NEOM close to 45% showing that agricultural SD can accumulate a large quantity of hardly degradable molecules such as lignin [48]. Thermo-alkaline post-treatment (Treated* SD in **Fig. 4**) had a strong effect on NEOM reducing it by 26% while SPOM content was increased by 123% (see **Table 5.**). Fluorescence spectroscopy analysis was used to have insight into the SPOM composition and the complexity ratio was slightly increased by 8% (from 1.42 to 1.54) due to higher lignin-like and humic acid-like compounds. This validates our previous hypothesis and indicates that NEOM get more accessible and potentially released soluble compounds as well as embedded holocelluloses explaining the 13% increase in methane yield. Mustafa et al., (2018) similarly obtained a delignification of 46% and a decrease in hemicellulose of 83% by a combination of a hydrothermal treatment at 180 °C and addition of 8.5% $Ca(OH)_2$ on sugarcane bagasse. On rice straw, 5% $Ca(OH)_2$ combined with 6 hours 80°C thermal treatment also lead to 31% lignin and 15% hemicellulose content reduction, as well as a 25% biogas production increase [49].

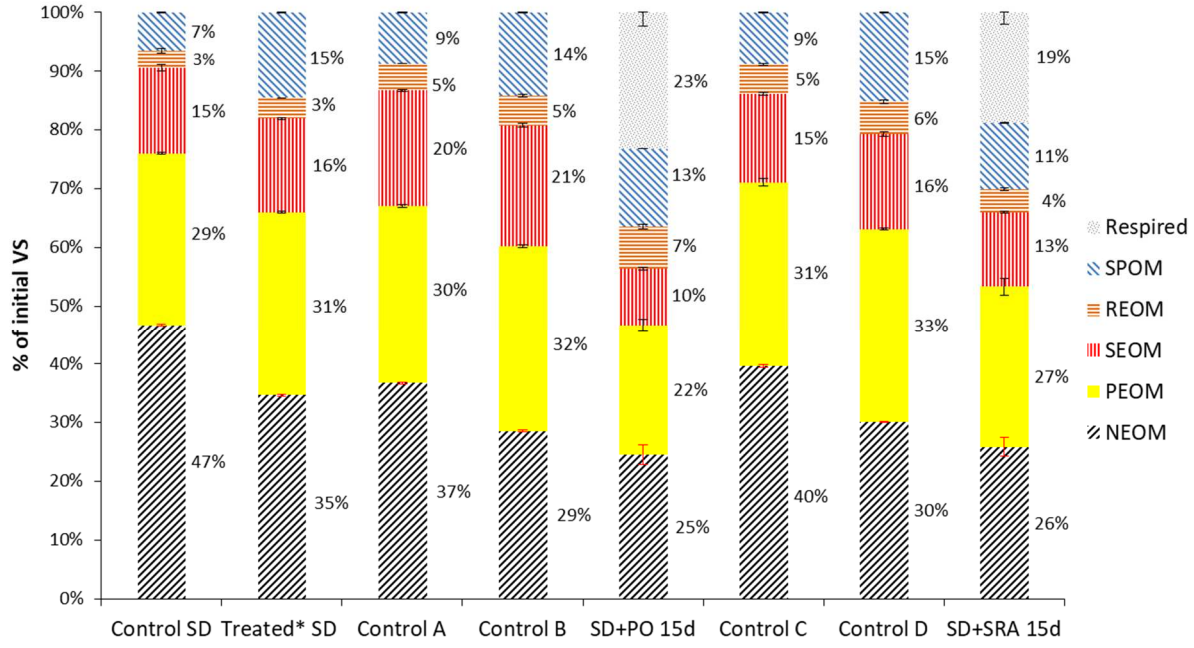



**Figure 4: Distribution of the different VS fractions in percentage of initial VS for various samples. (Treated* SD) SD CaO + autoclave + 5.5 aeration days; (Control A) untreated SD and *PO*; (Control B) Treated* SD + untreated *PO*; (Control C) untreated SD and *SRA*; (Control D) Treated* SD + untreated *SRA***

| | SPOM | REOM | SEOM | PEOM | NEOM |
|---|---|---|---|---|---|
| Thermo-alkaline post-treatment | +123% | +22% | +9% | +7% | -25,77% |
| *PO* colonization | -7% | +44% | -53% | -30% | -14% |
| *SRA* colonization | -26% | -28% | -21% | -17% | -14% |

436 **Table 5: Impact of treatments on the evolution of the different fractions of the VS in comparison to the untreated SD**

437 Control A and C have lower NEOM contents than SD which can be explained by the fact that *PO* and
438 *SRA* spawns had lower contents of lignin-like molecules. Between Control A and B as well as C and D,
439 decrease in NEOM contents and increase in SPOM contents are due to the thermo-alkaline post-
440 treatment on SD which degrades lignin-like molecules into soluble ones. When comparing control B
441 (treated SD and untreated *PO*) to the colonized digestate with *PO*, it can be observed in **Table 5** that
442 *PO* activity was not really selective toward lignin (only -14% for NEOM) but rather proteolytic (-53% for
443 SEOM) and hemicellulolytic (-30% for PEOM). This non-specific activity can explain the measured loss
444 in biodegradability and in methane yield. Similarly, for *SRA*, activities were not specific as all VS
445 fractions were used (between -14% and -28%). Contrary to *PO*, SPOM and REOM fractions were
446 degraded up to 25%. This could be linked to the activity of endogenous microorganisms such as
447 proteolytic bacteria. It might indicate that *SRA* probably did not entirely colonize SD and is still
448 competing or coexisting with endogenous SD flora.

