Università degli Studi di Torino

Tesi di Dottorato di Ricerca in Scienze Biologiche
e Biotecnologie Applicate

PhD Thesis in Biological Sciences
and Applied Biotechnologies

"Fungi and their enzymes in biomass pretreatment for biogas production"

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XXXIII Cycle: 2017 – 2020

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I) List of Abbreviations

ABP: Agricultural Biogas Plants

AD: Anaerobic Digestion

BMP: Biochemical Methane Potential

CS: Cow Slurry

Fur: Furfural

FYM: Farmyard Manure

GHG: Greenhouse Gases

Hba: 4-Hydroxybenzaldehyde

HRT: Hydraulic Retention Time

LME: Lignin-Modifying Enzymes

MS: Maize Silage

MST: Maize Stover

OFMSW: Organic Fraction of Municipal Solid Waste

OLR: Organic Loading Rates

PAH: Polycyclic Aromatic Hydrocarbons

PCWP: Plant Cell Wall Polymers

RS: Rice Straw

SFD: Solid Fraction of Digestate

SmF: Submerged Fermentation

SSF: Solid State Fermentation

Syr: Syringaldehyde

TS: Total Solids

Van: Vanillin

VFA: Volatile Fatty Acids

VS: Volatile Solids

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1. Introduction

1.1 Global warming and pollution: the role of fossil fuels

The Earth is an extremely fragile system that has been physically, chemically, and biologically altered in very short time by human activities. Industry, waste disposal, agriculture, deforestation, and especially fossil fuel (petroleum, coal, and natural gas) combustion have increased the emissions of several important greenhouse gases (GHG), such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and chlorofluorocarbons, resulting in concerns about global warming by 1–5°C over the next century (Wuebbles and Jain, 2001).

The major direct and indirect effects of climate change include:

- Increased droughts and heat waves (periods of abnormally hot weather), that are linked to increased wildfires and to decreased freshwater availability (Yilmaz et al., 2014).
- Changes in seasonality (lengthening of the frost-free season and the corresponding growing season) and precipitation patterns (heavy downpours and hail with consequent flooding and inundation); hurricanes will become more intense and frequent (Perera et al., 2020).
- Shrinking glaciers and thawing permafrost. Arctic is expected to become essentially ice-free in summer before mid-century (Overland et al., 2019).
- Increased sea level, costal erosion and ocean acidity (due to increased HCO_3 concentrations as a consequence of increased CO_2 concentrations). Global sea level has risen by about 20 cm since 1880 because of added water from melting land ice. It is assumed that the levels will rise another 30 to 240 cm by 2100 (Nicholls and Cazenave, 2010).
- Loss of biodiversity due to limited adaptability speed of flora and fauna and spread of infectious disease vectors, pests and pathogens (Lafferty, 2009).

Overall, these effects pose significant threats to health (increased illness, injury, and deaths from heat stress, floods, drought, intense storms, malnutrition and undernutrition, mental illness due to population displacement and social and political instability, etc.), economy (energy, infrastructure, transportation, agriculture, forestry, fisheries, etc.), environments (ecosystems, air and water quality, etc.) and more (Watkiss et al., 2005).

Unfortunately, the impacts and harms of climate change are exacerbated by those of the widespread pollution. In fact, the contamination with xenobiotics affects both human and ecosystem health, either directly or indirectly as a consequence of the impairment of biogeochemical cycles and ecological communities' structures and functions (De Marco et al., 2019). Fossil fuel combustion for energy, electricity, heat, transportation, and industry is recognized as a major cause of water, soil and especially air pollution (Perera, 2018). The anthropogenic emissions from fossil fuel-burning include several toxic pollutants, as fine particulate matter, black carbon, mercury (Hg), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), carbon monoxide (CO) and volatile chemicals that form ground-level ozone (O_3) (Granier et al., 2011). Emissions from fossil fuel-burning include also the polycyclic aromatic hydrocarbons (PAH), a class of semi-volatile, chemically stable and hydrophobic organic compounds with two or more fused aromatic rings. Soil contamination by PAH is widespread all-around the EU countries, being service stations and oil industries the main sources (Panagos et al., 2013). The human and environmental effects of PAH are severe, especially from the 4-6 rings derivatives, which are known to be potentially carcinogenic and mutagenic to humans and other living organisms (Lawal, 2017).

In order to mitigate the negative effects of climate change and pollution, a broad range of interventions have been implemented. In 1997, the United Nation Framework Convention on Climate Change (UNFCC) signed the Kyoto Protocol to

establish that GHG emissions had to be reduced of 8 % within the period 2008-2012 to limit global warming below 2 °C (COM, 2007). In 2007, the European Council adopted new environmental targets to reduce GHG emissions by 2020 (JEC, 2014). Despite this, recently GHG emissions reached worrisome peaks at global scale, and according to the increasing demographic growth and industrialization this trend is going to worsen. Before 2020, emissions of CO_2 (which is the most important human-produced climate-altering GHG) were rising by about 1% per year over the previous decade. In 2017, the CO_2 emissions increase of 2 % after three straight years of levelling off (IEA, 2020). As a result of the SARS-CoV-2 pandemic, the daily global CO_2 emissions decline by –17% by early April 2020 compared with the mean 2019 levels and they are expected to reach 30.6 Gt at the end of the year (Figure 1). This is one the lowest CO_2 level since 2010, but the present atmospheric concentrations of CO_2, are unprecedented in at least 800,000 years (IEA, 2020). The models drawn by the international scientific community suggest that, without major policy or technology changes, future concentrations of CO_2 will continue to increase, largely as a result of the massive fossil fuel usage (Liguori and Faraco, 2016).

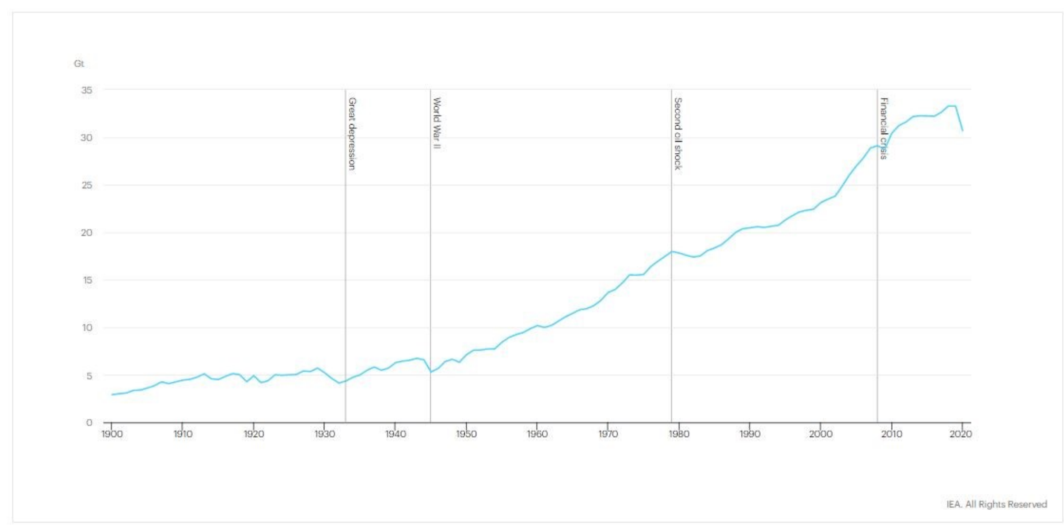


Figure 1: Global energy-related CO_2 emissions, 1900-2020 (IEA, 2020).

1.2 The alternative to fossil fuels: renewable energy sources

The majority of worldwide energy consumption depends on non-renewable fossil fuels, but their continuative use is unsustainable in meeting the worldwide needs for the future, since it has been established that depletion times for oil, coal and natural gas are about 35, 107 and 37 years, respectively (Liguori and Faraco, 2016). Moreover, modern society is facing the challenge to limit the dependence on fossil fuels for energy production in order to reduce a multitude of harmful consequences due to emissions of toxic molecules and gases that are major driver of climate change and pollution (Höök and Tang, 2013). The awareness about environmental damages caused by indiscriminate fossil fuels exploitation have led to a Green revolution in the energy production models, in favor of environmental-friendly and cost-effective technologies that employs renewables and sustainable sources (Garcia-Casals et al., 2019). The use and growth of renewable energy sources has many potential benefits: besides reduction in GHG emissions and pollution, it may stimulate the diversification of energy supplies and the employment through the creation of jobs in new technologies (Panwar et al., 2011).

The European Union (EU) introduced several directives aimed at reducing the dependence on fossil fuels and promoting renewable energies production (Scarlat et al., 2018). Among them, the Renewable Energy Directive (RED) 2009/28/EC on renewable energy sources promotion (Directive 2009/28/EC) defines national targets and provisions in order to extend green energy production to 20% of total EU energy production by 2020 (Scarlat et al., 2018). In detail, the 20/20/20 targets ratified in the European Directive 2009/28/EC included:

- reduction of 20 % in GHG emissions taking as reference 1990 emissions;
- improvement of energy efficiency in order to save 20 % of the EU energy consumption;
- reaching 20 % of renewable energy in the total EU energy production;
- reaching 10 % of biofuels in the total vehicle consumptions.

The European directive 2009/28/EC has been implemented by the Italian "National plan for renewable energy" which established that Italy has to reach 17 % of energy from renewables for heating and transport by 2020 (Malandrino et al., 2017).

By 2050, the EU targets to establish a competitive low carbon economy in which renewable energies would represent the 55-75% of total EU energy production (COM (2011) 885 final) and a GHG reduction of 80-90% would be reached (COM (2011) 112 final). In order to achieve this ambitious goal, the recent European Green New Deal (COM (2019) 640 final) aims at enhancing GHG emissions reduction targets to 50-55% compared with 1990 levels by 2030 and at supporting member states and economic sectors by boosting circular economy and providing financial assistance to reach a new climate-neutral economic model (COM (2019) 640 final). The Just Transition Mechanism is a plan that outlines provisions and a financial support scheme to help nations and economic sectors to face the transition towards this new green economic model and consists of the mobilization of at least €100 billion by 2021-2027 (COM (2019) 640 final).

Financial incentives for renewable energies production have turned out in boosting the sector worldwide (Vasco-Correa et al., 2018). In Europe, the main renewable energy sources are hydropower, wind, solid biomass, solar photovoltaic, geothermal and biogas. In 2011, the renewable energy from solid biomasses covered the 48 % of the total, followed by hydropower, liquid biofuels and all others (Bostedt et al., 2016). In 2014, in EU the electricity from renewable energy sources amounted to 27.5 %: hydropower contributed for 40 % to renewable electricity, followed by wind (25 %), solid biomasses, (which dropped to 12 %) and solar photovoltaic (11 %); all other technologies give smaller contribution, between 1 % and 7 % (EEA, 2017). In 2018 and 2019, renewable energy represented 18.9 and 19.7 % of energy consumed in the EU, on a path to the 2020

target of 20 %. Noteworthy, within the EU, only 12 Member States have already reached a share equal to or above their national 2020 targets (Figure 2). Further increases in the share of renewables are essentials to reach the EU climate and energy goals and to enable the European citizens and businesses to benefit from sustainable green transition.

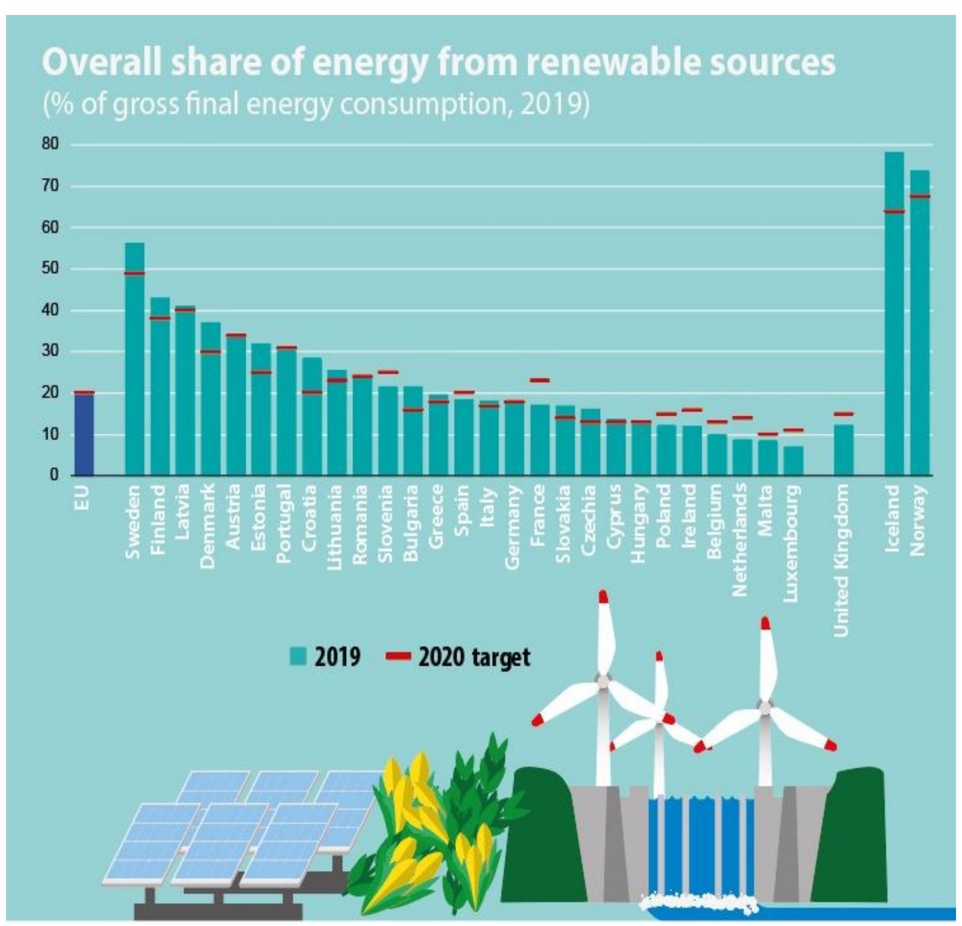


Figure 2: Share of energy from renewable sources in 2019 in the European Union (% of gross final energy consumption) (EUROSTAT, 2021).

1.3 Anaerobic Digestion

The production of biogas by anaerobic digestion (AD) is one of the most efficient and environmentally beneficial technologies for renewable bioenergy production (Rouches et al., 2016; Weiland, 2010). AD offers several advantages over other forms of bioenergy production. For instance, biogas production leads to a small amount of GHG emissions and may contribute to sustainable waste management, since a variety of substrates, as liquid and solid wastes, can be used as feedstocks (Weiland, 2010). Methane-rich biogas (biomethane) is a versatile renewable energy source that can be used to produce heat, steam, electricity, fuel, chemicals and materials. Moreover, the residues of agro-industrial AD (namely, the digestate) may be valorised as organic fertilizers (Monlau et al., 2015; Weiland, 2010). Table 1 summarized the main advantages and disadvantages of AD. Further details on this technology were discussed below.

Table 1: Advantages and disadvantages of the anaerobic digestion technology (Bond and Templeton, 2011).

ADVANTAGES	DISADVANTAGES
• improved sanitation (reduced pathogens and disease transmission); • low cost energy source: cooking, lighting etc.; • low cost fertilizer: improved crop yields; • improved living conditions; • improved air quality; • reduced GHG emissions; • reduced nitrous oxide emissions; • less demand for alternative fuels (conservation of woodland, less soil erosion, time saved collecting firewood).	• laborious operation and maintenance; • limited lifespan (~20 years for many plants); • construction costly; • less suitable in cold and arid regions; • negative perception where low functionality of existing plants; • requires reliable feed source; • requires reliable outlet for treated sludge; • poor hygiene of sludge from mesophilic digestion; • high construction costs relative to income of many potential users

1.3.1 Stages and Microbiology

AD is a multistep bioprocess in which microorganisms transform organic matter into biogas, a mixture of gases composed mainly of methane (CH_4) and carbon dioxide (CO_2) (Vasco-Correa et al., 2018). AD is constituted by four distinct subsequent steps carried out by heterogeneous microbial consortia in strictly oxygen-depleted environments (Figure 3) (Vasco-Correa et al., 2018). During hydrolysis, simple soluble monomers (i.e., amino acids, fatty acids, monosaccharides) are produced from the hydrolyzation of complex polymers (proteins, lipids and carbohydrates) from the feedstocks. In the acidogenesis step, the hydrolysis products are converted to volatile fatty acids (VFA: acetic, propionic, butyric acids) (Li et al., 2011). Subsequently acetogenesis leads to the conversion of the VFA to acetate (CH_3COOH), CO_2 and H_2 (Gerardi, 2003). During the fourth step, methanogenic archaea produce CH_4 and other compounds either combining H_2 and CO_2 ($CO_2+4H_2 \rightarrow CH_4+2\ H_2O$) or transforming acetate ($CH_3COOH \rightarrow CH_4+CO_2$) (Merlin Christy et al., 2014; Wang et al., 2018).

Each AD stage is performed by distinct microbial consortia (Figure 3), each of them having its own optimal growth and activity conditions (Wang et al., 2018). Since AD steps are closely related with each other, AD process performance and stability strictly depend upon the equilibrium between the various microbial communities (Franke-Whittle et al., 2014). Bacteria belonging to various phyla, among which Firmicutes, Bacteroidetes, Tenericutes, Actinobacteria, Proteobacteria, Spirochaetes, Lentisphaerae, Synergistetes and Fibrobacteres (rumen bacteria), are responsible for the performance of the first three AD steps, while methanogenesis is performed by two main kind of methanogenic Archaea: the acetotrophic/acetoclastic methanogens, which are able to turn acetate into methane, and the hydrogenotrophic ones (CO_2 reducing methanogens), which instead use CO_2 and H_2 as substrates for methane production (Traversi et al., 2011; Ziganshin et al., 2013).

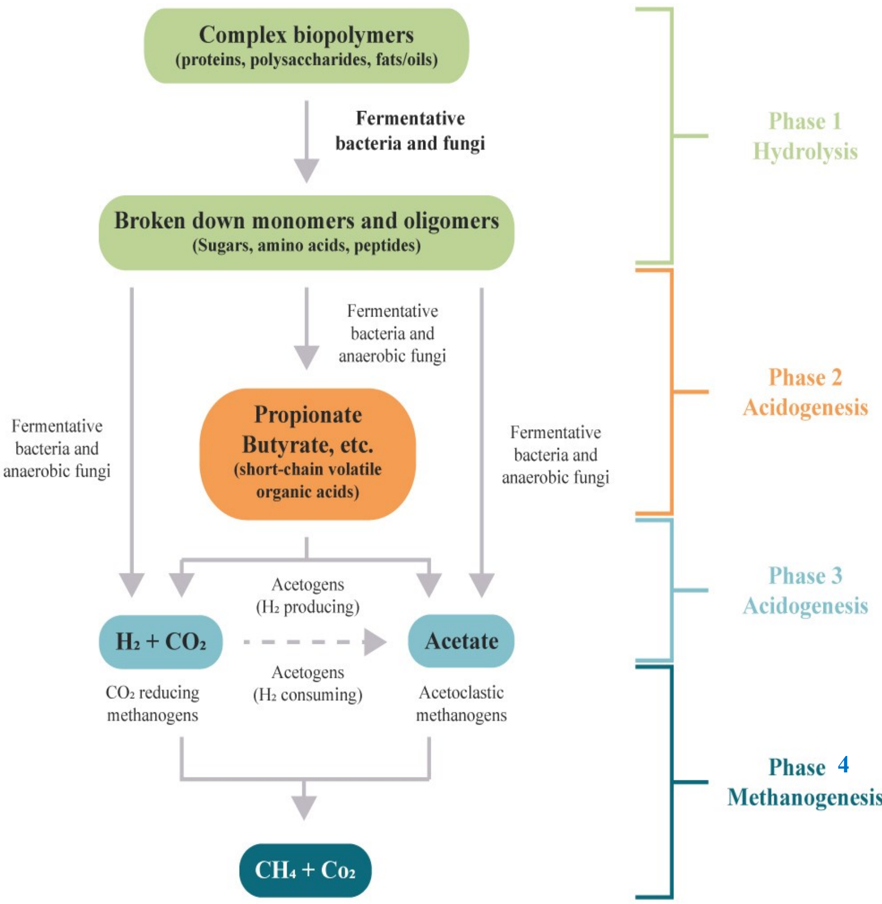


Figure 3: Phases of anaerobic digestion with the respective products formed and the microorganisms involved (https://www.e-education.psu.edu/egee439/node/727).

Concerning fungi, little is known on their role along the entire biogas production chain. Presumably, the strictly aerobic species are involved mainly during the hydrolysis since the change of pH values to alkaline conditions and the increasing oxygen depletion usually inhibit their survival. These fungi usually belong mainly to Ascomycota and Mucoromycota, while Basidiomycota are generally less represented; however, the fungal biodiversity strictly depends upon the characteristics of the used feedstocks (Young et al., 2018).

Furthermore, it is known that some yeast and mycelial microscopic fungi have the capacity for remain active under conditions of limited oxygen supply or in the absence of oxygen (Kurakov et al., 2008). Facultative and obligate anaerobic fungi can take part during hydrolysis and subsequent AD phases. Anyway, with the increasing of pH, their enzymatic activity generally decreases, since their optimal working requirements imply slightly acidic pH and mesophilic and microaerobic conditions (Young et al., 2018). Indeed, at alkaline pH values these fungi survive but are often metabolically inactivated. Obligate anaerobic fungi involved in the AD process usually belong to Neocallimastigomycota, which are typically associated to the digestive system of ruminants (Young et al., 2018). Indeed, as indicated by Fliegerová et al. (2010), their presence into anaerobic digesters depends on the introduction of ruminant manure for co-digestion with other feedstocks. Moreover, their community composition is strongly dependent upon physiological, physical and nutritional features of their animal host (Young et al., 2018).

1.3.2 Biogas Plants

AD is a natural process typical of many oxygen-free environments (Wang et al., 2018). Humans have learned to exploit and perform this natural process at industrial level in specific AD plants/bioreactors for biogas production (Figure 4), but also for wastes treatment in sewage treatment plants and landfills (Weiland, 2010). The microorganisms involved in the AD process are generally inoculated in the biogas plants trough fresh manure, sludge, or sewage sludge (i.e., inoculum source) (Liu et al., 2017). The operative conditions and the typology of feedstocks entering the biogas plant deeply affect the yields and quality of the AD products (Monlau et al., 2015; Vasco-Correa et al., 2018).

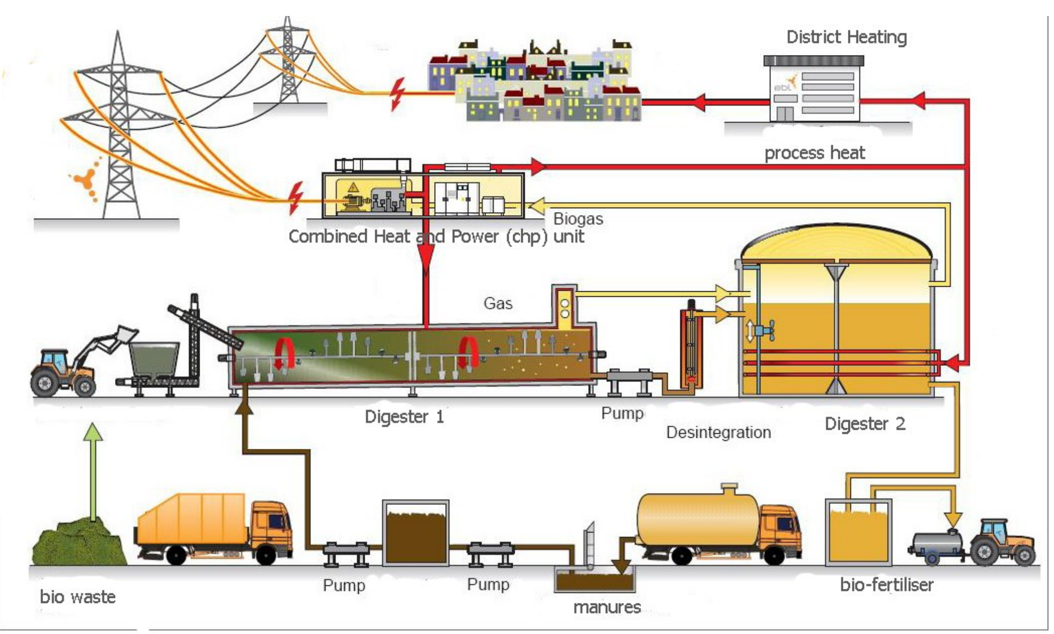


Figure 4: General scheme of an anaerobic digestion plant (http://www.fabbiogas.eu/en/home/).

The first European plant producing gas for the public supply was built in Germany in 1921 (Bond and Templeton, 2011). Intensive research began in the 1950s and after the 1970s oil crisis, and the AD process gained renewed appeal as a source of alternative energy (Carlsson et al., 2012; Vasco-Correa et al., 2018). Nowadays, AD is a well-established technology. In particular, Europe can be considered a world leader of this technology, with almost 18,000 AD plants and a total installed capacity of almost 10,000 megawatts electrical (MW) (EBA, 2017). Between 2009 and 2016, the total number of biogas plants rose from 6,227 to 17,662 installations (Figure 5). The growth was particularly strong from 2010 to 2012, mostly due to the increase in plants running with agricultural substrates (EBA, 2017). Concerning electricity generation from biogas, it grew from 1.1 million tons of oil equivalent (Mtoe) in 2005 to 5.0 Mtoe in 2014 in Europe. The annual growth rate for the period 2005-2014 in Europe was 18 %, among which

Germany, Italy and United Kingdom were the main producers sharing 54 %, 14 % and 12 %, respectively (EAA, 2017). In detail, in Italy the biogas plants were 1,555 at the end of 2015 with an electrical capacity of 1,200 MWel (EBA, 2016).

According to the most recent European Biogas Association (EBA) Statistical Report (2020), a total of 167 TWh of biogas are produced in Europe, equivalent to 15.8 billion cubic meters, and around 26 TWh of biomethane, equal to 2.43 billion cubic meters. At the end of 2019, this production corresponds to 18,943 biogas plants and 725 biomethane plants installed. The biomethane market has continued to grow significantly over the years: in 2019 production increased by 15% compared to the previous year. In addition, the EBA Statistical Report (EBA, 2016) has summarized the growth potential for biogas and there is a strong consensus that by 2030, the biogas-biomethane sectors can almost double their production and by 2050, production can more than quadruple. The potential biogas and biomethane production calculated for 2030 could reach up to 467 TWh. The predicted expansion of AD sector will bring significant economic and environmental implications and benefits for the European citizens.

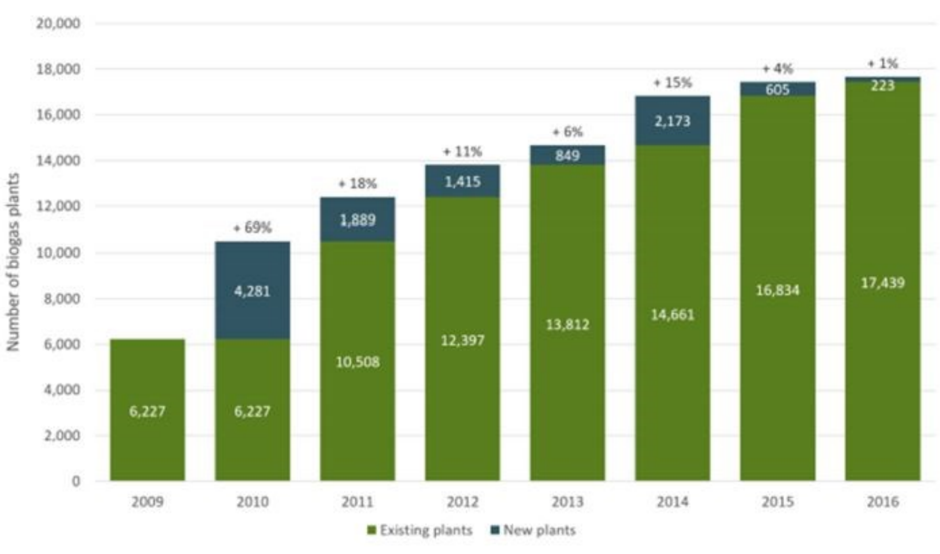


Figure 5: Number of biogas plants in Europe between 2009 and 2016 (EBA, 2017).

1.3.2.1 Bioreactors and Operational Parameters

Efficient bioreactors design and development are essential for the success of the AD process (Bouallagui et al., 2005). To be effective the operational parameters must allow maximum yields, while reducing process energy and heat loss, costs, and odour (Nasir et al., 2012). Usually, in order to reach this objective, reactors are designed in relation to the substrate quality (wet or dry, organic matter content, etc.), the microbial growth conditions (suspended or attached), and the kind of AD process to be performed (single- or two-stage AD) (Bouallagui et al., 2005; Nasir et al., 2012).

Anaerobic reactors and systems classification can be performed according to different criteria, including feedstock total solid content, the feeding configuration, the number of stages and the process temperature (Figure 6) (Monlau et al., 2015).

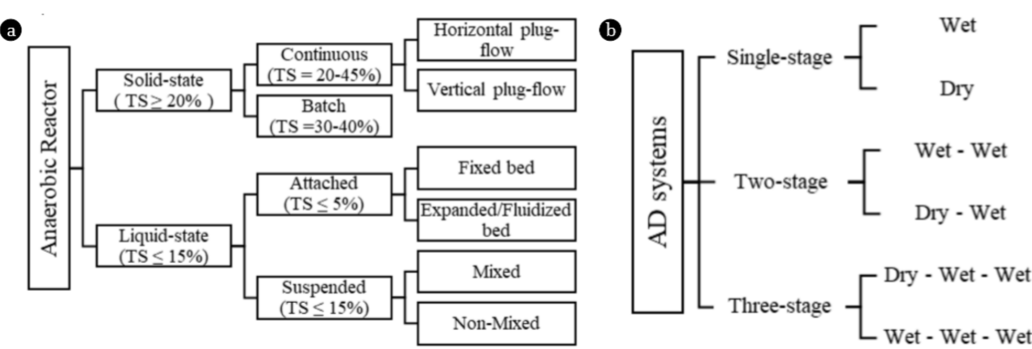


Figure 6: a) Classification of anaerobic reactor b) Classification of AD systems (Van et al., 2020).

The total solid (TS) content inside the anaerobic bioreactor is the parameter that most affects digesters design, performance, and costs (Van et al., 2020). Liquid-state (wet) digestion processes are operated with a TS concentration in the fermenter below 15% (Figure 6). In wet reactors the microorganisms can be

retained in two main different ways: suspended in the liquid by means of continuous mixing or attached to dedicated supports (Van et al., 2020). Instead, solid-state (dry) digestion processes are operated with a TS content inside the fermenter between 20 % and 35% (Figure 6). In this kind of digesters microorganisms can exploit the feedstocks particles as growth supports as well as source of nourishment (Van et al., 2020). The wet digestion processes dominate in the agricultural sector (Weiland, 2010). However, comparing dry and wet AD, the former is considered the most advantageous, since it allows smaller reactor volumes, lower energy inputs, higher organic loading rates (OLR) and lower costs, though it requires longer hydraulic retention time (HRT), which is an important limiting factor (Li et al., 2011; Sawatdeenarunat et al., 2015).

According to the feeding configuration, anaerobic bioreactors can be classified into batch and continuous systems (Figure 7). Batch digesters are fed only once, and then anaerobic digestion steps are performed sequentially till the end of the process (Figure 7a) (Bouallagui et al., 2005). Batch system is appealing for lab-scale experiments, since it is characterized by a simple design, easy process control and low costs, but it is rarely applied at industrial-scale process (Bouallagui et al., 2005). In contrast to the batch system, during continuous AD, digesters are continuously fed. According to the number of stages, continuous AD digesters can be classified into one-stage and multi-stage systems. In the single-stage system all four AD steps are performed in the same reactor (Figure 7b) (Van et al., 2020), thus compared with multi-stage AD it allows lower costs and easier design (Bouallagui et al., 2005). Moreover, arranging acidogens and methanogens in the same container helps to maintain digester equilibrium, since methanogens exploit H_2 formed by the acetogens for methane production, thus limiting the detrimental consequences of its accumulation, especially at high OLR (Bouallagui et al., 2005). Indeed, in such conditions acidogens activity increases at a higher rate than that of methanogens, leading to H_2 accumulation and, consequently, to AD inhibition

(Bouallagui et al., 2005). The aim of the multi-stage AD is to enhance the digester performance ensuring the refinement of each AD step by means of their physical separation, since each of them is performed by microorganisms with different environmental and nutritional needs (Van et al., 2020). Currently, there are available two-stage (Figure 7c) and three-stage systems. In the two-stage system, the first three AD steps are performed in one reactor, while methanogenesis is performed in a different one. Instead, in the three-stage systems, hydrolysis, acidogenesis/acetogenesis, and methanogenesis processes are physically divided into distinct reactors (Van et al., 2020). Among them, the two-stage AD was the most used and studied. However, though in many studies it was shown to perform better than one-stage AD, it is difficult to set the optimal conditions for both processes and harmonize them in order to enhance the efficiency of the whole system. Thus, in some cases, imbalances between the two processes and among microbial communities may occur, leading to lower methane yields (Ganesh et al., 2014).

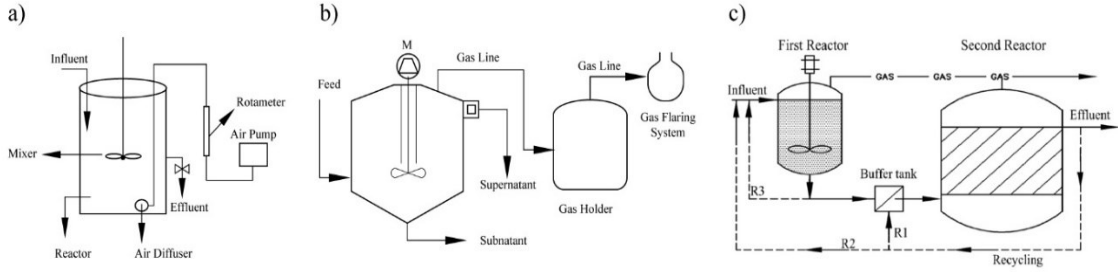


Figure 7: Different AD reactor schemes a) Batch reactor b) Continuous single-stage reactor c) Continuous two-stage reactor (Kushwaha et al., 2013; Van et al., 2020; http://www.lippsilos.com).

Based on the temperature set in the bioreactor, AD process is performed in mesophilic (30–40 °C), thermophilic (50–60 °C) or psychrophilic (below 20 °C) conditions. The mesophilic and thermophilic conditions are the most commonly employed (Wang et al., 2018). In comparison, thermophilic digestion allows higher metabolic rates and biogas yields than mesophilic AD (Wang et al., 2018). However, it also presents many drawbacks, such as lower stability, effluents richer in unprocessed VFA, higher expenses and energy inputs, and higher sensitivity towards acidification (Mao et al., 2015; Wang et al., 2018). Instead, mesophilic systems are able to sustain higher OLR and are characterized by higher stability and biodiversity, though they also show lower biogas yields (Mao et al., 2015; Wang et al., 2018). Temperature, together with the digester's operational parameters and feedstock quality, influences the efficiency of organic matter conversion, which usually ranges between 13–65% for mesophilic and thermophilic AD, due to its capability to affect substrate characteristics (i.e., solubility) and the composition of the AD microbial communities (Monlau et al., 2015; Van et al., 2020).

Other main operative parameters that influence AD are the pH, the OLR and the HRT. Microorganisms involved in AD are extremely sensitive towards pH changes (Mao et al., 2015). Among them, methanogens are the most vulnerable, since they can grow only in a relatively narrow range of pH (6.2-7.8) and are also significantly susceptible towards the presence of oxygen (O_2), free ammonia, hydrogen sulphide (H_2S), and VFA (Van et al., 2020). pH drops due to VFA accumulation during acetogenesis are extremely detrimental for the success of the whole AD process since they cause microbial inhibition and can lead to process failure. In order to prevent such negative consequences, the stability of the reactor could be improved by providing proper alkalinity, for instance, using additivities or alkaline biomasses (Hegde and Pullammanappallil, 2007).

The OLR represents a measure of the amount of organic matter supplied to the digester per unit of time (kg or m^3 per day), and it is strictly connected to the digester HRT, which represents the substrate processing duration (Van et al., 2020). The OLR parameter is particularly important for continuous feeding digesters. Increasing OLR usually results in higher biogas yields; however, too high OLR may lead to imbalances between microbial communities due to digester acidification (Mao et al., 2015). The resultant methanogens inhibition causes inefficient VFA conversion to biogas, thus digester effluents result to be richer in unprocessed VFA (Rincón et al., 2008).

The HRT represents the amount of time during which the feedstocks are retained into the reactor, thus in contact with AD microflora (Van et al., 2020). It is inversely proportional to the process temperature and depends on the feedstocks supplied (Vasco-Correa et al., 2018). In wet reactors, the HRT is divided into two different parameters: the liquid and solid HRT, since feedstocks dedicated to this kind of digesters are often composed by a separable liquid and solid phases. In order to maximize AD process efficiency, it is necessary to set optimum HRT. In fact, a long enough HRT is required to guarantee the accomplishment of all AD phases and maximize the process efficiency (Van et al., 2020). In fact, too short HRT results in VFA conversion inefficiencies and in consequent lower biogas yields (Mao et al., 2015; Van et al., 2020). Longer than optimal HRT, instead, leads to increased reactor volumes and thus to higher operational costs (Van et al., 2020).

In conclusion, in order to obtain maximal methane yields, AD operational parameters are often set with low OLR and long HRT (Mao et al., 2015).

1.3.3 Feedstocks

AD is a very flexible technology as, theoretically, every organic substrate could be exploited as raw material/feedstocks to produce biogas (Amon et al.,

2007). Thus, AD is a captivating technology for the treatment of a wide range of liquid and solid organic wastes and it can contribute to sustainable waste management (Vasco-Correa et al., 2018). However, the use of some feedstocks presents strong limitations related to process microbiology, AD plant technology, country legislation and biomass properties (Weiland, 2010). According to Vasco-Correa et al. (2018), the feedstocks used for AD may be grouped in five categories, whose main pros and cons are summarized in Table 2.

Table 2: Advantages and disadvantages of AD feedstocks (Vasco-Correa et al., 2018).

FEEDSTOCKS	ADVANTAGES	DISADVANTAGES
LIVESTOCK EFFLUENT	• low cost; • abundant availability; • waste valorisation; • complete source of nutrient and elements; • used in co-digestion; • easy use of digestate.	• low methane yield; • presence of antibiotics; • technical problems.
DEDICATED CROPS	• easily available; • easy storage; • high biogas yield; • high value digestate.	• high costs; • high lignocellulose content; • pretreatment needed; • high production of N rich digestate; • technical problems.
RESIDUAL CROPS + **ORGANIC FRACTION OF MUNICIPAL SOLID WASTE**	• low costs; • abundant availability; • contribute to sustainable waste management; • high biogas yield	• management and storage problems; • high lignocellulose content; • complex management of the process; • pretreatment needed; • high production of N rich digestate; • technical problems.
SEWAGE SLUDGE	• low costs; • contribute to sustainable waste management	• high solid, pathogen and nutrient content; • pretreatment needed; • low digestibility.

1. Livestock effluents: these substrates are derived from animal breeding. Effluents may be liquid or solid (slurry and manure, respectively) and their composition may be variable depending on the animal, management and seasonality. In particular, the presence of recalcitrant compounds as heavy metals or antibiotics may inhibit microbial activity decreasing the process performance. Nevertheless, the use of these substrates could be positive for farmers, because the digestate obtained from agrozootechnical feedstocks could be used as fertilizer, reducing waste disposal costs. Moreover, AD process may stabilize effluents, reducing malodorous emissions. However, they have generally low methane yields, and they can give technical problems (e.g., floating crusts that negatively affect microbial activity) during the process (Vasco-Correa et al., 2018).

2. Dedicated crops, as corn, sorghum, rye, etc. The use of dedicated crops is strongly criticized due to the food/energy competition. Since these crops are used both for human and animal nutrition, several objections were raised mostly concerning the over-exploitation of lands. They can be considered fine biomasses and their cost is very high compared to other substrates. Nowadays the use of dedicated crops is decreasing because new plant technologies allow the use of different substrates (Vasco-Correa et al., 2018). Moreover, the European (Directive 2018/2001) and Italian (DM 23/06/2016) legislation provides lower subsidy for biogas plants that use dedicated feedstocks for the process, trying to limit their use.

3. Residual biomasses, such as agricultural waste, agro-industrial by-products (corn stover, wheat straw, and rice straw); recently their use became relevant because of their economic sustainability. Nowadays the use of residual crops is strongly encouraged since they may contribute to sustainable waste management, adding value to these biomasses instead of discharging them (Vasco-Correa et al., 2018).

4. Organic fraction of municipal solid waste (OFMSW), as food, leaf, and yard waste. The composition of these substrates is considerably heterogeneous. Their use may contribute to sustainable waste management (Cesaro and Belgiorno, 2014).

5. Sewage sludge as by-products of wastewater treatment. Due to its low digestibility, sewage sludge may be used in combination with other substrates to increase biogas yields. These biomasses may have pathogens contents, which do not make them a favorable substrate (Vasco-Correa et al., 2018).

Co-digestion of two or more mixed substrates, such as dedicated crops (e.g., maize silage) and livestock effluents (e.g., animal manure/slurry), is usually employed in AD plants as it can enhance their performance by modifying limiting characteristics (pH, C/N ratio) or giving an adequate organic loading to the composed substrate (Tišma et al., 2018).

As feedstocks characteristics are highly variable and significantly affect AD parameters and efficiency, the aware choice of the feedstock is crucial for AD process optimization (Li et al., 2011; Weiland, 2010). The knowledge of the organic composition and the influence of the target biomass on process microbiology are mandatory also because substrate costs may affect for 70 % of the total costs of the process (Castelli and Sannazzaro, 2011). The choice depends on biomass properties as chemical composition, methanogenic potential, feedstock availability, environmental, and economic feasibility (Castelli and Sannazzaro, 2011). Thus, biomasses destined to AD should be evaluated for:

- Total Solids (TS) content: organic and inorganic compounds content;
- Volatile Solids (VS) content: organic matter content; this parameter represents the fermentable mass of a substrate;
- Nitrogen and Carbon contents;

- Lignocellulose (cellulose, hemicellulose and lignin) content;
- Biochemical Methane Potential (BMP): the highest amount of methane produced by a biomass; it is usually expressed as normal litres ((L_N) or cubic meters per kg of volatile solids (L_N/kg VS¸ m^3_N/kg VS) (VDI, 2006).

1.3.4 Products and by-products

During AD, the organic matter of feedstocks is transformed into biogas and the by-product digestate, whose amount and quality rely on feedstock features, inoculum source and reactor parameters (Monlau et al., 2015).

Biogas

Biogas is a gaseous mixture consisting mainly of methane, carbon dioxide, oxygen, hydrogen, nitrogen, carbon monoxide, hydrogen sulfide and water, but it may also contain other compounds such as siloxanes, ammonia, halogenated hydrocarbons, aromatic hydrocarbons and halogens (Vasco-Correa et al., 2018). An example of general biogas composition in reported in Table 3.

Table 3: General composition of biogas produced by AD (MJ/ m^3_N = MegaJoule/normal cubic meters; m^3_N = measure at 0 °C and 1 atm of pressure) (Weiland, 2010).

Methane (CH_4)	45-70 %
Carbon dioxide (CO_2)	25-55 %
Oxygen (O_2)	0.01-2%
Hydrogen (H_2)	1-10 %
Nitrogen (N_2)	0.01-5 %
Carbon monoxide (CO)	0.1 %
Hydrogen sulphide (H_2S)	0.005-2 %
Water (H_2O)	saturation
Lower calorific value	18.8-21.6 MJ/ m^3_N

Biogas can be exploited for energy production in different ways. Usually, before being utilized, biogas is cooled, dried and deprived of hydrogen sulphide (H_2S), which is detrimental for AD plants components due to its corrosivity (Holm-Nielsen et al., 2009; Weiland, 2010). Cogeneration (production of both heat and electricity) trough combined heat and power (CHP) units is the most common and effective way to exploit raw biogas for energy purposes, tough it can also be burnt to produce heat or steam (Vasco-Correa et al., 2018). Starting from raw biogas it is possible to obtain biomethane through CO_2 removal (Vasco-Correa et al., 2018). Since biogas has lower methane concentration in comparison with natural gas, such upgrading step is useful to enhance its heating value (Vasco-Correa et al., 2018). The resultant refined biomethane can be used for electricity production trough fuel cells or as transportation fuel (after being compressed) (Vasco-Correa et al., 2018). Furthermore, it can be converted into liquid drop-in fuels, after previous transformation into bio-syngas, or into other high value chemicals, such as methanol (Vasco-Correa et al., 2018).