449 A hypothesis to explain low lignin selectivity of fungi strains could come from the sterilization process
450 (thermo-alkaline post-treatment) that released soluble compounds such as sugar, which can be then
451 preferentially used by fungi. It has been previously shown that the addition of glucose can limit
452 delignification by fungi [46]. Besides, to our knowledge, it is the first time that effects of post-
453 treatments, different from composting, on SD lignin-like content are clearly evaluated using the
454 fractionation method. This method seems particularly adapted to better understand the effect of
455 various post-treatments on SD.

456 ### 3.6. Correlations between VS fractions and methane yield/biodegradability

457 Data on methane yield and biodegradability from **Table 4** as well as VS fraction percentages from **Fig.**
458 **4**, for control SD, CaO+autoclave+5.5d aeration SD, SD+PO 15d aeration and SD+SRA 15d aeration
459 samples, were used to perform a PCA analysis presented in **Fig. 5**. Looking at the PCA representation,
460 several observations can be made.

461 Only two major components are sufficient to describe 92.5% of the data variability. Component 1
462 explains 66% of the total variability. It can be shown that SD biodegradability is negatively correlated
463 with the NEOM content. Anaerobic biodegradability of lignin-like molecules are reported to be low,
464 therefore this correlation appears to make sense. SD control which has the highest lignin content (47%
465 initial VS) has logically the lowest biodegradability (157 mL $CH_4 \cdot g^{-1}$ final VS). Besides, SPOM content
466 appears to be positively correlated with SD biodegradability. On that point, similar observations were
467 already found in recent studies, pointing out that a significant positive correlation exists between
468 soluble content and BMP values [48,50]. For methane yield (once VS loss is taken into account), it can
469 be shown that it is negatively correlated with respiration. Indeed, during respiration, VS are lost that
470 would otherwise lead to additional methane production.

471 Regarding these results, a conclusion that can be drawn is that SD post-treatment that aims to improve
472 methane yield SD should avoid too important VS losses due to aeration and increase soluble molecules

(SPOM) content while reducing lignin-like (NEOM) content. Fungal SSF post-treatment does not follow these recommendations.

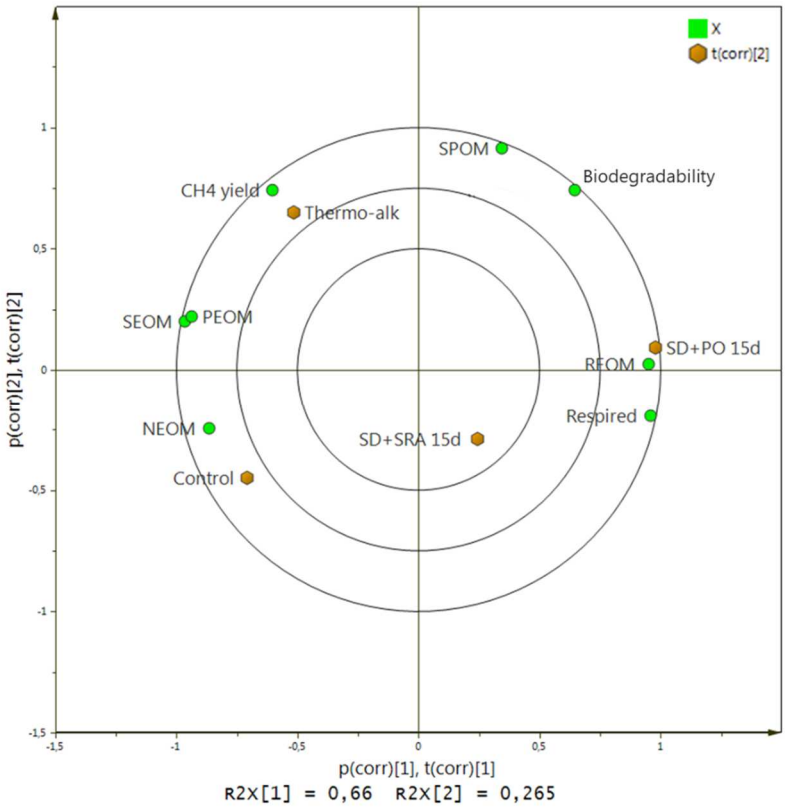



**Figure 5: Correlation circle (scores) obtained from PCA analysis of control SD, CaO+autoclave+5.5d aeration SD and fungal post-treated SD (SD+PO 15d & SD+SRA 15d) samples.**

### 3.7. Discussion over SD recirculation and the feasibility of fungal SSF post-treatment

**Table 6** gathers calculation results from equation (3) (see section 2.9) for a certain percentage of SD recirculated (20% to 80%) and applied for the control SD, the thermo-alkaline post-treated SD, the PO 15 days post-treated SD and the SRA 15 days post-treated SD. Additionally, equations (4) and (5) were applied to control SD recirculation.