Digestate

Digestate is the main by-product of AD; it is mainly composed by water, inorganic compounds (e.g., nitrogen, N; phosphorus, P; potassium, K) and the undigested organic matter (Monlau et al., 2015). The original feedstocks composition (e.g., C/N ratio) has a great influence upon digestate characteristics (e.g., ammoniacal nitrogen/total nitrogen content) (Dinuccio et al., 2013; Monlau et al., 2015). In fact, feedstocks characterized by low C/N led to higher ammonia (NH_4^+) production and thus to higher NH_4^+/total N ratios (Möller and Müller, 2012; Monlau et al., 2015). On the contrary, feedstocks characterized by higher C/N ratios, such as lignocellulosic residues, lead to lower ammonia production in the digestate (Möller and Müller, 2012; Monlau et al., 2015). Digestate also contain other macro-nutrients - i.e., P, K, etc. - and trace elements - i.e., cobalt (Co), iron

(Fe), nickel (Ni), etc. - which impact its quality, determining the potential applications (Monlau et al., 2015).

Digestate can be managed in its raw form, but it is often mechanically separated into a liquid and a solid fraction that have different characteristics (Monlau et al., 2015). The solid/liquid separation of digestate is advantageous, since it allows to reduce transportation and storage costs but also to lower volume, ease its storage and make both fractions easier to be handled and exploited for other applications (Guilayn et al., 2019). Currently, there are many digestate separation methods among which the most commonly utilized are decanting centrifuges, screw presses, filter presses and vibrating screens (Guilayn et al., 2019; Monlau et al., 2015). The employed separation technology affects the resultant solid and liquid fractions characteristics, since each of them present different separation efficiency, reliability and ability to provide adequate nutrients partitioning among liquid and solid fractions, as well as different operational costs which can limit some of these technologies' viability to high volume plants (Guilayn et al., 2019; Monlau et al., 2015). After separation, the liquid fraction retains a high amount of water, K and ammoniacal N, while the solid fraction of digestate (SFD) is mainly composed of P and residual fibers (Table 4). Since plant cell wall polymers (PCWP), as cellulose and lignin, undergo relatively little changes during AD, they are conveyed from the lignocellulosic feedstocks mainly into the SFD (Dinuccio et al., 2013; Monlau et al., 2015).

Complete digestate conditioning implies further treatments of both digestate fractions in order to obtain valuable end products, which usually consist of pure water, mineral nutrients, and fibers (Lukehurst et al., 2010). In fact, purified water may be obtained from liquid digestate by means of several treatment technologies, among which membrane filtration, evapo-concentration, N-stripping and struvite crystallization. Resultant water could eventually be reused in agriculture and in AD plants or be dismissed into sewage treatment plants or directly into water bodies

(Guilayn et al., 2019; Lukehurst et al., 2010). Solid digestate, instead, can be further treated in different ways in order to be valorised and used for other applications, thus making AD a more sustainable process (Guilayn et al., 2019).

Table 4: Example of chemical composition of whole digestate (D) and solid fraction of digestate (SFD) (Sambusiti et al., 2015).

	D	**SFD**
pH	8.1	8.7
TS (% = g/100 g wet biomass)	8.3 ± 0.2	21.6 ± 0.1
VS (% TS = g/100 g TS)	72.7 ± 0.2	83.8 ± 0.3
Cellulose (% TS = g/100 g TS)	13.5 ± 1.8	17.5 ± 0.9
Hemicelluloses (% TS = g/100 g TS)	15.1 ± 1.4	20.3 ± 1.9
Klason lignin (% TS = g/100 g TS)	21.2 ± 1.4	24.1 ± 0.6
Ash (% TS = g/100 g TS)	14.8 ± 0.5	12.9 ± 1.1
Total Nitrogen (TN) (% TS = g/100 g TS)	6.4 ± 0.5	1.0 ± 0.0
Total ammonia nitrogen (TAN) (% TS = g/100 g TS)	4.8 ± 0.2	n.d.

As both fractions of digestate still retain large amounts of nutrients, their use for agriculture purposes has raised particular interest (Monlau et al., 2015). In Italy, until 2012, digestate was considered a waste, therefore its use for agricultural purpose was not allowed. The Decree Law 134, art. 52, comma 2 bis of the 7/08/2012, established that digestate could be used as fertilizer after some conditioning and pretreatments. Recently the Decree Law 5046 of the 25/02/2016, regulated the production and the use of digestate depending on the biomasses used for the AD process. In particular, digestate derived from agricultural biogas plants (ABP), that use mixture of livestock effluents, agricultural wastes or dedicated crops as feedstocks, can be used as organic fertilizer and/or soil amendment; however, the application of digestate on soil must respects the limit imposed by the

European Union Nitrate Directive (91/676/EEC), that establish the maximum amount of N that can be applied per hectare in a year. This result advantageous for farmers, reducing digestate disposal costs and providing an added-value product. On the other hand, digestate derived from industrial AD plants, which use OFMSW and sewage sludge as feedstocks, needs an additional composting step to ensure its microbiological safety. Furthermore, according to EU Regulation EC1772/2002, some substrates, such as food waste, OFMSW need to be sterilized before AD to avoid the presence of pathogens (e.g., *Salmonella* and *Clostridium*). They also need further pretreatments to limit the presence of heavy metals and toxic compounds in by-products before AD (Ariunbaatar et al., 2014).

However, besides its benefits, digestate production and utilization in agriculture presents also some concerns related to its safe management and ever-increasing production (Monlau et al., 2015), which will be discussed below.

1.4 Challenges with Anaerobic Digestion

1.4.1 Use of energy crops and competition with the feed-food market

The increasing use of dedicated crops for energy purposes has raised concerns about the competition for arable land use with crops dedicated to feed and food production (Fritsche et al., 2010). The extensive land use changes in favor of energy crops plantations may affect global food prices and food security, but also ecosystem services, soil properties (i.e., carbon stocks) and increase the loss of habitats (López-Bellido et al., 2014; Shortall, 2013). The "food versus fuel controversy" concerned particularly the first-generation biofuels production, since food crops (i.e., corn, wheat, sugarcane) were grown specifically for energy production rather than for alimentation purposes (Fritsche et al., 2010; Valentine et al., 2012). Thus, their use for one purpose was in direct competition with their use

for the other (Valentine et al., 2012). Among first-generation energy crops, corn was the most criticized since its indiscriminate exploitation for energy purposes negatively affected the agro-ecosystems (Ziganshin et al., 2013). Currently, in European AD plants, energy crops, and especially maize silage (MS), are among the most employed lignocellulosic feedstocks (Hutnan, 2016). However, the competition for available land use occurs also for plantations of lignocellulosic crops dedicated to energy productions (i.e., miscanthus, switchgrass, willow, and eucalyptus) (Fritsche et al., 2010). In order to achieve a more sustainable exploitation of energy crops, their management should aim at improving crops quality and stress tolerance, thus limiting the land required for their production (Zegada-Lizarazu et al., 2010). Furthermore, they should be grown in sustainable crop rotations able to provide food, fodder, but also energy and other valuable products (i.e., oils, organic acids, etc.) (Amon et al., 2007). Thus, under such a scheme, it would be possible to reach a sustainable biogas production from energy crops if it would be based on maximum outcomes from the entire system rather than from single crops (Amon et al., 2007).

Improvements in bioenergy systems sustainability could also be reached by employing agricultural residues, such as corn stover and straw, since their utilization do not compete with food crops cultivation and could be even considered to be complementary to food production (Ziganshin et al., 2013). However, also the utilization of such lignocellulosic residues is quite controversial, since their presence onto the soil is necessary to maintain land fertility, preventing erosion, organic losses, and the consequent reduction of soil agronomic productiveness (López-Bellido et al., 2014). Therefore, before their utilization for bioenergy production it is necessary to estimate their impact on soil organic matter content, properties, and ecological services (Zegada-Lizarazu et al., 2010). Other materials which do not require land for their production, such as animal by-products, wood processing residues and food wastes can be used as feedstocks in order to improve the sustainability (Fritsche et al., 2010), although they are often

refractory to microbial degradation due to their recalcitrant physicochemical characteristics, such as imbalanced C/N ratios, high amount of lignocellulosic fibers and the presence of microbial hindering compounds (Ziganshin et al., 2013).

Marginal land exploitation for energy production is also considered an attractive way to overcome land use controversies, since it could represent an extra income for farmers without competing with food production (Shortall, 2013). However, it is necessary to consider the quality and extent of lands that are “really” available since many areas may be devoted to biodiversity preservation or may be unsuitable for cultivation due to soil characteristics or water deficiency. As a consequence, the areas that are potentially suitable for this application are fewer than previously presumed (Fritsche et al., 2010). Moreover, in order to obtain an effective agronomic productivity, this kind of soils often needs expensive reclamations in order to recover their fertility, which result in higher production costs (Fritsche et al., 2010). Hence, also the exploitation of marginal lands is quite controversial.

In conclusion, it is of increasing importance to find new sources of biomass able to yield high amount of energy while being economic, “green” and not in competition with food production (Ertem et al., 2017), also to achieve the objectives indicated by the European legislation. Indeed, the EU Directive 2015/1513 aims at reducing the competition for land use by limiting the consumption of biofuels derived from food or feed crops to 7% of the entire biofuels’ consumption in 2020.

1.4.2 Incentive policies

Financial incentives for renewable energies production have turned out in boosting the number of AD plants worldwide (Vasco-Correa et al., 2018). In fact, the spreading of this technology is inevitably linked with countries policies and incentives in different sectors, among which energy, environment and agriculture

(Vasco-Correa et al., 2018), but also with socio-economic barriers and other limiting factors (Scarlat et al., 2018). In general rules, countries with more stringent regulations present higher numbers of AD plants (Vasco-Correa et al., 2018).

In Italy the subsidization policy has caused an increment of biogas production from energy crops, especially in the Pianura Padana, which has the highest concentration of AD plants in the country (Figure 8) (Bartoli et al., 2016). This policy was based on the feed-in tariff (FIT) introduced in Italy in 2009 (Law 99/23 July 2009), which allowed all plants having an electric capacity lower than 1 Megawatt electric (MWe) to receive for 15 years the all-inclusive feed-in tariff (0.28 €/kilowatt-hour (kWh) (Bartoli et al., 2016). Such incentive scheme ensured to the farmers high profitability. As a consequence, the farmers started to exploit highly energetic feedstocks, such as corn and other cereals, in order to maximize the biogas yields (Vasco-Correa et al., 2018). The main detrimental consequences were land use changes in favor of energy crops cultivation (from 0.5% to 10% of arable land) and increased fodder prices, in a country where livestock production is one of the major trading forces (Bartoli et al., 2016). Thereby, agri-food supply chains were suffering from the competition with the bioenergy sector for corn production (Bartoli et al., 2016). Moreover, in order to bump up subsidies, farmers built oversized plants with capacities close to 1 MWe and dedicated to electricity production alone instead of cogeneration, which would have been a more energetically efficient process (Bartoli et al., 2016), leading to economic and environmental issues, such as the increasing of land required for corn production and of GHG emissions due to corn transport from farther areas for biogas plants feeding (Chinese et al., 2014). Furthermore, the public support to green energy decreased because of a contestation about presumed high costs of renewable energy support schemes demanded to end users (Chinese et al., 2014). Thus, changes in the Italian incentive policy were required in order to increase the sustainability of AD sector.

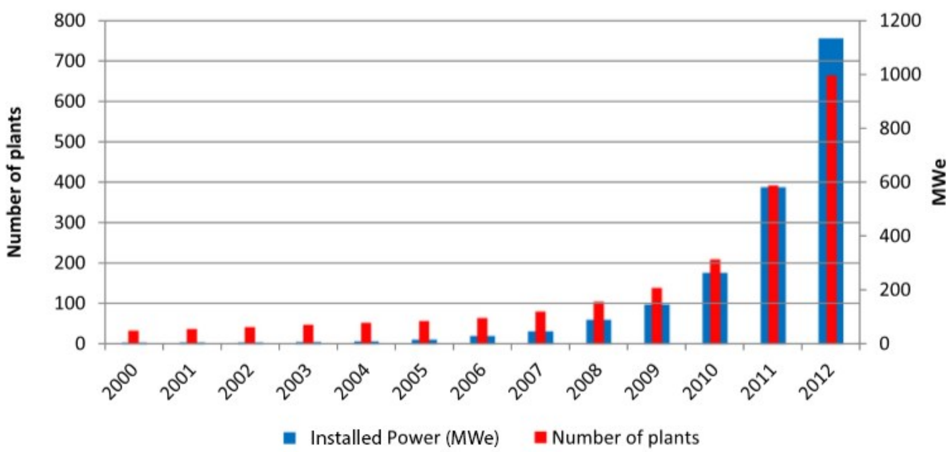


Figure 8: Trend of the number of biogas plants and the installed power (MWe) in Italy from 2000 to 2012 (Bartoli et al., 2016).

Actually, in 2012 a new subsidization policy (Decree of the Minister of Economic Development of 6 July 2012), more similar to those of the other European Countries, was adopted in Italy (Bartoli et al., 2016; Chinese et al., 2014). Under this new support plan (applied from January 2013) the plant owners were entitled to receive lower payments, cogeneration was encouraged trough higher bonuses and the adoption of smaller plants was promoted through the application of new criteria (Bartoli et al., 2016). Furthermore, in order to foster the substitution of energy crops with by-products and wastes as feedstocks, subsidies were associated with the chosen type of feedstock to feed the plants (Bartoli et al., 2016). Thereby, the adoption of this new incentive system allowed to lighten the competition for land use and lower maize price, since plant owners were motivated to choose different feedstocks rather than maize silage (Bartoli et al., 2016). However, the application of the new incentive scheme assures lower plants profitability (return on investment: ROI) and higher sensitivity towards maize price, if compared with the FIT policy. In fact, since subsidies are lower, plant owners cannot exploit maize silage as a feedstock when its price increases, while

with the past incentive system the ROI was sufficiently high even when expensive feedstocks were used (Bartoli et al., 2016). Overall, two main effects are likely to derive from this new incentive system: the deterrence towards investments on biogas production or the achievement of biogas sector and agri-food chains integration (Bartoli et al., 2016). Thus, in order to not discourage further investments on biogas sector there is the need to find more profitable feedstocks able to raise the ROI of biogas plants without affecting the agri-food sector.

1.4.3 Digestate issues

The AD plants continuously produce huge amounts of digestate that need proper storage and management. In fact, the agricultural digestate cannot be used immediately on farmlands due to its stabilization level, crop growth stage and soil type and the quantity that can be applied to the soil is limited according to regulations set by the European Nitrate Directive (Bartoli et al., 2016; Holm-Nielsen et al., 2009; Paavola and Rintala, 2008). Digestate is usually stored within aboveground uncovered tanks or platforms, and previous studies (Gioelli et al., 2011; Hansen et al., 2006; Menardo et al., 2011b) have reported that emission of different gases (i.e., N_2O, CO_2, CH_4 and NH_3) into the atmosphere may occur during storage and land use, as it retains high amount of undigested organic matter (VS). Moreover, the increasing number of ABP and their confluence in specific geographical area (e.g., Northern Italy) might lead to local oversupply (Bartoli et al., 2016) and the need to transport the excess to areas with nutrients deficits, increasing the overall costs of the process (Sambusiti et al., 2015). These problems ultimately lead to a consistent environmental impact (atmospheric contamination, increased plants GHG emissions and impacts on global climate) and in the loss of energetic efficiency (Kataki et al., 2017; Menardo et al., 2011b). Therefore, it is of growing importance to find innovative valorisation routes for digestate, in order to enhance AD process sustainability and avoid waste accumulation.

Due to its physicochemical characteristics, digestate was claimed to be a suitable organic fertilizer, since it is analogous to animal manure but enriched in N and P contents (Möller and Müller, 2012; Monlau et al., 2015). Its land application has demonstrated to be beneficial to enhance soils agronomic productivity and health (Kataki et al., 2017). However, besides its advantages, digestate land application brought up also some concerns (Monlau et al., 2015). In fact, digestate may result to be phytotoxic and even potentially harmful for the environment and human health due to its high ammonium content, which in some cases could reach that of landfill leachate (2266 mg/L), and the presence of organic acids and high saline concentrations (Kataki et al., 2017; Tigini et al., 2016). Other chemical compounds linked with digestate toxicity consist of high concentrations of copper (Cu) and zinc (Zn), which are often added to animal forage (Sawatdeenarunat et al., 2015), but also of manganese (Mn) and magnesium (Mg) (Bres et al., 2018), the presence of antibiotics, disinfectants (Lukehurst et al., 2010) and steroid hormones (Monlau et al., 2015). The presence of physical contaminants, such as plastic, glass, stones, etc., can negatively impact the environment as well, since it causes both pollution and aesthetic damages, but also detriments to AD plants components leading to increased maintenance costs (Lukehurst et al., 2010). However, it is possible to avoid their presence into the digestate by making an accurate selection of the biomass entering the bioreactor (Lukehurst et al., 2010). Eventually, the presence of pathogens in the digestate may result in health hazards for animals and human beings (Lukehurst et al., 2010). Therefore, in order to avoid soil contamination and health hazards, digestate needs to be subjected to strict controls according to the regulations established by countries. Limitations imposed by governments about digestate utilization, especially for agricultural purposes, encourage the research of alternative ways to treat and exploit digestate, in order to increase AD process sustainability (Monlau et al., 2015).

1.5 Anaerobic Digestion Biorefinery: valorise the by-products to enhance the sustainability

Since the number of AD plants, and the consequent production of digestate is constantly increasing worldwide, the necessity to find alternative ways to valorise and exploit it as an input material for other valuables production is growing of importance (Monlau et al., 2015). The AD biorefinery (Figure 9) is an integrative approach to biomass conversion into a wide range of valuables such as fuels, energy, but also chemicals, fertilizers and other materials (Monlau et al., 2015). The hallmark of this approach is the integration of outputs generated as raw materials for other resources production (Monlau et al., 2015; Vasco-Correa et al., 2018). Thereby, digestate becomes a profitable product potentially able to increase AD process efficiency and, consequently, its economic and environmental effectiveness (Monlau et al., 2015).

For instance, liquid digestate may be suitable for microalgal sustenance (Monlau et al., 2015). Indeed, in a biorefinery approach, microalgae may be potentially used as alternative feedstocks to energy crops, since they are characterized by high photosynthetic efficiency and rapid biomass production (Ertem et al., 2017; Monlau et al., 2015). Furthermore, before being utilized for biogas production, microalgae can provide oils and other raw materials that can be transformed into high-value chemicals and liquid and gaseous fuels (Ertem et al., 2017; Monlau et al., 2015). After its complete exploitation, spent algal biomass can also be applied as fertilizer (Ertem et al., 2017), contributing to the overall sustainability of AD process (Monlau et al., 2015). However, despite the benefits of growing microalgae in liquid digestate, some issues need to be mentioned: firstly, high operational costs of microalgal cultivation represent a limit in the spreading of this technology; secondly, liquid digestate allows lower biomass and fats yields than synthetic media due to its turbidity, ammonia inhibition, nutrient

availability and the presence of other inhibitory compounds (i.e., heavy metals) (Monlau et al., 2015). Moreover, bacteria contaminations may occur and induce nutrient competition, changes in digestate characteristics (i.e., turbidity, pH, etc.) and sanitary hazards (Monlau et al., 2015). Although there are still many aspects to be optimized in order to achieve a better viability of this technology, its coupling to AD is still an undoubtedly favorable solution for efficiency improvement (Monlau et al., 2015). For example, according to Simonazzi et al., (2019), the exploitation of CO_2 and liquid digestate derived from AD process could increment *Phaeodactylum tricornutum* biomass yields, improving its eicosapentaenoic acid production and allowing a better economic viability of this technology.

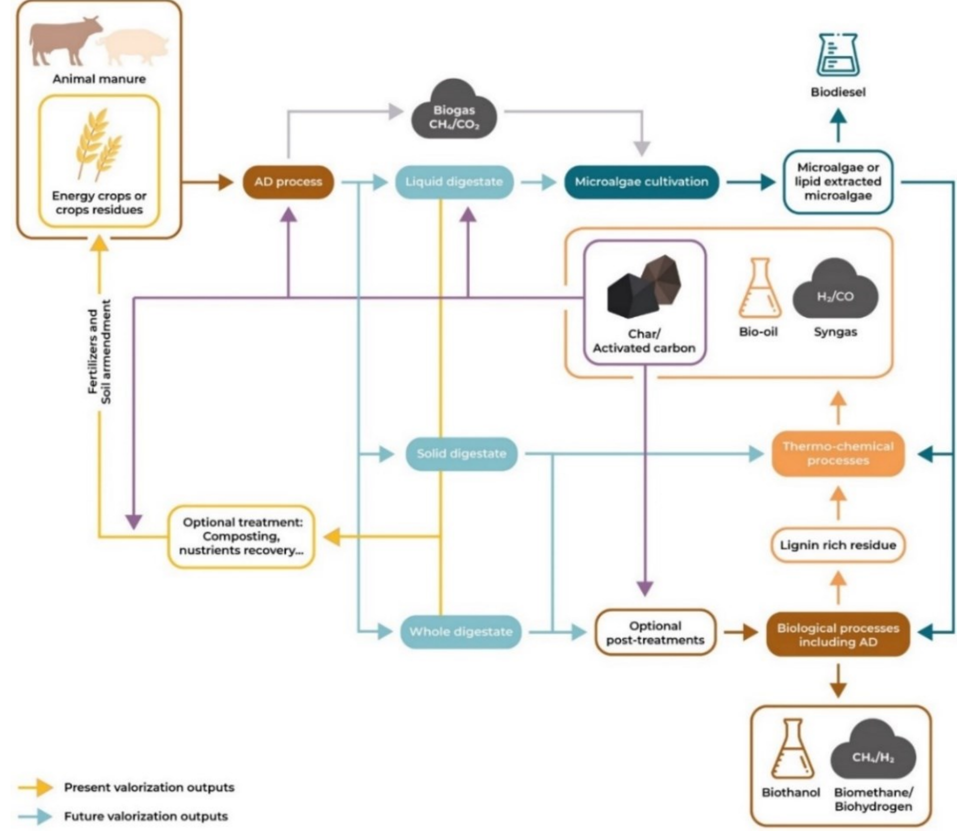


Figure 9: AD biorefinery scheme, showing AD products and their exploitation opportunities to increase AD sustainability (Monlau et al., 2015).

Solid digestate (SFD) valorisation, instead, may be performed through its conversion into different kinds of fuels by means of biological or thermo-chemical processes, since it is still rich of undigested organic matter (mainly fibers) (Monlau et al., 2015). A scheme with some examples of thermo-chemical solid digestate valorisation is displayed in Figure 10.

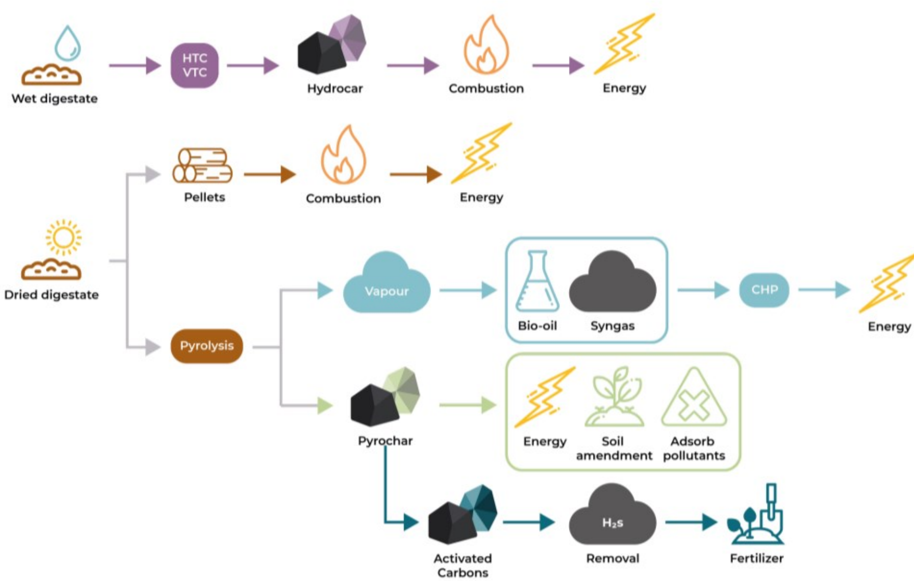


Figure 10: Examples of solid digestate valorisation through thermo-chemical processes (Monlau et al., 2015).

Among thermo-chemical processes, hydrothermal carbonization and vapo-thermal carbonization can be performed on wet organic biomasses, converting them into hydrochar, which is a carbon-rich solid fuels whose features are close to those of fossil coal (Monlau et al., 2015). Digestate conversion into hydrochar allows improvements in AD energetic efficiency, since it grants a better energy recovery, but also sanitization of the digestate and improvements of its fuel properties (Pawlak-Kruczek et al., 2020). Biochar performance is strictly linked with its original feedstock composition; for instance, according to Monlau et al.

(2015) digestates lead to the production of high performance hydrochar due to their high lignin content, while Parmar and Ross (2019) tested the fuel properties of four biochars derived from different digestates concluding that none of them was suitable to be used as solid fuel, but only as soil amendment (Parmar and Ross, 2019). Pawlak-Kruczek et al. (2020) instead, obtained, from digestate, biochar suitable both to be combusted for energy production, to be used as feedstock for pyrolysis and to be added as supplements into biogas plants, since their porosity could favor the immobilization of AD microorganisms (Pawlak-Kruczek et al., 2020). Similarly, Xu et al. (2018) observed that the addition of hydrochars into AD plants allowed to reach an increase in the methane yields of pig carcasses from 57.5% to 69.8%.

Pyrolysis and combustion, instead, need to be performed on dried up organic matter to operate correctly; therefore, digestate dedicated to these processes needs a drying pretreatment (Monlau et al., 2015).

Combustion process consists of burning pelletized digestate and it is considered an attractive method for digestate energy recovery since it can be performed through accessible burning technologies and pellets calorific value is close to those of the wood (Kratzeisen et al., 2010; Monlau et al., 2015). Anyway, pellets combustion produces large amounts of ashes that may suffocate the combustion by occluding furnace holes (Kratzeisen et al., 2010; Monlau et al., 2015).

Pyrolysis, instead, consists of combusting dry digestate under oxygen-depletion conditions. Pyrolysis products are the pyrochar, whose feature are analogous to hydrochar, and the vapour, whose cooling produces two distinct products: the bio-oil, which is a liquid composed by polar high molecular weight compounds, and the syngas, which instead is a gas composed by volatile compounds (Monlau et al., 2015). Liquor derived from this process can be also recycled for biogas production, increasing process sustainability and profitability (Hübner and Mumme, 2015). Pyrochar's features, such as their highly porous texture and almost alkaline pH, make them suitable for different applications (Monlau et al., 2015; Stefaniuk and

Oleszczuk, 2015). For instance, due to their porosity they may be used to adsorb AD inhibitory compounds (i.e., phenols and furans) or environmental pollutants (i.e., heavy metals, pesticides, herbicides etc.), or even to improve soils characteristics (Liu et al., 2019; Monlau et al., 2015). Indeed, their structure is suitable to provide refuge to desirable soil microorganisms and to improve soils water-holding ability, limiting soil evaporation (Liu et al., 2019; Monlau et al., 2015). Moreover, pyrolysis process allows to improve carbon immobilization and nutrient stability of SFD, reducing its leachability. In addition, pyrochar alkalinity helps acidic soils re-balance (Monlau et al., 2015; Stefaniuk and Oleszczuk, 2015). Pyrochar further conversion into activated carbons enhances their sorption ability, since they present even higher porosity than their original substrates (Monlau et al., 2015). Activated carbons may be employed in H_2S removal from biogas, often after a treatment with sodium hydroxide (NaOH) or potassium hydroxide (KOH) aimed to enhancing its sorption ability (Barelli et al., 2017). Then, the spent activated carbon can be recycled as soil amendments, since they catalyze H_2S oxidation to elemental sulfur (S_2) and sulfates (SO_4^{2-}), which can be beneficial to plants (Monlau et al., 2015). However, since pyrochar is generated from incomplete combustion, it can contains toxic compounds such as PAH and dioxins. Hence, before their application it is important to assess their possible leachability and bioavailability (Monlau et al., 2015). Syngas and bio-oil conversion into energy, through CHP systems, or into drop-in fuels and other chemicals contributes to the overall AD process efficiency (Monlau et al., 2015).

Biological processes for solid digestate conversion include mainly bioethanol and biogas production. In bioethanol production, AD is used as a biomass pretreatment in order to improve lignocellulose bioavailability and to reduce biomass particle size, thereby reducing grinding costs and improving subsequent residual sugars fermentation. Anyway, among all digestate exploitation opportunities, bioethanol production seems the most unprofitable (Monlau et al.,

2015), though more recently Logan and Visvanathan (2019) found that this strategy could be profitable for SFD management. Indeed, pretreating SFD could improve ethanol production, as proved by Stoumpou et al. (2020) who treated straw digestate with NaOH gaining ethanol yields up to 65%.
The biogas production process instead, implies digestate recirculation in AD plants as feedstock. During the storage, digestate naturally releases methane and ammonia into the atmosphere, thus its exploitation for biogas production allows both a better energy recovery from the original feedstock and the mitigation of GHG emissions (Dinuccio et al., 2013; Gioelli et al., 2011; Monlau et al., 2015). However, solid digestate obtained from AD of agrozootechnical residues is mainly composed of undigested lignocellulosic fibers which are extremely refractory to microbial digestion due to cellulose crystallinity, C/N ratio and high lignification degree (Auxenfans et al., 2017). The covalent bounds among lignin and the other cell-wall polysaccharides limit cellulose microbial accessibility (Carrere et al., 2016). Before its recirculation into the AD plants, the digestate needs to be pretreated in order to reduce its ammonia content, which is inhibitory for microorganisms involved in AD process, and to enhance cellulose bioavailability and to improve the biodegradability of recalcitrant compounds (i.e., lignin), since they may prevent microbial accessibility to sugars (Monlau et al., 2015; Mussatto et al., 2008).

1.6 Pretreatments

The pretreatments aim at increasing the feedstock's digestibility, and its consequent methane potential, through lignin decomposition, sugars solubilization and cellulose crystalline structure alteration (Carrere et al., 2016). In fact, partial modification of lignocellulosic biomasses can transform recalcitrant compounds (i.e., lignin, etc.), making the substrate more accessible for the microorganisms

involved in AD process (Shrestha et al., 2017). Several pretreatment methods can be employed for this purpose; in detail, they can be divided into four methods:

1. chemical,
2. physical,
3. physico-chemical,
4. biological.

The different methods can be used alone or in combination: i.e., lignocellulosic substrates may be treated combining physico-chemical and biological treatment to enhance substrate digestibility (Shrestha et al., 2017; Taherzadeh and Karimi, 2008; Wei, 2016).

Each pretreatment method have its own benefits and concerns (Sharma et al., 2019). In Table 5 the main advantages and disadvantages of the different pretreatment methods are summarized.

The choice of the most suitable pretreatment method depends mainly on the substrate selected for AD (Rouches et al., 2016). Table 6 shows the most common pretreatments currently used in relation to different kind of biomasses.

The economic feasibility of the pretreatment is evaluated considering the increase of methane yields and the additional costs of pretreatments (Rouches et al., 2016).

Notably, most of mechanical and thermal pretreatments have some full-scale application. Conversely, even though they are more environmental-friendly and cheaper, a lot of biological pretreatments are still used only at lab-scale since they are more difficult to optimize and to monitor (Carrere et al., 2016).

Table 5: Advantages and disadvantages of the different pretreatment methods (Akhtar et al., 2016; Shirkavand et al., 2016; Taherzadeh and Karimi, 2008).

PRETREATMENT	ADVANTAGES	DISADVANTAGES
CHEMICAL	• effective in breaking down bonds between lignin and hemicellulose.	• high-energy demand; • expensive equipment; • toxic/corrosive chemicals; • carbohydrate loss.
PHYSICAL	• size reduction; • decreasing polymerization and crystallinity of cellulose; • increasing the specific surface area; • negligible production of toxic material.	• high capital costs; • high-energy demands.
PHYSICO CHEMICAL	• removal of lignin and hemicellulose; • disruption of cellulose; • non-toxicity; • non-flammability; • easy recovery of the products (i.e., CO_2).	• possible cellulose loss; • possible high costs; • generation of toxic by-products (i.e., furanic and phenolic compounds).
BIOLOGICAL	• environmental friendliness; • cost-effectiveness; • mild operating conditions; • low energy requirement; • minimal waste production; • no generation of toxic compounds, which could compromise the subsequent fermentation; • generation of value-added co-products; • no steps are needed to neutralize strong acids or bases.	• prolonged treatment times; • low lignocellulosic biomass digestibility achieved in most cases; • lack of specificity.

Table 6: Most common pretreatment for different substrate type (Carlsson et al., 2012; Carrere et al., 2016).

BIOMASS	PRETREATMENT
DEDICATED CROP/ AGRICULTURAL WASTE	• chemical • thermal • wet oxidation • mechanical • microwave • biological
MANURE/SEWAGE	• thermal • chemical • wet oxidation • mechanical • microwave • ultrasonic • biological
OFMSW/SEWAGE SLUDGE	• mechanical • thermal • chemical • freezing • microwave • wet oxidation • biological

1.6.1 Chemical pretreatments

Chemical pretreatments are the most commonly used since they are usually really effective in PCWP (i.e., lignin) breakdown. However, they also present many economic and environmental issues, since they imply the use of toxic and corrosive chemical agents, high-energy and water requirement, and expensive equipment (Shirkavand et al., 2016). Furthermore, chemical pretreatments are eligible for lignocellulosic substrates because they can disrupt lignin fibers, while they are generally not suitable for carbohydrates-rich biomasses, because the fast degradation and VFA formation finally result in a decrease in methane yields (Table 5) (Ariunbaatar et al., 2014; Shirkavand et al., 2016).

Chemical pretreatments are divided into five main classes (Shirkavand et al., 2016; Taherzadeh and Karimi, 2008):

- Acid: an acid, such as sulphuric acid (H_2SO_4) or chloridric acid (HCl), hydrolyses lignin bonds and structural carbohydrates leading to enhanced cellulose bioavailability. However, hemicellulose degradation may result in furfurals generation, which are known to be inhibitory for microorganisms involved in AD process (Carrere et al., 2016).

- Alkaline: alkaline solutions such as sodium bicarbonate (NaOH), calcium hydroxide ($Ca(OH)_2$) or NH_3 effectively remove lignin and hemicellulose enhancing cellulose accessibility to microorganisms. Moreover, the alkalinity of the system may prevent detrimental acidification during AD process. Anyway, methanogens and other AD microorganisms may suffer from excessive sodium accumulation (Carrere et al., 2016).

- Organic solvents: they allow the break down of chemical bonds between lignin and hemicellulose, making cellulose more accessible to microorganisms' enzymes. However, after pretreatment, the solvents need to be separated from biomasses in order not to hinder microbial growth and activity during AD process (Akhtar et al., 2016; Shirkavand et al., 2016).

- Ozonolysis: ozone (O_3) acts as an oxidant decomposing lignin and hemicellulose. In contrast to other chemical pretreatments, it is the most environmentally safe since ozone has a good water-solubility and no toxic by-products are produced (Shirkavand et al., 2016).

- Oxidative: biomass is exposed to oxidizing compounds, like hydrogen peroxide (H_2O_2) or peracetic acid (CH_3CO_3H), in order to remove hemicellulose and lignin. However, organic matter losses due to non-selective cellulose oxidation and to the release of soluble aromatic compounds from lignin may occur leading to AD process inhibition (Hendriks and Zeeman, 2009).

1.6.2 Physical pretreatments

Mechanical pretreatments aim at feedstocks particle size reduction in order to release organic cell compounds and increase the specific surface area; the increase of surface area provided better contact between substrates and anaerobic bacteria, thus enhancing the AD process yields. Furthermore, mechanical pretreatments may also decrease the crystallinity and the polymerization of cellulose, enabling better and easier degradation. In contrast with chemical treatments, the physical ones are more environmental-friendly since they do not make use of toxic chemicals. However, their high cost, waste production and energy and water requirements represent undeniable drawbacks (Ariunbaatar et al., 2014; Lindner et al., 2015; Shirkavand et al., 2016; Taherzadeh and Karimi, 2008).

Shirkavand et al. (2016) classifies physical pretreatment in four categories:

- Milling and Grinding: they can increase the surface area of biomass particle.

- Freezing: the formation of ice crystals into the feedstock can generate forces that can disrupt the lignin matrix.

- Extrusion: during this process biomass undergoes shearing, mixing and heating, by means of an extruder, aimed to feedstock size reduction. It is commonly applied to lignocellulosic biomasses since it enhances lignocellulose depolymerization.

- Microwave: the substrates irradiation with microwaves for a short time produces effective lignin and hemicellulose removal; the advantages of microwave pretreatment over conventional heating processes are the short time and lower energy demand (Shirkavand et al., 2016).

1.6.3 Physico-chemical pretreatments

Physico-chemical pretreatments couple chemical and physical processes. These methods have moderate costs, low toxicity, easy products recovery and they are more environmental acceptable (Table 5). Five main categories may be identified (Shirkavand et al., 2016; Taherzadeh and Karimi, 2008):

- Steam explosion: this process implies biomass treatment with hot saturated steam under high-pressure conditions and subsequent explosive decompression which causes fibers disruption, improving enzymatic digestion. The addition of sulphurous anhydride (SO_2) enables both cellulose and hemicellulose fractions recovery (Akhtar et al., 2016; Auxenfans et al., 2017).

- Ammonia fibers explosion: during this process, the biomasses are exposed to liquid ammonia at relatively high temperature followed by immediate reduction of pressure; the process has a great specificity towards lignin. Indeed, it can modify or effectively reduce the lignin fraction of the lignocellulosic materials, while the content and quality of hemicellulose and cellulose fractions remain intact (Shirkavand et al., 2016).

- CO_2 explosion: biomasses are treated with supercritical CO_2 in the presence of water. Carbon dioxide is effective in enhancing the enzymatic digestibility of hardwood and softwood since its molecular size, analogous to water and ammonia, allows it to penetrate even the smallest pores of fibers, causing their breakdown (Shirkavand et al., 2016).

- Liquid hot-water: biomass is exposed to hot water under high pressure conditions. It results in higher cellulose accessibility to hydrolytic enzymes due to pretreatment effectiveness in removing hemicellulose and part of lignin. In addition, the process has lower chemicals request for the neutralization of the produced hydrolysate and produces lower amounts of neutralization residues compared to other processes, such as dilute-acid pretreatment. Higher pentosan

recovery and lower formation of microbial inhibitory components are the main advantages of this pretreatment method compared to steam explosion (Hendriks and Zeeman, 2009).

- Wet oxidation: it consists in biomass oxidation under high temperature and pressure conditions. It is commonly used for lignocellulosic biomass pretreatments; in fact, wet oxidation allows lignin decomposition, hemicellulose solubilization and cellulose crystalline structure alteration and loosening (Shirkavand et al., 2016).

1.6.4 Biological pretreatments

The use of biological pretreatments faced a wide expansion thanks to their several benefits and relatively few drawbacks compared to the traditional abiotic techniques (Table 5). Biological methods are eco-friendlier and more economical since they do not require the use of chemical compounds. Toxic compounds are not generated in the process and small amounts of wastes are produced. In addition, they do not have high-energy demand since they are carried out under mild operative conditions (e.g., ambient temperature and pressure) (Sindhu et al., 2016). However, despite their many benefits, biological pretreatments are usually time consuming, since process optimization requires the refinement of many parameters, such as the selection of the most suitable microbial strains and culture conditions (Sharma et al., 2019; Sindhu et al., 2016). Moreover, pretreatment times may be prolonged to ensure a complete depolymerization of recalcitrant compounds (Sindhu et al., 2016).

Biological pretreatments encompass the use of organisms "*in toto*" (whole cell systems) or their metabolites, as the enzymes. The choice of whole cell systems or more or less purified enzymes must be evaluated case by case based on the biomass features, process optimization and costs evaluation, since each biomass and experimental set-up have specific optimal work conditions (Soetaert and

Vandamme, 2010). Whole cell systems imply the exploitation of the metabolic machinery of the organism to transform the selected substrate. Moreover, these systems are captivating since different enzymes and their cofactors are produced without the need of an expensive cofactor regeneration system (Soetaert and Vandamme, 2010). On the other hand, the use of partially or entirely purified enzymes is advantageous to reduce pretreatment duration, but they require additional cofactors generation and purification steps, which are extremely expensive. Indeed, when purification costs exceed 5-10% of the total process costs they become economically unsustainable. Furthermore, enzymes may be affected by low stability in the working conditions; therefore, their application needs to be precisely refined (Soetaert and Vandamme, 2010).

Biological pretreatments can be classified in many categories according to the microorganism or the metabolites used:

- Fungi: filamentous fungi are among the most studied and applied microorganisms for biological pretreatment. Several authors described the exploitation of fungi for lignocellulose pretreatment since some of them naturally produce powerful enzymes able to degrade plant cell-wall polymers (Sindhu et al., 2016; Wei, 2016). Notably, few authors reported the possibility to use fungi under non-sterile conditions, allowing to reduce process costs since the sterilization step is quite expensive. For instance, Zhao et al. (2014) reported that methane yield obtained from unsterilized yard trimming pretreated with *Ceriporiopsis subvermispora* were comparable to those obtained with traditional (abiotic) pretreatment methods.

- Bacteria: bacteria pretreatments of lignocellulosic substrates are gaining importance since, in comparison with other microorganisms, they are characterized by faster growth rates and higher tolerance towards environmental stress (Woo et

al., 2014). Furthermore, many authors reported the ability of some bacteria to produce lignocellulolytic enzymes (Chen et al., 2010; Sindhu et al., 2016).

- Microbial consortia: these consortia are composed by mixture of different microorganisms (i.e., yeasts, filamentous fungi and many types of bacteria, as *Clostridium* sp., lactic acid, etc.) which work cooperatively producing a heterogeneous variety of enzymes that allow the degradation of lignocellulosic biomass into simpler products that can be easily degraded by the AD microbial community (Rouches et al., 2016; Shrestha et al., 2017).

- Ensiling: it consists of biomass lactic fermentation under oxygen-depletion conditions and acid pH (Shrestha et al., 2017). The acidic conditions of the environment hydrolyze hemicellulose and cellulose (i.e., up to 21 % after 83 days on wheat straw), enhancing the hydrolysis. On the other hand, during this pretreatment lignin is poorly degraded (less than 6 %) (Rouches et al., 2016).

- Enzymatic pretreatments: the biomass is exposed to the activity of partially or completely purified lignocellulolytic enzymes, able to alter lignin, hemicellulose and cellulose structure and enhance the digestibility. The most applied enzymes for lignocellulosic biomass treatment are laccases, peroxidases, cellulases and β-glucosidases (Akhtar et al., 2016). Compared to whole cell systems, the use of purified enzymes leads to a 20 % higher delignification, and they require simpler working conditions since they do not need nutrient for growth (Akhtar et al., 2016). Moreover, the enzymes can work in presence of inhibitors, bacteriophage and different toxins, and they act directly on the substrate (Wei, 2016). Since enzymes production and purification costs are expensive, a viable alternative may be the use of non-purified enzymatic extract, which is a mixture of different enzymes and mediators that act on lignin substrates. Since the purification step is avoided, their costs are lower. These methods have short treatment time, reduced waste volume and their management is easier (Asgher et al., 2016).

Interestingly, the actual mechanisms of biological pretreatments need still to be completely elucidated, thus further and exhaustive investigations are required to develop a new generation of environmental-friendly processes (Chen et al., 2010).

1.6.4.1 Pretreatments with filamentous fungi

Recently, fungal pretreatments have increasingly gained importance for lignocellulose pretreatment since they represent a valuable and environmentally sustainable alternative to traditional methods. Indeed, they have easy workout and low process costs since pretreatments can be carried out at room temperature, without the employment of toxic compounds, and reducing the production of waste or sludge (Rouches et al., 2016).

In the kingdom Fungi, several species mostly belonging to the phyla Basidiomycota and Ascomycota, followed by the subphylum Mucoromycotina, have been recognized for their abilities to transform lignocellulose through the use of a complex enzymatic pattern (Dashtban et al., 2009). The choice of the most suitable fungi for pretreatments is dependent on both substrate features and on microorganism's ecological characteristics (Wei, 2016).

Ligninolytic wood-rot fungi are distinguished into white (WRF), brown (BRF), and soft-rot (SRF) fungi, according to the appearance of the rotten wood, which is based on their capability to produce enzymes that target different lignocellulosic fractions. WRF can penetrate inside the wood by preferably breaking down the cellulose and the lignin, ultimately giving a white and fibrous appearance to the wood. These fungi produce an enzymatic arsenal that enables the direct oxidation of lignin; thereby they are the most commonly employed for pretreatment purposes (Rouches et al., 2016). WRF belong mainly to Basidiomycota phylum (i.e., *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Bjerkandera adusta, Pleurotus spp., Trametes spp*), though some of them belong to the Ascomycota phylum (i.e., *Xylaria* and *Hypoxylon* species) (Rouches et al., 2016). Brown-rot

fungi (BRF) efficiently degrade polysaccharides but are capable to attack lignin only slightly: at the end, the wood results brown and dry. They usually belong to Basidiomycota *(*as *Fomitopsis pinicola*) and Ascomycota phyla (Rouches et al., 2016). The soft-rot fungi (SRF) are able mainly to break down cellulose, leading to the softening of the wood. Many Ascomycota, such as *Chaetomium spp.*, belong to this group, which is effective in degrading biomasses characterized by low lignin content since they can only little affect phenolic structures (Rouches et al., 2016).

Many authors have described fungal lignocellulosic biomass pretreatment in order to obtain increments in the biogas production (Wei, 2016). The majority of them focused on the use of WRF Basidiomycota since they can allow the selective disruption of lignin without affecting cellulose, which is destined to biogas production (Baldrian et al., 2005; Ferreira et al., 2016; Dashtban et al., 2009; Rouches et al., 2016). For instance, Liu et al. (2014a) treated sterile corn stover with the basidiomycete *P. chrysosporium*, improving methane yield of 6.9-23.0 % compared with untreated samples. However also Ascomycota and even some Mucoromycotina species produce ligninolytic enzymes that can be exploited for lignocellulose pretreatment, though they are less employed than Basidiomycota (Baldrian et al., 2005; Ferreira et al., 2016; Dashtban et al., 2009; Rouches et al., 2016). Among the Ascomycota, numerous members of the subphylum Pezizomycotina, the most diverse group of the filamentous Ascomycetes, can attack lignocellulose (Stajich et al., 2009). Species belonging to the genera *Trichoderma* and *Aspergillus* are some examples of fungi that have been indicated as promising for lignocellulose pretreatments (Mutschlechner et al., 2015; Young et al., 2018). Mutschlechner et al. (2015) tested the ascomycete *Trichoderma viride* for non-sterile organic waste treatment and achieved a substantial difference in total gas and methane production compared to controls (+47% and +56%, respectively). Similar positive results were obtained by Wagner et al. (2013) after four days of fungal pretreatment with *T. viride*. In the subphylum Mucoromycotina,

part of the basal fungal lineage, the genera *Mucor* and *Rhizopus* may also include degraders of lignocellulosic material (Andlar et al., 2018).

In general, the fungal biomass production for pretreatment purposes is carried out under Submerged Fermentation (SmF) since the use liquid media allows to obtain higher biomass yields. The culture phase could be very expensive depending upon the chosen culture media. Indeed, the use of synthetic culture media on one hand allows to reach high growth rates, but on the other it is extremely costly (Aouidi et al., 2010). In order to reduce process costs, the exploitation of liquid agro-industrial wastes (i.e., cheese whey, olive mill water, beet molasse, etc.) as cheaper culture broths for biomass production is a valuable alternative (Costa et al., 2017; Vamvakaki et al., 2010). Conversely, the fungal pretreatments are usually performed under Solid State Fermentation (SSF), that allows to get higher delignification rates, presumably because the conditions and mechanisms are more similar to those existing in natural conditions (Rouches et al., 2016). The use of affordable feedstocks (i.e., food or green waste, cow manure or sewage, corn stover, rice straw, etc.) for fungal pretreatment and subsequent biogas production is desirable to diminish the costs (Enzmann et al., 2018; Yadav et al., 2017).

Notably, few publications reported the direct inoculation of fungi to unsterilized feedstock and generally, it is unsuccessful since the autochthonous microbiota prevails over inoculated microorganisms (Zhao et al., 2014). Thus, biomasses generally need a sterilization step before inoculation takes place. Biomass sterilization can be performed by either pressurized steam (autoclaving) or chemicals. However, these processes are not feasible for commercial-scale biogas production due to the significant increase in the process cost (Zhao et al., 2014). For these reasons, inoculum optimization is mandatory to pretreat unsterilized feedstocks. An efficient and well-established method is the use of pre-colonized sterile feedstock to inoculate unsterilized substrates (Zhao et al., 2014). In addition, the pre-colonization of feedstock may result in a pre-adaptation of the

microorganism to the substrate, promoting the colonization of unsterilized biomasses and allowing a decrease of pretreatment costs (Song et al., 2013; Zhao et al., 2014).

In conclusion, fungal pretreatments are giving very promising results, but the majority of the experiments and applications are still nearly exclusively limited to sterile substrates and lab-scale trials (Wei, 2016). Further optimizations are necessary in order to provide higher feedstock digestibility and methane potential. The selection of strains able to act selectively against lignin and to grow on unsterilized feedstocks is fundamental on this purpose. However, the fungal selection is often arduous due to feedstocks features and the presence of competitive indigenous microbiota (Singh et al., 2014; Vasco-Correa et al., 2016).

1.6.4.2 Fungal enzymes

Lignocellulolytic enzymes produced by fungi can be attributed to two different enzymatic systems: an oxidative ligninolytic system, which enables direct lignin oxidation, and a hydrolytic system, which transform cellulose and hemicellulose (Castoldi et al., 2014). The key lignin-modifying enzymes (LME), belonging to the oxidative systems, include lignin peroxidases (E.C. 1.11.1.14), manganese peroxidases (E.C. 1.11.1.13) and laccases (E.C. 1.10.3.2). LME transform lignin making cellulose and other compounds more bioavailable to microrganisms that produce biogas (Chen et al., 2010; Rouches et al., 2016). In WRF Basidiomycetes, the oxidoreductases laccases (EC 1.10.3.2) and peroxidases (EC 1.11.1.x) are the most important enzymes involved in the degradation of lignin. Laccases are copper containing extracellular enzymes belonging to group of blue oxidases that use copper as cofactor and molecular oxygen as co-substrate. Laccases are capable of oxidizing most of the phenolic and non-phenolic compounds (Baldrian, 2006; Majeau et al., 2010). The lignin-modifying class II

heme peroxidases includes the extracellular enzymes lignin peroxidase, manganese peroxidase, versatile peroxidase, dye-decolorizing peroxidase and the newly discovered peroxygenases of the heme–thiolate peroxygenase–peroxidase superfamily (Hofrichter et al., 2010; Lundell et al., 2010; Martínez, 2002). Lignin and manganese peroxidases are heme peroxidases that require the presence of hydrogen peroxide and manganese for activity; they are mostly reported for degradation of lignin and other compounds by WRF Basidiomycetes. Versatile peroxidase enzymes are broad substrate specific enzymes capable of oxidizing both phenolic and nonphenolic compounds (Harms et al., 2011). The heme–thiolate peroxygenases produced by certain wood-colonizing Basidiomycetes of the order Agaricales (i.e., *Cycloybe aegerita*) catalyse H_2O_2-dependent hydroxylations of various molecules, including aromatic, heterocyclic, and aliphatic compounds (Hofrichter et al., 2010). Laccases and peroxidases are frequently expressed in many isoforms, and the presence of several functionally related enzymes helps the maintenance of their activity under varying environmental conditions (Deshmukh et al., 2016).

Fungi that lack of extracellular oxidoreductases use a different hydrolytic mechanism for degrading lignocellulose. Enzymes involved in the hydrolytic system target cellulose, hemicellulose and chitin. These enzymes include proteases, carbohydratases (e.g., cellulases, amylases, hemicellulases, xylanases, β-glucosidases, etc.), esterases, phosphatases, phytases and chitinases (Mutschlechner et al., 2015). Among cellulose degrading enzymes, endo-glucanases target hydrolyze the inner glycosidic bonds of cellulose chains, while cellobiohydrolases (exo-glucanases) act on the cellulose chain ends (Baldrian et al., 2011; Dashtban et al., 2009). Due to their intrinsic low substrate specificity, hydrolases may play a pivotal role in the degradation of lignocellulose and other recalcitrant macromolecules (Anastasi et al., 2013).

2. Aims of the Work

Humanity is approaching all the harmful consequences of the indiscriminate fossil fuels exploitation for energy production, such as global warming, increased pollution and the run out of fossil carbon sources. The awareness about environmental concerns and the increase of global population lead to the necessity of a revolution in energy production models, in favor of new Green energy systems. On this purpose, EU introduced many directives aimed at reducing the dependence on fossil fuels for energy production by 2050. Among them, the European Green New Deal is the most recent and its objectives are to achieve GHG emissions reduction of 50-55% by 2030 and to reach a new climate-neutral economic model, supporting member states and economic sectors by boosting circular economy and providing financial assistance (COM (2019) 640 final).

Biogas production through anaerobic digestion (AD) represents an attractive solution since it would provide both renewable energy resources (i.e., biomethane) and a sustainable waste management strategy. Indeed, several organic wastes (i.e., agro-industrial residues) can be exploited and valorised as feedstocks, reducing wastes storage and disposal issues and costs. However, the presence of recalcitrant (i.e., lignin) or inhibitory (i.e., antibiotics, tannins, furans and phenols) compounds, may interfere with the microbial metabolism and the accessibility to feedstock, leading to lower biogas yields. Therefore, before AD of refractory lignocellulosic feedstocks (i.e., solid digestate), a pretreatment step is fundamental to increase the digestibility and the overall energetic potential. Traditional pretreatment methods include physical and chemical processes, that are extremely costly and environmentally unsustainable since they require high-energy inputs and amount of water and imply the utilization of toxic reagents. Instead, biological pretreatments are more cost-effective, less energy consuming, and they also do not produce toxic effluents or great amounts of wastes. Consequently, the interest for these pretreatments has recently grown of importance in recent years. Among the

biological process, the most promising are those performed with fungi, since their nonspecific lignocellulolytic enzyme system enables them the decomposition of a broad range of complex substrates, including lignin, phenols, and several xenobiotic molecules. By reducing the lignin content and transforming or removing the lignocelluloses-derived inhibitors (i.e., furans and phenols), fungi have the potential: i) to enlarge the feedstocks useful for biogas production; ii) to increase the bioavailability of more biodegradable components as cellulose, hemicellulose and fermentable sugars; iii) to improve the performance of several bioprocesses, such as the production of biogas, ethanol, biohydrogen, xylitol, butanol, and lipids.

The PhD project focused on the use of fungi to develop processes aimed to improve the performances and the sustainability of the AD. In detail, the main aims of the work were:

1. to isolate and select a promising and pre-adapted microbial resource capable of growing in the presence of non-sterile lignocellulosic biomasses, favorable to develop a low-cost and high-efficiency approach to biomass pretreatment.

2. to assess whether solid-state pretreatments with selected fungi may modify the plant cell-wall polymers composition and improve the biogas-methane production by AD of recalcitrant substrates.

3. to study the methanogenic potential of fungal biomass and the possible role and activity of fungi during AD.

4. to investigate the potential of fungi for removal or detoxification of molecules that can inhibit the AD and other biotechnological processes, such as the phenolic aldehydes and furfural.

2.1 Project outline

The first part of the work (Chapter 3, Figure 11) aims to partially fill a knowledge gap concerning the mycobiota inhabiting AD plants. The autochthonous fungi from agrozootechnical biogas plant feedstocks and by-products were isolated and molecularly identified. Presumably, these fungi are pre-adapted to the specific physicochemical and environmental conditions, thus they may be promising candidate for in field application of the pretreatment.

Subsequently, the study focused on the screening and exploitation of (autochthonous and allochthonous) fungal biodiversity to develop innovative processes aimed to increase the energy recovery from AD of agro-industrial products and wastes.

A qualitative screening was performed to select fungi that can be used as inoculants for pretreatment of non-sterile maize silage and solid fraction of digestate, in the future perspective of enhancing their valorisation and the (re)use as AD feedstocks (Chapter 4, Figure 11). After, the effects of fungal pretreatment performed with different selected strains on plant cell wall polymers (cellulose, hemicellulose and lignin) and subsequent anaerobic production of biogas and methane were evaluated (Chapter 5, Figure 11). Besides, it was tested the methanogenic potential of the fungal biomass to evaluate its suitability as biogas feedstocks.

Finally, the (autochthonous and allochthonous) fungal biodiversity was screened also for tolerance towards phenolic aldehydes and furfural, which have been previously reported to inhibit various bioprocesses, including the AD. Based on the growth performance, a fungal strain was selected to analyse its degradation ability against single molecules and mixes of the toxic compounds (Chapter 6, Figure 11).

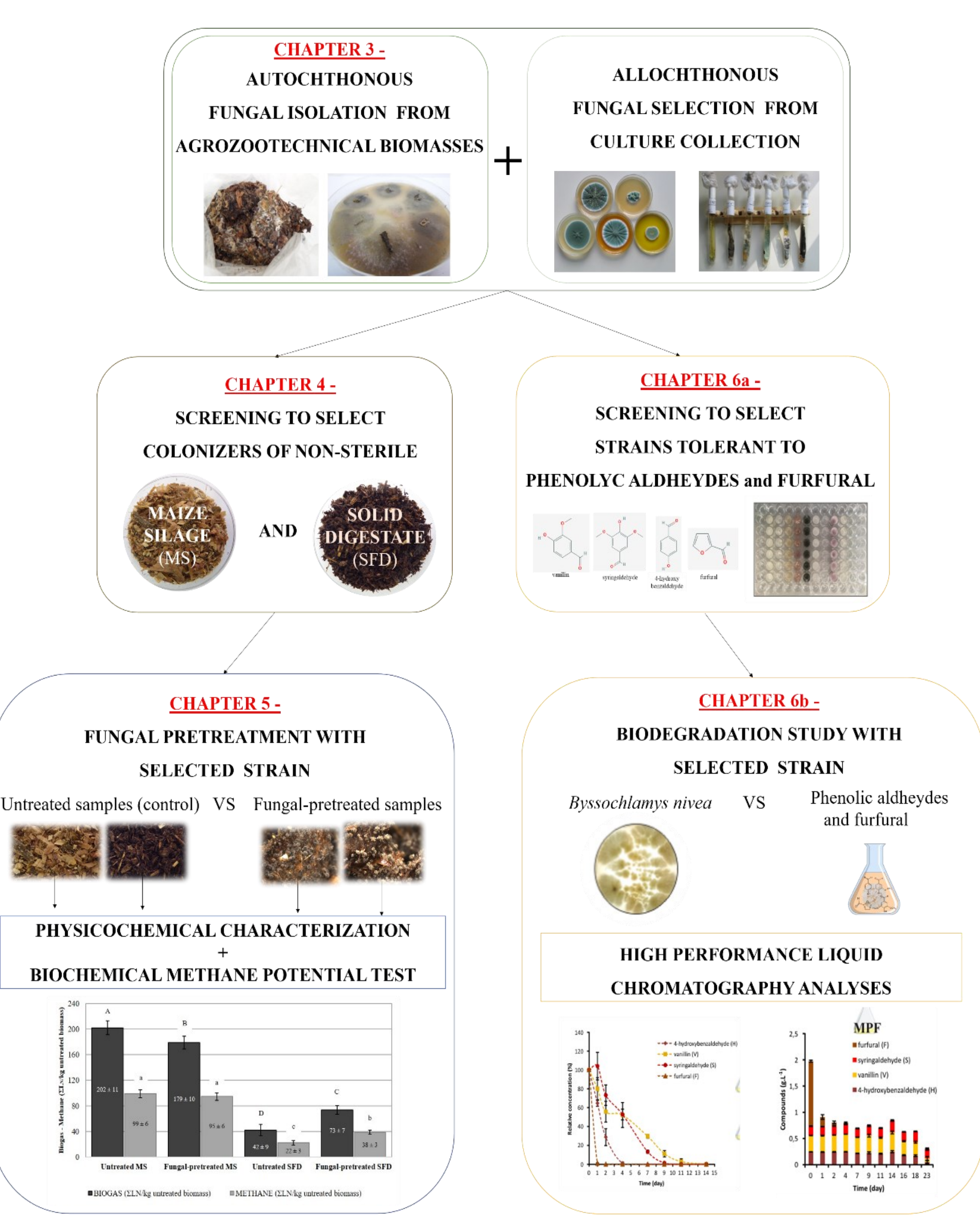


Figure 11: The overall PhD project outline.

3. Isolation and characterization of the mycobiota from biogas plants feedstocks and by-products

3.1 Introduction

Fungi are ubiquitous organisms, able to colonize all matrices (soil, water, air) and survive in a variety of natural environments (including the extreme ones), in which they play key roles in maintaining the ecosystems equilibrium (Anastasi et al., 2013). Fungi are known as the main decomposers of plant biomass in nature: many have developed a specific mechanism that employs few enzymes and molecules with high oxidizing power, able to cause partial or total degradation of lignin (Rouches et al., 2016). Thanks to their mechanical fibers penetration and lignocellulolytic capabilities, fungi have great potential to facilitate the use of recalcitrant lignocellulose-rich biomass (Dashtban et al., 2009; Dollhofer et al., 2015). The use of fungi to improve the conversion of lignocellulosic biomass is extensively reported in literature (Liu et al., 2014a; Mutschlechner et al., 2015; Tian et al., 2012). Notably, most of the studies are based on the use of allochthonous fungi, often obtained from culture collection. However, the introduction and application of allochthonous organisms in environments which differ from their natural habitats often proved to be inefficient (Baldrian, 2008; Harms et al., 2011). This phenomenon might be either due to their inability to compete with indigenous microbiota or to their non-adaptation to these new environments. For example, studies on the degradation of organic pollutants have shown a greater degradation potential of autochthonous fungal strains isolated from contaminated soils than those from similar uncontaminated environments (Garon et al., 2004).

Autochthonous fungi isolated from biogas plant feedstocks and by-products could offer interesting microbial resources to develop pretreatment processes on lignocellulosic biomasses destined to AD. Being adapted to the peculiar ecological niches, autochthonous fungi could efficiently colonize the matrix and transform the recalcitrant components, even in field applications. Despite this, little is known on this mycobiota and its possible involvement in the AD (Young et al., 2018). The aim of the study was to partially fill a knowledge gap concerning the mycobiota inhabiting biogas plant and to study such biological resource to develop biotechnological processes aimed to improve the performance of AD process. Different kind of agrozootechnical feedstocks and by-products, collected from two agricultural biogas plant (ABP) plants and a local farm, were used as source of fungal strains. Selective media and different culture condition were used to favor the development of a fungal communities adapted to the target biomass samples. A polyphasic approach was then employed to taxonomically identify all the isolated strains. The qualitative and quantitative structure of the isolated fungal communities in relation to the different sampling sites, biomasses and temperature of incubation were determined, providing new insights on the fate and possible contribution of fungi along the entire biogas production chain.

3.2 Materials and Methods

3.2.1 Biomass sampling, storage and preliminary characterization

Agrozootechnical biomasses used for fungal isolation were collected from storage facilities of two mesophilic full-scale ABP operating in the Piedmont region (Italy) and fed with a mixture of zootechnical effluents, maize silage (MS) and crop residues. Samples of maize silage (MS), maize stover (MST), cow slurry (CS), farmyard manure (FYM) and solid fraction of digestate (SFD) were taken from ABP-1 (Figure 12). Samples of MS, MST, CS, FYM and wheat straw (WS) were taken from ABP-2 (Figure 12). In addition, a sample of rice straw (RS) was taken

from a local farm. Examples of the agrozootechnical biomasses used for fungal isolation were reported in Figure 13. The collected biomasses were stored individually at room temperature until use (within 1 month).

For each biomass used for fungal isolation, the pH and humidity content were measured. pH in water was determined with a pH-meter (Basic20, Crison). Sample's aliquots were dried in an oven at 60°C for almost 24 h to determine the dry weights. Percent humidity was determined by calculating the amount of weight lost during the drying procedure.

Figure 12: The two biogas plants that provided biomass samples for fungal isolation.

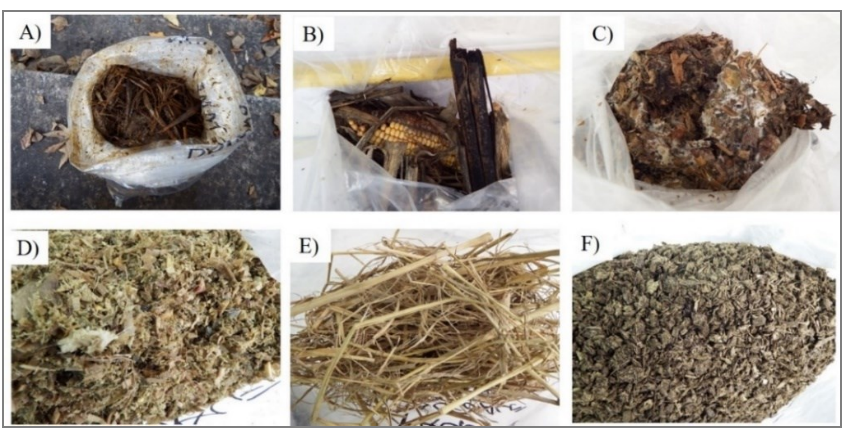


Figure 13: Examples of biomasses used for fungal isolation: A) FYM from ABP-2; B): MST from ABP-1; C) MS from ABP-1; D) MS from ABP-2; E) RS from the local farm; F) SFD from ABP-1.

3.2.2 Fungal isolation

Isolation was carried out with selective media containing the agrozootechnical biomasses as sole source of nourishment, in order to maximize the isolation of samples-specific fungi. In detail, the methods were adapted according to the characteristics of the biomasses. For zootechnical samples (CS and FYM), isolation was performed by directly plating portion of biomasses onto Petri dishes (9 cm Ø) lined with sterilized moist filter-paper, recreating a moist chambers technique (Figure 14) (Makhuvele et al., 2017; Misra et al., 2014). As for the lignocellulosic samples (SFD, MS, MST, WS and RS), biomass extracts were prepared by autoclaving them with deionized water (1:9 fresh weight/volume ratio) and used as agarized (18 g/L agar) culture media (Figure 14) (modified methods reported by Kim et al., 2013; Lee et al., 2011).

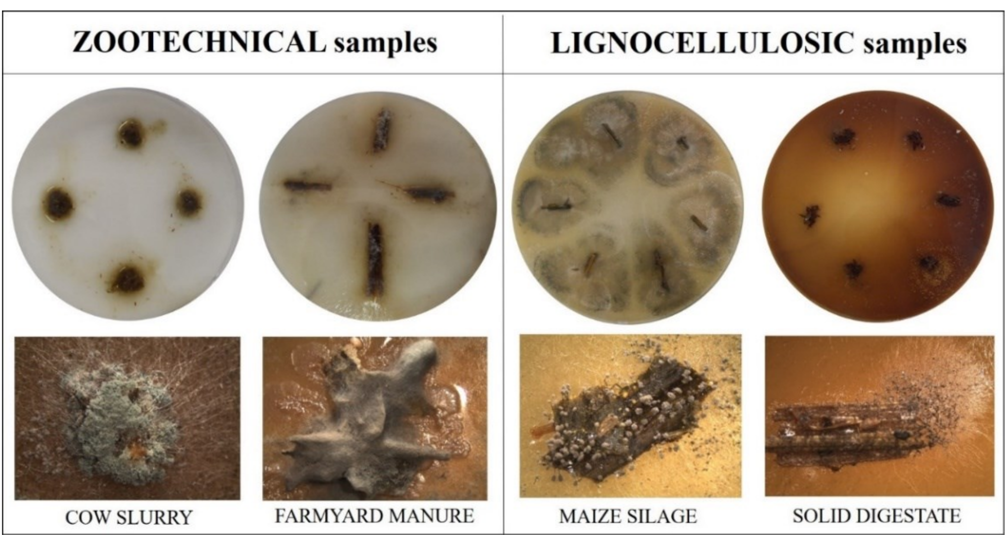


Figure 14: Examples of moist chambers prepared for isolation from zootechnical samples (left) and selective media used for isolation from lignocellulosic samples (right). Stereomicroscope images of fungi grown on the samples were also reported below.

All culture media were supplemented with a mix of antibiotics (gentamicin sulphate 40 mg/L, piperacillin plus tazobactam 11 mg/L) to limit the bacterial growth. Ten replicates for each biomass were performed and incubated in the dark at three different temperatures: 15 - 25 - 37 °C. Plates were inspected every 3 days for 30 days under a stereomicroscope (Leica EZ4) and the presence of fungi was recorded. The growing fungal colonies were picked up with a sterilized needle and sub-cultured onto solid Malt Extract Agar (MEA: 20 g/L malt extract, 20 g/L glucose, 2 g/L peptone, 18 g/L agar) to obtain pure cultures. A representative of each taxa isolated during the study is preserved at *Mycotheca Universitatis Taurinensis* (MUT – www.mut.unito.it) of the Department of Life Sciences and Systems Biology, University of Turin (Italy).

3.2.3 Identification

Fungal isolates were identified by means of a polyphasic approach. Morphological identification was performed according to the macro- and microscopic features (e.g., colony color, type, texture, shape and growth pattern). The morphological observations, performed using a stereomicroscope (Leica EZ4) and optical microscope (LEICA DM4500 B), were useful to group isolates into similar morphotypes and to guide the choice of specific primers for subsequent molecular identification (Bovio et al., 2018).

Genomic DNA was extracted from mycelium pre-grown at 25 °C for 1-3 week on MEA medium using the NucleoSpin Plant II kit (Macherey-Nagel GmbH, Duren, DE, USA), according to the manufacturer instructions. The quality and quantity of extracted DNAs were spectrophotometrically measured by using Infinite M200 (TECAN Trading, Austria). All reagents for molecular analyses an PCR reaction were supplied by Sigma-Aldrich (Saint Louis, USA). Details on the amplified gene loci, primers and PCR programs used can be found in Table 7.

Table 7: DNA regions sequenced, specific primers for molecular analysis and PCR programs (modified from Bovio et al., 2018).

Fungal Taxa	DNA regions sequenced[a]	Primers Forward and Reverse	PCR amplification Conditions
Alternaria	*GAPDH*	GPD1 - GPD2	96 °C: 2 min, (96 °C: 1 min, 50 °C: 1 min, 72 °C: 50 sec) × 35 cycles; 72 °C: 5 min
Aspergillus* and *Penicillium	*TUB*	BT–2a and BT–2b	94 °C: 4 min, (94 °C: 35 sec, 58 °C: 35 sec, 72 °C: 50 sec) × 35 cycles; 72 °C: 5 min
Cladosporium	*ACT*	ACT–512F and ACT–783R	94 °C: 8 min, (94 °C: 15 sec, 61 °C: 20 sec, 72 °C: 40 sec) × 35 cycles; 72 °C: 10 min
Fusarium	TEF	EF1- EF2	95 °C: 3 min (94 °C: 30 s, 53 °C: 1 min, 72 °C: 50 s) × 35 cycles, 72 °C: 8 min
Trichoderma	TEF	EF1- EF2	95 °C: 10 min (94 °C: 30 s, 55 °C: 30 s, 72 °C: 1 min) × 40 cycles, 72 °C: 10 min
Yeast and yeast-like fungi	D1-D2	NL1-NL2	94 °C: 4 min, (94 °C: 1 min, 52 °C: 35 sec, 72 °C: 1.5 min) × 35 cycles; 72 °C: 5 min
Taxa for whom no specific primers are required	ITS	ITS1- ITS4	95 °C: 5 min, (95 °C:40 s, 55 °C: 50 s, 72 °C: 50 sec) × 35 cycles; 72 °C: 8 min

[a] GAPDH: partial glyceraldehyde-3-phosphate dehydrogenase gene; TUB: partial beta-tubulin gene; ACT: partial actin gene; TEF: translation elongation factor-1α; D1-D2: D1-D2 region of the nuclear ribosomal DNA large subunit; ITS: internal transcribed spacer regions and intervening 5.8S nrRNA gene.

Amplification products were visualized under UV light (BIO-RAD Universal Hood II) on 1.5 % agarose electrophoresis gels stained with ethidium bromide. PCR products were purified and sequenced at Macrogen, Inc. Europe Lab (Madrid, Spain). Consensus sequences were obtained by using the software Sequencer 5.0 (Gene Code Corporation). Taxonomic assignments were based on high percentage homologies (similarity values equal or higher than 98 %; e-value > e-100) between the newly generated sequences and those available in public nucleotide databases (GenBank - NCBI database and CBS-KNAW Collection, Westerdijk Fungal Biodiversity Institute). Newly generated sequences were deposited in GenBank with the following accession numbers: MT151627 - MT151658; MT159813 - MT159827; MT180133 - MT180148; MT216344; MT219960; MT271821 - MT271828; MT274498 - MT274504.

3.2.4 Dereplication analyses

Fungi belonging to the same species, isolated from biomass samples coming from the same area of collection, were subjected to dereplication analyses in order to: i) identify unique genotype-strain, thus possibly reducing the number of fungi to be tested in the subsequent analyses; ii) investigate the presence and/or persistence of a same fungal strain in different ABP compartments and biomasses (i.e., upstream and downstream biomasses of the AD processes). Dereplication analyses were based on a PCR fingerprinting technique that has been successfully applied to amplify hypervariable repetitive DNA sequences in a wide range of fungal genomes (Meyer and Mitchell, 1995; Zézé et al., 1997). The analyses were performed as described by Poli et al. (2016), using a specific single primer to amplify the minisatellite core sequence derived from the wildtype phage M13. Amplification products were loaded on agarose gel and different fragments band profiles were detected by staining with ethidium bromide. The electrophoretic bands were visualized on a Gel Doc™ XR system (Bio-rad) equipped with the commercial software BioNumerics version 7.2 (Applied Maths) for the analysis of electrophoresis patterns. Fungi that showed identical fingerprint/profile have the same genomic sequence thus they were considered as unique genotype-strain.

3.2.5 Statistical analyses

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

3.3 Results and Discussion

3.3.1 Characteristics of the biomasses: pH and humidity

The pH and humidity content were measured for each biomass used for isolation in order to better understand some environmental conditions to which the autochthonous fungi were exposed. The results are presented in Table 8.

Table 8: Provenance and characteristics of biomass samples used for fungal isolation.

Provenance	**ABP-1**					**ABP-2**					**Regional Farm**
Samples	**CS**	**FYM**	**MS**	**MST**	**SFD**	**CS**	**FYM**	**MS**	**MST**	**WS**	**RS**
pH	8.9	7.2	5.5	6.0	9.7	8.8	7.8	4.5	5.5	6.1	5.7
Humidity (%)	94.0	89.0	62.0	24.0	84.0	96.0	83.0	67.0	12.0	7.0	61.0

The zootechnical samples (CS and FYM) showed alkaline pH (≥ 7.2) and high humidity content (≥ 83 %). As expected, CS had a higher pH and humidity content in comparison with FYM, since cattle slurry is typically generated in systems where little or no plant material (often straw) used as bedding is added to the excreted manure/urine (Font-Palma, 2019).

The lignocellulosic samples (MS, MST, WS and RS) were generally characterized by a lower pH value and humidity content in comparison to the zootechnical ones. Maize samples (MS and MST) had both acid pH, ranging from 4.5 to 6.0, but MS was characterized by a higher humidity in comparison to MST. The difference in humidity can be attributed to the different methods of production and management of MST and MS. The MST indeed consists of the residues (leaves, stalks, and cobs) of maize plants left in the field following the harvest, thus its moisture content is highly dependent on the soil and the environmental

conditions. Instead, for the production of MS, maize plants are chopped, moistened, well compacted, and stored in order to achieve a lactic fermentation in oxygen-depletion conditions and acid pH (Shrestha et al., 2017).
Straw samples (WS and RS) showed comparable pH values, ranging from 5.7 to 6.1, but RS was characterized by a higher humidity in comparison to WS. Also in this case, the differences in the moisture content could be linked to the storage methods and the final use of the biomasses. WS are often dried and stored in covered platforms to avoid rotting and allow the subsequent use as animal bedding. On the other hand, the RS are usually used for soil conditioning though composting, thus the characteristics of moisture can vary according to the soil and environmental conditions (Helal, 2005).

Interestingly, SFD showed pH value and humidity content more similar to those of the zootechnical samples respect to the lignocellulosic ones. The pH of SFD is generally higher compared to the parent biomasses used as feedstocks, and the increases in pH values during the AD is generally caused by the formation of ammonium carbonate $(NH_4)_2CO_3$ (Möller and Müller, 2012).

The characteristics of pH and humidity of the biomass may significantly influence the fungal community. In fact, excessive alkalinity could represent a limiting factor for fungal growth since the optimum pH usually ranges around acidic conditions (5 to 6.5) (Musatti et al., 2017) Besides, the water content is one of the most important parameters regulating biological activities in the natural environments. Changes in water availability influence the organisms through complex interactions with nutrient, temperature, and atmosphere conditions (Schnürer et al., 1986). However, several fungi, especially belonging to the phylum Ascomycota (e.g., *Aspergillus, Penicillium, Eurotium* spp.), are known for their ability to tolerate environments with low water activity (Pitt and Hocking, 2009, 1977).

3.3.2 Isolated mycobiota

In this study, both the biomasses and the methods used for fungal isolation were selected to favor the isolation of strains promising for pretreatment application. In fact, lignocellulosic and zootechnical biomasses are widely considered as a natural source for the isolation of organisms that can encode enzymes needed for the hydrolysis of lignocellulose (Makhuvele et al., 2017; Zmitrovich et al., 2014). Moreover, selective media containing the different agrozootechnical biomasses as sole source of nutrient were used to maximize the isolation of fungi that could have the potential to utilize the target biomasses.

Overall, 255 fungal strains, assigned to 89 taxa (50 Genera), were isolated. By means of molecular dereplication analyses, they were referred to 172 unique genotype-strains, of which 72 were isolated from ABP-1 samples, 75 from ABP-2 samples and 25 from the local farm (i.e., RS sample).

One hundred and sixty-two (162) strains were identified at species level and assigned to 80 species (41 Genera). Of the remaining 10 strains, 5 were identified at Genus level (*Chaetomium* sp., *Didymella* sp., *Massarina* sp., *Sodiomyces* sp., *Westerdykella* sp.), 3 at Family or Order level (Chaetomiaceae sp. Pezizaceae sp., Pleosporales sp.), and 2 black-microcolonial fungi remained unidentified.

The complete list of the isolated fungal taxa, with respective sampling site, biomasses and temperature of isolation is presented in Table 9.

Most (approx. 91 %) of the isolates were characterized by a filamentous habitus, which constitute a significant advantage for spatial colonization of solid biomasses (Baldrian, 2008). However, 15 strains (8 species) of yeasts (e.g., *Candida ethanolica* and *Yarrowia lipolytica*) and yeast-like organisms (e.g., *Galactomyces candidum*) were isolated, too.

Table 9: List of isolated fungal taxa, with respective number of strains, sampling site, biomass, and temperature of isolation.

Isolated Species/Taxa	N° of strains	Sampling site	Biomasses	Temperature (°C)
Acremonium antarcticum	1	ABP-1	FYM	25
Alternaria alternata	7	ABP-2; Regional Farm	FYM; WS; RS	15; 25
Alternaria slovaca	1	ABP-2	WS	25
Alternaria tenuissima	1	Regional Farm	RS	25
Aspergillus alabamensis	2	ABP-2; Regional Farm	FYM, RS	37
Aspergillus cejpii	2	ABP-1	MS; SFD	25
Aspergillus flavus	2	ABP-1; Regional Farm	MS; RS	25; 37
Aspergillus fructus	1	ABP-2	FYM	25
Aspergillus fumigatus	11	ABP-1; ABP-2; Regional Farm	CS; FYM; MS; MST; WS; RS; SFD	25; 37
Aspergillus giganteus	1	ABP-2	CS	25
Aspergillus latus	3	ABP-2	MST; WS	37
Aspergillus nidulans	1	ABP-2	WS	37
Aspergillus niger	6	ABP-1; ABP-2; Regional Farm	MS; MST; WS; RS	25; 37
Aspergillus niveus	2	ABP-1; ABP-2	FYM; WS	25; 37
Aspergillus tabacinus	1	ABP-2	FYM	25
Aspergillus terreus	4	ABP-1; ABP-2	CS; FYM; WS	25; 37
Aspergillus tubingensis	1	ABP-1	MST	25
Beauveria felina	1	ABP-1	MS	25
Bipolaris spicifera	2	Regional Farm	RS	37
Botryotrichum piluliferum	3	ABP-1; ABP-2	CS	15; 25
Botrytis cinerea	1	ABP-2	CS	25
Byssochlamys nivea	2	ABP-1	MS; SFD	25
Candida ethanolica	2	ABP-2	MS	37
Cephalotrichum microsporum	1	ABP-1	MS	15; 25
Cephalotrichum stemonitis	3	ABP-1; ABP-2	CS; FYM	15; 25
Chaetomiaceae sp.	1	ABP-2	CS	37
Chaetomium sp.	1	ABP-2	CS	15
Chrysosporium lobatum	1	ABP-2	FYM	25

Papulaspora equi	1	ABP-2	MST	37
Cladosporium aggregatocicatricatum	1	ABP-2	FYM	25
Cladosporium cladosporioides	3	ABP-1; ABP-2	SFD; MST; WS	15; 25
Cladosporium pseudocladosporioides	4	ABP-2	FYM; MST; WS	15; 25
Cladosporium sphaerospermum	1	ABP-2	FYM	25
Coprinopsis cinerea	4	ABP-1; Regional Farm	CS; SFD; RS	25; 37
Corynascus verrucosus	1	ABP-2	WS	37
Didymella sp.	1	ABP-1	SFD	15
Epicoccum nigrum	3	ABP-2; Regional Farm	FYM; RS	15; 25
Fusarium andiyazi	3	ABP-1; Regional Farm	MST; RS	25
Fusarium commune	1	Regional Farm	RS	15; 25
Fusarium fujikuroi	1	Regional Farm	RS	25
Fusarium graminearum	1	ABP-2	WS	15
Fusarium incarnatum	3	ABP-2; Regional Farm	MST; RS	15; 25
Fusarium lichenicola	1	ABP-1	CS	25
Fusarium proliferatum	2	ABP-1; Regional Farm	MS; RS	15; 25
Fusarium subglutinans	2	ABP-2; Regional Farm	MS; RS	25
Fusarium tricinctum	1	ABP-2	WS	25
Fusarium verticillioides	4	ABP-1; ABP-2	CS; FYM; MS; MST	15; 25
Galactomyces candidum	2	ABP-1; ABP-2	CS; FYM; MS; MST	15; 25
Lichtheimia ramosa	1	ABP-1	MS	25
Massarina sp.	1	Regional Farm	RS	37
Microdochium nivale	1	ABP-2	WS	15
Mortieriella indohii	1	ABP-1	FYM	15
Mucor circinelloides	7	ABP-1; ABP-2	CS; MS; MST	15; 25
Mucor fragilis	1	ABP-1	MS	15
Parastagonospora nodorum	1	ABP-2	WS	15
Penicillium crustosum	1	ABP-1	MS	15; 25
Penicillium italicum	1	ABP-1	CS	15
Penicillium oxalicum	1	ABP-2	MST	25

Penicillium paneum	1	ABP-2	MS	25
Penicillium roqueforti	1	ABP-2	MS	15; 37
Penicillium simplicissimum	1	ABP-1	MST	25
Pezizaceae sp.	1	ABP-2	CS	15; 25
Pichia kudriavzevii	1	ABP-1	MS	37
Pichia membranifaciens	2	ABP-1	MS	15; 25
Pleosporales sp.	1	ABP-1	SFD	15
Pseudeurotium bakeri	1	ABP-1	MS	15
Remersonia thermophila	1	ABP-1	CS; SFD	25; 37
Rhizomucor pusillus	2	ABP-2	MST; WS	37
Rhizopus oryzae	1	ABP-1	MS	37
Sarocladium strictum	1	Regional Farm	RS	25
Scedosporium apiospermum	3	ABP-1; ABP-2	CS; SFD	25
Scedosporium aurantiacum	1	ABP-1	CS	37
Scedosporium boydii	2	ABP-1; ABP-2	FYM	25
Scedosporium prolificans	1	ABP-2	FYM	25
Scopulariopsis brevicaulis	2	ABP-1; ABP-2	FYM; SFD	15; 25
Scytalidium thermophilum	4	ABP-1; ABP-2	CS; FYM; MS; SFD	15; 25; 37
Sodiomyces sp.	1	ABP-2	FYM	25
Talaromyces wortmannii	1	ABP-1	MST	25
Thermoascus crustaceus	4	ABP-2	MST; WS	37
Thermomyces lanuginosus	5	ABP-1; ABP-2	FYM; MS; SFD; WS	37
Thermothelomyces thermophilus	1	Regional Farm	RS	37
Trichoderma asperellum	1	ABP-2	FYM; MST	25
Trichoderma harzianum	2	ABP-1; Regional Farm	MST; RS	25
Trichoderma longibrachiatum	1	ABP-1	CS	25
Trichosporon asahii	2	ABP-1; ABP-2	CS; FYM	25
Westerdykella sp.	1	Regional Farm	RS	37
Wickerhamomyces anomalus	1	ABP-1	MS	15
Yarrowia lipolytica	2	ABP-1	MS	15; 37
Unidentified Fungi sp.	2	ABP-1	CS	25

The isolated mycobiota were dominated by the Ascomycota (153 strains, 81 species, 43 genera). This is not surprising considering that Ascomycota represents the fungal phylum with the largest diversity of species, capable of colonizing almost all ecological niches (Peay et al., 2016). Mucoromycota were also often found (13 strains, 6 species, 5 genera), whereas Basidiomycota were rarely isolated (6 strains ascribable to 2 species, *Coprinopsis cinerea* and *Trichosporon asahii*). Similarly, Young et al. (2018), studied the presence of fungi along the biogas production chain of one-phase and two-phase biogas plants in Germany and, among the 17 aerobic species isolated, the occurrence of Ascomycota and Mucoromycota was higher in comparison to the Basidiomycota.

The isolated mycobiota was characterized by few abundant genera and a long tail of many rarely isolated taxa. A similar result was observed also by Shrestha et al. (2011) studying the diversity and distributions of cultivable fungi associated with decaying *Miscanthus* and sugarcane plants in nature. The most frequently isolated genus was *Aspergillus* (13 species, 37 strains), the only one found in all sampling sites and biomasses analysed. The findings agree with other studies, in which *Aspergillus* spp. have been often observed as dominant in animal manure-slurry (Makhuvele et al., 2017) and lignocellulosic biomasses used as ABP feedstocks, as the silage (Alonso et al., 2013; Young et al., 2018). The genus *Aspergillus* includes highly sporulating fungi, thus, their occurrence could be overestimated due to the culture dependent method used for the isolation (Evans and Seviour, 2012). However, their presence in all sampling site and biomasses may indicate that the genus *Aspergillus* has evolved mechanisms of adaptations and resistance to extremely different environmental conditions (Bhabhra and Askew, 2005). *Aspergillus fumigatus* was the most abundant species (11 strains), and the only one found in in all sampling sites and biomasses. The ability to grow on different substrates and to produce thermostable lignocellulolytic enzymes (Adav et al., 2013), make this species potentially useful for pretreatment of lignocellulosic biomasses (Ferreira et al., 2016). However, *A. fumigatus* is an opportunistic human

pathogen and a mycotoxin producer (Dos Santos et al., 2003), and its abundant presence in biomasses used to feed ABP and cattle, as well as in digestate used as fertilizer, may constitute a safety problem. Other *Aspergillus* species isolated in this study might be better suited for pretreatments to improve the biogas production. Examples of promising species were *Aspergillus niger* (6 strains) and *Aspergillus terreus* (4 strains), which have been already reported for the production lignocellulolytic enzymes and are widely used in biotechnological application (Adav et al., 2013; Young et al., 2018).

Other frequently isolated fungal genera were *Fusarium* (10 species, 19 strains) and *Penicillium* (6 species, 6 strains). Unlike *Aspergillus*, the occurrence of *Fusarium* and *Penicillium* species was almost restricted to the lignocellulosic feedstocks, while they were sporadically found in zootechnical samples (CS and FYM). In line with our results, *Fusarium* and *Penicillium* species were often isolated from agricultural residue, compost and manures (Helal, 2005; van Asselt et al., 2012). Interestingly, all the collected *Fusarium* species (*Fusarium andiyazi, Fusarium commune, Fusarium fujikuroi, Fusarium graminearum, Fusarium incarnatum, Fusarium lichenicola, Fusarium proliferatum, Fusarium subglutinans, Fusarium tricinctum* and *Fusarium verticillioides*) were potential plant pathogenic fungi who can damage hosts from field to harvest. In this study, other plant pathogenic fungi, such as *Alternaria* spp. (3 species, 9 strains) and *Cladosporium* spp. (4 species, 9 strains) were frequently isolated. Plant pathogens could be interesting for pretreatment application, in fact this eco-physiological group of fungi have evolved strategies to naturally deconstruct PCWP (cellulose, hemicelluloses, and pectin) using extracellular enzymes with diversity and activity best suited to bioconversion of host plants (Lee et al., 2011). In some cases, the pathogen-host specificity results in a more effective bionconversion of the parasitized plants. For example, fungi that parasitize monocotyledons bioconvert these plants more effectively than fungi parasitizing dicotyledons, and viceversa (Shrestha et al., 2015). However, considering that many phytopathogens are

potentially mycotoxin producers and that most ABP are located near cultivated fields, their use in pretreatment processes should be carefully evaluated.