Firstly, direct recirculation of SD into the digester could compensate up to 2.4% of the total plant methane yield in the case of a feedstock shortage or when feedstocks need to be economized. By assuming that methane production and TS from feedstock is evenly distributed, SD recirculation could replace up to 320 tons of feedstock, while only increasing digester TS content up to 12.8% (+6.75%). In a context where prices of feedstocks is increasing due to competition among existing biogas plants, such strategy can be a good supporting solution. It has following advantages: reduce costs, increase flexibility in feedstock management and enhance biogas plant efficiency.

Sterilization post-treatment (CaO 2%w + autoclaving) allows to get a slightly higher methane yield compensation, up to 2.6%. However, economic interest remains uncertain due to chemical costs (CaO costs around 150 $\$\cdot ton^{-1}$), additional heating energy required as well as the accompanying heating structure or device (tank…). Nevertheless, further optimization of this post-treatment may enhance its interest (notably lower heating temperature or even no heating).

Finally, fungal SSF post-treatment appears to give higher methane production recovery, up to 3.6%, but this is due to the addition of 20% inoculum that is also producing methane. Besides, it has been shown that higher methane yield could be obtained by direct co-digestion of inoculum and SD without SSF colonization. Thus, additional inoculum costs, sterilization process as well as VS losses due to fungal respiration make fungal SSF post-treatment non-viable for full-scale agricultural biogas plant.

Alternatives to thermo-alkaline sterilization treatment might be explored to reduce costs and potentially get a higher specific activity toward lignin-like fraction: (i) Trials performed directly on-site, using fresh SD from thermophilic plants would be of interest to determine if long term anaerobic and thermophilic conditions can create an available ecological niche in SD for subsequent aerobic mesophilic fungal SSF. (ii) Spawn could be propagated on-site by colonizing low-cost substrate such as weed plants without sterilization [51] or miscanthus pellets [42] in order to apply higher spawn levels at an affordable cost. However, it is likely that extensive labor would counterbalance the economic gains. (iii) Explore mild alkaline or acidic treatment without sterilization on SD as lower or higher pH may inhibit endogenous bacterial activity while favoring fungal growth [52]. Nevertheless, it is foreseeable that optimization of the fungal SSF at full scale will be difficult to manage and remain too costly. Tracks that explore production of higher value products than methane (e.g. enzymes, biomolecules…) is likely to be the only way to enhance economic profitability and render viable this strategy.

| | 20% SD recirculation | 50% SD recirculation | 80% SD recirculation |
|---|---|---|---|
| Tons of SD recirculated per year | 360 | 900 | 1,440 |
| $CH4_{production}$ offset by direct SD recirculation (% of plant methane yield) | 0.6% | 1.5% | 2.4% |
| $\Delta_{feedstock}$ (tons) for direct SD recirculation | 80 | 200 | 321 |
| New Digester_$TS_{recirculation_SD}$ for direct SD recirculation | 12.2% | 12.5% | 12.8% |
| $CH4_{production}$ offset by thermo-alkaline post-treated SD recirculation | 0.7% | 1.6% | 2.6% |
| Tons of SD + fungal inoculum recirculated per year (80% SD + 20% inoculum) | 432 | 1,080 | 1,728 |
| $CH4_{production}$ offset by PO post-treated SD (15d) recirculation | 0.9% | 2.3% | 3.6% |
| $CH4_{production}$ offset by SRA post-treated SD (15d) recirculation | 0.7% | 1.7% | 2.7% |

**Table 6: Evaluation of the effect of various amounts of SD (post-treated or not) recirculated on plant methane yield compensation and digester TS content.**

## 4. Conclusion

In the case of a feedstock shortage or when feedstocks need to be economized it has been shown, for the agricultural biogas plant we studied, that direct recirculation of SD into the digester could compensate up to 2.4% of the total plant methane yield (equivalent of 320 tons feedstock). It is a low-cost strategy that increase flexibility in feedstock management and allow a higher plant methane yield

recovery. Concerning fungal solid-state fermentation of SD before recirculation into agricultural biogas plants, it has never been evaluated until now. It appears not to be a viable strategy to enhance energy recovery from SD within the tested conditions. For the two strains studied, sterilization and a high spawn level were needed to ensure fungal colonization. Besides, fungal activities during SSF were not specific to the most complex fraction, leading to uncontrolled VS losses and subsequent decrease of biodegradability and methane yield from SD. Overall, looking at the energetic loss (sterilization and lower methane yield) and the additional economic cost (mycelium and labor) this strategy will not be profitable at full scale. Finally, sterilization process, consisting of a thermo-alkaline post-treatment, showed an increase in energy recovery from SD of 13%. Further optimization of this post-treatment may enhance efficiency of SD recirculation strategy to offset plant methane production losses.

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**Declaration of interest**

Declarations of interest: none

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691