Unfortunately, members of the mycobiota, and especially those isolated from CS, FYM and SFD, included also emerging opportunistic human and animal pathogens, such as *Scedosporium* spp. (4 species, 7 strains), often associated with human impacted areas (Rougeron et al., 2018). Moreover, *Chrysosporium lobatum* (1 strain) and *Scopulariopsis brevicaulis* (2 strains) strains were isolated, which frequently demonstrate the ability to invade and parasitize cornified tissues through keratinolytic activity (Tigini et al., 2018). The spread of this emerging pathogens in agricultural biomasses is particularly alarming on the account of their increasing impact on animals and humans and raise safety issues for the operators of ABP and for the potential final uses of the digestate (Picco et al., 2011).

On the other hand, many isolated fungi are known for their ability to produce lignocellulolytic enzymes, a skill that can be crucial for improving the bioconversion of lignocellulosic feedstocks. The *Trichoderma spp.* (3 species, 4 strains), that were isolated from both zootechnical (CS and FYM) and lignocellulosic samples (MST and RS), are worthy of mention for their biotechnological potential. *Trichoderma spp.* are: i) soft-rots fungi commonly found in agricultural biomasses (Ja'afaru, 2013), as well as in dung of herbivores (Makhuvele et al., 2017), sewage sludge and industrial wastewater (More et al., 2010); ii) effective lignocellulolytic enzymes producer, used as main industrial source of cellulases and hemicellulases (Kim et al., 2013; Schmoll and Schuster, 2010). Their oxidative and hydrolytic enzymatic potential makes isolated *Trichoderma* strains good candidates for improving the bioconversion of lignocellulosic agricultural feedstocks. Since an abundant presence of plant and human pathogens has been found in the biomasses, *Trichoderma spp.* results interesting also for their ability as biocontrol agent, capable of impair growth of phytopathogens and opportunistic human pathogens by competition for nutrients,

antibiosis, production of lytic enzymes, and increasing plant resistance (Kausar et al., 2010; Schmoll and Schuster, 2010).

Noteworthy is also the isolation of thermophilic and thermotolerant species like *Thermomyces lanuginosus, Thermoascus crustaceus*, *Scytalidium thermophilum*, *Thermothelomyces thermophilus*, *Remersonia thermophila*. These fungi gained particular attention due to their capability of producing thermostable extracellular lignocellulolytic enzymes (such as endoglucanases and xylanases) that can find several biotechnological applications (Shrestha et al., 2015; Singh et al., 2016). As expected, thermophilic species was more frequently isolated from higher temperature of incubation (37 °C), and their presence in ABP feedstocks and digestate can be explained by the high temperatures achieved in stockpile during the biomass storage (Alonso et al., 2013).

3.3.2.1 Effect of samples origin

In the present work, isolation of fungi was conducted from samples of agrozootechnical biomasses mainly collected from two distinct mesophilic full-scale biogas plant, namely ABP-1 and ABP-2. In detail, 49 taxa (72 strains) were retrieved from ABP-1 and 48 taxa (75 strains) from the ABP-2. 33 taxa were exclusively found in ABP-1 and 32 in ABP-2 (Table 10); only 16 taxa were common to the two ABP sampled, including: i) ubiquitous airborne contaminants, commonly sampled from outdoor environments (i.e. *A. niger, A. terreus, Aspergillus niveus, Cladosporium cladosporioides*, *Mucor circinelloides)* (Skóra et al., 2014); ii) human (i.e. *A. fumigatus, Scedosporium apiospermum*, S*cedosporium boydii, S. brevicaulis*) and plant (*F. verticillioides*) pathogens; iii) thermophilic fungi (i.e. *S. thermophilum* and *T. lanuginosus*). Other common taxa in ABP-1 and ABP-2 were *Botryotrichum piluliferum, Cephalotrichum stemonitis, G. candidum* and *T. asahii* (Table 10).

Table 10: Lists of isolated taxa in relation to the sampling sites (ABP-1 or ABP-2).

Taxa exclusive of ABP-1	Common Taxa between ABP-1 and ABP2	Taxa exclusive of ABP-1
Acremonium antarcticum	*Aspergillus fumigatus*	*Alternaria alternata*
Aspergillus cejpii	*Aspergillus niger*	*Alternaria slovaca*
Aspergillus flavus	*Aspergillus niveus*	*Aspergillus alabamensis*
Aspergillus tubingensis	*Aspergillus terreus*	*Aspergillus fructus*
Beauveria felina	*Botryotrichum piluliferum*	*Aspergillus giganteus*
Byssochlamys nivea	*Cephalotrichum stemonitis*	*Aspergillus latus*
Cephalotrichum microsporum	*Cladosporium cladosporioides*	*Aspergillus nidulans*
Chaetomiaceae sp.	*Fusarium verticillioides*	*Aspergillus tabacinus*
Chaetomium sp.	*Galactomyces candidum*	*Botrytis cinerea*
Coprinopsis cinerea	*Mucor circinelloides*	*Candida ethanolica*
Didymella sp.	*Scedosporium apiospermum*	*Chrysosporium lobatum*
Fusarium andiyazi	*Scedosporium boydii*	*Papulaspora equii*
Fusarium lichenicola	*Scopulariopsis brevicaulis*	*Cladosporium aggregatocicatricatum*
Fusarium proliferatum	*Scytalidium thermophilum*	*Cladosporium pseudocladosporioides*
Lichtheimia ramosa	*Thermomyces lanuginosus*	*Cladosporium sphaerospermum*
Mortieriella indohii	*Trichosporon asahii*	*Corynascus verrucosus*
Mucor fragilis		*Epicoccum nigrum*
Penicillium crustosum		*Fusarium graminearum*
Penicillium italicum		*Fusarium incarnatum*
Penicillium semplicissimums		*Fusarium subglutinans*
Pichia kudriavzevii		*Fusarium tricinctum*
Pichia membranifaciens		*Microdochium nivale*
Pleosporales sp.		*Parastagonospora nodorum*
Pseudeurotium bakeri		*Penicillium oxalicum*
Remersonia thermophila		*Penicillium paneum*
Rhizopus oryzae		*Penicillium roqueforti*
Scedosporium aurantiacum		*Pezizaceae sp.*
Talaromyces wortmannii		*Sodiomyces sp.*
Trichoderma harzianum		*Rhizomucor pusillus*
Trichoderma longibrachiatum		*Scedosporium prolificans*
Unidentified Fungi sp.		*Thermoascus crustaceus*
Wickerhamomyces anomalus		*Trichoderma asperellum*
Yarrowia lipolytica		

The statistical analyses showed that the isolated mycobiota was significantly affected by the area of collection of the biomasses. In fact, the mycobiota of the two ABP sampled showed an average intragroup similarity of about 76.1 % (PERMANOVA, $p<0.05$; SIMPER), while the average intergroup dissimilarity was of about 75.5 % (PERMANOVA, $p<0.05$; SIMPER). These similarity/dissimilarity values indicated a clear difference in fungal communities between the two sampling sites and suggest that there may be a relationship between geographic distance and species diversity. The PCO analyses allowed to visualize the differences in the fungal community between ABP-1 and ABP-2 (Figure 15). The species that mostly contributed to the intergroup dissimilarity (SIMPER tests), each with a respective contribution in percentage of 2.72 %, were: *Acremonium antarcticum, F. lichenicola, Penicillium crustosum, Pichia membranifaciens, Pleosporales sp.* and *R. thermophila* from ABP-1, and *Alternaria alternata, Aspergillus giganteus, C. ethanolica, Cladosporium pseudocladosporioides, F. graminearum, Penicillium paneum, Penicillium roqueforti* and *Rhizomucor pusillus* from ABP-2.

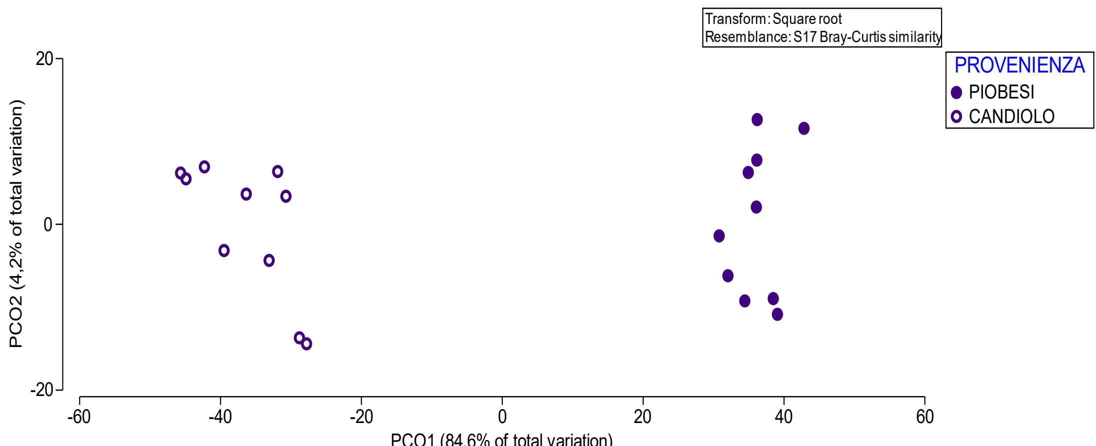


Figure 15: Graphical representation, obtained through PCO analyses, of similarity/dissimilarity within and between the two ABP analysed (ABP-1 = PIOBESI and ABP-2 = CANDIOLO).

Considering only the mycobiota from the ABP-1 samples, the dereplication analyses revealed the presence of unique genotype-strains in upstream and downstream biomasses of the biogas production processes. In fact, a unique genotype-strain of *R. thermophila* isolated from CS and one of *A. fumigatus* from MS were found also in the SFD obtained by co-digestion of CS and MS. The occurrence of a same strain in both feedstocks and digestate of the same ABP could be due to aerial diffusion; in fact, biomasses are accumulated in an open-air storage, giving rise to de-novo colonization (Young et al., 2018). On the other hand, the finding could suggest that such *R. thermophila* and *A. fumigatus* strains may survive to the extreme condition occurring during the AD process (i.e., high temperature, alkaline pH, and anaerobiosis). The possibility that *R. thermophila* can be present along the whole AD process and compartments is very promising for its potential in bioaugmentation systems, since this is a thermophilic species with substantial lignocellulolytic potential, capable of producing thermostable extracellular endo-β-1,4-xylanases (McPhillips et al., 2014). On the contrary, if the same hypothesis will be confirmed also for the pathogen and mycotoxigenic *A. fumigatus*, a serious human and environmental risk should be considered.

Notably, this is one of the few studies focusing on the characterization of the cultivable mycobiota associated with biogas plant feedstocks and by-products. Only recently, an investigation focused on the fungal community isolated from four biogas plants in Germany was carried out (Young et al., 2018). Interestingly, 12 out of the 17 taxa isolated by Young et al. (2018) were also isolated in the present study, namely *Lichtheimia ramosa, M. circinelloides, R. pusillus, Rhizopus oryzae, Alternaria* sp. *A. fumigatus, Geotrichum* (*Galactomyces*) sp. *Penicillium* sp. *R. thermophila*, *Trichoderma* sp., *C. cinerea* and *T. asahii*. In this study, the species *M. circinelloides, A. fumigatus, Galactomyces* sp. and *T. asahii* were found on both ABP-1 and ABP-2 samples. The retrieval of common taxa in different and distant geographical sites (as ABP from different country), could indicate a great adaptation of such fungi to the ecological niches constituted by the ABP biomasses.

3.3.2.2 Effect of biomasses

The qualitative composition of the isolated mycobiota showed significant differences among the different agrozootechnical biomasses (PERMANOVA, $p<0.05$, Figure 16).

On average, the intragroup similarity within substrates ranged from 62.4 to 81.7% (SIMPER, Table 11), indicating that each kind of biomass constitute a distinct and homogeneous group. The analyses highlighted the presence of ubiquitous species, that were isolated from and contributed to the intragroup similarity of different biomass samples; for instance, *A. alternata* in WS and RS, or *A. fumigatus* in CS, FYM, MS, MST and RS. On the other hand, some species resulted associated with a unique biomass: for example, *F. lichenicola* and *F. graminearum* were found on and contribute only to the intragroup similarity of CS and WS, respectively. The specificity of substrate is a positive finding for targeted pretreatment, indicating the isolation of specialized fungi.

The average intergroup dissimilarity between the different biomasses ranged from 70.9 % to 93.7 % (SIMPER, Table 11), suggesting that maize samples (MS and MST), WS, RS, CS, FYM and SFD are distinct ecological niches, each inhabited by a unique fungal mycobiota (Figure 16). The differences in the retrieved fungal species could be associated to the different physicochemical characteristics of the substrates (i.e., pH and humidity, see Table 8).

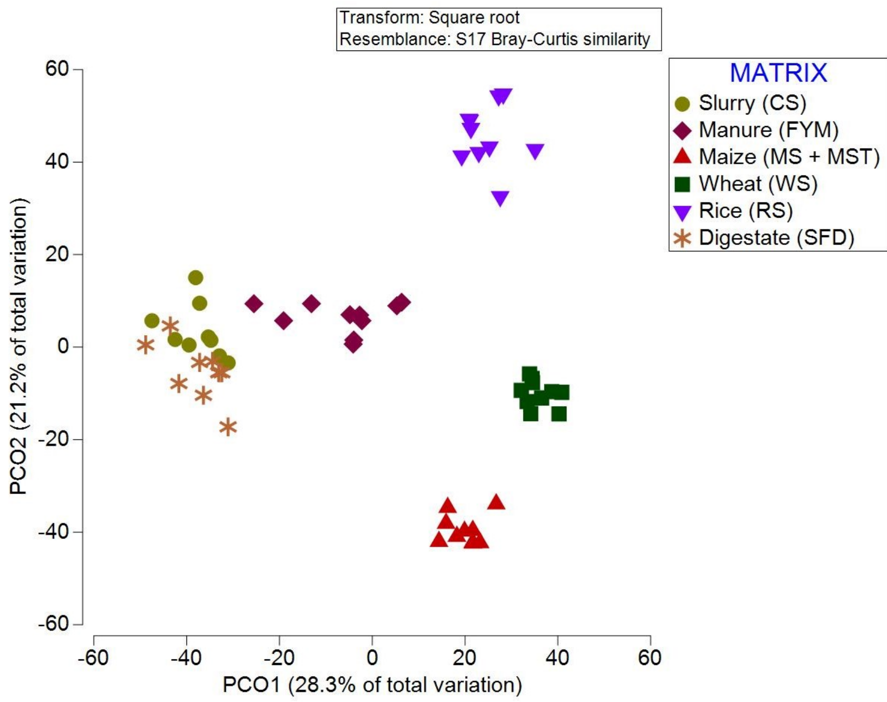


Figure 16: Graphical representation, obtained through PCO analyses, of similarity/dissimilarity within and between the different substrates used for fungal isolation.

Table 11: Percentage of similarity and dissimilarity within and between the different biomasses used for fungal isolation. Color legend: yellow = average similarity % between groups; green = average similarity % within groups ; orange = average dissimilarity % between groups.

Average Similarity and Dissimilarity Within and Between Biomasses						
Biomass samples	CS	FYM	MS + MST	WS	RS	SFD
CS	68.6	80.4	84.7	93.2	90.8	72.9
FYM	19.6	62.4	84.4	83.6	88.8	86.0
MS + MST	15.3	15.6	81.7	70.9	91.5	87.3
WS	6.8	16.4	29.1	74.0	79.7	86.4
RS	9.2	11.2	8.5	20.3	63.7	93.7
SFD	27.1	14.0	12.7	13.6	6.3	80.2

The use of different agrozootechnical biomasses significantly affected the isolation of fungi also from a quantitative point of view. 40 taxa (56 strains) were isolated from the zootechnical biomasses (CS and FYM), while 61 taxa (104 strains) from the lignocellulosic ones (MS and MST, WS, RS). 47 taxa were exclusively found in the lignocellulosic samples, while 26 in the zootechnical ones. The higher fungal biodiversity isolated on lignocellulosic biomasses in comparison to the zootechnical ones could be related to the higher number and kind of samples or to the different methods used for isolation. Only the following (14) species were common between zootechnical and lignocellulosic biomasses: *A. alternata*, *Aspergillus alabamensis*, *A. fumigatus*, *A. niveus*, *A. terreus*, *C. pseudocladosporioides*, *C. cinerea*, *Epicoccum nigrum*, *F. verticillioides*, *G. candidum*, *M. circinelloides*, *S. thermophilum*, *T. lanuginosus* and *T. asperellum.*

Focusing on the zootechnical samples, 23 taxa (29 strains) were retrieved from CS and 24 taxa (29 strains) from FYM. 16 taxa were exclusively found in CS and 17 in FYM. The average intragroup similarity was of 68.6 % and 62.4 % for CS and FYM, respectively (Table 11). Only 7 taxa were in common between the two zootechnical samples, and the species *A. terreus* (5.0 %)*, F. verticillioides* (5.0 %)*, G. candidum* (4.6 %)*, S. thermophilum (*3.7 %) and *C. stemonitis* (3.1 %) contributed mostly to the intergroup similarity between CS and FYM. The average dissimilarity between the fungal communities of CS and FYM was of 80.4 % (Table 11), indicating that, despite comparable origin and composition, cattle slurry and farmyard manure are inhabited a different fungal community. The species *A. antarcticum* (6.2 %), *C. pseudocladosporioides* (5.5 %)*, Scedosporium prolificans* (4.9 %), *Cladosporium sphaerospermum* (3.0 %), *Cladosporium aggregatocicatricatum* (2.4 %) from FYM, and *B. piluliferum* (6.17 %), *A. giganteus* (6.2 %), *F. lichenicola* (6.2 %), *Trichoderma longibrachiatum* (3.7 %) from CS*,* contribute mostly to the intergroup dissimilarity between FYM and CS.

Concerning the lignocellulosic biomasses, 41 taxa (58 strains) were isolated from the maize samples (MS and MST), while 34 taxa (46 strains) from the straw

(WS and RS). 27 taxa were exclusively found in maize and 20 taxa in the straw; The (14) species that were common to both were: *Aspergillus flavus, A. fumigatus, Aspergillus latus, A. niger, C. cladosporioides, C. pseudocladosporioides, F. andiyazi, F. incarnatum, F. proliferatum, F. subglutinans, R. pusillus, T. crustaceus, T. lanuginosus* and *Trichoderma harzianum.*

Comparing the straw samples, 17 taxa (21 strains) were retrieved from WS and 20 taxa (25 strains) from RS. 14 taxa were exclusively found in WS and 17 in RS. The average intragroup similarity was of 74.0 % and 63.7 % for WS and RS, respectively (Table 11). Only the species *A. alternata, A. fumigatus* and *A. niger* were common to both straw samples. The average intergroup dissimilarity between WS and RS was about 79.7 % (Table 11) and it is mostly determined by the following species: *Aspergillus nidulans* (6.4 %), *C. cladosporioides* (7.3 %)*, C. pseudocladosporioides* (4.4 %) *F. graminearum* (6.64 %)*, F. tricinctum* (5.7 %)*, R. pusillus* 4.5 %) and *T. crustaceus* (5.1 %) from WS*,* and *C. cinerea* (4.9 %), *F. andiyazi* (7.3 %), *F. commune* (5.1 %) and *F. proliferatum* (7.3 %) from RS.

The average intragroup similarity among the lignocellulosic biomasses was of 81.7 %, 74.0 % and 63.7 for the maize (MS + MST), wheat (WS) and rice (RS) samples, respectively (Table 11). On average the dissimilarity between the different lignocellulosic samples ranged from 70.9 to 91.5 % (Table 11), indicating that each botanical species (maize, wheat and rice) is inhabited by a distinct mycobiota. The most similar fungal communities where those of the maize and the wheat (average similarity = 29.1 %), which shared the following 8 species: *A. fumigatus, A. latus, A. niger*, *C. cladosporioides, C. pseudocladosporioides*, *R. pusillus, T. crustaceus* and *T. lanuginosus.* The intergroup dissimilarity between maize and wheat was determined mostly by the species, *A. alternata* (4.6 %)*, A. nidulans* (4.1 %)*, F. graminearum* (4.6 %)*, F. tricinctum* (3.7 %) from WS, and *C. ethanolica* (4.6 %)*, F. incarnatum* (4.2%)*, F. subglutinans* (4.6 %)*, G. candidum* (4.6 %)*, M. circinelloides* (4.6 %)*, Mucor fragilis* (4.1 %)*, P. crustosum* (4.6 %)*, P. paneum* (4.6 %)*, P. roqueforti* (4.6 %)*, P. membranifaciens* (4.6 %)*, Y. lipolytica*

(4.6 %) from MS. On the contrary, the mycobiota recovered from maize and rice were the most different (average similarity: 9 %), though they share the following 8 species: *A. flavus, A. fumigatus, A. niger, F. andiyazi, F. incarnatum, F. proliferatum, F. subglutinans* and *T. harzianum.* The intergroup dissimilarity is mostly determined by the following species: *C. ethanolica* (3.9 %)*, Y. lipolytica* (3.9 %)*, G. candidum* (3.9 %)*, C. cladosporioides* (3.9 %)*, M. circinelloides* (3.9 %)*, P. crustosum* (3.9 %)*, P. paneum* (3.9 %)*, P. roqueforti* (3.9 %)*, P. membranifaciens* (3.9 %) from MS, and *R. pusillus* (3.9 %)*, T. lanuginosus*, *A. alternata* (3.9 %) and *F. proliferatum* (3.9 %) from RS.

Interestingly, the fungal community of SFD showed the highest similarity with that of zootechnical samples (Figure 16). Seven out of 12 taxa isolated from SFD were also found on CS and FYM, namely: *A. fumigatus, C. cinerea, R. thermophila, S. apiospermum, S. brevicaulis, S. thermophilum* and *T. lanuginosus*. In detail, SFD showed the highest similarity whit CS (SIMPER; 27.1 % average intergroup similarity, Table 11). The species that mostly contributed to the intergroup dissimilarity between CS and SFD were *A. giganteus* (8.7 %)*, B. piluliferum* (8.7 %)*, F. lichenicola* (8.7 %)*,* from CS, and Pleosporales sp. (8.7 %) and *S. brevicaulis* (5.8 %) from SFD. The high similarity between CS and SFD could be due to the fact that both constitute by-products of a similar digestion process: the peculiar conditions (i.e., high temperature, alkaline pH, and anaerobic environment) occurring in both the digestive tract of ruminant animals and ABP (Bryant, 1979) may have favored the selection of a similar mycobiota.

In conclusion, the highest number of taxa was retrieved from biomasses rich in nutrients easily accessible for fungi, such as maize and zootechnical samples. Lower fungal loads and biodiversity were found on the more recalcitrant biomasses, i.e., straw and SFD. In such substrates, the lack of easily degradable carbon sources may have led to the reduction of biodiversity, fostering the development of species that can grow in limiting conditions (Tigini et al., 2018).

3.3.2.3 Effect of incubation temperature

Incubation temperature was another culture parameter that significantly influence both fungal load and biodiversity. The greatest number of taxa (59) and strains (87) were isolated at 25 °C, which seems the optimal temperature for fungal growth. Lower fungal biodiversity was achieved at other temperatures: 31 taxa (34 strains) were isolated at 15 °C, while 27 taxa (51 strains) at 37 °C. This finding indicate that mesophilic fungal community was dominant in the biomass samples.

S. thermophilum was the only species isolated from all the three temperatures (Figure 17) Besides, 17 taxa were isolated at both 15 and 25 °C, including *A. alternata, B. piluliferum, Cephalotrichum microsporum, C. stemonitis, C. cladosporioides, C. pseudocladosporioides, E. nigrum, F. commune, F. incarnatum, F. proliferatum, F. verticillioides, G. candidum, M. circinelloides, P. crustosum, Pezizaceae sp., P. membranifaciens, S. brevicaulis.* The species *A. flavus, A. fumigatus, A. niger, A. niveus, A. terreus, C. cinerea, R. thermophila* showed a more thermophilic behaviour, being isolated from incubation of both 25 and 37 °C. Only the species *P. roqueforti* and *Y. lipolytica* were found at both 15 and 37 °C, but not at 25 °C. On the other hand, several fungi were found exclusively at one specific temperature. In detail:

- 11 taxa were isolated only at 15 °C, among which *Microdochium nivale, Mortieriella indohii, Parastagonospora nodorum, Pseudeurotium bakeri* and *Wickerhamomyces anomalus;*
- 34 taxa were isolated only at 25 °C, as *A. antarcticum, Beauveria felina, Byssochlamys nivea, L. ramosa, Sarocladium strictum* and *T. asahii.*
- 17 taxa were isolated only at 37 °C, as the thermophilic species *T. crustaceus, T. lanuginosus, T. thermophilus, Westerdykella sp.*

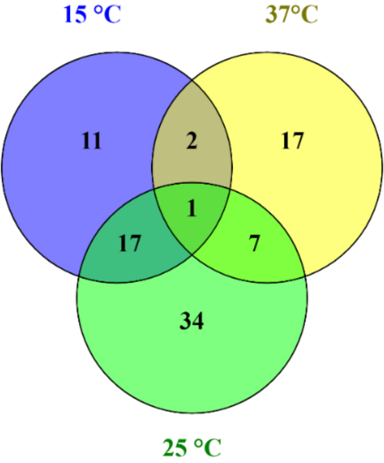


Figure 17: Veen diagram showing the relation between the number of isolated taxa and the three temperatures of incubation.

Considering each substrate individually, differences in the fungal biodiversity determined by the use of different temperature were always statistically significant (PERMANOVA, $p<0.05$; Figure 18). As reported in Table 12, the intragroup average similarity percentages were high (> than 40%) within all substrates. Among the different substrates, the intergroup similarity was on average 24 % between 15 and 25 °C, 10 % between 25 and 37 °C and 1 % between 15 and 37 °C. This low similarity, especially between the extreme temperatures (15 and 37 °C), derives from the isolation of a clearly distinct mycobiota between the different temperatures.

In line with the study of Anastasi et al. (2005), the results suggest that the employment of different temperatures allowed the isolation of rare and less competitive species, with different and peculiar physiological features.

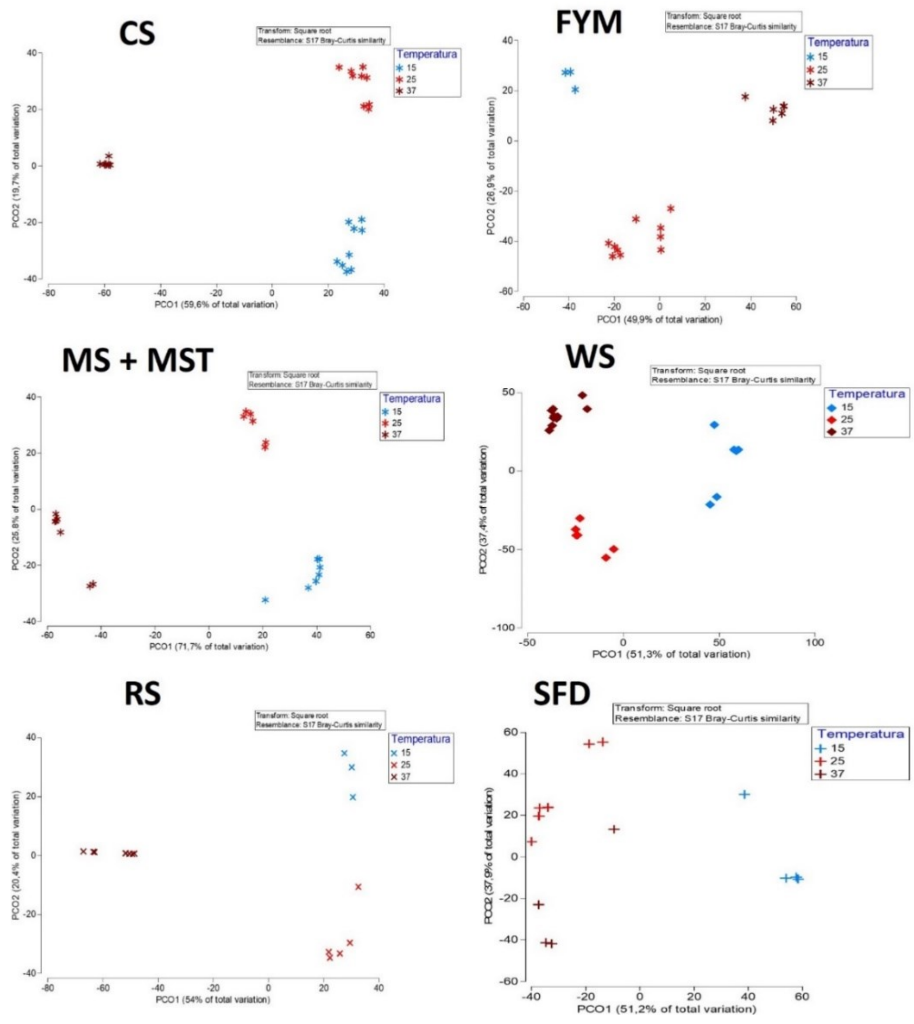


Figure 18: Graphical representation, obtained through PCO analyses, of similarity/dissimilarity within and between the different temperatures for each substrate.

Table 12: Average similarity percentage between/within groups temperatures for each substrate.

Substrates		*CS*			*CM*			*MS + MST*			*WS*			*RS*			*SFD*		
Temperatures		15	25	37	15	25	37	15	25	37	15	25	37	15	25	37	15	25	37
Average Similarity (%) between/within groups	15	54			89			85			78			83			73		
	25	31	68		20	56		40	87		9	75		37	73		4	75	
	37	0	0	73	0	8	55	5	19	85	2	16	62	0	0	42	0	18	62

3.4 Conclusions

The results highlight the importance of a fungal biodiversity investigation in order to identify promising strains for the development of efficient bioprocess for conversion of lignocellulosic biomass. The knowledge of a fungal community is at the base of a successful bioaugmentation strategy.

Overall, the zootechnical and lignocellulosic biomasses collected from biogas plants can be considered a valid environmental sample for the isolation of a culturable mycobiota potentially promising for pretreatment and other interesting biotechnological applications.

The recovery of several taxa potentially producers of plant cell wall degrading enzymes provide a strong justification for evaluating the potential of isolated fungi for pretreatment of target lignocellulosic feedstocks.

4. Fungal screening to assess the colonization of non-sterile lignocellulosic biomasses

4.1 Introduction

Several studies have investigated the impact of fungal pretreatment on the methane potential of different lignocellulosic biomasses (Wei, 2016). The results generally show a significant increase in specific methane potential, up to 50% and even higher in the case of feedstocks with very low initial biodegradability (Ge et al., 2015; Mutschlechner et al., 2015; Ramarajan and Manohar, 2017). On the other hand, other studies reported decrease in bioenergy production after fungal pretreatment due to excessive degradation of organic matter (i.e., holocellulose) (Liu et al., 2016; Vasco-Correa and Li, 2015). For this reason, fungus selection is one of the key points for an efficient biological pretreatment (Alexandropoulou et al., 2017). Other crucial parameter is the optimization of the cultivation conditions, including inoculation, moisture, temperature, aeration, particle size of biomass and supplements, during the solid-state fermentation of biomass (Alexandropoulou et al., 2017). The screening strategy for fungal selection should be preferably, cheap, rapid and sensitive, and assumes to minimize the possibility of losing the potent strains (Narkhede, 2013).

In this study, a screening was developed and performed to investigate the potential of autochthonous and allochthonous fungi for colonization and pretreatment of non-sterile lignocellulosic biomasses. In detail, the screening was focused on colonization of the main income (feedstock) and outcome (by-product) of the European agricultural biogas plant (ABP), namely maize silage (MS) and the solid fraction of digestate (SFD), with the future perspective of (re)use and valorise them as AD feedstocks. The screening was conducted on the basis of a visual evaluation of the growth and competitive ability under non-sterile conditions. The

fungal species and strains that showed the best performance were selected to be used as inoculants for further pretreatment experiments.

4.2 Materials and Methods

4.2.1 Biomass sampling and storage

Fresh samples of MS and SFD (Figure 19) were collected periodically at a third selected agricultural biogas plant (ABP-3) operating in the Piedmont region (Figure 20). ABP-3 is a completely stirred tank reactor with 1 MWel of installed power. It is usually fed with MS (75 %), triticale silage (13 %) and other cereals (12 %). The organic loading rate is 2.25 kg volatile solids (VS) m^3 digester/day. Hydraulic retention time is approximately 60 days. The resulting digested slurry (approximately 70 t/day) is processed through a screw-press (CRIMAN® mod. SM260) to separate SFD (approximately 5 t/day) from the liquid fraction (approximately 65 t/day). The SFD was then subjected to drying and N-stripping and it was stored on-site in a static heap on an uncovered platform (Figure 20).

The collected samples were stored under vacuum at 5 °C for maximum 1 month before use.

Figure 19: Samples of non-sterile maize silage (MS) and solid fraction of digestate (SFD) use for the fungal screening.

Figure 20: a) Agricultural biogas plant (ABP-3) that provided biomass samples for the experimental work; b) Silobag used for maize silage storage; c) Solid fraction of digestate on the storage platform.

4.2.2 Fungal screening

For safety reasons, 22 (out of 172 isolated) autochthonous strains belonging to (7) species classified as potentially harmful for human and animals (e.g., H2, keratinolytic species or mycotoxigenic fungi as *A. flavus, A. fumigatus, C. lobatum, S. apiospermum, S. boydii, S. prolificans, S. brevicaulis*) were excluded from the screening and further analyses.

Preliminary trials also led to the exclusion of the following fungi:

- the yeast and yeast-like organisms (15 strains belonging to 8 species, namely *C. ethanolica, G. candidum, P. kudriavzevii, P. membranifaciens, B. piluliferum, T. asahii, W. anomalus* and *Y. lipolytica)*, because inadequate for solid-state fermentation pretreatment;

- slow growing fungi (11 strains belonging to 7 species, namely, *R. thermophila*, *S. thermophilum,* Chaetomiaceae sp. *Chaetomium* sp., Pezizaceae sp., *Sodiomyces* sp. and the black microcolonial-like unidentified fungi), being inadequate for inoculum production.

Overall, 124 autochthonous strains, belonging to 67 species (34 genera), were screened for their ability to grow on non-sterile MS and SFD, being colonization of feedstocks fundamental for the establishment of a whole cell pretreatment. Besides, fifteen (15) species-strains of allochthonous wood-rot basidiomycetes (preserved at the MUT) were also included in the screening as potential lignocellulose-colonising organisms.

To produce the fungal pellet used for inoculation, fungi were pre-grown on MEA plates incubated at 25°C for 7-10 days. Fungi were then inoculated through agar plugs (0.5 cm^2) and grown in submerged fermentation in 500 mL Erlenmeyer flasks containing 350 mL of diluted (1:10) Malt Extract Broth (MEB, as MEA without agar) with 10 g/L of MS. Adding solid agro-supports to the liquid media had several purposes: pre-adapt fungi to lignocellulosic substrate, provide a

different nutrient source and mechanically disrupt the mycelia to obtain a pellet morphology optimal for homogeneous colonization (about 2-3 mm diameter). For each fungus, almost two replicate flasks were prepared and incubated at 25 °C (37 °C for thermophilic species) in agitation at 120 rpm. After about 7-10 days, the mycelium was filtered and inoculated into Petri dishes (9 cm Ø) with 10 g (fresh weight) of non-sterile MS (4.1 g dry weight) and SFD (3.3 g dry weight), to obtain a fungal inoculum/biomass ratio of about 1:20 w/w. No other nutrients were added, but SFD was supplemented with sterile deionized water (2:3 ratio w/v) to balance its moisture with that of MS (about 65-70 %). For each fungus and biomass, three replicates were set up. Negative controls consisted of not-inoculated biomass. Plates (inoculated and controls) were incubated at 25 °C (37 °C for thermophilic species) in the dark, for 3 weeks. The grow rate, percentage of plates colonization and predominance of inoculated fungi over indigenous microorganisms were visually evaluated every 3 days up to 21 days. For the evaluation of growth, fungi were categorized into the following qualitative classes (C) (Figure 21):

- C0 = no plates colonization (0-25 %);
- C1 = low plates colonization (25-50 %), whit dominance of indigenous microorganisms;
- C2 = partial plates colonization (50-75%), with low presence of indigenous microorganisms;
- C3 = total plates colonization (75-100%), with absence of visible indigenous microorganisms.

The fungi that showed the best growth performance and competitive ability under non-sterile conditions were selected to perform whole cell pretreatment. The selection targeting mainly fast-growing strains, as rapid growth means shorter treatment times, but also more slowly growing, which potentially cause less loss of fermentable carbon than the faster ones (Singh et al., 2014).

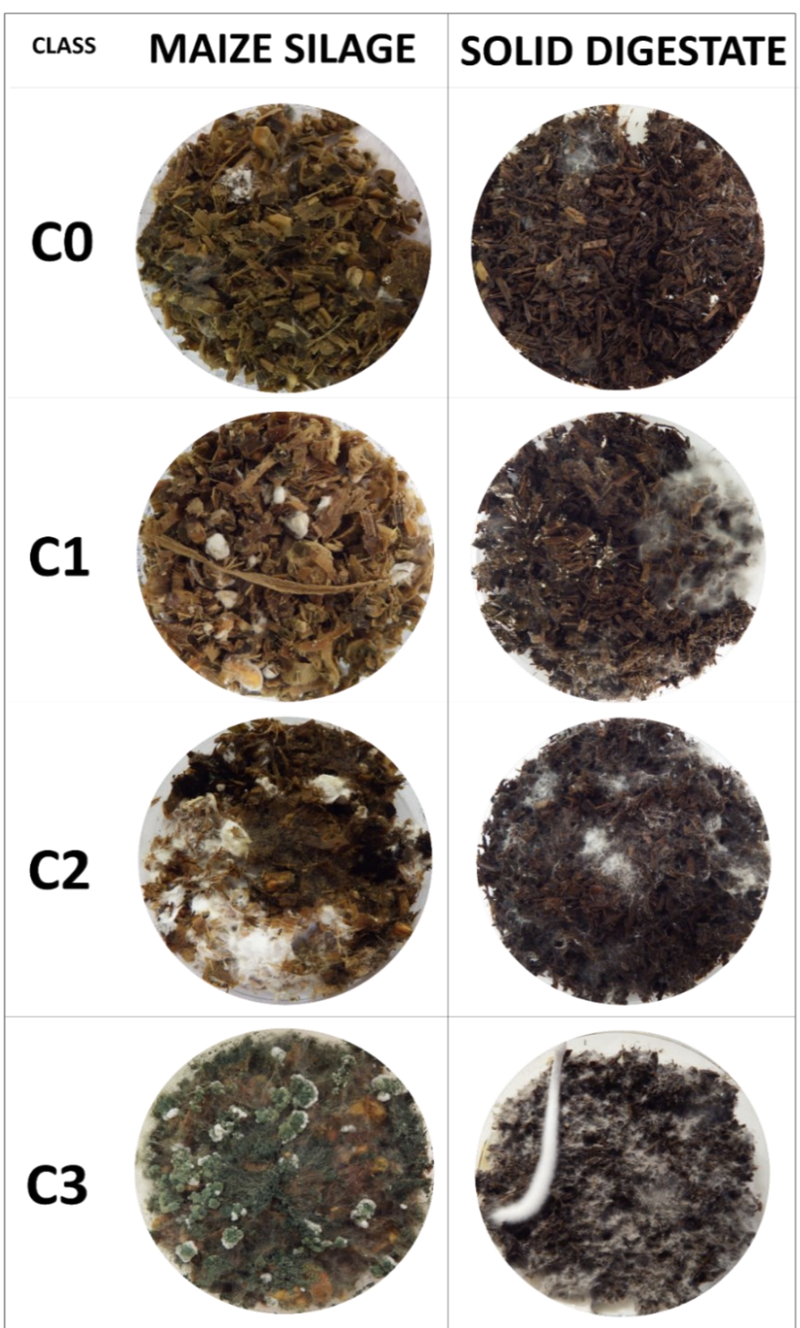


Figure 21: Visual evaluation of the fungal biomass development and colonization on non-sterile maize silage (MS) and solid fraction of digestate (SFD). C0 = no plates colonization (0-25 %); C1 = low plates colonization (25-50 %); C2 = partial plates colonization (50-75%); C3 = total plates colonization (75-100%).

4.3 Results and Discussion

The choice of the most effective fungi is a fundamental and critical step in order to develop an efficient biotechnological process (Zhou et al., 2015). It is necessary to screen a wide number of fungi to enhance the probability of finding the few strains that have the characteristic of interest. In this study, a wide fungal biodiversity (including 124 autochthonous strains, 67 species, isolated from ABP-related biomasses and 15 allochthonous Basidiomycota strains-species) was investigated for the ability to colonize non-sterile MS and SFD.

As regard MS, 55 % of the screened strains (76 strains - 49 species – 36 genera) shown no signs or very little growth on this biomass (C0). Interestingly they include all the species of allochthonous Basidiomycota, which had previously demonstrated to be sensitive and not competitive enough in non-sterile lignocellulosic substrates (Tian et al., 2012; Zhao et al., 2014). On the other hand, 63 strains belonging to 33 species (11 Genera) shown the ability to colonize MS to varying extents (C1-3) (Table 13). However, only 31 % of the total strains (43 strains - 20 species – 9 genera) were massively growing on this non-sterile biomass and, by the end of the experiments, were assigned to the C3 class (Table 13).

The fastest fungi were the strains that belonged to the genera *Lichtheimia (L. ramosa)*, *Mucor* (*M. circinelloides* and *M. fragilis*) and *Trichoderma (T. asperellum, T. harzianum, T. longibrachiatum):* they showed distinct growth after 3 days and completely colonized MS after only 5 days, while the other fungi needed more time, e.g., 7-14 days, to achieve the class C3 (Table 13, Figure 22). Despite the colonization ability indicate fungal utilization of MS components for growth, Mucoromycota (also called sugar fungi), like *Lichtheimia*, and *Mucor,* can utilize only readily available carbon source, lacking the necessary array of enzymes to degrade the complex plant cell-wall polymers (lignin and cellulose) (Andlar et al., 2018). This characteristic could make these Mucoromycota species less interesting for pretreatment application on lignocellulose. In fact, an ideal fungal

isolate for a successful process on lignocellulose would be highly selective toward lignin and cellulose degradation (Narkhede, 2013).

Table 13: List of species and strains which showed the ability to growth on MS (C1-3).

Species	N° Strain	Qualitative Categorization - Class at				
		3 days	5 days	7 days	14 days	21 days
Aspergillus alabamensis	2	0	1	2	3	3
Aspergillus fructus	1	0	0	0	1	2
Aspergillus cejpii	2	0	0	0	1	3
Aspergillus giganteus	1	1	2	3	3	3
Aspergillus latus	3	0	1	2	2	2
Aspergillus nidulans	1	0	0	1	2	2
Aspergillus niger	6	0	1	3	3	3
Aspergillus niveus	2	0	1	2	2	2
Aspergillus tabacinus	1	1	2	2	2	2
Aspergillus terreus	4	0	1	2	3	3
Aspergillus tubingensis	1	0	1	2	3	3
Bauveria felina	1	0	1	3	3	3
Byssochlamys nivea	2	0	0	1	2	3
Cephalotrichum microsporum	1	0	0	1	1	2
Cephalotrichum stemonitis	3	1	1	3	3	3
Fusarium andiyazi	3	0	0	1	1	1
Fusarium fujikuroi	1	0	0	1	2	2
Fusarium incarnatum	3	0	1	2	2	2
Fusarium verticillioides	4	0	1	2	2	3
Lichtheimia ramosa	1	1	3	3	3	3
Mucor circinelloides	7	1	3	3	3	3
Mucor fragilis	1	1	3	3	3	3
Penicillium italicum	1	1	1	2	2	2
Penicillium crustosum	1	1	1	2	2	2
Penicillium oxalicum	1	0	0	2	2	3
Penicillium paneum	1	0	1	2	2	3
Penicillium roqueforti	1	1	2	2	3	3
Penicillium semplicissimums	1	0	1	1	3	3
Pseuderotium bakeri	1	0	0	0	1	2
Scedosporium aurantiacum	1	0	1	2	2	2
Trichoderma asperellum	1	1	3	3	3	3
Trichoderma harzianum	2	1	3	3	3	3
Trichoderma longibrachiatum	1	1	2	3	3	3

On the other hand, *Trichoderma* spp., widely known as effective lignocellulolytic fungi, represent promising candidates for pretreatment of MS. In fact, previous studies (Mustafa et al., 2016; Mutschlechner et al., 2015) have reported the use of *Trichoderma* species for the pretreatment of different biomasses, and in all experiments, their application led to an enhanced biogas production. For instance, Mutschlechner et al., (2015) tested *T. viride* on non-sterile organic household waste with garden residues and obtained a clear distinction in total gas and methane production compared to controls (+47 % and +56 %, respectively). Similar positive results were obtained by Mustafa et al., (2016) on rice straw after fungal pretreatment with *Trichoderma reesei*.

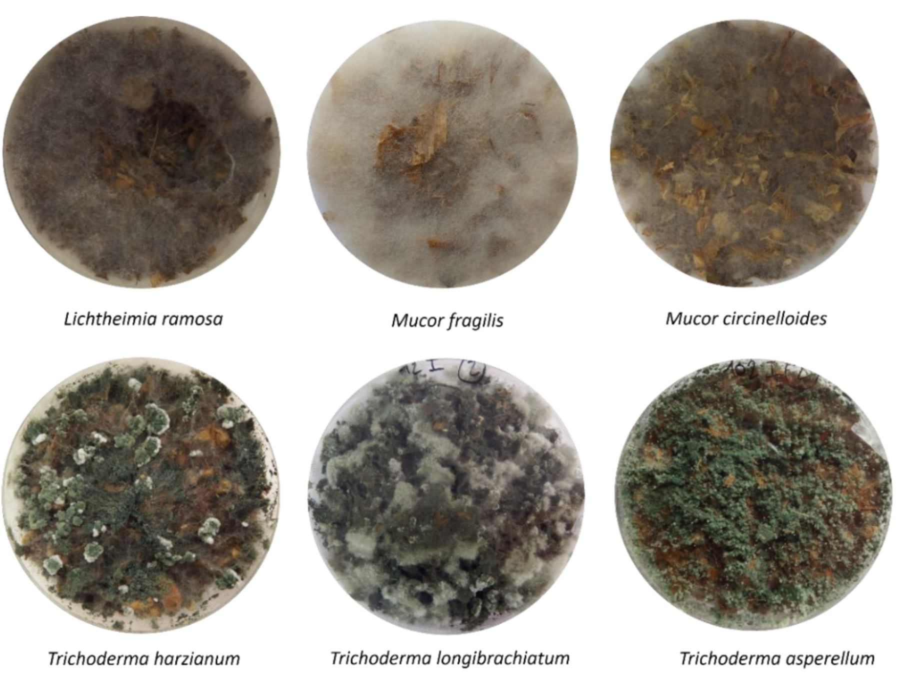


Figure 22: Species that showed the fastest growth on MS.

A particularly marked ability to grow on MS were also observed for all the species of *Aspergillus* and *Penicillium* tested, but those that grew faster and competed better with native microflora (C3 class) were *Aspergillus alabamensis, Aspergillus cejpii, Aspergillus giganteus, Aspergillus niger, Aspergillus terreus, Aspergillus tubingensis, Penicillium oxalicum, Penicillium paneum, Penicillium roqueforti* and *Penicillium semplicissimum* (Figure 23). The colonizing abilities of MS observed among *Aspergillus* and *Penicillium* spp. is not surprising considering that these fungal taxa: i) are well-known for their ability to colonize lignocellulosic materials (Young et al., 2018); ii) are among the prevalent genera in corn silage samples (Alonso et al., 2013); iii) possess an extensive range of plant cell-wall degrading enzymes (Ja′afaru, 2013). Especially noteworthy for pretreatment of MS is the species *A. niger,* a thermo-tolerant ligninolytic fungus, known to be effective in the biodegradation of lignocellulosic materials by producing oxidative and hydrolytic enzymes (Kausar et al., 2010; Young et al., 2018).

Figure 23: *Aspergillus* and *Penicillium* species that showed efficient growth on MS (C3).

Among the genus *Fusarium*, only 4 species, namely, *F. andiyazi, F. incarnatum, F. fujikuroi* and *F. verticillioides*, out of the 10 screened species showed growth on MS (C1-3). However, only *F. verticillioides,* reach the complete colonization of plates (C3). Noteworthy *F. verticillioides* is the most commonly reported fungal species infecting maize (Oren et al., 2003). The efficiency in the ability to colonize the MS within the genus *Fusarium* seems therefore linked to a species-specificity with respect to their natural habitat.

The Ascomycota species *B. nivea*, *B. felina* and *C. stemonitis* also showed massive colonization of MS (C3). *B. nivea* has already been reported to colonize MS, being one of the toxigenic fungi found with a high incidence in silages (Alonso et al., 2013). Instead, to the best of the author's knowledge, the species *B. felina* and *C. stemonitis* have not yet been studied for the colonization and the bioconversion of plant biomass.

Figure 24: Other species that showed efficient growth on MS (C1-3).

As for the SFD, 91 % of the tested strains (127 strains - 76 species – 41 genera) failed to growth (C0) and only 12 strains - 7 species (6 genera) showed the ability to colonize this biomass to varying extents (C1-3) (Table 14). However, only 7 strains, belonging to 3 species (3 genera) reached an efficient and full colonization on SFD plates (C3). The fastest were the Basidiomycota *C. cinerea* and *Cyclocybe aegerita*, that reached the full colonization in only 3-5 days of incubation and maintaining it until the end of the experiment (21 days). *C. cinerea* and *C. aegerita* are species of wood-decay fungus, previously found to colonize SFD (Musatti et al., 2017; Young et al., 2018). Besides, these species are described as efficient producers of enzymes involved in the degradation of plant cell wall components as laccases, peroxydases cellulases and pectinases (Isikhuemhen et al., 2009; Nuchdang et al., 2015). Their ability to growth on SFD and to potentially produce enzymes involved in lignocellulose degradation are reasons to consider these species promising candidates for pretreatment of digestate fibers. Other (3) Basidiomycota strains and species (*Bjerkandera adusta, Lopharia spadicea* and *Pleurotus ostreatus*) showed an initial growth on SFD, but they were progressively overwhelmed by autochthonous microorganisms. Such Basidiomycetes could have the potential to colonize and degrade the recalcitrant fibers of SFD but unfortunately many WRF have demonstrated to be not aggressive enough to compete with indigenous microorganisms in unsterilized lignocellulosic biomasses (Tian et al., 2012; Zhao et al., 2014).

The only Ascomycota species that showed efficient growth performance on SFD was *C. stemonitis*; the mycelia of this species was visually evident after 3 days of incubation and from the 7th days the substrate appeared heavily colonized. In literature, very little is known on the ability of such fungal species to grow on SFD resulting from biogas production, which is still rich in carbohydrates and might be further exploited through AD biorefinery.

Table 14: List of species and strains which showed the ability to growth on SFD (C1-3).

Species	N° Strain	Qualitative Categorization - Class at				
		3 days	5 days	7 days	14 days	21 days
Cephalotrichum stemonitis	3	1	2	3	3	3
Cephalotrichum microsporum	1	1	1	2	2	2
Coprinopsis cinerea	4	2	3	3	3	3
Bjerkandera adusta	1	0	1	1	2	2
Cyclocybe aegerita	1	2	3	3	3	3
Lopharia spadicea	1	0	0	1	1	1
Pleurotus ostreatus	1	0	1	1	2	2

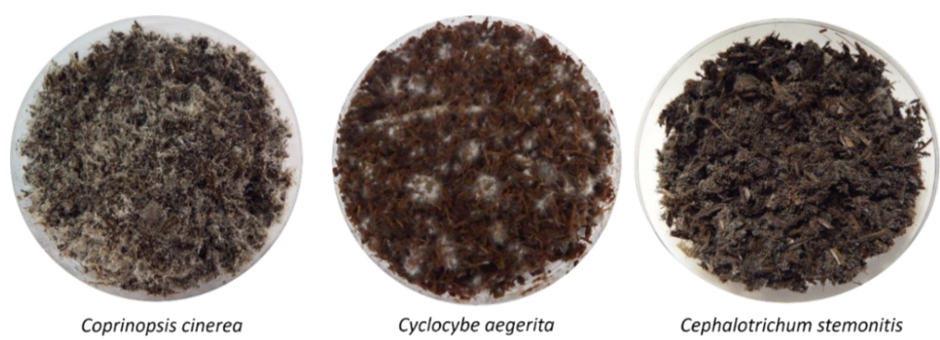


Figure 25: Species that showed fast and efficient growth on SFD (C3).

Overall, the observations made within the study suggests that the potential for the lignocellulose colonization is a species-specific feature, since strains of the same species showed similar ability, regardless of the agrozootechnical biomass or sampling sites from which they were isolated. However, eventual intraspecific differences in lignocellulosic biomass bioconversion need to be assessed by more in-depth quantitative screening and analyses (Berrin et al., 2012; Zhou et al., 2015).

The results suggest also that SFD is more recalcitrant to fungal growth than MS, since the number of species that showed colonization of MS was much higher

than that of species growth in the presence of SFD. The findings are in line with the research of Chen et al. (2012), which reported that digestate derived from AD of maize was more recalcitrant to biodegradation than maize straw. It seems plausible that the different composition caused the dissimilar fungal growth between the different substrates. SFD is commonly characterized by a high lignin content and pH level (8.5), which can represent limiting factor for fungal growth (Musatti et al., 2017). The SFD could be also particularly enriched in ammonia, and fungi are known to be sensitive towards its high concentrations (Chen et al., 2012; Musatti et al., 2017). Therefore, the lack of easily degradable carbon source and other inhibitory factors may have limited or slow down fungal growth on SFD. Contrariwise, MS, resulted more easier to colonize by fungi, due to the higher availability of nutrients and the more favorable conditions (i.e., acid pH).

Notably, most of the studies on pretreatment of lignocellulosic feedstocks focused on the use of white-rot basidiomycetes inhabiting wood, while fungi of other taxonomic groups or habitats have only scarcely been investigated (Singh et al., 2014). In our screening, Basidiomycota result efficient in the colonization of the more recalcitrant SFD, but not of MS. On the contrary, Mucoromycota showed excellent colonization abilities only on MS, while on SFD they did not grow. Ascomycota also seems to prefer MS as substrate, but one species, *C. stemonitis,* was found capable to growth efficiently on both non-sterile biomasses. The findings emphasize the importance of a screening step on the whole fungal biodiversity in order to identify new and promising species for the conversion of lignocellulosic biomasses (Chang et al., 2012; Ja'afaru, 2013).

Although observational rather than experimental, the adopted screening strategy resulted a powerful tool to select a restricted number of fungi suitable for the pretreatment of non-sterile MS and SFD (Table 15). The selection of fungi has prioritized growth performance and competitive ability of the inoculated fungi; therefore, our strategy differs from the traditional approach in which microorganisms were selected based on their enzyme activity or taxonomic status

(Hart et al., 2002). Besides being simple and inexpensive, the adopted strategy has the advantages that allowed the simultaneous screening of a large number of fungi on different biomass feedstocks, and it could be applied to a wide variety of solid lignocellulosic substrates. Moreover, the development of process relying on autochthonous strains has the advantage of being generally more accepted by public authorities and society, since it excludes the use of exogenous or genetically modified strains. However, the major innovative aspect of the work is that the screening was carried out by culturing fungi in non-sterile conditions. Currently, few papers report the direct inoculation of fungi into unsterilized feedstocks (Song et al., 2013; Zhao et al., 2014) and according with our results, the colonization results unsuccessful for most of screened strains, probably due to the high competition with the indigenous microflora (Zhao et al., 2014). The selection of fungi that can efficiently grow in the presence of non-sterile biomass is a prerequisite for future industrial application. Significant energy input may be reduced by developing a pretreatment technology that does not require sterilization of feedstocks (España-Gamboa et al., 2017; Zhao et al., 2014).

Based on the results, 20 species could be considered promising candidates for pretreatment of MS, and 3 species for pretreatment of SFD (Table 15). However, the most promising strains for the development of a pretreatment on both ABP feedstock (MS) and by-product (SFD) belonged to *C. stemonitis* species, being the only ones capable of widely colonizing both non-sterile biomasses. Interestingly, *C. stemonitis* strains were isolated only from zootechnical samples (FYM and CS), indicating their coprophilous behaviour. Indeed, fungi that inhabit herbivore feces are known to secrete enzymes capable of degrading recalcitrant PCWP still present after the digestion process (Peterson et al., 2011). Noteworthy, very scattered information are available on the skills of *C. stemonitis* in lignocellulosic biomass transformation. Peterson et al. (2011) analysed the secretome of *C. stemonitis,* revealing its ability to produce several enzymes involved in the degradation of cellulose, hemicellulose, pectin, lignin (e.g., cellobiohydrolases endoglucanases,

glucosidases, xylanases, xylosidases, oxidoreductases). The ability to produce enzymes which can degrade recalcitrant PCWP, makes this species even more attractive for pretreatment applications. Therefore, one *C. stemonitis* (MUT 6326) strain was the first fungus selected to be used in the next experimental phase, which consist in the scale-up of pretreatment on MS and SFD and in the assessment of its effects in terms of PCWP degradation and BMP by means of batch trials.

Table 15: Fungi selected for pretreatment of non-sterile MS and/or SFD.

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

4.4 Conclusions

Natural fungal diversity offers a fundamental source of new fungal strains with high biotechnological potential. In this study, a large number of (autochthonous and allochthonous) fungi were evaluated for lignocellulosic biomass colonization. The qualitative screening method described is a powerful tool to preselect relevant fungi suitable for whole cell biological pretreatment. The method resulted also to be helpful for performing preliminary assays for culture condition optimization (moisture, inoculum/substrate ratio, etc.,). The selected strains will undergo to a scale-up of the solid-state pretreatment to further select the best combination of fungi and biomasses and to optimize the process.

5. Fungal pretreatments and Biochemical Methane Potential tests

5.1 Introduction

Anaerobic digestion (AD) is among the most efficient technology for renewable energy production, but there is still room for some significant improvements. The biogas production can be indeed hindered by the presence of:

- chemicals (i.e., antibiotics, tannins, phenols and furans in feedstocks) that inhibit microorganisms during AD (Monlau et al., 2014);
- recalcitrant compounds, as lignin, which decrease the bioavailability of the more biodegradable components, as hemicellulose and cellulose (López et al., 2013).

Moreover, the increased use of energy crops for biogas production shows some drawbacks: i) the use of cereal and other starch-rich crops may come into competition with land use and food-feed production (Fritsche et al., 2010); ii) the economic feasibility of the AD process is challenged due to the rising price of energy crops and the future reduction of financial incentives (Young et al., 2018).

The enhancement of the process efficiency and/or the use of alternative and sustainable lignocellulosic feedstocks, such as agro-industrial wastes and by-products, may contribute to improve the environmental and economic sustainability of AD (Bond and Templeton, 2011; Young et al., 2018). According to a circular economy approach, previous studies have suggested the possibility of exploiting the solid fraction of digestate (SFD) as a feedstock for a further AD step (Gioelli et al., 2011; Menardo et al., 2011b; Monlau et al., 2015). The integration of this option could reduce GHG and ammonia emissions that occur during digestate storage and could allow the concomitant recovery of economically attractive amounts of biogas-methane, reducing the environmental impact and improving the profitability of AD process (Monlau et al., 2015; Sambusiti et al., 2015).

Unfortunately, the SFD from agrozootechnical residues has been widely recognized as a recalcitrant feedstock and its use for biogas production is not generally a feasible option (Zhong et al., 2016). The cellulose crystallinity and the high lignin content decrease the digestibility and limit the theoretical biogas yields (Rouches et al., 2016).

In order to enhance the anaerobic degradability of refractory lignocellulosic biomasses and improve the biogas recovery, biological pretreatment processes can be applied. Among biological treatment, the use of fungi to improve the conversion of lignocellulose have increasingly gained importance (Liu et al., 2014a; Mutschlechner et al., 2015; Tian et al., 2012). Indeed, many fungi are known for the ability to produce lignocellulolytic enzymes capable of effectively degrading the structural components of plant cells: by inducing mechanical and biochemical modifications of lignocellulose, fungi can make structural polysaccharides more accessible to the microorganisms involved in AD process, with a consequent improvement in energetic yields (Merlin Christy et al., 2014; Sindhu et al., 2016).

Despite the great potential, most of the fungal pretreatment studies are performed at lab-scale, with conditions that are difficult to apply at the industrial level (e.g., sterile conditions). Any application of fungal pretreatment at a large scale is prevented by the lack of knowledge about how to control the process, the operative parameters to be monitored, the microbial resources to be exploited, etc. The present work wants to partially fill this gap, focusing the attention on processes that could lead to an efficient (re)use of maize silage (MS) and the solid fraction of digestate (SFD) as AD feedstock. Biological techniques based on filamentous fungi were then the tool used to achieve this goal. The role of different fungal strains, belonging to the Basidiomycota and Ascomycota phyla, and different pretreatment times were investigated. The biomethane potential from co-digestion of fungal biomass was also studied, increasing the information about the fate and possible contribution of fungi along the entire AD process.

5.2 Materials and Methods

5.2.1 Biomass sampling

The MS and SFD samples were collected from the storage facility of the ABP-3. For further details, see chapter 4.2.1.

5.2.2 Fungal strains

Three fungal strains were selected to perform the whole cell pretreatment on non-sterile MS and/or SFD. They were the Ascomycota *Cephalotrichum stemonitis* MUT 6326 and the two Basidiomycota *Coprinopsis cinerea* MUT 6385 and *Cyclocybe aegerita* MUT 5639. *C. stemonitis* and *C. cinerea* were isolated during the PhD project from feedstocks and by-products of the AD, i.e., cow slurry and solid digestate, respectively; *C. aegerita* is allochthonous and it was previously isolated from fruiting bodies in Piedmont woods and preserved at the MUT collection. The nucleotide sequences of *C. cinerea* and *C. stemonitis* were deposited at the GenBank NCBI database under the accession numbers MT151631 and MT151633, respectively.

5.2.3 Experimental set-up

Three different experiments were performed:

- **Experiment 1:** one fungal strain (*C. stemonitis* MUT 6326) was used to perform 10 days of whole cell pretreatment on both non-sterile MS and SFD. The aim was to obtain preliminary data about the effects of the fungal pretreatment on biomasses with different physicochemical features (easy-accessible MS vs recalcitrant SFD), that constitute the main income (feedstock) and outcome (by-product) of local agricultural biogas plant.

- **Experiment 2:** three fungal strains (*C. cinerea* MUT 6385, *C. aegerita* MUT 5639 and *C. stemonitis* MUT 6326) were used to perform the pretreatment on non-sterile SFD. For each strain, three pretreatment durations were tested:

- T0: fungal inoculum is added to the lignocellulosic biomass immediately before BMP tests start; thus, the pretreatment did not occur, and the fungal biomass was co-digested with SFD;
- T10: pretreatment duration of 10 days;
- T20: pretreatment duration of 20 days.

The main aims of the study were: i) to evaluate the performances of pretreatment with different fungal species; ii) to study the influence of different pretreatment duration; iii) to investigate the effects on biogas production of the co-digestion of fungal biomass and SFD.

- **Experiment 3:** one fungal strain (*C. stemonitis* MUT 6326) was used to perform the pretreatment on non-sterile SFD. Two pretreatment durations were tested (T0 and T10). For each thesis, a further variable was added, consisting in a combination of biological and thermal pretreatment with autoclave. In thermally pretreated samples the fungus was dead and not active during BMP, while, in the not-autoclaved ones, the fungus is presumably alive and active, almost on the first phase of the AD process. Besides, it was tested the methanogenic potential of the fungal biomass only, thermally pretreated (autoclaved - dead) and not thermally pretreated (not-autoclaved - alive).

The aims were: i) to confirm the reproducibility of the results obtained in previous experiments; ii) to evaluate the effects of a combination of fungal and physical pretreatment (autoclaving); iii) to study the possible role and activity of fungi during AD; iv) to investigate the methanogenic potential of fungal biomass (alone and during co-digestion with SFD) and its suitability as a feedstock for AD.

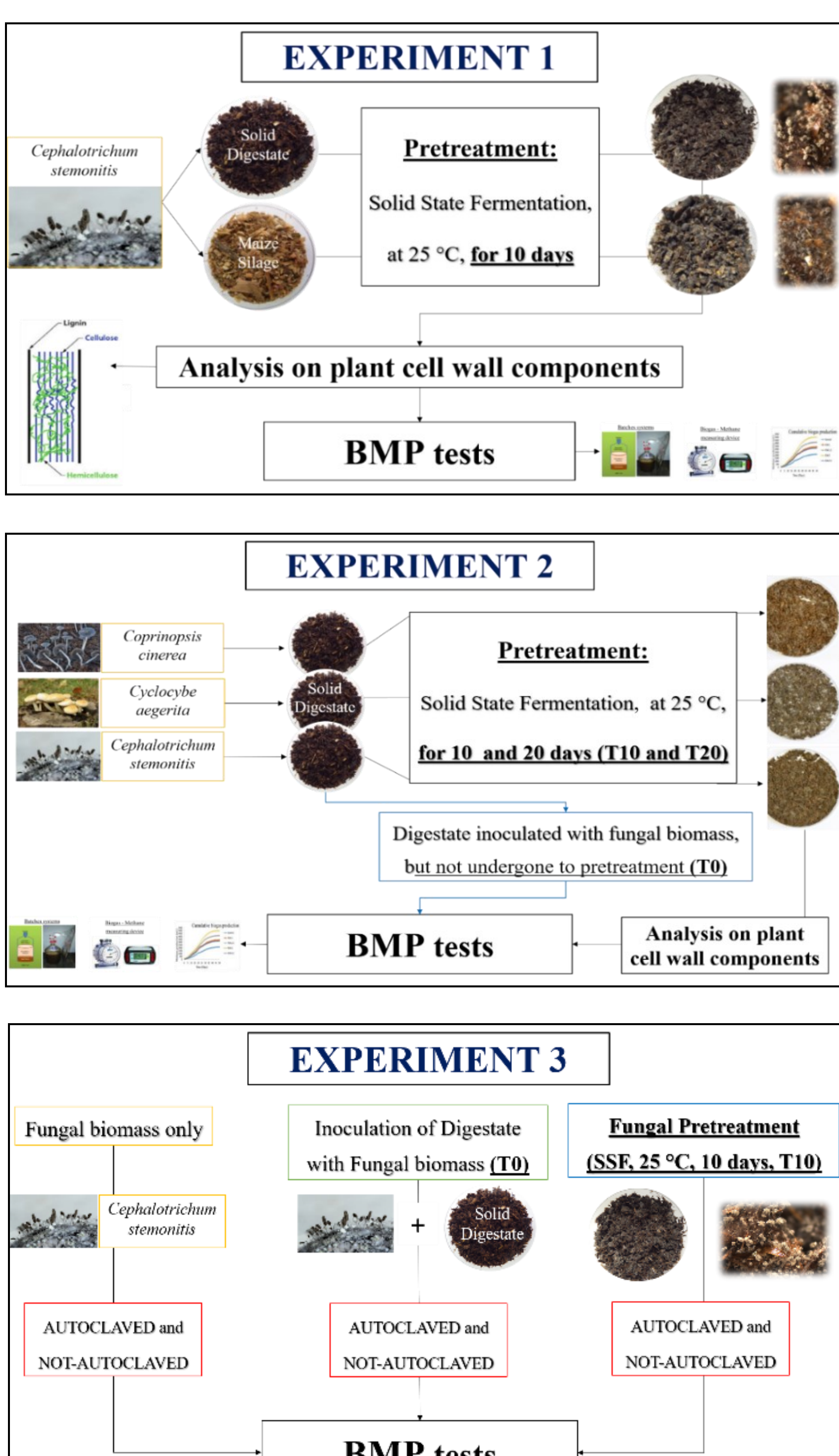


Figure 26: Graphical representation of the three experiments performed.

5.2.4 Fungal inoculation and pretreatment

To produce the mycelial biomass used as inoculum, fungi were pre-grown in MEA plates and incubated at 25 °C for 10 days. Agar cultures were then used to produce mycelium pellets under submerged fermentation into 500 mL Erlenmeyer flasks containing 350 mL of liquid medium. The fungal inoculum was produced in submerged fermentation (SmF) since compared with the solid-state ones (SSF), it has a higher efficiency in the production of fungal biomass, requires shorter time and enables a proper scale-up and an easier process control, thus it is reported as the best choice to develop a large-scale pretreatment (Tian et al., 2012). In order to optimize the growth conditions of each fungal strain, different culture broths were tested:

- Malt Extract Broth (MEB, as MEA without agar),
- MEB diluted (1:10 v/v),
- MEB with 10 g/L maize silage,
- MEB diluted (1:10 v/v) with 10 g/L maize silage.

Ultimately, MEB was selected to produce the inocula of Basidiomycota strains while MEB diluted (1:10 v/v) with 10 g/l maize silage for the Ascomycota.

Flasks' inoculation was also carried out in different ways for Ascomycota and Basidiomycota strains. For Ascomycota, (25) agar plugs (0.5 cm^2) were added into the flasks, while for Basidiomycota the inoculation was performed by putting into each flask 4 mL of mycelium homogenate, obtained from blending fungal agar colonies and sterile deionized water (1 cm^2 agar/mL) (Spina et al., 2018).

Inoculated flasks were incubated at 25 °C in agitation (120 rpm) in the dark. After 7-10 days, mycelial pellets were filtered from the liquid medium using sterile sieves (mesh: 150-300 µm). In Experiment 1 and 2, the fungal biomasses were washed with sterile deionized water in order to avoid adding to the biomasses any nutrients residues of the exhaust medium and/or the fungal metabolites produced

during the liquid fermentation. On the other hand, in the Experiment 3 the fungal biomasses were not washed, and any residual component of exhausted media was inoculated onto the lignocellulosic biomass.

The inoculation was conducted as reported in Section 4.2.2, scaling up the system to almost 400 g. Thus, 20 g dry weight (dw) of fungal biomass was inoculated in 400 g dw of non-sterilized MS and/or SFD. The content of total solids (TS) and volatile solids (VS) of the mycelial biomass used as inoculum was determined according to Dinuccio et al. (2010); the fungal inoculum had an average TS content of 4.3-7.3%, of which 97.4- 98.2-% were VS. When necessary, water was added (2:3 ratio w/v) to obtain a final moisture content of about 70-75%, indicated in literature as optimal for lignin decomposition (Wan and Li, 2011). Fungal and lignocellulosic biomasses were then mixed with a sterile spatula and portion of the homogeneous mix were transferred into bioreactors (15 cm diameter Petri dishes) where the pretreatment processes occurred.

Fungal pretreatments were performed at 25 °C, in the dark, under solid-state fermentation and non-sterile condition, for 0, 10 or 20 days. According to the literature data (Mustafa et al., 2016; Phutela et al., 2011) and preliminary trials, a few weeks of pretreatment is optimal for fungal growth and should ensure extensive colonization of the biomass. During cultivation, the plates were supplied with sterile deionized water every 3-4 days, to compensate for evaporation (moisture content was kept around 65-75 %). Biomass without fungal inoculum (untreated) was set-up as negative control.

At the end of aerobic pretreatments, fungal-pretreated biomasses and the respective samples without fungal inoculum (untreated controls) were processed to determine the main physicochemical characteristics and changes induced by the processes and to be used as feedstocks for subsequent AD in lab-scale experiments (BMP tests in batch trials).

5.2.5 Biomass characterization

Analyses on untreated and fungal-pretreated samples were conducted on homogenous composite samples obtained by mixing different replicates.

In order to estimate the autochthonous microbial load of the non-sterile and untreated samples, microbial cell density was measured using a light-microscope (no staining needed). In detail, 1 g of each biomass was suspended in 6 mL sterile water and incubated at 25 ◦C and 150 rpm for 1 h in order to release all the microbial propagules in the supernatant: the microbial cells (i.e., bacteria, yeasts and fungal conidia or spores) was determined in a Bürker chamber, calculating the concentration of cells per gram of biomass (cells/g) (Camacho-Fernández et al., 2018; Gömöri et al., 2018).

The total solids (TS), volatile solids (VS), total nitrogen (TN), total ammoniacal nitrogen (TAN) and the pH were analysed according to Association of Official Analytical Chemists (AOAC, 2000) and Dinuccio et al. (2010). pH in water was determined with a pH-meter (Basic20, Crison). TS content calculation occurred after aliquot samples were heated for 24 hours at 105 ° C, while VS calculation occurred after a 4 hours' incineration in a muffle oven at 550 ° C (AOAC, 2000; Dinuccio et al., 2013).

The total fibers composition was estimated as neutral detergent fibers (NDF), acid detergent fibers (ADF) and acid detergent lignin (ADL) using the detergent Van Soest methods (Van Soest et al., 1991). The hemicelluloses and celluloses content were calculated as the difference between NDF and ADF, and ADF and ADL, respectively (Dinuccio et al., 2010). It should be considered that the applied Van Soest method, as with others presented in the literature (Nayan et al., 2019), is not actually capable of discriminating the contribution of fungal and plant insoluble components. Hence, the normalization with the contribution of the fungal biomass (2.7 - 6.7% of TS) was not performed and the data presented include a marginal fraction of NDF, ADF, and ADL possibly ascribable to the fungal mycelium.

The TS, TN, and TAN data were expressed as percentages of the raw wet biomass. On the contrary, the VS, NDF, and PCWP data were expressed as percentages of the TS content of the dry biomass, avoiding any bias due to samples with different water contents (Dinuccio et al., 2013, 2010).

The percentage loss of biomass components (e.g., TS, VS, NDF and PCWP) during the pretreatments was calculated according to Zhao et al. (2014), using the following equation:

$$\text{Loss \%} = [(\text{Concentration}_{untreated} - \text{Concentration}_{fungal\text{-}treated})/\text{Concentration}_{untreated}] \times 100 \%.$$

These parameters are expressed as variation percentage comparing data obtained after the pretreatment with those of the untreated samples, namely control.

5.2.6 Enzymatic analyses (Experiment 2 only)

During the fungal pretreatments of Experiment 2, samples of the colonized SFD were periodically collected to assess the production of cellulolytic (endo-glucanases and exo-glucanases) hemicellulolytic (xylanases) and ligninolytic (laccases) enzymes under solid-state fermentations.

5.2.6.1 Enzymes recovery

Enzymes' recovery was performed after 3 – 5 – 7 – 10 – 14 - 17- 20 days by placing 2 g of colonized SFD into a 50 mL Falcon with sodium citrate (0.05 M, pH 5). Different biomass:buffer ratios were tested in order to optimize the enzyme extraction protocols (1:2 - 1:5 - 1:10 – 1:20 w/v). The Falcons were then put onto a rotatory shaker (120-130 rpm) at room temperature (22 ± 2°C) for two different incubation times (30 min and 3 h) in order to solubilize the enzymes produced by fungi. Falcons were centrifuged at 10000 x g, 4 ° C for 20 min and the resulting aqueous supernatants was separated and transferred into sterile 2 mL Eppendorf. These crude enzyme extracts were used for the enzyme assays.

5.2.6.2 Enzyme assays

Enzyme assays protocols were optimized starting from the literature data (Bailey et al., 1992; Dutta et al., 2018; Gao et al., 2008; Ghose, 1987; Gutiérrez-Soto et al., 2015). All enzymatic analyses were carried out in 96-wells, flat-bottom, microtiter plates, using a spectrophotometer (TECAN Infinite M200, Austria).

Laccase assay

Laccase activity was measured at 25 °C, observing the oxidation rate of 2.20-azinobis (3- ethylbenzothiazoline-6-sulfonic acid (ABTS) at 420 nm, in 0.10 M of sodium citrate buffer (pH 3), by means of a spectrophotometer (Niku-Paavola et al., 1988). The total reaction volume was 200 µL and the reagents were: 20 µL of ABTS (0.05 M in water), 160 µL sodium citrate buffer (100 mM, pH 2) and 20 µL of crude enzyme (Anastasi et al., 2012). The reaction started by the addition of the crude enzyme sample; hence the absorbance was measured immediately in one-minute intervals. Laccase activity was expressed as IU/L, where 1 IU is defined as the amount of enzyme catalyzing the conversion of 1 µmole of the target substrate per minute (Anastasi et al., 2012; Niku-Paavola et al., 1988).

Xylanase and Cellulases assays

Xylanase and cellulases activity were measured by determining the amount of reducing sugar released by the crude enzymes from specific substrates, which is different for each assay. The substrates consisted of:

- Oat-spelt Xylan (1 % in sodium citrate buffer, 50 mM, pH 5) for xylanase assay (Bailey et al., 1992);
- Carboxymethyl Cellulase (CMC, low viscosity) (2 % in citrate buffer, 50 mM, pH 5) for endo-glucanase assay (CMCase) (Gao et al., 2008; Ghose, 1987);

- Microcrystalline Cellulose (AVICEL PH-101) (2 % in citrate buffer, 50 mM, pH 5) for exo-glucanase assay (AVICELase) (Ghose, 1987; Gutiérrez-Soto et al., 2015).

The reaction mixture consisted of a combination of crude enzymes and substrate and the total reaction volume was 1 mL. In order to optimize the tests performance, different crude enzyme:substrate ratios were tested (1:9, 1:4, 1:1, 2:1 v/v). Blanks consisted in sodium citrate buffer (50 mM, pH 5) alone, while enzyme controls and substrate controls were composed by mixtures of buffer and crude enzyme and by buffer and substrate, respectively. Both enzyme controls and substrate controls were tested at the same ratio of their correspondent assay.

All reaction mixture, including standard curves, blanks and controls, were incubated at 50 °C for 30 min. After incubation, the release of reducing sugars, as xylose or glucose, was evaluated through the dinitrosalicylic acid (DNSA) assays (Bailey et al., 1992; Ghose, 1987). Eppendorf were centrifuged at 10000 g for 1-2 min. Then, 100 µL of each reaction mixture was transferred into a new Eppendorf (1.5 mL), together with 500 µL of DNSA reagent. Eppendorf were incubated at 95 °C for 5 min and cooled in fresh water for 5 min. The amount of released reducing sugars was measured trough spectrophotometric analysis (OD 540 nm).

The data obtained from xylanase and cellulase analyses were normalized using either blank and controls (enzyme and substrate), in order not to consider reducing sugars derived from the culture media. A glucose standard curve was used to calibrate analyses results and was prepared diluting a glucose stock solution (10 g/L) into sodium citrate buffer (50 mM, pH 5) in order to obtain different known concentrations (0 – 0.125 – 0.25 – 0.5 – 0.75 – 1 – 2 – 3 – 4 – 5 – 6 - 7 g/L). Xylanase and cellulase enzymes activity were defined as IU/L, where 1 IU corresponds to the quantity of enzyme able to release 1 µmol of reducing sugars from the substrates per min (Bailey et al., 1992; Das and Ray, 2016; Ghose, 1987).

5.2.7 Biochemical Methane Potential tests

Biochemical Methane Potential (BMP) tests were performed through batch trials, according to Dinuccio et al. (2010) and Verein Deutscher Ingenieure (VDI 4630, 2006). The batch reactors were set up by filling 2 L capacity digesters with a mixture of feedstock, inoculum (800 g) and deionized water (700 g) to obtain a final feedstock to inoculum ratio of 1:2 based on VS content. This mean that, for each experiment, inside each batch reactor the same amount of total VS was added. The inoculum source used for the BMP trials was the separated liquid fraction of digested slurry produced by selected ABP. Batches containing untreated MS and/or SFD were used as control and they were used to evaluate the performance of the batches run with biomass that had undergone to fungal pretreatment: the two trial conditions (control and pretreated) were then compared, and the results are expressed as variation percentage. Blank batches trials with inoculum only were also carried out to determine its productivity and correct the yields from the tested biomass. The potential biogas production derived from the addition of VS from fungal biomass was not compensated, and it was included in the calculations of the final yields. For each trial condition at least three replicate reactors were run.

Immediately after batches preparation the anaerobic conditions were allowed by nitrogen flushing (Figure 27). Each batch reactor was then sealed with glass taps connected, by means of tygon tubing, to a Tedlar bag (3 L capacity) for biogas collection. Trials were performed under mesophilic conditions (40 °C ± 2 °C) in a temperature-controlled incubator (Figure 28), for at least 50 days (max 75 days).

The biogas volume and composition were monitored every 3-4 days during the first 2-3 weeks, and then weekly until no more biogas production was detected. The volume of biogas produced was measured by means of a Ritter Drum-type Gas volume meter (TG05/5, Ritter Apparatebau GmbH & Co. KG, Bochum, Germany). The biogas composition and the methane concentration in the biogas was determined with a gas analyser equipped with infrared sensors (model XAM 7000, Drägerwerk AG & Co. KgaA, Lübeck, Germany) (Figure 29) (Dinuccio et al.,

2013). Batches headspaces were also calculated based on feedstock mixture density in order to correct the produced biogas yields.

Figure 27: Batches prepared with mixture of inoculum, water and biomass and subjected to nitrogen flushing to provide the anaerobic conditions necessary to start AD process.

Figure 28: Batches ready for BMP trials inside the climatic chamber used to keep constant the process temperature at mesophilic conditions (40 °C ± 2 °C)

Figure 29: The equipment used to measure the volume and the chemical composition of biogas produced during the BMP tests.

The recorded data were normalized at standard temperature and pressure (0 °C and 1013 hPa) according to Verein Deutscher Ingenieure (VDI 4630, 2006) and the specific yields of biogas and methane were expressed as normal liters (L_N) per kg of VS. The daily rate (expressed as L_N per kg of VS per day, L_N/kg VS d) and the cumulative rate (expressed as cumulative L_N per kg of VS, ΣL_N/kg VS) of biogas and methane production were calculated according to the procedure described by Dinuccio et al. (2010).

5.2.8 Statistical analysis

The data for which replicas were available (TS, VS, enzymes production, cumulative biogas-methane yields from BMP tests) were statistically analysed by one-way analysis of variance (ANOVA) followed by Tukey's means grouping tests using the Software package RStudio Version 3.4.3.

5.3 Results and Discussion

5.3.1 Fungal inoculum growth optimization

Producing an adequate and competent inoculum source is fundamental for the success of biological pretreatments (Zhao et al., 2014). In this work, standardization and maximization of the fungal inoculum production was performed testing:

- two different methods of flasks inoculation (agar plugs and mycelium homogenate obtained from blending fungal agar colonies);
- four different culture broths (MEB; MEB with 10 g/l maize silage; MEB diluted 1:10 v/v; MEB diluted 1/10 v/v with 10 g/l maize silage).

Both parameters influence inoculum characteristics and, therefore, its effectiveness.

Concerning the inoculation methods, the Basidiomycota strains showed to produce more biomass and an adequate mycelium pellets morphology when the agar colonies were blended, and the mycelium homogenates were used for flasks inoculation. Indeed, Basidiomycota do not produce conidia, and when they were inoculated with agar plugs, the obtained fungal pellets size was inadequate (too big) to provide an efficient and homogeneous colonization of the substrate (Figure 30). Instead, blending Basidiomycota colonies allowed to obtain smaller mycelium pellets in SmF (Figure 30). As regards the Ascomycota *C. stemonitis*, the inoculation of flasks was conducted indiscriminately with agar plugs or mycelium homogenate since it produced small pellets in both conditions. However, considering a cost-benefit analysis, it is more efficient to use agar plugs as the blending phase would require additional materials, time, and costs (mainly related to the use and sterilization of supplementary materials).

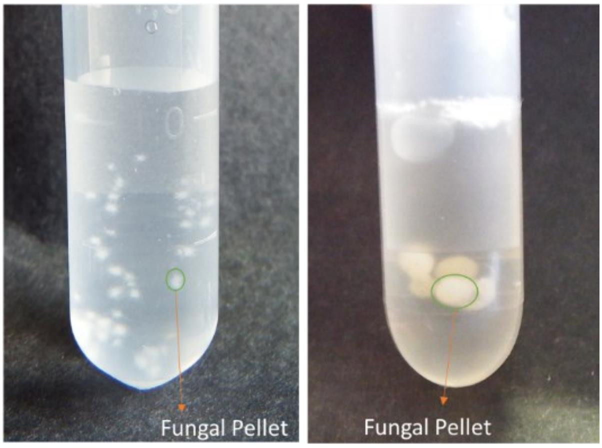


Figure 30: Difference in the morphology of the fungal pellet of *C. cinerea* inoculated trough mycelium homogenate (left) or agar plugs (right) into the liquid media. The use of the mycelium homogenate results in the formation of smaller pellets.

In this work the production of fungal inocula was performed and optimized under SmF based on the literature data and previous trials performed at the MUT laboratory (Romagnolo, personal communication). According to literature, the SmF culture method requires shorter time, allows to produce higher amounts of inoculum and enables a proper scale-up and an easier process control (Farinas, 2015; Saqib et al., 2010) in comparison with SSF, though the latter allows fungi to pre-adapt to biomass, allowing a potential better PCWP degradation during pretreatments (Rouches et al., 2016). One drawback of the SmF is that it could become expensive due to the utilization of synthetic culture media, which are highly performing, but also extremely costly (Aouidi et al., 2010). An alternative to synthetic media is to exploit cheaper culture broths, such as liquid agro-industrial wastes (i.e., cheese whey, etc) (Vamvakaki et al., 2010).

Among the tested culture media, MEB was identified as the most suitable for the growth of Basidiomycota strains (Figure 31-32). MEB is currently one of the most frequently employed culture media for aerobic fungi, due to its suitability

for growing most of fungal species (Wu et al., 2000). Instead, MEB diluted (1:10 v/v) with 10 g/l maize silage resulted the most performing for the Ascomycota *C. stemonitis* (Figure 31 and 32). This new medium was designed at MUT trying to develop a cheaper methodology for a vigorous fungal biomass production. The use of the solid agro-supports into the liquid medium has several purposes: pre-adapt fungi to a lignocellulosic substrate, modify the pellet morphology practicing a mechanical force on mycelia helping to induce the production of smaller pellets, and provide a different carbon source. In the literature, growing fungi under SmF with the addition of solid substrates was especially applied for enzymes production. For examples, Songulashvili et al. (2015) used wheat bran to grow *Carrena unicolor* for the production of laccases and other interesting enzymes. Similarly, Emtiazi et al. (2001) obtained cellulase production by *A. terreus* grown on wheat straw, while Xu et al. (2017) cultivated *Inonotus obliquus* using straw biomass obtaining production of both ligninolytic and cellulolytic enzymes.

Since the crude extract of fungi grown in SmF with lignocellulosic substrates may contain several lignocellulolytic enzymes, it could be potentially useful as supplements for degrading PCWP during pretreatment processes. On this aspect, it should be noted that different approach was used in this work. In Experiments 1 and 2, only fresh fungal biomass, deprived of excess water and nutrients, were inoculated into the lignocellulosic biomass. Thus, the crude enzymes extracts were not inoculated on the substrates to not introduce further variables in the experiments, as residual nutrients (i.e., sugars) or metabolites present in the culture broth. On the other hand, in the Experiment 3, part of the exhausted media was inoculated together with the fungal biomass, to both humidify the lignocellulosic biomass and to evaluate its potential contribution in the pretreatment.

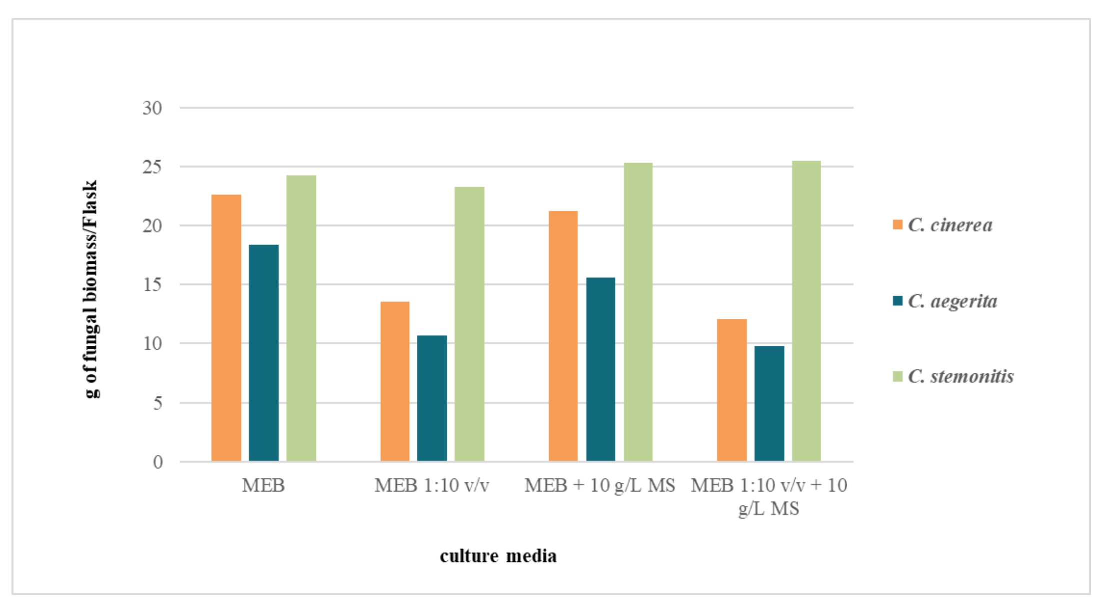


Figure 31: Net fresh weight (g) of fungal biomass produced per flask in the four different media tested.

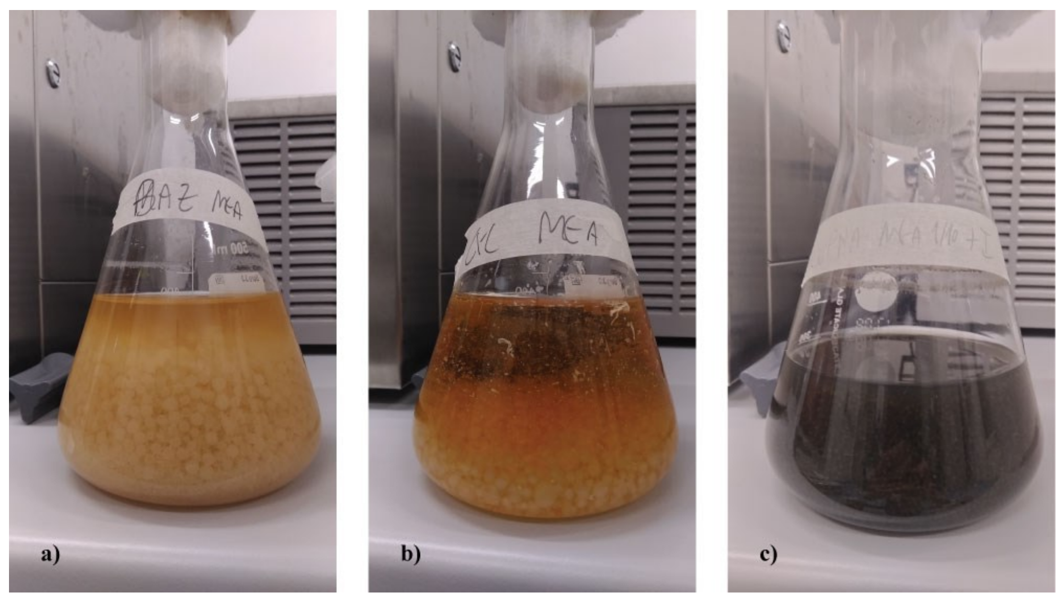


Figure 32: Fungal colonies grown in the selected culture media a) *C. cinerea* growing into MEB b) *C. aegerita* growing into MEB c) *C. stemonitis* growing into MEB diluted (1:10 v/v) with 10 g/l maize silage.

5.3.2 Pretreatment of non-sterile biomasses

In lab-scale experiments, the sterilization of feedstocks is a routine step prior to fungal pretreatment to kill indigenous undesired microbes and assure inoculated microorganisms the best working conditions. However, sterilization is extremely expensive and requires additional energy inputs, materials, and time, preventing its application to an industrial-scale level (Carrere et al., 2016; Zhao et al., 2014).

The proposed technology, which directly uses fungal inocula to pretreat unsterilized biomasses, represents a fundamental advantage for future industrial application (Zhao et al., 2014). Despite processes developed in non-sterile conditions would allow an easier scale-up and the reduction in the productivity expenditure, they also present some drawbacks. For instance, it is extremely challenging to set the optimal conditions necessary to get an efficient colonization and to keep inoculated fungi prevailing over indigenous microflora (Song et al., 2013; Zhao et al., 2014). For this reason, very few works about it can be found in the literature about pretreatments in non-sterile conditions. Moreover, pretreatments applied on unsterilized substrates are often destined to fail (Song et al., 2013; Zhao et al., 2014). For instance, the pretreatment performed by Zhao et al. (2014) on unsterilized yard trimmings inoculated with *C. subvermispora* was unsuccessful. Similar results were also described by Reid (1989), who used *Phlebia tremellosa* to inoculate unsterilized aspen wood. A fundamental factor that could influence pretreatments performance is the fungal strain sensitivity towards the presence of other organisms into the biomass (Rouches et al., 2016). For instance, Vasco-Correa et al., (2016) described that *C. subvermispora* is particularly sensitive towards the closeness to indigenous microorganisms. Instead, Akin et al. (1995) reported that *C. subvermispora* and *Cyathus stercoreus* inoculated on unsterilized bermuda grass were quite insensitive towards the presence of autochthonous microorganisms, since no alteration of the pretreatments efficacy were observed. The different results reported by these authors for *C. subvermispora* and the results obtained in this study emphasize the key role played by the right

experimental set up, working condition, and type and origin of the feedstock in pretreatment success, and also on the intrinsic saprotrophic competitive capabilities of each fungal strain in fighting the autochthonous microorganisms. In this regard, autochthonous fungi, isolated from the same biomass intended for pretreatment, certainly represent a competitive and promising resource.

A valuable alternative to the direct use of fungal inocula to pretreat unsterilized biomasses could be inoculating fresh biomass with pre-colonized sterile substrates. Zhao et al. (2014) proved that this strategy was effective since inoculating non-sterile yard trimmings with pre-colonized material they obtained a PCWP degradation comparable to that obtained from sterilized substrate. The effectiveness of this method is conceivably due to microorganisms' pre-adaptation to the biomass, that allows fungi to readily colonize fresh substrates and to outcompete indigenous microflora (Zhao et al., 2014). Another solution could be treating biomasses with hot water or formic acid in order to wash out indigenous microorganisms, as demonstrated by Del Pilar Castillo et al. (2001) who obtained good results when pretreating straw with *P. chrysosporium*. However, both these solutions imply the utilization of supplementary energy inputs and chemical reagents, which represent an additional cost and therefore could limit the application of fungal pretreatments at industrial scale.

In the present work, the selected fungal strains' hallmarks included the ability to efficiently colonize non-sterile biomasses without being outcompeted by the indigenous microorganisms. As results, the contamination rates of unsterilized biomasses accountable to indigenous organisms (other microorganisms and nematodes) were negligible (< 3 %), demonstrating that selecting the right fungal strains and experimental conditions allows to get results comparable to those obtained starting from sterilized biomass, avoiding economic and environmental issues, and therefore allowing a better process sustainability and scalability.

5.3.3 Experiment 1

5.3.3.1 Fungal pretreatment

The fungal pretreatment was carried out under SSF for 10 days at 25 °C. During the process, the fungal colonization of the substrates was monitored through macro and microscopic observations (Figure 33). After one week, the mycelium of *C. stemonitis* MUT 6326 was abundant in both biomasses, and the asexual reproductive structures (conidia) were produced (Figure 33).

Figure 33: *C. stemonitis* grown for 10 days on MS and SFD.

5.3.3.2 Characteristics of untreated and fungal-pretreated biomasses

Untreated MS and SFD showed pH, TS, VS and fibers composition (Table 16) comparable with the data ranges reported in literature (Liu et al., 2014b; Zhong et al., 2016). About half of the TS of untreated MS was composed by fibers (NDF)

and the prevailing components were cellulose and hemicellulose (Table 16). The NDF content in the untreated SFD was higher than in MS samples. Lignin and cellulose were the main components of the TS of SFD (Table 16), confirming its higher recalcitrance respect to MS (Zhong et al., 2016).

The fungal pretreatment greatly affected the characteristics of both biomasses (Table 16). *C. stemonitis* MUT 6326 transformed PCWP of both MS and SFD, potentially increasing their digestibility during the subsequent AD (López et al., 2013). However, according to the results of Wan and Li (2011), different degradation profiles were observed depending on the tested biomass. The differences observed between MS and SFD reflected their different physicochemical features. The recalcitrant nature and the alkaline conditions of SFD could have been detrimental for ligninolytic enzymatic production and activity, leading to lower PCWP degradation than the one observed in MS (Musatti et al., 2017; Young et al., 2018). In comparison with the untreated sample, fungal-pretreated MS showed decrease in lignin content up to 55.2 %. The degradation of cellulose and hemicellulose in MS was 25.0 % and 24.5 %, respectively, indicating that *C. stemonitis* preferred lignin rather than holocellulose. However, the results indicated that other than the recalcitrant fractions, also the easily accessible MS fibers had been substantially metabolized by the fungus. When grown on SFD, *C. stemonitis* caused a selective degradation of lignin and hemicellulose (8.7 % and 10.9 %, respectively) while cellulose loss was minimal (0.6 %). This is an important process outcome. Indeed, degradation of lignin is one of the main factors for a successful fungal pretreatment, as it increases the accessibility of AD microorganisms to the more easily degradable structural carbohydrates, directly contributing to the enhancement of anaerobic digestibility (Vasco-Correa et al., 2016). Cellulose and hemicellulose degradation are also correlated with improved biomass digestibility, although an excessive loss during the pretreatment could be undesirable, since they constitute the main sources of sugars for microorganisms involved in AD (Vasco-Correa et al., 2016).

The TS concentration decreased up to 22.1 % and 20.5 % in fungal-pretreated MS and SFD, respectively. Most likely, this result was a consequence of the dilution due to the water content in fungal inoculum (95.7 %) and to the VS and PCWP degradation during the pretreatment (Mustafa et al., 2016; Zhao et al., 2014). However, small variations (< 1 %) in VS content were observed for both fungal-pretreated MS and SFD respect to the untreated groups. These data suggest that although during pretreatment PCWP were degraded by the fungus (Table 16), the inoculated fungal biomass (4.2 % VS content) and the grown mycelia may have compensated the loss in VS. It is known that, in the presence of easily degradable biomass as MS, the growth of fungi could lead to considerable TS and VS losses due to consumption of PCWP, proteins, soluble sugars and volatile fatty acids. For instance, Liu et al. (2014a) reported that the fungal pretreatment of corn stover silage decreased the TS content up to 55.3 %. On the contrary, as regards recalcitrant materials such as SFD or *Albizia moluccana* (albizia) biomass residues, the loss of TS and VS is generally lower and not so relevant for the economic efficiency of the downstream process (Ge et al., 2015).

Table 16: Characteristics and fibers composition of the untreated and fungal-pretreated MS and SFD.

	Untreatred MS	**Fungal-pretreated MS**	**Untreatred SFD**	**Fungal-pretreated SFD**
pH	3.7	6.9	8.4	7.9
TS %	34.4	26.8	34.2	27.2
VS (% TS)	96.4	95.5	87.3	87.0
NDF (= Fibers) (% TS)	49.5	35.4	70.6	66.1
ADF (% TS)	29.4	20.2	54.0	51.4
ADL (= Lignin) (% TS)	6.1	2.8	28.8	26.2
Hemicellulose (% TS)	20.0	15.1	16.6	14.8
Cellulose (% TS)	23.3	17.5	25.3	25.1

5.3.3.3 Biogas and methane yields

The daily biogas yields and the respective methane concentration recorded during BMP tests are illustrated in Figure 34. During the first 7 days of AD, untreated MS showed higher daily biogas and methane production rates than fungal-pretreated samples (Figure 34 a, b). From day 9, the average daily biogas production rates recorded from untreated and pretreated MS were comparable, although fungal-pretreated samples produced biogas with higher methane concentration (maximum peak of about 60 % maintained up to the end of the trial) (Figure 34 a, b). In detail, the fungal pretreatment on MS increased the daily methane concentration in biogas up to about 10 percentage points (Figure 34 a, b). As a result, fungal-pretreated MS exhibited a significant ($p<0.05$) decrease (- 16 %) of the cumulative biogas yield compared to the untreated sample, while the cumulative methane yield was comparable between control and fungal-pretreated MS (- 9 %; $p>0.05$) (Figure 35). In detail, fungal-treated MS led to cumulative biogas and methane yields of 514 ± 33 L_N/kg VS (= 179 ± 10 L_N/kg untreated biomass) and 271 ± 20 L_N/kg VS (= 95 ± 6 L_N/kg untreated biomass) respectively, while data with untreated MS were 609 ± 37 L_N/kg VS (= 202 ± 11 L_N/kg untreated biomass) and 299 ± 22 L_N/kg VS (= 99 ± 6 L_N/kg untreated biomass), respectively. In agreement with these findings, other authors reported a decrease in AD productivity after fungal pretreatment on lignocellulosic biomass (Liu et al., 2016; Vasco-Correa and Li, 2015). The lower yields obtained with fungal-pretreated MS compared to the untreated samples could be explained by the excessive consumption of cellulose and hemicellulose (presumably lost as CO_2) during pretreatment (Table 16) in comparison to the amounts of recalcitrant compounds became available for anaerobic bacteria (Liu et al., 2016; Vasco-Correa and Li, 2015). On the other hand, the fungal pretreatment on MS improved the daily methane concentration in biogas of approximately 10 percentage points (Figure 34 a, b) and the cumulative biogas quality (Figure 35). This finding has positive implications, as it is well known that the energy content of biogas is in direct

proportion to the methane concentration (Angelidaki et al., 2018). Furthermore, by increasing methane level in biogas produced during the AD process, energy consumption and related economic costs of biogas upgrading to biomethane can be effectively reduced (Angelidaki et al., 2018). We may hypothesize that the lignin degradation may have provided the microorganisms involved in the AD process with organic components with a higher potential in terms of methane production. Another hypothesis is that the inoculated fungal biomass and the grown mycelium has increased the total fat and protein content in the feedstock, boosting AD microorganisms, and ultimately leading to the increase of methane level. However, based on technological progression, data of BMP test in 2 L batch cannot be exhaustive for the future scale up of the process. As regards the actual economic feasibility of the process, continuous 5 L trials would be necessary to clearly define the precise biogas and methane productivity (Koch et al., 2020).

Concerning SFD, the results of batch trials confirmed that it still contained residual biogas and methane potential (Figure 34 c, d; Figure 35) (Menardo et al., 2011b). However, a large fraction of its organic matter is not readily biodegradable, as demonstrated by the poor biogas and methane yield obtained from untreated samples, which were about 141 ± 36 L_N/kg VS (= 42 ± 9 L_N/kg untreated biomass) and 75 ± 14 L_N/kg VS (= 22 ± 3 L_N /kg untreated biomass), respectively (Figure 35). The fungal pretreatment affected the anaerobic digestibility of SFD, leading to a higher daily biogas and methane production rates from fungal-pretreated SFD than from the untreated control (Figure 34 c, d). However, in contrast to the findings with MS, fungal pretreatment of SFD did not vary the methane concentration in daily produced biogas (Figure 34). As a result, cumulative biogas and methane yields from fungal-pretreated SFD, which were about 233 ± 25 L_N/kg VS (= 73 ± 7 L_N/kg untreated biomass) and 122 ± 12 L_N/kg VS (= 38 ± 3 L_N/kg untreated biomass), respectively, resulted approximately 60% and 62 % higher ($p<0.05$) than the control, respectively (Figure 35). Ultimately, the fungal

pretreatment seems a cost-effective and environmental-friendly option to increase the digestibility and yields of SFD. The improved performance can be associated to the reduced biomass recalcitrance due to selective lignin and hemicellulose removal (Table 16), as confirmed by other studies (Sindhu et al., 2016; Singh et al., 2014). Besides, the fungal biomass added to the system may have also played a role on the final BMP value. In fact, the fungal biomass could be profitably used as a substrate in AD processes as it contains lipids, proteins and other organic molecules that may be exploited by anaerobic microorganisms. However, literature is scarce on this subject, thus a lack of knowledge must be filled with exhaustive and targeted investigations on the precise contribution of the fungal biomass to the AD. Hom-Diaz et al. (2016) reported that the methane production of fungal biomass could vary from 281 to 595 L_N/kg VS, depending on the inoculum (digested slurry) used to start the methanation. Considering the average methane productivity reported in literature per g of fungal VS (about 387.5 ± 123.8 L/kg VS) (Hom-Diaz et al., 2016) and the amount of fungal VS introduced inside each batch reactor (around 0.23 g VS), in this experiment, the fungal biomass could have contributed to approximately 0.9-2.5 % to the total methane productivity.

Interestingly, previous studies (Brémond et al., 2020; Sambusiti et al., 2015) have investigated biological and/or physicochemical pretreatments to get a higher energy recovery from SFD, but they often obtained weak results compared to that here reported. For instance, Bremond et al. (2020) tested on solid digestate the effectiveness of two different Basidiomycota strains (*P. ostreatus* and *Stropharia rugoso-annulata*), but the experimental conditions were not optimized and led to uncontrolled organic matter losses and to subsequent decreases in the methane yield (up to 50%). Besides, Sambusiti et al. (2015) revealed that thermal and alkaline treatments did not enhance methane potentials of SFD, while enzymatic treatment only slightly increased the methane yield (13 %). The promising results obtained in our work encourage to continue the experimentation and to focus the attention on recalcitrant SFD pretreatment and valorisation.

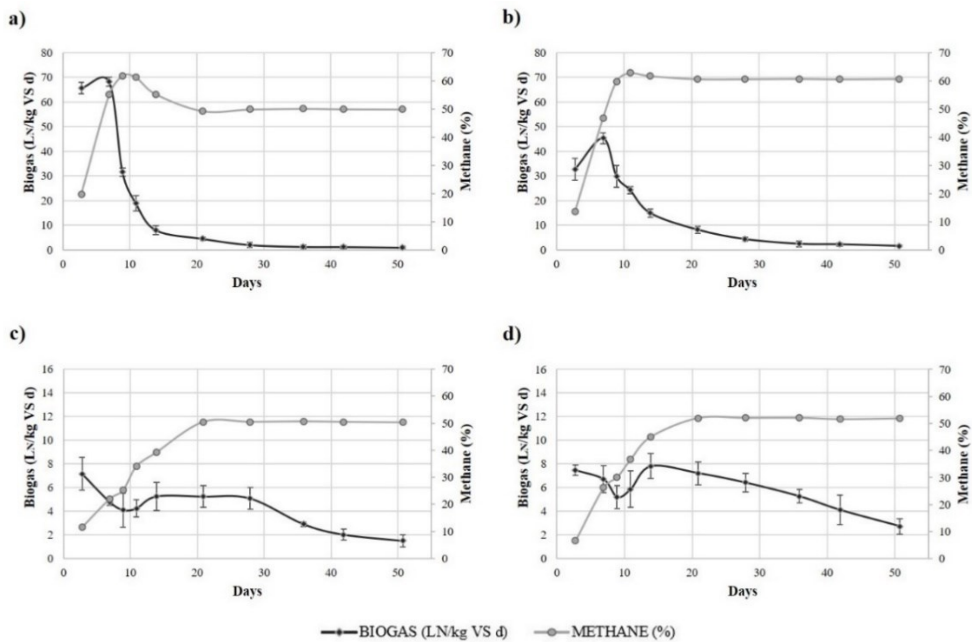


Figure 34: Daily biogas yield (L_N /kg VS d) and methane concentration (%) in biogas of: a) Untreated MS; b) Fungal-pretreated MS; c) Untreated SFD; d): Fungal-pretreated SFD. Results are expressed as mean ± standard deviation from four replicates.

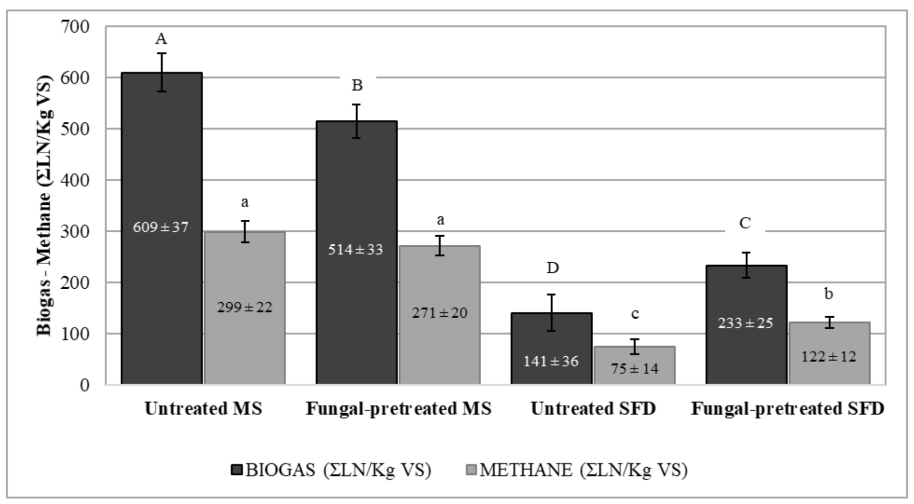


Figure 35: Cumulative biogas and methane yields (ΣL_N/kg VS) of untreated and fungal-pretreated MS and SFD. Results are expressed as mean ± standard deviation from four replicates. The letters were used to show the results of statistical analyses.

5.3.4 Experiment 2

5.3.4.1 Fungal pretreatment

The fungal pretreatments were carried out in SSF, at 25 °C, for 10 days and 20 days, in order to test the impact of different pretreatment durations on PCWP biodegradation and biogas production. During the process, the fungal colonization of the substrates was monitored through macro and microscopic observations (Figure 36). After 5 days, the fungal colonies were already visible, and after one week, the mycelium was abundant and hyphal cordons, devoted to nutrient transfer, were observable, as well as asexual reproduction structures. In all cases, a vigorous colonization was maintained until the end of the trials (20 days) (Figure 36).

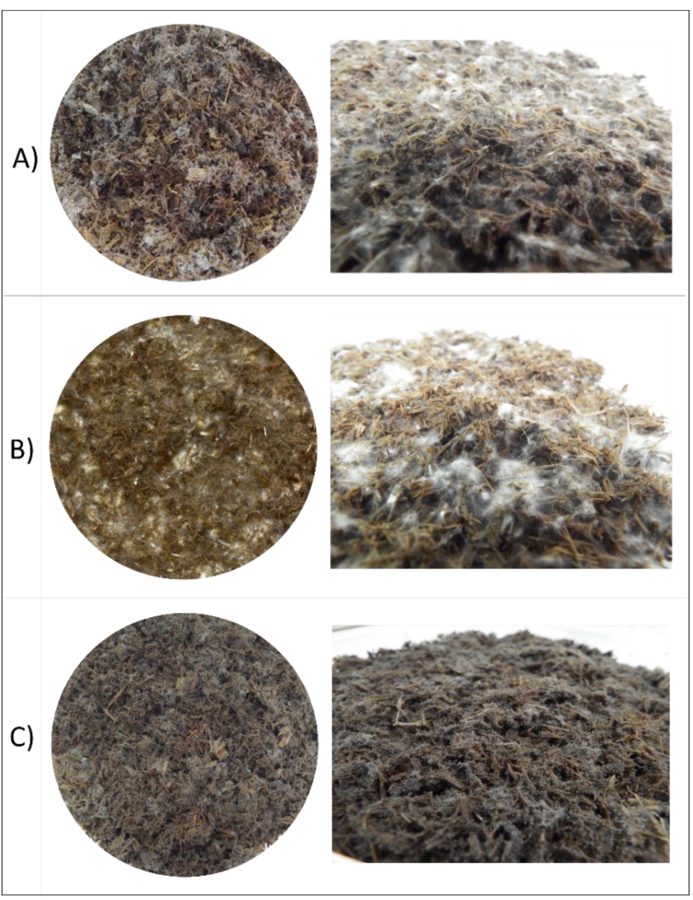


Figure 36: SFD colonized by A) *C. cinerea*; B) *C. aegerita*; C) *C. stemonitis*.

5.3.4.2 Enzymatic Assays

In literature it is possible to find many enzymatic assays protocols but there is not a standardized method for analyses on solid digestate (SFD); it is therefore extremely complicated to find out which one better suit our experimental conditions. Moreover, in comparison with SmF, enzymes extraction from complex matrices under SSF may result more difficult; indeed, the extraction is one of the most important steps for down-stream processing and requires the right solvents to loosen the non-covalent bonds between enzymes and mycelium or substrate (i.e., hydrogen bond, hydrophobic and Van-der-walls forces) (Kar et al., 2013). Other substrates' intrinsic features that could negatively impact enzymes recovery and also alter enzymatic characteristics, production, activity and stability are the heterogeneity, pH, moisture content, particle size, porosity and adsorption ability (de Castro and Sato, 2015; Kar et al., 2013; Young et al., 2018).

The parameters tested in this work for protocols optimization of enzymatic assays were:

- the biomass: buffer ratios (1:5 - 1:10 w/v)
- the incubation times necessary to solubilize the enzymes produced by fungi (30 min and 3 h), and,
- for cellulase and xylanase assays, the crude enzyme:substrate ratios (1:9, 1:4, 1:1, 2:1 v/v).

Significant differences among results obtained changing the different parameters were not found ($p>0.05$, data not shown), therefore, among the conditions tested and mostly reported in the literature, those that allowed a better practicality (e.g., shortest time, minor reagents consumption) were chosen, namely:

- biomass: buffer ratio of 1:5 w/v,
- 30 min incubation time and
- for cellulase and xylanase assays, a crude enzyme: substrate ratio of 1:1 v/v.

Among the tested fungi, *C. cinerea* was the most productive in terms of cellulases and xylanases yields (Figure 37). In comparison with the other two strains, *C. cinerea* yielded a significantly higher ($p<0.05$) amount of endo-glucanases 33.76 IU/L (0.17 IU/g) and xylanases 263.27 IU/L (1.32 IU/g) during almost all pretreatment duration. Concerning exo-glucanases, instead, the yields of all fungal strains ranged between 11.00 IU/L and 22.15 IU/L (0.06-0.11 IU/g; $p>0.05$) during almost the pretreatments duration. Only on day 20 *C. cinerea* yielded a significantly ($p<0.05$) higher amounts of enzymes (50.95 IU/L), suggesting that longer treatments duration could increase its exo-glucanase productivity. This is in line with the results of Nuchdang et al. (2015), which reported higher cellulolytic and hemicellulolytic enzymes production for *C. cinerea* during a 30 days' pretreatment on lignocellulosic grass (0.03-0.18 U/mL and 0.90-1.11 IU/mL). On the other hand, too long pretreatments could cause excessive loss of organic matter and may represent an obstacle for industrial-scale applications.

C. aegerita, instead, was the less productive fungus in terms of cellulases and xylanases (Figure 37): its xylanases production (33.61 IU/L; 0.17 IU/g) was significantly lower ($p<0.05$) than those of *C. cinerea* and *C. stemonitis*, while it yielded an amount of endo-glucanases (2.83 IU/L; 0.01 IU/g) comparable to that of *C. stemonitis* (4.07 IU/L; 0.02 IU/g). According to Musatti et al. (2017), who grew *C. aegerita* under SmF with the addition of SFD, this species is able to produce high amounts of xylanases (3.10 IU/g), while the data concerning cellulase production are in accordance with our observations (endo- and exo-glucanase yields lower than 0.02 IU/g and 0.10 IU/g, respectively). However, in another work performed by Isikhuemhen et al. (2009), *C. aegerita* showed to produce endo-glucanases, and an overall higher lignocellulolytic enzymes production on digestate derived from poultry litter (15.70 U/g of endo-glucanase, 11.40 U/g of exo-glucanase and 14.87 U/g xylanase).

Compared with other well-studied WRF, both the Basidiomycota strains tested in this work yielded less cellulases and xylanases. Indeed, Zeng et al. (2010) described for *P. chrysosporium* a cellulase production that reached 5000 IU/g and a xylanase yield of almost 3000 IU/g. Similar differences between cellulolytic and hemicellulolytic enzymes production were usually reported for WRF in literature. For instance, Singh et al. (2013) found that cellulase and hemicellulase production of *Trametes versicolor* grown on oil palm trunk chips was of 53.30 U/L and 1.50 U/L, respectively. An explanation of these differences in enzymatic productions is that not all WRF are able to produce effective ligninolytic, cellulolytic and hemicellulolytic enzymes. Some fungal species and strains can degrade lignin selectively, without affecting the cellulose content (i.e., *C. subvermispora*) (Yoon et al., 2014). Therefore, it seems plausible that the Basidiomycota used in this study are more selective towards lignin decomposition. Alternatively, it is possible that the enzymes released in the lignocellulosic matrix have remained adsorbed into the substrate; another possibility could be that the tested fungi produce isoforms of enzymes with biochemical characteristics (pH optimum, temperature, etc.) different from those used in the works. Although it is also possible that the fungi had minimal enzymes production, poorly detectable with the applied techniques, it is known that even small quantities of enzymes are also able to activate large structural changes thanks to the enzyme cascade mechanism (Baldrian, 2006).

As regard the laccase, the fungi *C. aegerita* and *C. cinerea* were the most productive strains (Figure 37). These results were not surprising since both are WRF, thus, they were expected to produce lignin-degrading enzymes (Rouches et al., 2016). Their laccase production however followed a different curve during the treatments: during the first 10 days the laccase production of *C. cinerea* was significantly higher ($p<0.05$) than that of *C. aegerita*, while that of *C. aegerita* was constantly increasing and, during the second half of pretreatment duration, it was the highest ($p<0.05$). *C. stemonitis* instead, produced a significantly lower ($p<0.05$)

amounts of laccases during the whole pretreatment. The results suggests that it may be worthy to test whether forming a consortium with *C. cinerea* and *C. aegerita* would provide a better lignin removal, considering that one of them produces more laccases at the beginning and the other more from the second half of pretreatment. However, laccase production was lower than data reported in literature for other WRF. For instance, Levin et al. (2008) described a laccase production of 901 IU/g for *Trametes trogii* on poplar wood. The laccase production by *T. versicolor* was also studied and distinct authors reported different data in their works; for example, Singh et al. (2013) and Jing et al. (2007) described a laccase yields of 218.66 IU/L and 110.90 IU/g, respectively. According to literature, lignocellulolytic enzymes production is extremely variable based on the fungal strains (intraspecific difference), the culture conditions and the specific lignocellulosic substrate (Rouches et al., 2016; Songulashvili et al., 2015). Therefore, it is quite expected that different strains and experimental conditions reached different enzymatic yields.

In the literature, many works about Ascomycota ability to produce lignocellulolytic enzymes can be found. For instance, different species belonging to the genera *Aspergillus* and *Trichoderma* are currently industrially exploited for enzymatic production (Carrere et al., 2016). In particular, *A. niger, A. fumigatus, A, nidulans* and *A. terreus* are reported to produce mainly cellulases and xylanase (Jabasingh and ValliNachiyar, 2011; Miao et al., 2015; Olanbiwoninu and Odunfa, 2016; Sherief et al., 2010; Sridevi et al., 2016). For example, Sherief et al. (2010) reported a cellulase and xylanase production of 14.71 and 42.70 IU/g for *A. fumigatus* grown on rice straw and wheat bran, respectively, while Miao et al. (2015) described for *A. fumigatus* a xylanase production of 21.45 IU/ml.

Concerning *C. stemonitis,* in literature can be found only a work regarding its secretome (Peterson et al., 2011), but none about the effectiveness of its enzymes in terms of PCWP degradation and lignocellulose bioconversion. According to

Peterson et al. (2011), *C. stemonitis* can produce both endo-glucanases and exo-glucanases (in particular cellobiohydrolases). However, in this study the cellulase production by *C. stemonitis* was almost null (Figure 37), while it showed to produce higher amounts of xylanases (72.03 IU/L). It is conceivable that to obtain a higher cellulase production different cultivation and treatment conditions should be tested. Further studies on the lignocellulolytic potential of this fungus should be performed.

Based on the results obtained and the comparison with the literature data it could be concluded that SFD "as it is" is not a particularly suitable substrate to produce high amount of lignocellulolytic enzymes by the tested fungi. A factor that could have potentially negatively influenced the enzymatic production, activity and detection was that SFD have usually an alkaline pH (Menardo et al., 2011a). Indeed, optimal pH values for cellulase and xylanase activity range between 3 and 6 (Bansal et al., 2012; Nichawee Wipusaree, 2011). For instance, in order to get the maximum lignocellulolytic enzymes production from *T. trogii,* Levin et al. (2008) and Singh et al. (2013) set the pH at about 4.8 - 5. Laccase activity could be also inhibited by alkaline conditions; moreover, laccase activity is usually enhanced by the presence in the substrate of co-factor as copper (Cu), as demonstrated by Baldrian et al. (2005) and Songulashvili et al. (2015) for *P. ostreatus* and *C. unicolor,* respectively. Therefore, the addition of co-factor could have probably resulted in higher enzymes yields. Although the enzymatic productions have not been very high and there is still space for improvements, the lignocellulolytic fungal enzymes can be active against PCWP even at relatively low concentrations (Shraddha et al., 2011). A further optimization of fungal cultivation conditions and experimental set-up (as pH values) could potentially favor a higher fungal enzymatic production from such cheaper substrate, and consequently a higher lignocellulose degradation.

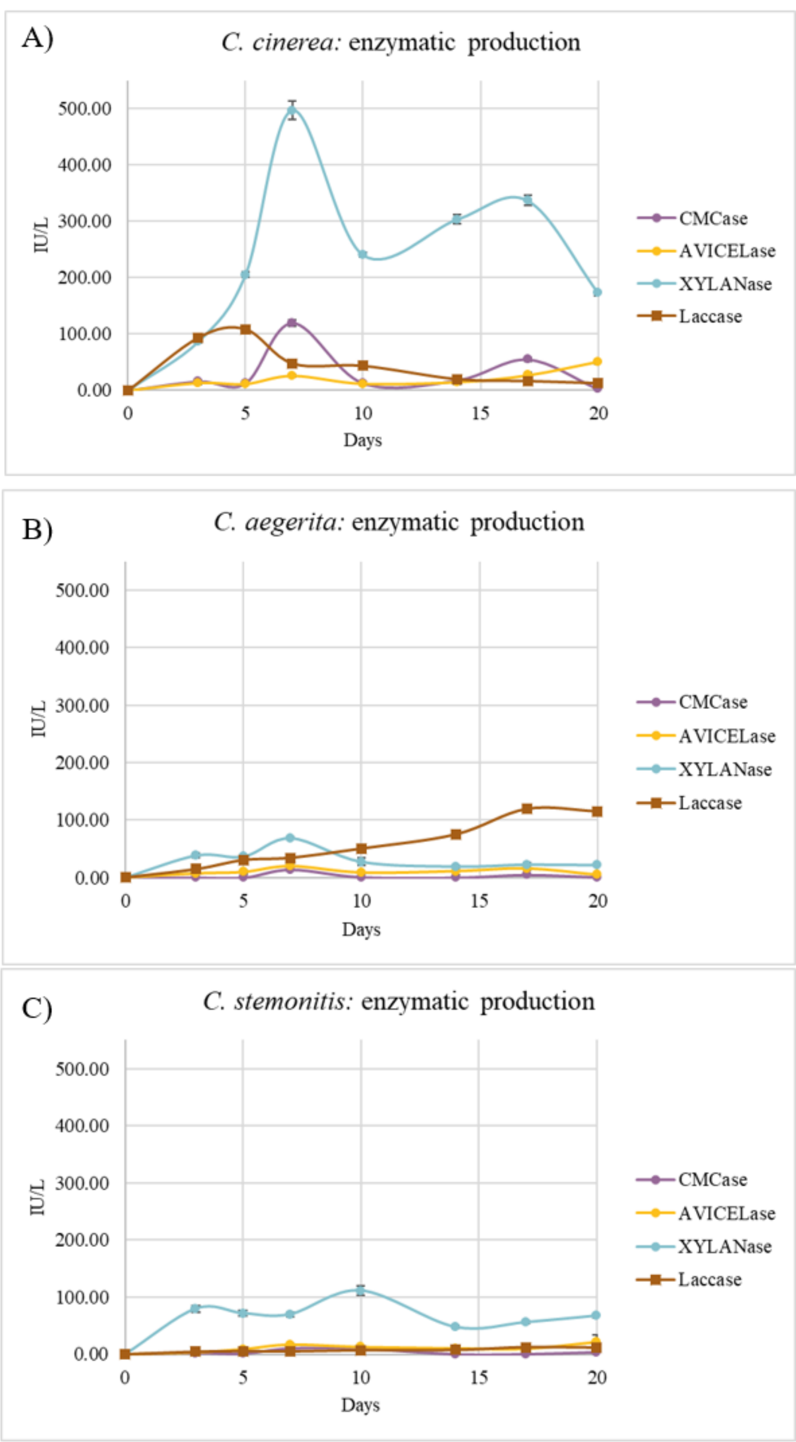


Figure 37: Lignocellulolytic enzymes production of *C. cinerea* (A), *C. aegerita* (B) and *C. stemonitis* (C), during solid-state fermentation on SFD.

5.3.4.3 Characteristics of untreated and fungal-pretreated biomasses

The chemical characterization of the untreated and fungal-pretreated SFD is presented in Table 17. It should be noted that the biomasses that have undergone pretreatments under T0 conditions were not analysed for TN and TAN contents and for fibers (NDF) and PCWP composition since it is assumed that these features are identical to those of the untreated SFD (control).

Table 17: Characteristics and fibers compositions of the SFD, untreated and pretreated with *C. cinerea, C. aegerita* and *C. stemonitis*, for 0, 10 and 20 days (T0, T10 and T20, respectively). Statistical significance for TS and VS data is expressed as results of Tukey's grouping.

	Untreated SFD (Control)	**Fungal-Pretreated SFD**								
		C. cinerea			***C. aegerita***			***C. stemonitis***		
		T0	**T10**	**T20**	**T0**	**T10**	**T20**	**T0**	**T10**	**T20**
pH	9.4	8.3	8.2	8.5	8.7	8.8	9.0	8.5	9.0	9.1
TN [%]	0.8	-	0.6	0.6	-	0.7	0.6	-	0.7	0.6
TAN [%]	0.01	-	0.01	0.01	-	0.01	0.01	-	0.01	0.01
TS [%]	31.7^{a}	25.8^{c}	22.7^{ef}	22.6^{f}	28.4^{b}	24.1^{de}	24.2^{d}	27.7^{b}	23.9^{ef}	23.4^{f}
VS [% TS]	88.1^{a}	88.9^{a}	87.4^{a}	87.7^{a}	89.2^{a}	88.7^{a}	88.2^{a}	88.5^{a}	87.8^{a}	89.1^{a}
Fibers - NDF [% TS]	82.4	-	80.6	79.9	-	81.1	79.7	-	79.6	73.8
ADF [% TS]	79.7	-	78.4	77.8	-	79.3	78.1	-	77.7	72.7
ADL (Lignin) [% TS]	31.1	-	30.6	30.1	-	30.8	29.9	-	29.9	28.1
Hemicellulose [% TS]	2.7	-	2.2	2.1	-	1.8	1.6	-	1.9	1.1
Cellulose [% TS]	48.6	-	47.8	47.7	-	48.5	48.2	-	47.8	44.6

The characteristics of SFD can widely vary according to different parameters, such as the feedstock type and quality, organic loading rate, hydraulic retention time, storage parameters, and type of separator used on the raw co-digested slurry (Hansen et al., 2006; Monlau et al., 2015). According to the literature (Dinuccio et al., 2013; Sambusiti et al., 2015), the currently studied SFD has alkaline pH (>8) (Table 17). Menardo et al. (2011b) also reported pH values ranging from 8.6 to 9 for the SFD of three Italian biogas plants treating mainly manure and energy crops.

All the samples showed negligible TN and TAN content (0.01%) (Table 17). These low concentrations were related to the N-stripping and drying process performed on-site before storage (Guilayn et al., 2019). Biomass alkalinity without the presence of ammonia (TAN) could be a favorable condition for the here-suggested SFD reuse in AD, since it prevents acidification and ammonia inhibition inside the digester tanks (Carrere et al., 2016; Mao et al., 2015; Monlau et al., 2015; Wang et al., 2018). However, excessive alkalinity could represent a limiting factor for biological pretreatment, since the optimum pH for fungal cultivation and growth usually ranges around acidic conditions (5 to 6.5) (Musatti et al., 2017). According to the results, the SFD alkaline pH did not affect fungal colonization ability but could have affected the enzymatic production and activity, as suggested by the enzymatic analyses described in Section 5.3.4.2, with potential negative consequences for the PCWP degradation (Young et al., 2018).

Several works reported that SFD could be characterized by a high BMP mainly due to undigested organic matter (VS) (Monlau et al., 2015; Vasco-Correa et al., 2018). In this study, the SFD was confirmed a biomass that still contained a high VS content (approximately 88% of the TS), mainly due to the presence of not-degraded lignocellulosic fibers (NDF) (around 80% of the TS). The findings confirmed that SFD could be further exploited for energy production (biogas, bioethanol, etc.) and justified its potential recirculation into the AD plant. In comparison to easily accessible lignocellulosic biomass, as MS, the SFD organic matter seems more recalcitrant to biodegradation, as suggested by the lower VS

content (95.5-96.4 % of TS for MS used in Experiment 1). However, the observed TS and VS contents (Table 17) were comparable to those reported by Zhong et al. (2016), who indicated for the SFD derived from the co-digestion of animal manure and food wastes an average TS and VS contents of 30.6 ± 2.1% and 89.2 ± 0.3% on TS, respectively. In comparison with the TS and VS data obtained from digestate utilized in this study, the digestate described by Bauer et al. (2009), derived from co-digestion of animal manure and energy crops, was characterized by a lower TS content (19.3%) and by a comparable VS content (80.0%). Besides, the SFD used in this study was characterized by a higher VS content when compared to SFD samples described by Sambusiti et al. (2015) (83.8 ± 0.3 % on TS) and Musatti et al. (2017) (80 % on TS).

The hemicellulose content in SFD was minimal (1.1–2.7% of TS), whereas cellulose and insoluble lignin (ADL) were the main fibrous components (around 30% and 48% of the TS, respectively). These findings agree with the literature data (Musatti et al., 2017; Vasco-Correa et al., 2016; Zhong et al., 2016). It is known indeed that polymeric lignin is not biodegradable under anaerobic conditions, while cellulose is degraded at a slower rate than hemicellulose, since cellulose degradation is negatively affected by its degree of crystallinity and interconnection with other PCWP (Monlau et al., 2015; Musatti et al., 2017). This results in an accumulation of more refractory cellulose and lignin in SFD, while hemicellulose can be largely exploited during AD (Sambusiti et al., 2015). Considering that the SFD fibers composition mainly depends on the original feedstock pattern (Monlau et al., 2015), the obtained results were not totally unexpected. In fact, the AD of energy crops (maize silage) usually leads to SFD with a higher cellulose and lignin content and a lower hemicellulose content than that obtained from organic food wastes. For instance, Opatokun et al. (2016) reported for the digestate from food wastes a fibers composition characterized by lignin, hemicellulose, and cellulose contents of 13.4%, 33.5% and 32.3% of TS, respectively. The SFD analysed by Zhong et al. (2016), derived from the co-digestion of animal manure and food-

processing wastes, was characterized by a comparable lignin content (30.3 % TS), while the cellulose content was lower (26.5% TS) and hemicellulose was higher (13.3% TS). In comparison with other agro-industrial wastes and by-products exploitable for biogas production, such as wheat straw (lignin 11.2%, hemicellulose 30%, and cellulose 40.2%) (Zhi and Wang, 2014) and rice straw (14.1 ± 0.5% lignin, 27.9 ± 1.3% hemicellulose, and 36.3 ± 1.2% cellulose) (Kainthola et al., 2019b), the SFD used in this work was characterized by a higher lignin and cellulose content and a lower hemicellulose content. In general, higher cellulose and hemicellulose contents are desirable, as they constitute the main sources of sugars available from lignocellulosic feedstocks for microorganisms involved in AD (Vasco-Correa et al., 2016).

Interestingly, the analysed SFD showed characteristics (Table 17) comparable and representative of those obtained in most of European mesophilic full-scale ABPs, such as those collected in the regions of northern Italy (Dinuccio et al., 2013; Menardo et al., 2011a; Sambusiti et al., 2015), which are areas characterized by a local oversupply of digestate (Bartoli et al., 2016). In this context, the importance of SFD (re)use as an AD feedstock is even higher, since it could permit a better and complete exploitation of the original feedstocks into added-value product (methane) and the reduction of GHG emissions, contributing to improve the economic and environmental efficiency of the AD process.

The fungal inoculum addition and the application of the fungal pretreatments greatly affected the characteristics of non-sterile SFD (Table 17). Decreased values of pH and TN content were observed in comparison with the untreated SFD. It was assumed that fungi had alkaline tolerance, but during the pretreatment they caused a pH lowering, probably to establish an environmental niche more closely related to their physiological needs (i.e., acidic conditions), including the optimal conditions for their oxidative enzymes to operate (Musatti et al., 2017).

The TS content significantly ($p<0.05$) decreased in all pretreatment conditions, especially during the first 10 days. The T0 treatments generally caused a lower TS reduction in comparison with the other pretreatments, while the TS reduction induced by the T10 and T20 treatments were comparable with each other ($p>0.05$). The TS concentration decreased by 23.8%, 25.4%, and 28.5% in samples pretreated with *C. aegerita, C. stemonitis*, and *C. cinerea*, respectively. The decreases in TS after the pretreatments were mostly caused by the dilution due to the fungal inoculum water content and by the PCWP degradation (Mustafa et al., 2016; Zhao et al., 2014). In detail, the addition of water from the fungal inoculum caused an approximately 20.6% reduction in the TS. Similar results of TS losses were obtained by Nuchdang et al. (2015) when pretreating lignocellulosic grass with *C. cinerea* (27%). Instead, Carrere et al. (2016) and Baldrian et al. (2005) reported a slightly lower decrease (10 - 20 % and 14.9 ± 0.6 %, respectively). In general rules, to obtain a successful pretreatment it is necessary to find out the experimental conditions that allows to reduce at minimum the TS losses, although it is known that the decreases in TS for recalcitrant materials are generally not so relevant for the efficiency of the downstream AD process (Ge et al., 2015).

On the other hand, the VS losses were very low (< 1.0 %), and no statistical differences ($p>0.05$) could be detected between values of the untreated sample and those of the fungal-pretreated samples. Noteworthily, the data reported in the literature for other fungal pretreatments showed higher VS losses than those obtained in this work. For instance, Ge et al. (2015) described a VS degradation of 11.2% on albizia chips pretreated with *C. subvermispora*. Similar results were also reported by (Kainthola et al., 2019a) with *P. ostreatus* and *Ganoderma lucidum* on rice straw (17.2% and 11.6% VS loss, respectively). Since VS content correspond with those of the organic matter, an excessive VS degradation during pretreatment could negatively affect the AD process, leading to lower biogas production (Kainthola et al., 2019a). Therefore, it was fundamental for pretreatment success

having found suitable microorganisms and experimental conditions that allowed the reduction in these losses (Carrere et al., 2016). Interestingly, in the samples pretreated with *C. cinerea* and *C. stemonitis* the VS content resulted slightly ($p>0.05$) increased compared to untreated controls, probably due to fungal inoculation, growth, and metabolism. In fact, the addition of fungal biomass as inoculum was the most probable cause of enhanced VS content of T0 treatments. During the first 10 day, fungi may have consumed the easily degradable compounds (i.e., hemicellulose) together with the more recalcitrant PCWP (lignin and cellulose) to sustain their growth and metabolism, lowering the TS and VS content of SFD. This is in line with Muthangya et al. (2009), who reported that rot fungi preferably devour simple sugars instead of lignin and cellulose. When the pretreatment lasted longer (20 days), the VS losses accountable to fungal degradation of PCWP may have been compensated by the grown mycelial biomass, causing the increase in the overall VS content. This compensation mechanism allowed to obtain an efficient pretreatment maintaining a high biomass VS and, therefore, a high potential in biogas and methane production.

Fungal pretreatments also changed the concentration of lignocellulosic fibers. The percentage of NDF losses ranged from 1.6% to 10.4% in samples pretreated with *C. cinerea* for 10 days and with *C. stemonitis* for 20 days, respectively. All the fungal strains affected also the PCWP composition. This was not completely unexpected, since the selected fungal species may produce lignocellulolytic enzymes (Isikhuemhen et al., 2009; Nuchdang et al., 2015; Peterson et al., 2011) potentially useful for improving the biodegradation and bioconversion of lignocellulosic feedstocks during subsequent AD (López et al., 2013). The different strains showed a similar behaviour towards the PCWP, causing a higher reduction in hemicellulose (18.5–59.3%) as compared to lignin (1.0–9.6%) and cellulose (0.2–8.2%). However, as expected, distinct fungal species caused a different level of conversion of each PCWP. Noteworthy, the highest reductions in all the PCWP

components were obtained with pretreatment with *C. stemonitis* for 20 days, which resulted in 59.3%, 9.6%, and 8.2% hemicellulose, lignin and cellulose reductions, respectively. Compared to *C. stemonitis*, the Basidiomycota strains gained a lower lignin reduction (1.6–3.2% and 1.0–3.9% with 10 and 20 days of pretreatment with *C. cinerea* and *C. aegerita*, respectively). The pretreatments with *C. aegerita* achieved lower reductions in cellulose (from 0.2% to 0.8% with 10 and 20 days, respectively), while those performed with *C. cinerea* reached a lower hemicellulose reduction (from 18.5% to 22.2% for 10 and 20 days, respectively).

The easily accessible, but less abundant, hemicellulose fraction was the main target by all tested strains (Table 17). These data are in accordance with enzymatic assays results, in which xylanases resulted the most produced enzymes by all fungi during the SSF pretreatment. The hemicellulose reductions obtained in this study fall within the range of those observed in the literature. For instance, Nuchdang et al. (2015) reported the ability of *C. cinerea* to reach a hemicellulose degradation of about 27% after 30 days on lignocellulosic grass. Similar results were reported for *P. ostreatus* and *T. reesei* on rice straw, which reduced the hemicellulose content by about 23% (Mustafa et al., 2016). A similar hemicellulose reduction was reached also by *P. chrysosporium* on wheat straw and corn stover silage (31.2% and 32.4-48.4% respectively) (Liu et al., 2014a; Zhi and Wang, 2014). In the work of Isikhuemhen et al. (2009), *C. aegerita* appears to degrade hemicellulose components from 14% to 53.9%, depending on the combinations of solid waste from digester effluent, wheat straw, and millet used as substrate. Surprisingly, the highest hemicellulose degradation was obtained with *C. stemonitis*, though *C. cinerea* produced the highest IU/L of xylanases (Figure 37). The levels of hemicellulose degradation obtained with *C. stemontis* were even higher than those observed in the literature with well-known white-rot fungi. For example, Zhi and Wang (2014) and Liu et al. (2014a) obtained with *P. chrysosporium* a hemicellulose degradation rate lower than 50% on wheat straw and corn stover

silage (31.2% and 32.4–48.4%, respectively). Chen et al. (2007) stated that some levels of hemicellulose removal can enhance digestibility, since the process increases the porosity of lignocellulosic material. However, an excessive degradation of this heteropolymer can lead to a decrease in the biogas yields, as it is a fundamental source of AD microorganisms (Rouches et al., 2016; Vasco-Correa et al., 2016). Considering the initial poor hemicellulose content in SFD, the relatively high losses obtained in this study could be neglectable for the downstream AD process.

The more recalcitrant and abundant lignin and cellulose were also reduced by all fungi, but less efficiently compared to hemicellulose. When comparing the different strains, the SFD pretreated with *C. aegerita* and *C. cinerea* showed the lowest lignin reduction. A similar result was reported by Nuchdang et al. (2015), which observed that lignin degradation of lignocellulosic grass treated with *C. cinerea* was not significantly different from that obtained by natural decay. Isikhuemhen et al. (2009) reported variable levels of delignification for *C. aegerita*, obtaining values similar to those observed in this work (0.6%) and values far higher (21.8%), depending on the mix of substrates used. Although Basidiomycota species are reported as the most powerful organisms in delignification (Rouches et al., 2016), the pretreatment with Ascomycota *C. stemonitis* leads to an almost triple delignification compared to *C. cinerea* and *C. aegerita* strains. It was quite surprising that the highest lignin removal was performed by *C. stemonitis*, despite *C. aegerita* and *C. cinerea* being the most productive strains in terms of laccases yields (Figure 37). This suggests that a little correlation can be found between fungal enzymatic yields and the PCWP degrading ability. This phenomenon was also reported by Stepanova et al. (2003), who observed that a relatively high laccase production and accumulation by *Carrena maxima, Coriolus hirsutus* and *Coriolus zonatus* corresponding to a low lignin disruption rates on oat straw, and by Levin et al. (2008), who grew *T. trogii* on poplar wood. An explanation could be

that the alkaline pH values inhibited the laccase activity of Basidiomycota strains. In fact, laccases are known to operate at lower pH values (3-6) (Baldrian, 2006; Kong et al., 2017; Liers et al., 2011). On the other hand, the laccases produced by *C. stemonitis* may be more efficient under the selected pretreatment conditions. Indeed, it is reported that many Ascomycota laccase present higher optimum of pH than the Basidiomycota ones (Shraddha et al., 2011). In addition, laccase usually produce a cascade of oxidative reactions, therefore even low enzymes concentrations could result in high delignification rates (Baldrian, 2006; Shraddha et al., 2011). Another explanation of the results could be that *C. stemonitis* produces peroxidases or other enzymes involved in lignin disruption or exploits still unknown non-enzymatic lignin oxidative mechanisms.

Anyway, the maximum lignin reduction obtained with *C. stemonitis* (9.6%) was relatively scarce respect to the data reported in literature. For instance, Zhi and Wang (2014) reported that *P. chrysosporium* was able to reduce wheat straw lignin content by 36%. *P. chrysosporium* was studied also by (Liu et al., 2014a), leading to a lignin reduction of 22.6-39.0%. A similar delignification rate (35.3%) was described also by Mustafa et al. (2016), pretreating rice straw with *P. ostreatus*. Analogous delignification rates were obtained also by pretreatments done with *C. subvermispora* on different substrates. Ge et al. (2015) obtained a delignification rate of 24% pretreating albizia chips with *C. subvermispora* and similar results were also obtained by Wan and Li (2011) by pretreating corn stover and switchgrass. Zhao et al. (2014), instead, reported a slightly inferior delignification rate of 20.9 % for sterilized yard trimmings, comparable to that obtained by Wan and Li (2011) when treating hardwood with the same fungal species.

Since lignin removal is a key process for pretreatments success, it is fundamental to further investigate how to stimulate higher delignification rates. It could also be worthy to optimize fungal growth and pretreatment conditions by modifying (lowering) biomass pH and to assess whether pre-adaptation of fungi to the specific biomass could positively affect PCWP modifications, as suggested by

Zhao et al. (2014). However, according to Muthangya et al. (2009), even a small depletion of lignin may lead to a significant increase in methane production during subsequent AD. In fact, lignin degradation is reported as the main factor for a successful fungal pretreatment, as it increases the accessibility of AD microorganisms to the more easily degradable structural carbohydrates and directly contribute to the enhancement of anaerobic digestibility (Zhao et al., 2014).

Notably, the cellulose reduction rates obtained in this study were lower than those generally described in the literature. For instance, Nuchdang et al. (2015) reported a 16% cellulose degradation for *C. cinerea* grown on lignocellulosic grass. Higher cellulose reductions (ranging from 27.7 to 55.2%) were described also by Isikhuemhen et al. (2009) using *C. aegerita*. Conversely, lower cellulose losses, comparable to those obtained in this study, were reported using specific Basidiomycota characterized by a selective delignification system, hence capable to selectively remove lignin without affecting the cellulose content (Yoon et al., 2014). For example, Wan and Li (2011) reported that *C. subvermispora* lacks a complete cellulolytic system, thus produces negligible cellulose degradation (<5%) in all the different lignocellulosic substrates tested. Similarly, Zhao et al. (2014) observed a reduction in cellulose content of only 7.4 % on yard trimmings after fungal pretreatment with *C. subvermispora*. Low cellulose decomposition rates are generally desirable when operating pretreatment processes, as this polysaccharide constitutes one of the main sources of sugars for microorganisms involved in AD (Rouches et al., 2016; Vasco-Correa et al., 2016).

As expected, the concentrations of biomass components (TS, VS, NDF, and PCWP) generally decreased as the duration of the pretreatments increased, confirming that incubation period could be one of the most important factors influencing treatment efficiency. However, most of reductions occurred during the first 10 days, while minor changes were observed when prolonging the

pretreatments to 20 days. For instance, *C. cinerea* and *C. aegerita* exhibited a relatively limited variation in the TS and PCWP content between 10 and 20 days of pretreatment.

On the whole, the changes in SFD can be ascribable both to the addition of fungal biomass and to the fungal activity during pretreatment. As also indicated by literature data (López et al., 2013), the PCWP modifications could increase the digestibility of SFD during AD. However, the heterogenicity of lignocellulosic biomass and the complicated nature of AD prevent to predict the performance of the process solely based on the composition of feedstocks (Liew et al., 2012). BMP tests in batches were then carried out to assess the actual effect of fungal pretreatments on biogas and methane production.

5.3.4.4 Biogas and methane yields

Figure 38 shows the daily biogas production rates and respective methane concentrations of untreated and fungal-pretreated SFD. All the samples showed a similar profile of daily biogas yields, with a peak at day 4, followed by a progressive decrease, which dropped to zero after about 75 days. The SFD samples inoculated and pretreated with fungi did perform better than the untreated control. In particular, the samples pretreated with *C. cinerea* for 10 days produced the highest daily biogas yield (15.6 ± 0.8 L_N/kg VS d), which was approximately double with respect to the untreated SFD (8.8 ± 0.1 L_N/kg VS d).
The average daily methane concentration in biogas was comparable among all samples (Figure 38): the profile showed a gradual increase (from about 20–30% up to 55%) during the first 10 days of AD, when the plateau was reached and then maintained until the end of the trials.

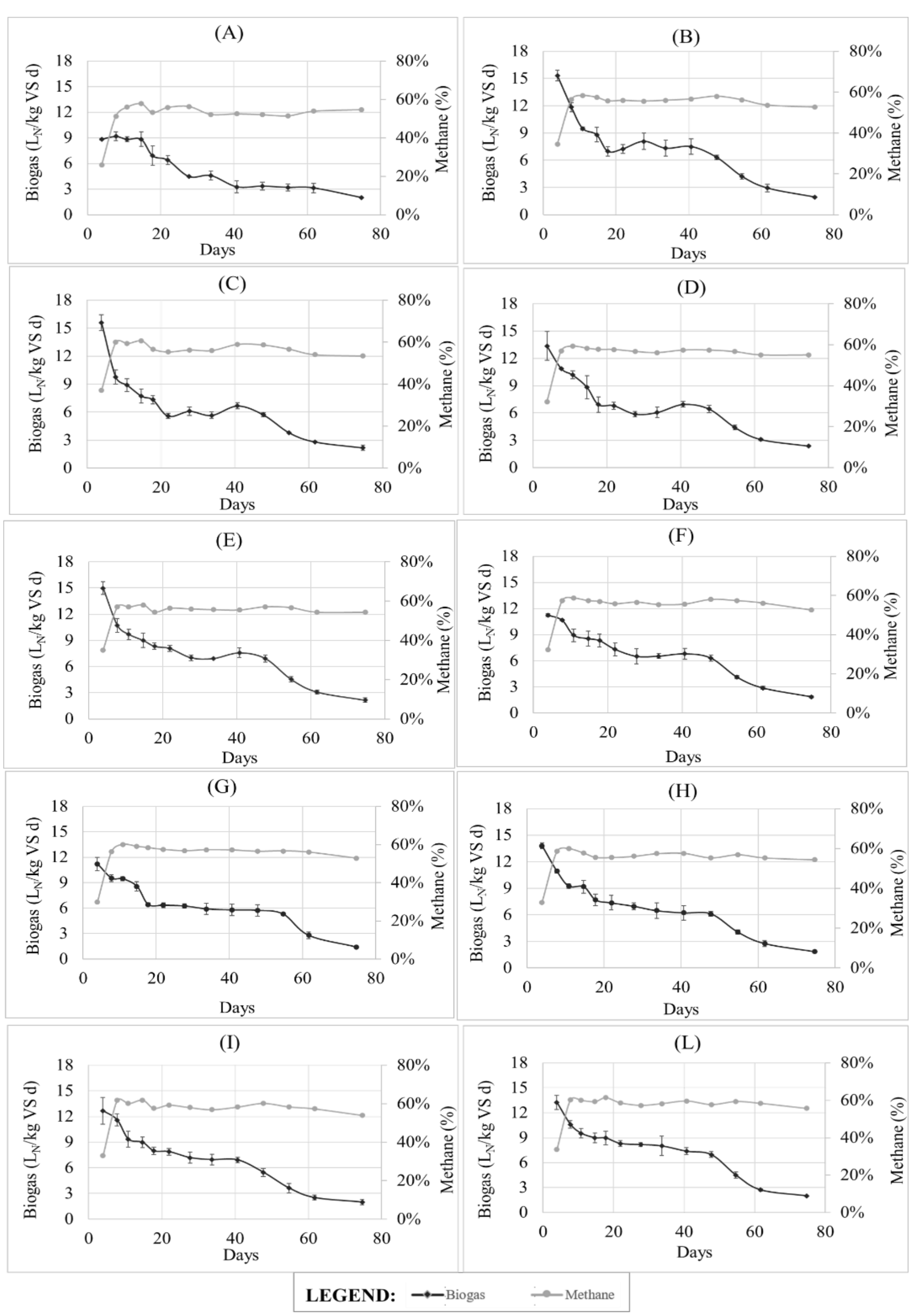


Figure 38: Daily biogas yield (L_N/kg VS day) and methane concentration (%) of the SFD, untreated (A) and pretreated with *C. cinerea* (B,C,D), *C. aegerita* (E, F, G) and *C. stemonitis* (H, I, L) for 0 (B, E, H), 10 (C, F, I) and 20 (D, G, L) days.

At the end of the anaerobic incubation, the cumulative biogas and methane yields from all the SFD samples pretreated with fungi were significantly (p<0.05) higher than those of the untreated control (Figure 39), indicating their effectiveness in enhancing biogas and methane yields of this recalcitrant biomass. The AD worked better with SFD treated by *C. stemonitis* for 20 days, which led to approximately three-fold higher biogas and methane yields (+182% and +214%, respectively) than the untreated SFD. The biogas yield obtained with pretreatment with *C. stemonitits* for 20 days resulted statistically higher (p<0.05) from that achieved with *C. aegerita* for 20 days and *C. cinerea* for 10 days, whose increment in biogas respect to the untreated samples ranged from 82 % to 100 %, respectively. However, comparable (p>0.05) cumulative biogas yields were achieved with pretreatments by *C. stemonitis* and *C. aegerita* for 10 days and *C. cinerea* for 20 days (Figure 39). On the other hand, the cumulative methane produced with *C. stemonitis* was significantly higher (p<0.05) than that obtained with *C. aegerita* and *C. cinerea* for both 10 and 20 days.

Notably, within a process carried out by the same fungal strain, no significant differences (p>0.05) in the cumulative yields were observed between 10 days and 20 days of pretreatment.

The results obtained in the BMP tests highlighted that SFD still contains residual biogas and methane potential, confirming that it could be considered a suitable feedstock for biogas plant feeding (Menardo et al., 2011b). However, the poor biogas and methane yields obtained from the untreated samples (Figure 39) confirmed that a large fraction of SFD organic matter is not readily biodegradable as lack of easily degradable carbon source. The fungal pretreatments resulted a promising biotechnology to increase SFD hydrolysis and BMP yields during the subsequent AD, though also the simply co-digestion of fungal biomass (i.e., T0 process) led to considerable and comparable (p>0.05) increase in biogas and methane yields.

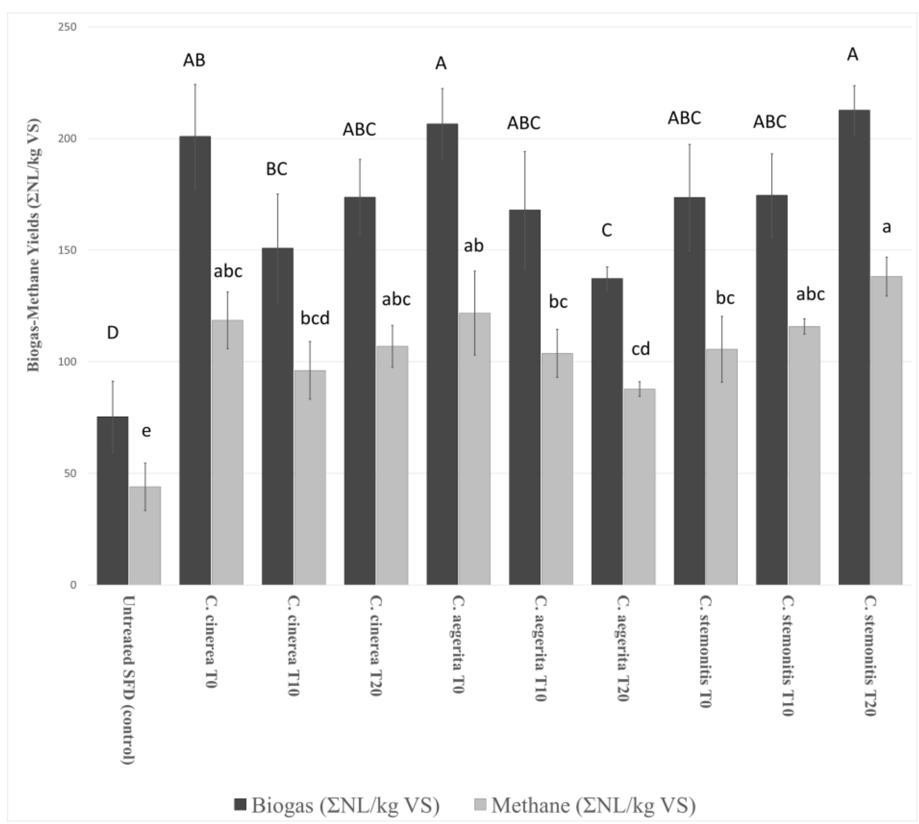


Figure 39: Cumulative biogas and methane yields (ΣL_N/kg VS) of SFD, untreated and pretreated with *C. cinerea*, *C. aegerita*, and *C. stemonitis* for 0, 10 and 20 days (T0, T10 and T20, respectively). Results are expressed as mean ± standard deviation from three replicates. The letters were used to show the results of statistical analyses.

In the case of pretreated samples, the improved digestibility can be explained mainly to the reduced biomass recalcitrance caused by the PCWP degradation (Table 17). A higher level of PCWP degradation seems indeed correlated with higher biogas and methane production. However, the fungal biomass added to the system may have also played a role. The increase in biogas and methane yields obtained also with samples T0 could be accounted to AD microorganisms digesting the inoculated fungal biomass. In fact, in the T0 condition the fungal biomass was added to SFD immediately before the BMP tests' starting, thus the inoculated

fungal biomass has only increased the total TS and VS content in the feedstock, without causing organic matter losses due to their growth and metabolism. Based on these results, it was hypothesized that the fungal biomass can contains some organic components, as lipids, proteins and other molecules, that may boost the microorganisms involved in AD providing organic components with a good potential in terms of biogas-methane potential. Hom-Diaz et al. (2016) demonstrated, by means of BMP tests batch assays at mesophilic temperature that the exhausted fungal biomass could be profitably used as a substrate in AD processes, producing 281-595 L methane/kg VS. Furthermore, Jasko et al. (2012) tested the BMP of fungal biomass of *Paxillus involutus* and *Phaeolus schweinitzii* and they obtained a biogas production of 607.3 L/kg VS and 137.9 L/kg VS, respectively. Considering the average methane productivity reported in literature per g of fungal VS (about 387.5 ± 123.8 L/kg VS) (Hom-Diaz et al., 2016) and the amount of fungal biomass introduced inside each batch reactor (0.71 g VS) in this experiment, it could have contributed to approximately 5–10% of the total methane productivity. Interestingly, the difference between the results obtained by Jasko et al. (2012) with the different fungal species was accounted to the difference in VS composition or to the production of bioactive compounds able to inhibit or enhance the AD process (Jasko et al., 2012). The same type of mechanism could be involved in this study and may explain the different yields obtained with the distinct fungal species. Overall, new questions have been arisen, such as and the precise contribution of the fungal biomass itself to the AD process. This aspect certainly deserves to be further explored. Indeed, the exploitation of spent fungal biomass as an alternative AD feedstock would bring benefits in terms of renewable energy production and sustainable waste disposal, since it is produced in large volumes by many industrial processes.

In the literature, the time of pretreatment is reported as one of the most critical factors for the efficiency of AD performance (Mutschlechner et al., 2015).

Mustafa et al. (2016) tried the effects of different pretreatments duration (10, 20 and 30 days) on rice straw with *P. ostreatus* and *T. reesei* and the best process performances were obtained with 20 days pretreatment, with higher biogas and methane yields in comparison with the shorter and longer treatments. In fact, shorter incubation times did not lead to enough PCWP degradation, while a longer period would lead to excessive organic matter losses due to fungi consuming sugars released from the PCWP decomposition (Mustafa et al., 2016). Different results were obtained by Phutela et al. (2011) treating rice straw with *T. reesei* and *Coriolus versicolor* for 5, 10, 15, 20, and 25 days. According to these authors (Phutela et al., 2011), the best treatment last 10 days, while in the cases of longer incubation, the biogas and methane yields decreased (up to 50.90% on day 25). In this work, the samples pretreated with *C. cinerea* and *C. stemonitis* gave higher yields with a longer process (20 days). On the contrary, the pretreatment with *C. aegerita* achieved higher performance with 10 days of process. Thus, in general, the least effective treatments appeared to be those that last for 10 days. Interestingly, T10s conditions were those characterized by the lowest VS content and PCWP reduction. Therefore, biogas-methane productivity seems to be related to biomass VS and PCWP content. However, surprisingly, within a pretreatment carried out by the same strain, no significant ($p>0.05$) differences were observed in the cumulative biogas and methane yields between 10 days and 20 days of processing. It would probably take longer to see significant differences, but, as observed for *C. aegerita*, longer treatment times could cause increased loss of organic matter and a consequent decrease in the biogas production during AD (Carrere et al., 2016; Muthangya et al., 2009). Considering that one of the main disadvantages of biological pretreatments is that they are often time consuming (Sindhu et al., 2016) it is a positive finding that the developed process allowed to obtain a significant increase in the AD yields also with relatively short pretreatment times (T10) or even without any pretreatment process (T0).

The use of different fungal strains and species for pretreatment turned out to be the most important factor affecting the AD performance, with *C. stemonitis,* producing almost triple cumulative biogas and methane with respect to the untreated samples (Figure 39). In comparison with other authors who performed fungal pretreatments on digestate and other recalcitrant biomass, the improvement in biogas and methane yields obtained in this study were far higher and, therefore, encouraging. For instance, López et al. (2013) concluded that the treatment of lignocellulosic anaerobic digestate with the white-rot fungus *Phanerochaete flavido-alba* was not useful to increase the biogas production. Liu et al. (2014b) treated corn stover with *P. chrysosporium* and obtained an improvement of biogas production by only 10.5% to 19.7% and methane yield by 11.7% to 21.2%, though a pretreatment duration that was longer (30 days). Similarly, Phutela et al. (2011) reported an augmentation in biogas yields of only 20.8% and 26.2% treating rice straw with *T. reesei* and *C. versicolor*, respectively.

Previous studies have also investigated physical and chemical pretreatments to get a higher energy recovery from SFD and other recalcitrant lignocellulosic by-products and wastes, but surprisingly they often obtained poor results compared to those that are here obtained with fungal pretreatments. For instance, Song et al. (2019) proved to get an increment of only 14-22 % in methane production treating SFD with free ammonia. Similarly, Menardo et al. (2011a) assessed on four different mechanically separated SFD the effects of a high temperature thermal pretreatment, and they obtained no statistically significant effect on the daily yields of thermal pretreated samples but reported a significant increase in the cumulative methane yields that ranged from 35% to 171% depending on nature of the organic matter of the considered samples. Lindner et al. (2015) instead, tested the effects of milling on digestates derived from different AD processes and feedstocks, and observed a triplication in methane yields for digestate derived from a full-scale plant fed with hay/straw, and lower increments (9-17%) for digestates deriving from two-stages AD fed with maize silage digestate and hay/straw digestate

respectively. Ultrasound treatments have also been applied to digestates in order to get higher biogas-methane yields; according to Garoma and Pappaterra (2018) and Boni et al. (2016) increase of 1.6-2.3 times in methane production and a gain of 30% in biogas yield, respectively, could be reached with ultrasonication. Chowdhury et al. (2020) tested the efficacy of ultrasound treatments as well, obtaining an increment in methane production of 1.94 times respect to that obtained from untreated SFD. However, considering the energy requirements of the ultrasonication process, the net energetical gain was lower and the effective economic and environmental feasibility should be evaluated. For instance, according with Azman et al. (2020), who performed ultrasonication on digestate and gained an increment in methane production of 18%, the ultrasound energy demand was higher than the energy that could be recovered from pretreated SFD. Comparing these literature works with our experiment it is possible to assume that fungal pretreatments allow the highest net energy recovery when applied on SFD. Moreover, since the process do not require high energy inputs or chemical reagents, it result to be greener and economically feasible.

In other studies, biological pretreatments with fungi were confirmed as more efficient respect to the abiotic ones, and the results obtained were comparable to those observed in this work. For instance, Zhao et al. (2014) pretreated unsterilized yard trimmings with *C. subvermispora* obtaining a biogas yield 2.3 times higher than that obtained from untreated samples. Similarly, Lalak et al. (2016) pretreated *Agropyron elongatum* (a type of lignocellulosic grass) with *Flammulina velutipes* and obtained an increase of 134 % in biogas yields. However, it should be noted that most of the literature work on the pretreatment of lignocellulosic feedstocks is focused on the use of WRF basidiomycetes inhabiting wood. The Ascomycota and fungi of other taxonomic groups or habitats have only scarcely been investigated (Singh et al., 2014). According to the results, the Ascomycota species could be equally able or even more competent in disrupting recalcitrant PCWP and enhance AD yields. Indeed, *C. stemonitis* was the most efficient in reducing hemicellulose,

lignin, and cellulose and increasing the biogas and methane yields. Other works also confirmed the effectiveness of fungal pretreatments performed with Ascomycota. For instance, the study of Mutschlechner et al. (2015), Wagner et al. (2013), and Deng et al. (2018), reported a significant increase in the AD yields when pretreating biowaste with *Trichoderma* species. In detail, Deng et al. (2018) reported an increase in biogas yields of 318 % when pretreating rice straw and soybean straw with *T. reesei*. Wagner et al. (2013) reached a boost in biogas production of 400% treating bio-waste with *T. viride*. Unfortunately, studies on Ascomycota focusing mainly on a restricted number of well-studied fungal taxa (e.g., *Trichoderma* and *Aspergillus*), while this work demonstrated that it would be worthy to expand the research to other potentially suitable fungal species. At present, this is the only available information on the potential of *C. stemonitis* to improve the AD bioconversion of lignocellulosic biomass. The findings emphasize the importance of investigating fungal biodiversity to identify new and promising species suitable for the development of effective pretreatment processes.

In conclusion, the results obtained in this work could be considered as a fundamental step towards the development of a cost-effective and environmentally friendly pretreatment technology/strategy to ultimately makes the SFD more susceptible to a further digestion step and increase its biogas and methane yields. Favoring the (re)use of SFD as a feedstock for AD, the fungal pretreatments contribute to the development of a next-generation by-product management strategy. Ultimately, the integration of the fungal pretreatment of SFD and its subsequent reuse in the anaerobic digester has the potential to allow GHG emissions abatement and concurrent energy recovery, leading to environmental and economic benefits that make the AD technology even more attractive and effective.

5.3.5 Experiment 3

5.3.5.1 Characteristics of untreated and fungal-pretreated biomasses

On the whole, the results of Experiment 3 confirm the data obtained in the previous experiments. The pH of SFD ranging around alkaline values (Table 18). On the contrary the fungal biomass of *C. stemonitis* had acid pH (4.4 - 4.7). The addition of exhausted media (pH 4.9) and fungal biomass to SFD caused the pH lowering, from 8.9 to around 7.8 (untreated and pretreated samples, respectively).

The SFD showed TS and VS (Table 18) comparable with literature data (Zhong et al., 2016). About 91 % of the TS was composed by VS, confirming that SFD retained a high organic matter content. In comparison to the SFD, the fungal biomass was characterized by a significantly ($p<0.05$) lower TS content (about 4 %) and a higher VS content (around 98 % of TS). The high VS content of fungal biomass suggest the feasibility of an AD step to valorise this kind of substrate.

The fungal inoculum addition and pretreatment significantly ($p<0.05$) lower the TS content in most of the pretreatment conditions, with the only exception of the fungal-treated and autoclaved SFD ($p>0.05$). The TS reductions obtained in this study (from 0 to 16 %) fall within the range of those observed in the literature (Carrere et al., 2016; Baldrian et al., 2005) and those obtained in the previous experiments. The decrease in TS were mostly attributed to the dilution due to the water content in fungal inoculum (around 96 %) and to the biomass degradation during the pretreatment (Mustafa et al., 2016; Zhao et al., 2014).

On the other hand, small decrease (< 1 %; $p>0.05$) in VS content were observed for the fungal-pretreated SFD respect to the untreated samples. Therefore, the involvement of a compensation mechanism of VS losses, accountable to inoculation and growth of the mycelial biomass, seems to be confirmed.

Interestingly, little variations ($p>0.05$) in TS and VS content were observed between the autoclaved and not autoclaved (i.e., alive) samples, with the only exception of the TS content of the fungal-treated SFD (T10) ($p<0.05$).

Table 18: Characteristics of pH, TS and VS of the SFD, untreated and fungal pretreated with *C. stemonitis* for 0 and 10 days (T0 and T10, respectively), and of its fungal biomass. Each thesis was tested in the presence and absence of thermal pretreatment with autoclave (autoclaved and alive, respectively). Statistical significance for TS and VS data is expressed as results of Tukey's grouping.

	pH	TS %	VS (% TS)
Untreated SFD + Water	8.9	28.1^{a}	91.9^{b}
Untreated SFD + Exhausted Media	7.9	28.1^{a}	91.7^{b}
SFD + Fungal biomass (T0) - AUTOCLAVED	7.8	23.6^{c}	91.2^{b}
SFD + Fungal biomass (T0) - ALIVE	7.9	24.8^{bc}	92.0^{b}
Fungal-treated SFD (T10) - AUTOCLAVED	7.9	28.1^{a}	91.2^{b}
Fungal-treated SFD (T10) - ALIVE	7.9	25.5^{b}	$92.^{b}$
Fungal Biomass - AUTOCLAVED	4.7	3.6^{d}	98.5^{a}
Fungal Biomass - ALIVE	4.4	4.0^{d}	98.2^{a}

5.3.5.2 Biogas and methane yields

The average daily biogas production and the respective methane concentration obtained in BMP are presented in Figure 40. The untreated SFD added of water showed similar trend of daily biogas yields respect to the samples added of the exhausted media from fungal inoculum production, with a maximum peak at 29 days, followed by a progressive decrease in production (Figure 40). However, the samples with water showed a higher peak of daily biogas production rates than SFD added of exhausted media: the first yielded a maximum of 10.3 ± 1.5 L_N/kg VS d, while the second produced 7.5 ± 0.5 L_N/kg VS d. The profile of daily methane concentration showed a gradual increase and the maximum peak (about 55-60 %) was reached at 29 days, but the SFD added of the exhausted media produced biogas with higher methane concentration. In detail, the daily methane

concentration in biogas was increased up to 5 percentage points at 29 days (Figure 40). As a result, the cumulative biogas and methane yields were comparable between SFD plus water and the SFD with exhausted media ($p>0.05$) (Figure 41). In detail, SFD added of water led to cumulative biogas and methane yields of 167 ± 14 L_N/kg VS and 97 ± 8 L_N/kg VS, respectively, while the data with samples added of exhausted media were 193 ± 8 L_N/kg VS and 115 ± 2 L_N/kg VS, respectively. Thus, the addiction of exhausted media to SFD did not have a significant effect in enhancing the cumulative biogas and methane yields, probably because it contains just few nutrients (since most of them were consumed by the fungus during the SmF) or because the SFD did not have the optimal conditions for the enzymes or other metabolites to operate (Musatti et al., 2017).

Compared to the SFD samples added of water and exhausted media, all the SFD inoculated and pretreated with *C. stemonitis* did perform better (Figure 40 and 41). Focusing on the T0 treatment (i.e., co-digestion of SFD and fungal biomass), the maximum peak of daily biogas yields was achieved at 17 days (Figure 40), indicating a greater and faster digestibility of the substrate respect to the untreated ones, probably due to the presence of the more easily accessible fungal biomass. The T0 autoclaved produced a maximum of 12.4 ± 1.1 L_N/kg VS d, while the T0 not autoclaved (alive) produced 8.0 ± 0.9 L_N/kg VS d. As regard the average daily methane concentration in biogas, the T0 samples, autoclaved and not, were almost comparable (Figure 40); the profile showed a gradual increase (from about 6-7% up to 48-53%) during the first 14 days of AD, then the maximum values (about 56-57 %) were reached at 29 days.

The fungal-pretreated samples (T10) generally achieved higher daily biogas methane yields respect to the untreated samples, but the yields are often lower compared to the T0 treatments (Figure 40). Nevertheless, in comparison to the untreated and T0 treatments, the profile of daily biogas yield obtained with fungal pretreated samples seems more regular and constant over time, especially from day

14 to 41, in which the maximum peak of daily biogas production was reached (Figure 40). The fungal-pretreated and autoclaved samples produced a maximum of 8.2 ± 1.4 L_N/kg VS d, while the not autoclaved (alive) produced 7.3 ± 1.5 L_N/kg VS d. The profile of daily methane concentration in biogas was similar among the autoclaved and not autoclaved samples, but different values were obtained (Figure 40); in fact, at day 29, the autoclaved samples achieved a maximum peak of 55 % while the not autoclaved, with the fungus alive, produced biogas with a methane concentration of about 61 % (Figure 40).

At the end of the anaerobic incubation, the cumulative biogas and methane yields from all the SFD samples pretreated with *C. stemonitis* were significantly ($p<0.05$) higher than those of the untreated control (Figure 41), confirming the effectiveness of this biological process. The AD worked better with T0 treatment, which led to more than two-fold higher biogas and methane yields (+142% and +128%, respectively) than the untreated SFD with water. In detail, the T0 autoclaved led to cumulative biogas and methane yields of 404 ± 21 L_N/kg VS and 220 ± 14 L_N/kg VS, respectively, while the data for the T0 not autoclaved (alive) were 261 ± 52 L_N/kg VS and 148 ± 25 L_N/kg VS, respectively. The cumulative biogas and methane yields obtained with fungal-pretreated and autoclaved SFD were of 351 ± 39 L_N/kg VS and 188 ± 21 L_N/kg VS, respectively, while the data with the not autoclaved samples were 278 ± 23 L_N/kg VS and 157 ± 12 L_N/kg VS, respectively. Notably, within the same kind of process, significant differences in the cumulative biogas and methane yields were observed between the autoclaved and the not autoclaved (alive) samples: the biogas and methane yields obtained with autoclaved T0 and fungal-pretreated SFD (T10) resulted statistically higher ($p<0.05$) compared to the not autoclaved ones. However, comparable ($p>0.05$) cumulative biogas and methane yields were achieved with T0 and fungal-pretreated autoclaved samples, and between T0 and fungal pretreated not autoclaved samples (Figure 41).

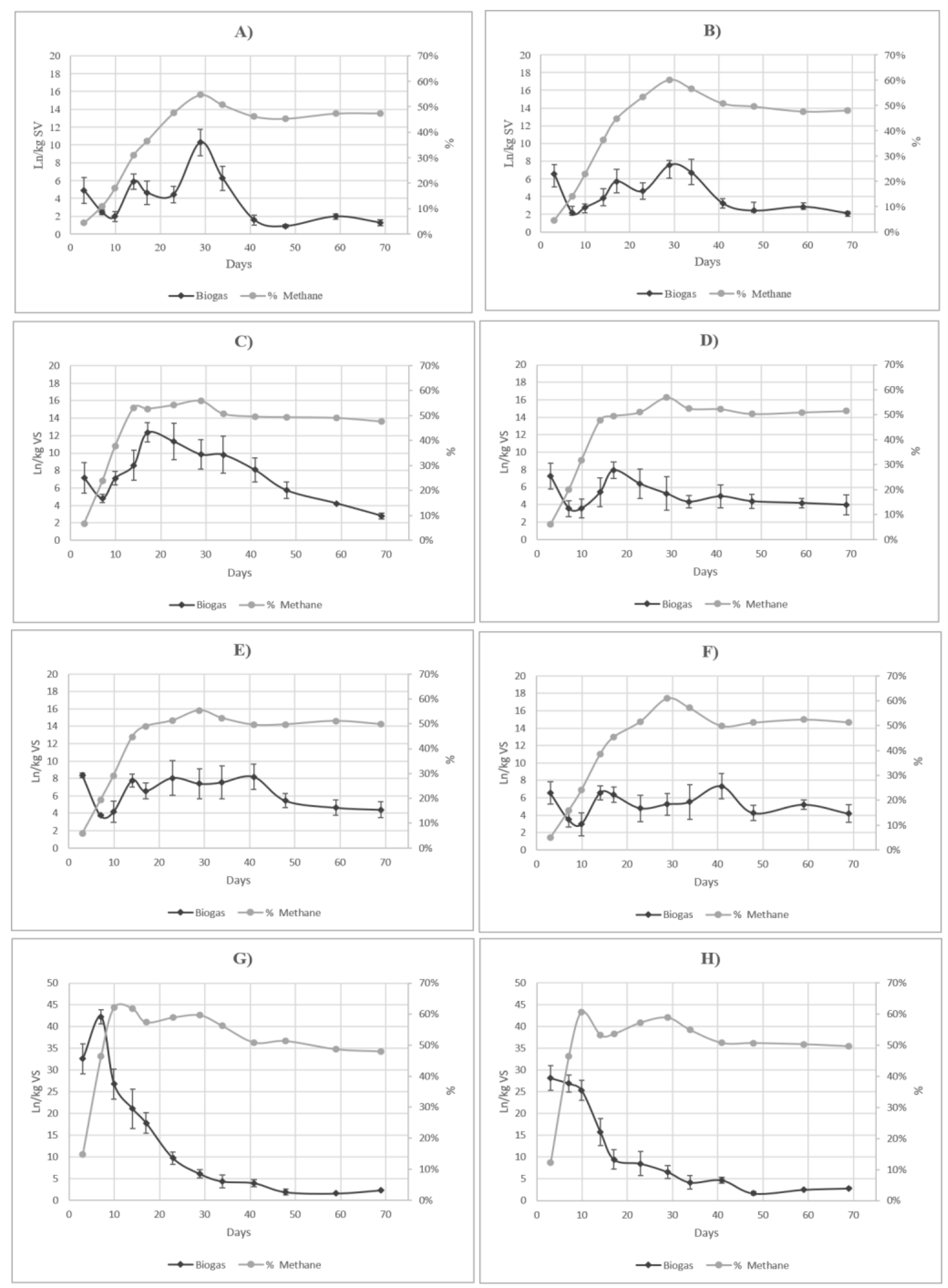


Figure 40: Daily biogas yield (L_N /kg VS d) and methane concentration (%) of: A-B) Untreated SFD plus Water (A) and Exhausted Media (B); C-D) SFD plus fungal biomass (T0) – autoclaved (C) and alive (D); E-F): Fungal-treated SFD - autoclaved (E) and alive (F); G-H): Fungal Biomass only - autoclaved (G) and alive (H).

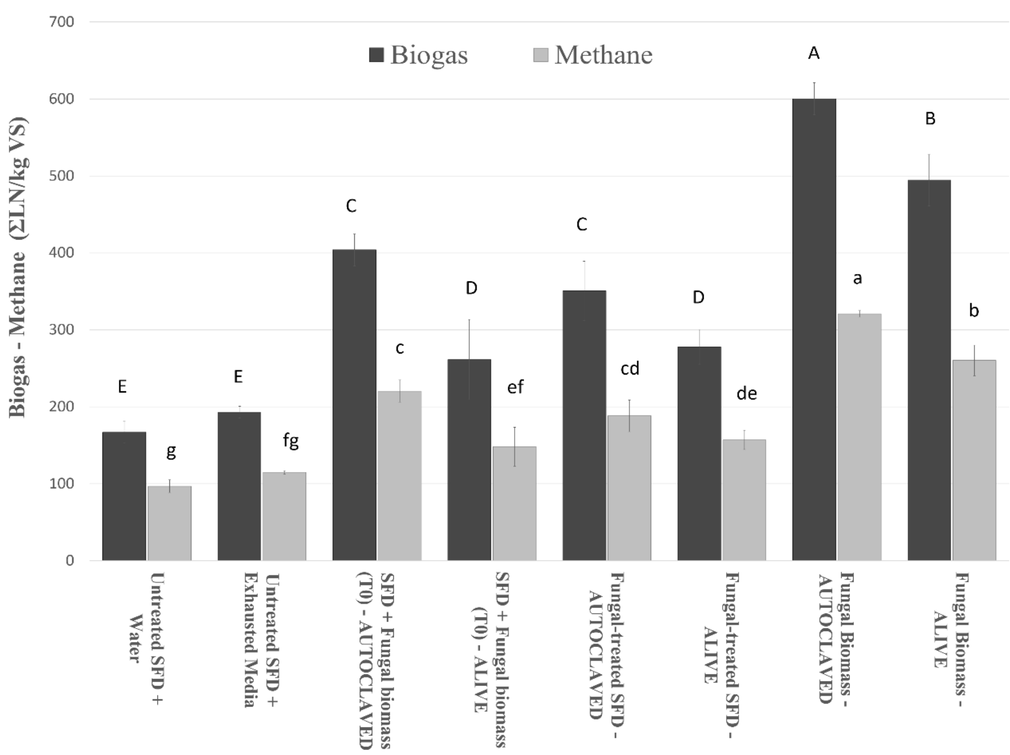


Figure 41: Cumulative biogas and methane yields (ΣL_N/kg VS) of the SFD, untreated and fungal pretreated with *C. stemonitis* for 0 and 10 days (T0 and T10, respectively), and of its fungal biomass. Each thesis was tested in the presence and absence of thermal pretreatment with autoclave (autoclaved and alive, respectively). Results are expressed as mean ± standard deviation from three replicates. The letters were used to show the results of statistical analyses.

The combination of thermal (autoclave) and biological (fungal) pretreatments seems to have had positive effects on the AD yields. In order to obtain increments in biogas and methane production, a combination of different kinds of pretreatments is widely applied also in the scientific literature. For instance, cumulative biogas increments similar to those obtained in this work were reported by Mustafa et al. (2017), using on rice straw a combination of physical and biological pretreatments: they obtained a cumulative biogas augmentation of 165 % combining milling and fungal pretreatment with *P. ostreatus*. However, the

beneficial income gained from the enhanced biogas yields need to be evaluated together with the higher energy requirements, operational costs, and the environmental issues linked to abiotic pretreatments (Akhtar et al., 2016; Shirkavand et al., 2016; Taherzadeh and Karimi, 2008). In fact, in some cases the enhancement of biogas yields results in higher process costs due to energy and reagents consumption and to the disposal of effluents. For instance, Jiang et al. (2016) found that abiotic treatment (liquid hot water and alkaline) of giant reed biomass was not energetically convenient due to the excessive energetic requirements, thus its application is not justified. Moreover, the energy consumption for sterilization (e.g., 886 kJ/kg dry basis for decontamination of wood chips using low pressure steam) is one major concern in industrial application of fungal pretreatment (Zhao et al., 2014). In the case of chemical pretreatments, instead, the major drawback is the production of toxic effluents that be safely disposed (Shirkavand et al., 2016). Considering the high inputs required by the abiotic pretreatment technologies and the environmental risks, the biological pretreatments appear to be far more convenient and environmentally friendly. In addition, the results obtained in this study were comparable or even higher than those reported by other authors (Brémond et al., 2020; Sambusiti et al., 2015) with biological and/or physicochemical processes, highlighting the higher efficiency of the developed fungal pretreatments and their higher sustainability from both an economical and environmental point of view. Overall, the co-digestion of SFD and fungal biomass (T0) resulted the most appliable and profitable condition in terms of yields and practicability, considering also that this process did not required particular operative conditions and time for fungal cultivation. Further studies on the co-digestion of fungal biomass with other types of fungi and/or recalcitrant lignocellulosic biomasses could be worthwhile and interesting.

Very promising results have been also obtained by digesting the fungal biomass alone. In comparison to the SFD (untreated and pretreated), the daily

biogas yield of the fungal biomass was significantly ($p<0.05$) higher (Figure 40). The highest peak of daily biogas yields was achieved during the initial stages of the AD (3-7 days), confirming that the fungal biomass is easily digested by the microorganisms involved in AD. In detail, the autoclaved fungal biomass achieved maximum daily biogas yields of 42.2 ± 1.6 L_N/kg VS d, while the biomass alive reached a maximum of 28.2 ± 0.8 L_N/kg VS d (Figure 40). The profile of daily methane concentration showed a rapid increase and the maximum peak (about 61-62 %) was reached at 10 days (Figure 40). Significant ($p<0.05$) higher cumulative biogas and methane yield were achieved in the reactors containing the autoclaved fungal biomass (600 ± 21 LN/kg VS and 321 ± 4 LN/kg VS, respectively) compared with the reactors containing the fungus alive (494 ± 33 LN/kg VS and 260 ± 20 LN/kg VS, respectively) (Figure 41). Thus, also in this case, the thermal pretreatment significantly ($p<0.05$) enhanced the AD yields, probably because it makes more accessible the intracellular fungal components. On the other hand, the alive and active fungus may have produced specific bioactive components that could partially inhibit or slower the anaerobic fermentation (Jasko et al., 2012).

The methane yields obtained with the fungal biomass were in the range of those obtained by Hom-Diaz et al. (2016) (from 281 to 595 L/kg VS) and Jasko et al. (2012) (from 137.9 to 607.3 L/kg VS). However, because of the lack of other studies on the BMP of fungal biomass, the results can only be compared with work that used other organic substrates, such as food wastes and algae. For instance, Elbeshbishy et al. (2012) obtained comparable methane yields, ranging from 440 to 790 L/kg VS, when food waste was anaerobically degraded by an inoculum obtained from a digester treating source separated organics. Dinuccio et al. (2010) evaluated the biogas potential of different Italian agro-industrial biomasses and obtained that the most productive biomasses were the whey and the maize drying up residues, which reached a specific methane yields of 501 and 317 L_N/kg VS, respectively. Instead, barley and rice straw gave a lower specific methane yield (229 and 195 L_N/kg VS, respectively). Noteworthy, lower AD yields were obtained

also when algae have been used as AD feedstock in BMP tests. For instance, Costa et al. (2012) used macroalgae *Ulva* spp. and *Gracilaria* spp. in co-digestion with waste activated sludge and they observed methane yields lower than 200 L_N/kg VS. Yen and Brune (2007) also obtained low yields (150 L/kg VS) through the co-digestion of microalgal sludge (with mainly *Scenedesmus* spp. and *Chlorella* spp.) and wastepaper. The absence of cellulose in the cell walls of fungi might leads to higher methane yields than using algae as substrate, although also the fungi contain recalcitrant macromolecules, as the chitin (glucosamine polymer) (Hom-Diaz et al., 2016). Nevertheless, according to the results of this study, the fungal biomass is confirmed to be an even better AD feedstocks than some lignocellulosic ones.

Currently there is a lack of information about the fate and possible contribution of fungi along the entire AD process and their possible use as feedstocks. Ultimately, this study partially fills this knowledge gap, confirming the possible valorisation of the fungal biomass through the AD. In fact, the fungal biomass contains lipids, proteins, and other macro- and micro-molecules, that can be profitably used as a source of biogas and methane. This is confirmed also by the fact that the yields obtained with fungal biomass were higher than those obtained from algae and within the range of values achieved using organic wastes or commonly used AD feedstocks.

5.4 Conclusions

The fungal pretreatment with *C. stemonitis* appears to be detrimental on the nutrient rich and easily biodegradable MS. Further studies are needed to optimize the experimental conditions in order to enhance the biogas and methane production from this substrate. The exploitation of other selected fungi (e.g., *Aspergillus* and *Trichoderma* spp.) may offer an alternative solution, as well as the use of crude

enzyme cocktails, which can avoid the sugar loss that typically occurs with whole cell processes.

On the contrary, the co-digestion and the fungal pretreatments with *C. stemonitis, C. cinerea and C. aegerita* demonstrated to be a cost-effective and environmental-friendly tool to increase AD performances with the recalcitrant SFD. This finding primarily opens an alternative valorisation scenario of this by-product and favor the development of next-generation by-product management strategy, that could address some environmental issues that often mine the disposal of AD by-products. Moreover, the integration of fungal pre-treatment on SFD and its subsequent reuse into the anaerobic digester has also the potential to allow concurrent gaseous loss abatement and better energy recovery, leading to environmental and economic benefits that make even more attractive and effective the overall sustainability of AD technology. According to the literature (Balsari et al., 2010), the reuse of SFD in the anaerobic digester has the potential to improve the total methane production of the AD plant by between 4% and 8%. The results of the BMP tests suggest that under the specific conditions of ABP-3 plant, the fungal pretreatment of SFD could improve the daily methane production of the AD by at least 192.3 m^3_N/day. Considering the average hourly methane consumption (approximately 240 m^3_N/h) of the combined heat and power system of ABP-3 plant, the latter value allows the production of approximately 0.80 additional MWel per day (approximately 290 MWel per year). This extra electrical production could correspond to a significant economical income to ABP owners. Considering the low inputs required for pretreatment (e.g., use of diluted culture media for inoculum production, cultivation in non-sterile condition), the economical income could allow the coverage of the process costs. However, further studies are required to evaluate the actual techno-economic feasibility of the process in full-scale application.

6. Screening and evaluation of phenols and furans degrading fungi for the biological pretreatment of lignocellulosic biomass

6.1 Introduction

Lignocellulosic biomasses, such as agricultural and forest residues, represent abundant, renewable, and low-cost resources to produce biofuels, chemicals, and polymers (Baruah et al., 2018). Lignocellulose is made up of cellulose, hemicellulose, and lignin fractions, linked by strong hydrogen and covalent bonds, that provide the structure its peculiar resistance to chemical and biological breakdown (Sun et al., 2016). The recalcitrant nature of lignocellulosic biomass is the major issue for its exploitation in biotechnological processes (Baruah et al., 2018). To overcome this drawback and obtain a valuable utilization of lignocellulose-rich biomasses, pretreatment processes can be applied (Sun et al., 2016). Physical and chemical processes are the most commonly employed methods to improve lignocellulosic bioconversion. Unfortunately, they often involve the use of high pressure and temperature, strong acids, and bases, etc. (Chen and Liu, 2015), with the consequent generation of undesired by-products, including furan derivatives (e.g., furfural and 5-hydromethylfurfural), organic acids (e.g., acetic acid, formic acid, and levulinic acid), and phenolic compounds (e.g., vanillin, syringaldehyde, 4-hydroxybenzaldehyde, coniferyl aldehyde, ferulic acid, and cinnamic acid) (Kumar et al., 2019; Palmqvist and Hahn-Hägerdal, 2000). Plant biomasses also have a plethora of phenolic compounds. The chemical structure and abundance of these secondary metabolites vary among species (Kumar et al., 2020). These compounds have been reported to inhibit various bioprocesses, such as the production of methane, ethanol, biohydrogen, xylitol, butanol, and lipids

(Monlau et al., 2014). Their removal could enhance the performance of several biotechnological processes, such as the anaerobic digestion (AD) and fermentation processes (Baruah et al., 2018). Different physical and chemical methods that use membranes, ion-exchange resins, neutralization, liquid–liquid extraction, have been developed for their removal or at least the abatement of the undesired effects (Kumar et al., 2020). Biological approaches could also be used for lignocellulose detoxification thanks to the activity of microorganisms (either single or in co-culture) or enzymes (pure or cocktails) (Zabed et al., 2019). Transformation of inhibitors into less toxic compounds using biological methods provides several advantages over the physical and chemical methods, such as low costs, mild operative conditions, high degradation efficiency, and low water and energy consumption (He et al., 2016; Jönsson and Martín, 2016). Several microorganisms have already been assessed to degrade the lignocelluloses-derived inhibitors (Ran et al., 2014). Most of the research on biodegradation of phenolic compounds has focused on bacteria, especially *Pseudomonas* genus, and fungi, i.e., Basidiomycota (Al-Khalid and El-Naas, 2012). White-rot fungi (WRF) possess a nonspecific lignocellulolytic enzyme system that allows them to oxidize a wide range of substrates, including lignin, phenols, and other aromatic compounds (Elisashvili et al., 2018; Martínková et al., 2016). Fungi belonging to other taxonomic groups have been scarcely investigated, even though Ascomycota such as *Aureobasidium*, *Candida, Penicillium, Aspergillus, Fusarium,* and *Graphium* are capable of mineralizing aromatic compounds (Al-Khalid and El-Naas, 2012; dos Santos et al., 2009). Likewise, the biodegradation of furans has been extensively studied in bacteria such as *Cupriavidus basilensis* and *Pseudomonas putida,* which have been characterized at physiological and genetic levels (Guarnieri et al., 2017; Koopman et al., 2010). Furans degradation has also been observed in fungi, including *Amorphotheca resinae* (He et al., 2016; Jönsson and Martín, 2016; Ran et al., 2014; Wang et al., 2015; Yi et al., 2019), *Coniochaeta ligniaria* (Cao et al., 2015; López et al., 2004; Nichols et al., 2008), *A. niger* (Rumbold et al., 2009), *T. reesei* (He et

al., 2020), *A. nidulans* (Yu et al., 2011), *Paecilomyces* sp. (Nakasaki et al., 2015), *Chaetomium globosum, Cunninghamella elegans, Mucor plumbeus, Mortierella isabellina* (Ruan et al., 2015; Zheng et al., 2012), *P. ostreatus* (Feldman et al., 2015), and *T. versicolor* (Kudahettige Nilsson et al., 2016). Even though many fungal species can degrade furan and phenolic compounds, for biotechnological applications it would be advantageous to find a single organism harbouring the whole variety of enzymes needed for the total elimination of these inhibitors. It is necessary to screen a wide number of fungi to enhance the probability of finding those few strains with outstanding performances against lignocelluloses-derived inhibitors. For this reason, this study aims to investigate the potential of fungi to detoxify phenolic aldehydes and furfural, as single molecules and a combinations of them. A miniaturized screening was developed to evaluate the capability of 40 fungal strains to grow in the presence of inhibitors such as furfural (Fur), vanillin (Van), syringaldehyde (Syr), and 4-hydroxybenzaldehyde (Hba). Based on the growth performance, *Byssochlamys nivea* MUT 6321 was selected to analyse its degradation ability against these toxic compounds.

6.2 Materials and Methods

6.2.1 Chemicals

Furfural (Fur), vanillin (Van), syringaldehyde (Syr), 4-hydroxybenzaldehyde (Hba), gallic acid, and guaiacol were 98-99 % pure and purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Stock solutions were prepared in ethanol. Ultrapure water, ethanol, methanol, and acetic acid were High-Performance Liquid Chromatography (HPLC) grade (Biopack, Argentina). HPLC solvents were filtered through 0.45 μm nylon membrane and degassed in an ultrasonic bath before use.

6.2.2 Fungal strains

Forty fungal strains from the culture collection of *MUT* (University of Turin, Italy) were selected to be studied in the high-throughput microplate screening. They included 26 Ascomycota, 11 Basidiomycota, and 3 Mucoromycota, and the selection was based on different criteria (e.g., the taxonomy and phylogenetic relationships, the adaptability to different and adverse environmental conditions, the substrate of isolation, and the potential of producing lignocellulolytic enzymes).

6.2.3 High-throughput microplate screening

Fungi were pre-grown on Petri dishes (9 cm diameter) with MEA and incubated at 25 °C for 7-14 days. For Ascomycota and Mucoromycota, a conidia suspension was prepared. For Basidiomycota, mycelia homogenate was prepared using a sterile mixer (Spina et al., 2018). The fungi were suspended in a mineral medium (2 g/L $NaNO_3$, 1 g/L NH_4Cl, 0.01 g/L $ZnSO_4.7H_2O$, 0.005 g/L $CuSO_4.5H_2O$, 0.5 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$, and 0.01 g/L agar). A turbidimeter (Biolog Inc., Hayward, USA) was used to standardize the concentration of the inoculum: the optical density was set at 60-70 % and 40-60 % transmittance for conidia suspension and mycelium homogenate, respectively.

Fungi were screened in 96-wells microplates (Sarstedt, Nümbrecht, Germany) to evaluate their tolerance towards Fur, Van, Syr, Hba, and a mix of the four molecules (MPF mix). Single-molecule trials were run at different concentrations, namely 1 and 2 g/L for Fur, 0.5 and 2 g/L for Van, and 0.25 and 2 g/L for Hba and Syr. MPF included 1 g/L Fur, 0.5 g/L Van, 0.25 g/L Hba, and 0.25 g/L Syr. Target molecules were used as the sole carbon source or in combination with 2 g/L glucose as co-substrate. Two biotic controls were performed to evaluate the growth capacity of each isolate in the presence of glucose (0.25, 0.5, 1, 2 g/L) or without the addition of an external carbon source. Abiotic control was carried

out as blank using the mineral medium supplemented with the molecules. Microplates were stored in sealant boxes and incubated at 25 °C in the dark for 3 weeks. Every 2 days, the mycelial growth was spectrophotometrically measured at 750 nm (Infinite M2000 with Magellan V 6.5 software, TECAN).

6.2.4 Degradation assays of inhibitor compounds

B. nivea MUT 6321 (GenBank Accession Number: MT151630) conidia suspension was prepared and adjusted to a concentration of 1×10^6 spores/mL. The suspension was used to inoculate 150 mL flasks containing 60 mL sterile mineral medium supplemented with 2 g/L glucose, chloramphenicol, and gentamicin to a final concentration of 1 µg/mL. Flasks were incubated at 25 °C in agitation at 120 rpm. After 4 days, the molecules were added as i) single compounds (1 g/L Fur; 0.5 g/L Van; 0.25 g/L Syr; 0.25 g/L Hba), ii) MPF mix, iii) MP mix: MPF mix without the addition of 1 g/L Fur. Each treatment was performed in triplicate. An aliquot of each culture broth and abiotic control were harvested every day and centrifuged at 10.000 x g, 10 min, 4 °C. Supernatants and standard calibration solutions of Fur, Van, Hba, and Syr were filtered through 0.22 µm nylon membrane and used for HPLC determinations. Glucose concentration was measured in supernatants using an enzymatic assay kit (Wiener lab., Argentina). After 23 days, mycelia were filtered and lyophilized to determine the dry weight.

HPLC analyses on cell-free supernatants were performed according to Canas et al. (2011), with modifications. A Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Massachusetts, USA) and the software Chromeleon Chromatography Data System (Thermo Fisher Scientific, Massachusetts, USA) were used for the acquisition and management of data. The ProntoSIL Spheribond ODS C18 5 µm column (150 x 4.6 mm) (Bischoff Chromatography, Leonberg, Germany) was used as the stationary phase. The following chromatographic conditions were selected:

column temperature of 25 °C, a flow rate of 1 mL/min, and an injection volume of 20 μL. Concerning the elution program, a binary gradient was selected, using solvent A water/acetic acid (98:2 v/v) and solvent B methanol/water/acetic acid (70:28:2 v/v/v). Separations of single molecules were carried out isocratically at 50 % B. For MP and MPF mixes, the following elution program was used: 0 % B isocratic in 3 min, linear gradient from 0 to 40 % B in 22 min, from 40 to 60 % B in 18 min, 60 % B isocratic in 12 min, linear gradient from 60 to 80 % B in 5 min, 80 % B isocratic in 5 min. Simultaneous detection was done at wavelengths of 232 nm (for furoic acid and furfuryl alcohol), 278 nm (for Fur, Van, and Hba), and 309 nm (for Syr). The identification of compounds was made by comparing the retention time and UV-Vis spectra of the peaks with those obtained by injection of standards solutions. Quantification was carried out at their maximum absorption by referring to a calibration curve in the range of 0-2 g/L of the standards.

6.2.5 Data and statistical analyses

Four-way Venn diagram analyses were used to display all the 15 possible overlaps among datasets corresponding to the growth percentage of fungi in the presence of inhibitors compared to the respective controls where glucose was used as the sole carbon source (Oliveros, 2007-2015).

Experimental data were subjected to analysis of variance (ANOVA) and Dunnett´s or Tukey's post hoc test to detect significant differences. A p-value of less than 0.05 was considered statistically significant. All experiments were done at least in triplicate.

6.3 Results and Discussion

6.3.1 High-throughput microplate screening

The concentration and the composition of lignocellulose-derivative compounds formed during physicochemical pretreatments usually differ according to the lignocellulosic biomass used and the applied treatment. For instance, He et al. (2016) reported that the corn stover hydrolysate contained 0.75 g/L Fur, 0.57 g/L Van, 0.25 g/L Syr, and 0.06 g/L Hba. Srilekha Yadav et al. (2011) showed that in the rice straw hydrolysate, Fur concentration was 0.21 g/L and the total phenolic concentration reached up to 1.58 g/L. It should be noted that in most of the literature studies, the inhibitory effects of phenolic and furanic compounds have been assessed at higher concentrations than those actually present in the lignocellulosic hydrolysates (e.g., 5 g/L) (Palmqvist and Hahn-Hägerdal, 2000). On the contrary, the molecules concentrations used in this study were comparable to those found in physicochemical pretreated corn stover and rice straw (He et al., 2016; Srilekha Yadav et al., 2011). In detail, forty strains from the *MUT* collection were analysed for their potential to grow on Fur (derived from dehydration of pentoses) and the phenolic aldehydes Hba, Van, and Syr (lignin derivatives of p-hydroxyphenyl, guaiacyl, and syringyl groups) (Palmqvist and Hahn-Hägerdal, 2000). Two different concentrations of Fur, Van, Syr, or Hba were assayed, as single molecules or mixture, and in the presence or not of glucose as co-substrate. Figure 42 shows a general overview of the number of species-strains and the respective percentage of growth inhibition (I %) after 21 days of growth in the presence of single molecules and the MPF mix, in comparison with the respective control growing with glucose.

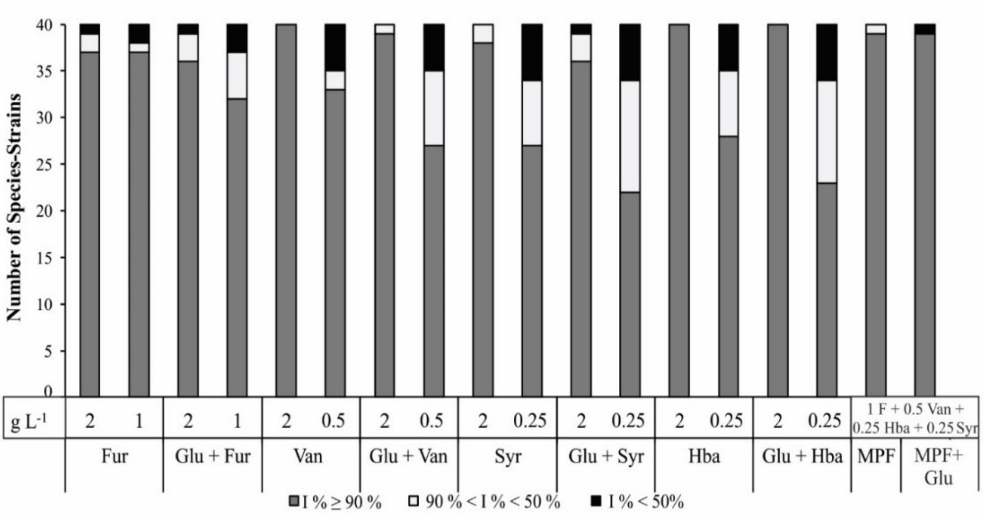
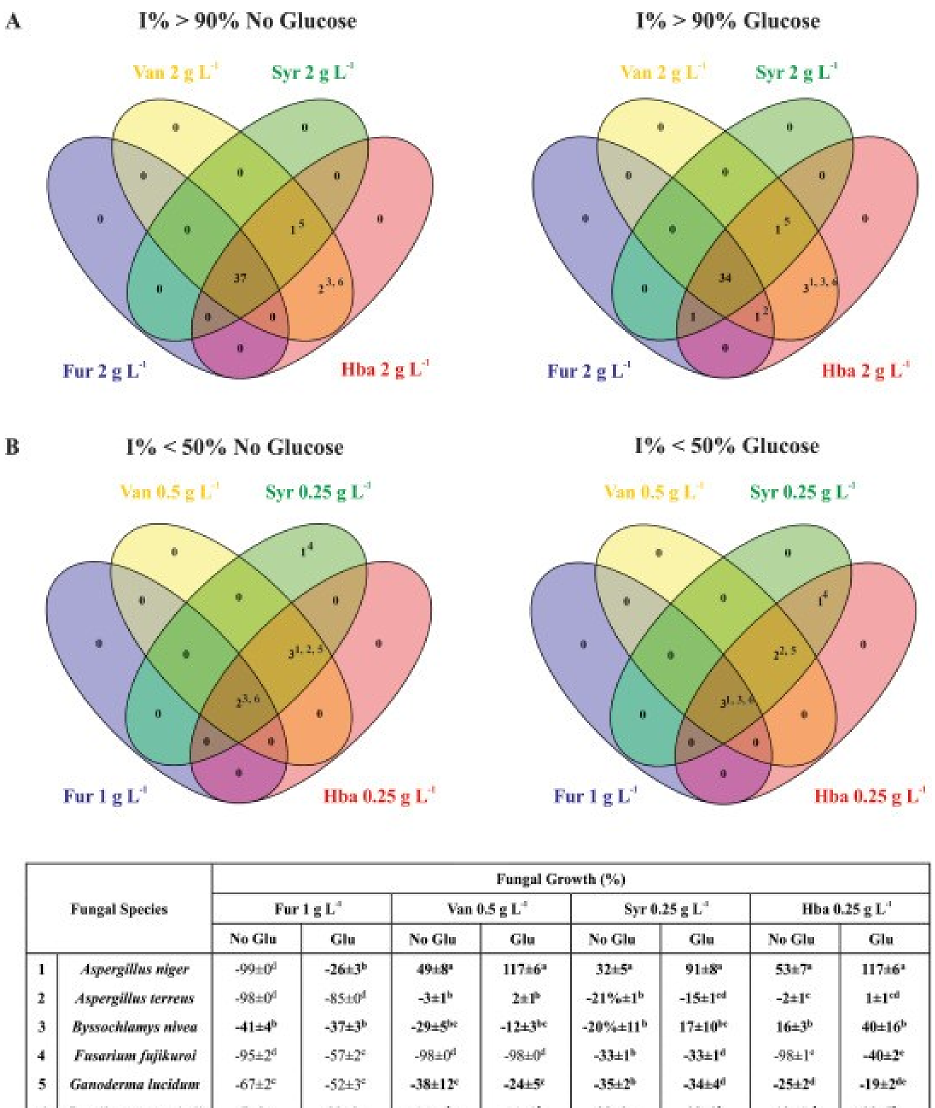


Figure 42: Number of species-strains and respective percentage of growth inhibition (I %) after 21 days of growth in the presence of Fur, Van, Hba, or Syr, and the mix of the four molecules (MPF), in comparison with the respective control growing with glucose only. The molecules tested as inhibitors were used at the different concentrations shown in the table and in the presence or not of 2 g/L glucose (Glu) as co-substrate.

Four-way Venn diagrams were used to perform comparison analyses among datasets of those fungi that displayed a growth inhibition higher than 90 % in the presence of 2 g/L of the inhibitors (Figure 43A). Ellipsis's intersections show those fungi that were inhibited by four compounds (one overlapping area composed of four ellipses), three compounds (four overlapping areas composed of three ellipses), two compounds (six overlapping areas composed of two ellipses), and those that were inhibited by one compound (the four areas where ellipses are not intersected). In the absence of glucose, 37 strains were strongly inhibited (above 90 %) in all tested conditions, while in the presence of glucose, 34 strains showed this behavior (Figure 42 and 43A). *B. nivea*, *Paecilomyces variotii*, and *A. niger* growth was inhibited above 90 % in the presence of Van or Hba (Figure 42 and 43A). *B.*

nivea growth was almost halved by Fur or Syr, regardless of the presence of glucose. *P. variotii* had the best performance on Fur, showing inhibition values of 1 and 18 %, with or without glucose, respectively. As regards Syr, *P. variotii* had an inhibition on biomass production of 42 and 83 % in the presence or absence of glucose, respectively. *A. niger* had a growth inhibition of 62 % by Fur and 73 % by Syr when glucose was also present in the media. *G. lucidum* showed an inhibition higher than 90 % when grown in the presence of the three phenolic aldehydes as single molecules but showed a better growth performance in the presence of Fur (inhibition around 75 %), regardless the presence of glucose (Figure 43A). In the presence of glucose, the inhibition was almost complete (above 90 %) for *A. terreus* with Fur, Hba, or Van (Figure 43A).

Growth performance of fungal strains was also analysed at lower concentrations, namely 1 g/L Fur, 0.5 g/L Van, 0.25 g/L Hba, and 0.25 g/L Syr. Figure 43B shows the fungi that displayed a growth inhibition lower than 50 % in the absence or presence of glucose. *B. nivea* and *P. varioti* were poorly affected by the four compounds, regardless of the presence of glucose as a co-substrate. Moreover, *P. varioti* was the least affected fungus by Fur, followed by *B. nivea* (Figure 43B, Tukey's test $p<0.05$). *A. niger*, *A. terreus*, and *G. lucidum* were inhibited less than 50 % in the presence of the phenolic aldehydes, with or without glucose. Furthermore, *A. niger* always showed the best growth performance in the presence of Van, Hba, or Syr (Figure 43B, Tukey´s test $p<0.05$). As regards Fur, growth inhibition of 26 % was observed in *A. niger* in the presence of glucose whereas this inhibitor deeply affected *A. terreus* and *G. lucidum* growth (Figure 43B). *F. fujikuroi* showed the worst growth performance in most of the conditions, with the only exception of Syr (Figure 43B, Tukey´s test $p<0.05$).



	Fungal Species	Fungal Growth (%)							
		Fur 1 g L^{-1}		Van 0.5 g L^{-1}		Syr 0.25 g L^{-1}		Hba 0.25 g L^{-1}	
		No Glu	Glu	No Glu	Glu	No Glu	Glu	No Glu	Glu
1	*Aspergillus niger*	-99±0^{d}	**-26±3^{b}**	**49±8^{a}**	**117±6^{a}**	**32±5^{a}**	**91±8^{a}**	**53±7^{a}**	**117±6^{a}**
2	*Aspergillus terreus*	-98±0^{d}	-85±0^{d}	**-3±1^{b}**	**2±1^{b}**	**-21%±1^{b}**	**-15±1^{cd}**	**-2±1^{c}**	**1±1^{cd}**
3	*Byssochlamys nivea*	**-41±4^{b}**	**-37±3^{b}**	**-29±5^{bc}**	**-12±3^{bc}**	**-20%±11^{b}**	**17±10^{bc}**	**16±3^{b}**	**40±16^{b}**
4	*Fusarium fujikuroi*	-95±2^{d}	-57±2^{c}	-98±0^{d}	-98±0^{d}	**-33±1^{b}**	**-33±1^{d}**	-98±1^{e}	**-40±2^{e}**
5	*Ganoderma lucidum*	-67±2^{c}	-52±3^{c}	**-38±12^{c}**	**-24±5^{c}**	**-35±2^{b}**	**-34±4^{d}**	**-25±2^{d}**	**-19±2de**
6	*Paecilomyces variotii*	**7±2^{a}**	**23±2^{a}**	**-21±4^{bc}**	**11±2^{b}**	**28±2^{a}**	**28±2^{b}**	**-19±4^{cd}**	**33±7^{bc}**

Figure 43: Four-way Venn diagrams showing the relationship among fungi with a growth inhibition (I %) higher than 90 % (A) or lower than 50 % (B) in the presence of Fur, Van, Hba, and Syr, as the sole carbon source or in the presence of 2 g/L glucose (Glu) as co-substrate. (A) Fur, Van, Hba, and Syr were used at 2 g/L (B) Inhibitors were tested at different concentrations: 1 g/L Fur, 0.5 g/L Van, 0.25 g/L Hba, and 0.25 g/L Syr. The table shows the average growth percentage ± SE (*n=6*) of fungi growing with the inhibitors at the lower concentrations (B) in comparison with their respective control. Negative values indicate inhibition, while values equal to or higher than 0 indicate growth comparable or higher than controls. In bold, fungi inhibited less than 50 %. Different lower-case letters (a, b, c, d, or e) show significant differences among fungi within each treatment (Tukey´s test, p<0.05). The numbers that appear as superscripts in the Venn diagrams correspond to the fungi that are numbered in the Table.

Figure 44 shows the growth curves of those fungi that had the best growth performance at the highest and the lowest inhibitor concentrations. At 2 g/L of each inhibitor, fungi showed a clear growth inhibition, which was reflected by longer lag phases and lower OD values than controls (Fig. 44A and C). In some cases (i.e., *A. terreus*, *A. niger*, and *P. variotii*), at the lower concentration, the growth curves reached a stationary phase as in the control (Figure 44B and C). In general, glucose did help fungi to tolerate the presence of the inhibitors: OD values were higher in the presence of glucose than in its absence (Figure 44B). *P. variotii* grew in the presence of Fur (2 and 1 g/L) as well as with glucose, also exhibiting a similar growth profile (Figure 44). On the other hand, the phenolic aldehydes deeply affected its growth: at 2 g/L, Van and Hba led to complete growth inhibition, whereas it grew similar to the controls in the presence of Syr and glucose (Figure 44A). The threshold inhibition limit is just below 2 g/L: the lowest concentrations tested did not have a significant impact on fungal growth. As regards *B. nivea*, Fur and Syr did not create a major impact since the fungus grew even though the start of the exponential phase was delayed compared to the control (Figure 44). Decreasing the concentration of Van and Hba, they end up being almost harmless, showing OD values similar to those obtained in the presence of glucose.

Since lignocellulosic hydrolysate generally contains multiple inhibitors, a mix of the four molecules at the lowest concentrations was tested (MPF mix). Most of the fungi (39 out of 40 strains) almost did not grow (inhibition above 90 %) (Figure 42). *B. nivea* was the only exception: the starting inhibition (78 %) was even halved in the presence of glucose (35 %) (Figure 42).

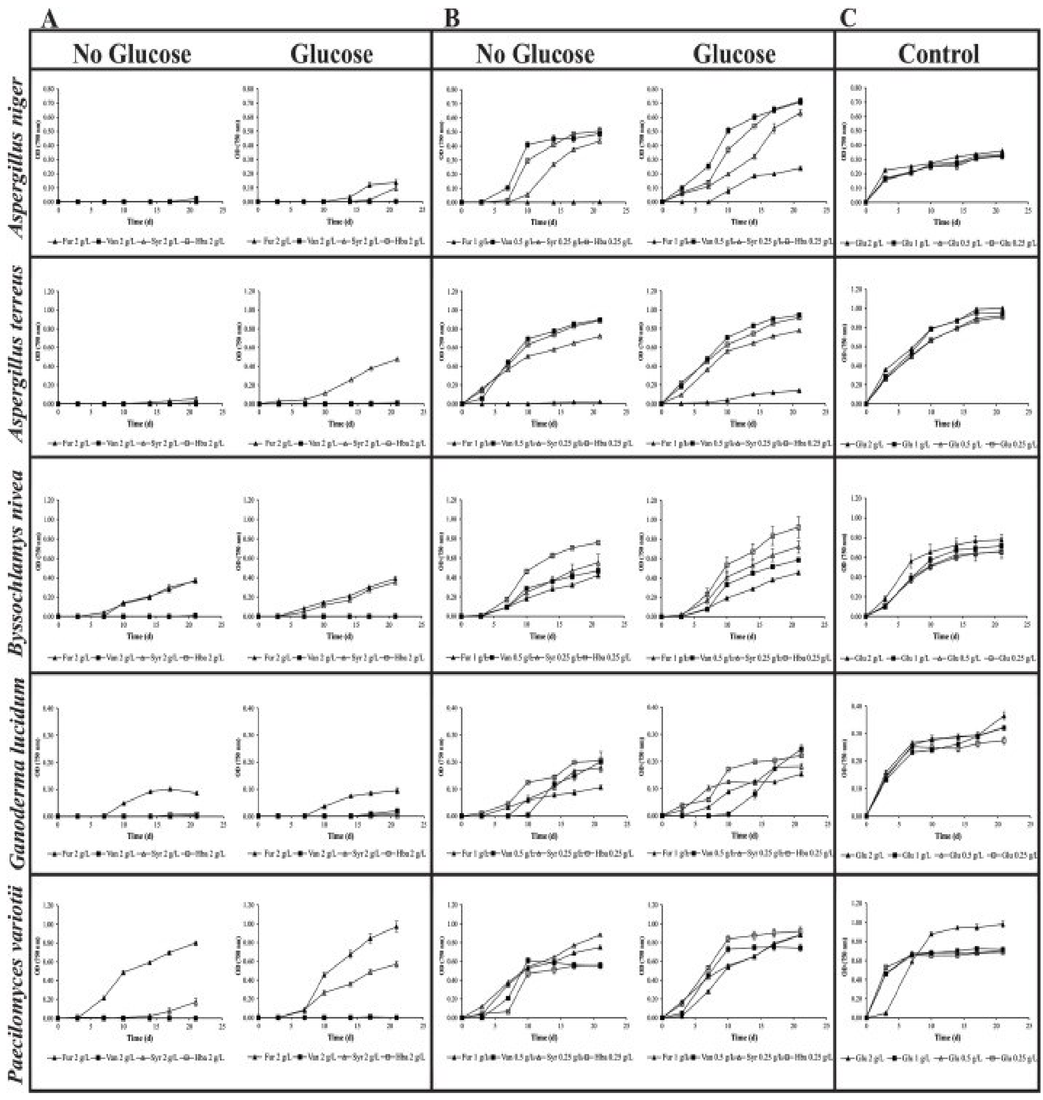


Figure 44: Growth curves of *A. niger*, *A. terreus*, *B. nivea*, *G. lucidum*, and *P. variotii* in the presence of 2 g/L of inhibitors (A) and at lower concentration (B), namely 1 g/L Fur, 0.5 g/L Van), 0.25 g/L Hba, and 0.25 g/L Syr. Each concentration was performed in the presence or not of 2 g/L glucose (Glu). Controls correspond to the five fungi grown with 0.25, 0.5, 1, and 2 g/L of glucose as the sole carbon source (C). The data represent the mean of the OD values measured at 750 nm at each time point. The error bar indicates the standard error (*n*=6).

The high-throughput screening performed with the 40 fungal strains confirmed the strong toxicity of phenolic aldehydes and furanic compounds, even at low concentrations (i.e., 0.25 g/L). Results showed that in the presence of single-molecule solutions, the growth inhibition depends not only on the nature and concentration of the inhibitors but also on the presence of glucose as co-substrate (Figure 42). *P. variotii*, *B. nivea, A. niger, A. terreus*, and *G. lucidum* had a high tolerance in most of the tested conditions, even when the inhibitors were used as the sole source of nourishment (Figure 43). The profile of the growth curves was different according to the fungal strain, but it was also dependent on the presence of glucose as co-substrate, the type and concentration of the inhibitors (Figure 44). In a fungal fermentation, many phenotypic, physiological, and molecular changes occur to adapt the cell to stressful conditions, having a direct influence on the lag phase length, the slope of the curve, or both (Hamill et al., 2020). As regards the strains tested in this study, no data are available about growth inhibition using the assayed molecules. Literature reports are scarce with just a few examples. For instance, *A. resinae* ZN_1 could grow with Fur as the sole carbon source and its degradation was significantly accelerated in the presence of glucose (Ran et al., 2014). *Candida tropicalis* was capable of using phenol as the sole carbon source up to a concentration of 2 g/L (Komarkova et al., 2003). On the other hand, many fungi can metabolize lignin-related phenolic compounds only in the presence of a co-substrate (Kirk and Farrell, 1987; Kowalczyk et al., 2019). According to the screening datasets, *P. variotii*, *B. nivea, A. niger, A. terreus*, and *G. lucidum* did grow more when glucose was present (Figure 43B, Figure 44). These results confirmed that glucose could promote fungal growth, favoring their survival or activating a co-metabolism suitable for the degradation.

Interestingly, some fungal species tested in this study were previously found capable of tolerating furans and phenols, but they were highly inhibited under the screening conditions used in the present work. For instance, the white-rot fungus *T. versicolor* CBS 109428 metabolized phenols and furans when used as single

molecules or in a mixture, and at a range of concentrations between 0.2-0.6 g/L (Kudahettige Nilsson et al., 2016). On the contrary, the *T. versicolor* strain used in this study was very sensitive to Fur, Syr, Hba, and Van (inhibition ranging from 70 to 90 %). Similarly, *M. isabellina* ATCC 42613 can tolerate 1 g/L Fur (Ruan et al., 2015) while the strain used in this study did not grow (97 % inhibition). These findings underline that the tolerance and adaptation skills are strain-specific features. Considering that fungi are less studied than bacteria (Al-Khalid and El-Naas, 2012), this study emphasizes the underexploited potential of fungal biodiversity for the development of improved bioprocesses.

6.3.2 Degrative ability of *Byssochlamys nivea*

Among the tested fungi, *B. nivea* showed the best growth performance in the presence of the inhibitors alone or mixed (Figure 42, 43, and 44). It was then selected to perform quantitative studies, using the inhibitory compounds as single molecules or mixes of them. Glucose was always added to the culture media to better mimic the real operative conditions. Indeed, alongside the formation of toxic compounds, during physicochemical lignocellulose biomass pretreatment, partial hydrolysis of cellulose into glucose usually takes place (Ran et al., 2014).

The ability of *B. nivea* to degrade Fur and the phenolic aldehydes was analysed by monitoring the residual concentration of each compound in the media (Figure 45). None of the compounds suffered abiotic degradation in negative controls without the fungus (Figure 46). Despite the high concentration tested (1 g/L), Fur was completely transformed within the first 24 h. As regards the phenolic aldehydes, 99 % of Hba, Syr, and Van were removed after 4, 9, and 11 days, respectively. Even though the concentrations of Syr and Hba were the same (0.25 g/L), the degradation of Syr was slower than Hba. Van was the most recalcitrant substrate since the transformation rate was slower than the other phenols and a residual 30 % of Van was still present after 7 days of fungal growth (Figure 45).

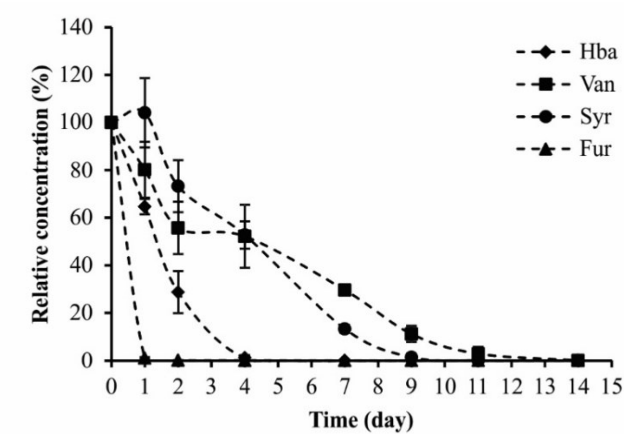


Figure 45: Time course profiles of fungal degradation of Fur, Van, Hba, and Syr. *B. nivea* was grown in the presence of the inhibitors as single molecules, at the following concentrations: 1 g/L Fur, 0.5 g/L Van, 0.25 g/L Hba, and 0.25 g/L Syr. The data represent the mean of the relative concentrations of the molecules at each time point in comparison to their initial concentrations. The error bar indicates the standard error (n=3).

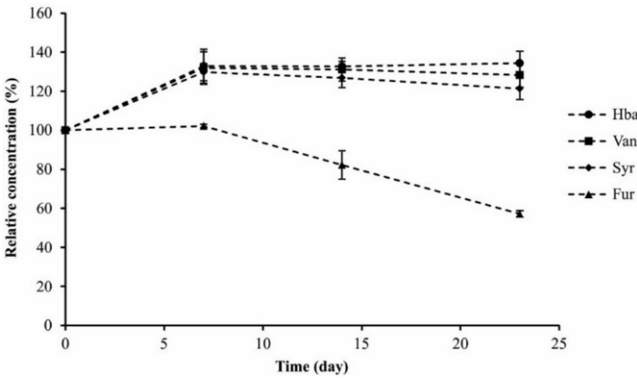


Figure 46: Time course profiles of abiotic degradation of Fur, Van, Hba, and Syr. This control was performed in parallel and under the same conditions as biotic degradation experiments, except that *B. nivea* was not present in the culture media. Inhibitors were added to the culture media as single molecules, at the following concentrations: 1 g/L Fur, 0.5 g/L Van, 0.25 g/L Hba, and 0.25 g/L Syr. The data represent the mean of the relative concentrations of the molecules at each time point in comparison to their initial concentrations. The error bar indicates the standard error (n=3).

To analyse the combined effect of the toxic molecules, two different mixes were tested. The three phenolic aldehydes were used as a mixture in the absence (MP) or the presence of Fur (MPF). Phenolic aldehydes were almost completely metabolized in the MP mix (Figure 47A). Hba and Van concentrations decreased faster than Syr, being these two molecules undetectable in the culture media after 16 days of treatment (Figure 47A). On the other hand, traces of Syr (2 %) were still present at the end of the experiment (Figure 47A). In the presence of MPF, at first, the fungus degraded Fur, with a complete removal within 4 days (Figure 47B). In comparison with MP, phenolic aldehydes degradation was slowed down: a consistent transformation was detectable only after 2 weeks. At the end of the experiment, the residual concentration of Van and Hba was approximately 20 %, but Syr was unaltered (Figure 47B).

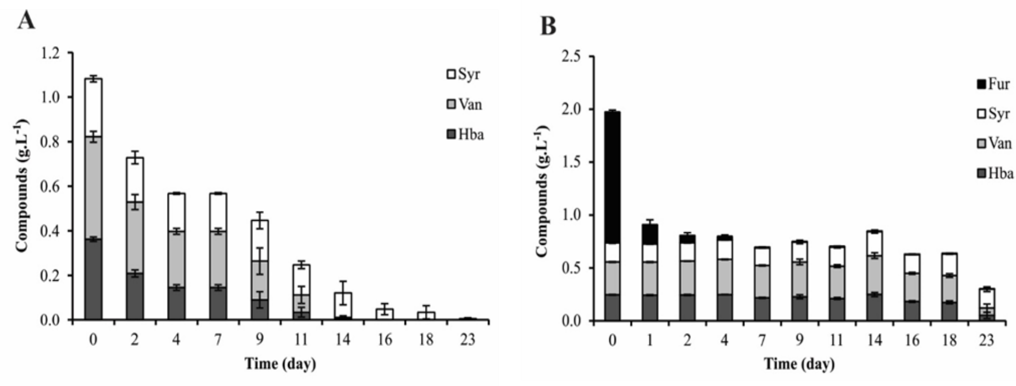


Figure 47: Time course profiles of fungal degradation of (A) MP mix: Van, Hba, and Syr and (B) MPF mix: MP with the addition of Fur. *B. nivea* was grown in the presence of the three phenolic compounds, with the addition or not of 1 g/L Fur, at the following concentrations: 0.5 g/L Van, 0.25 g/L Hba, and 0.25 g/L Syr. The data represent the mean of molecules concentrations at each time point. The error bar indicates the standard error (*n*=3).

In the bioconversion experiments performed with *B. nivea*, it was observed that when Fur was completely degraded, 87 % of the initial glucose was still present in the culture media (Table 19). By the time Hba, Syr, and Van were completely removed, the residual concentration of glucose was 35 %, 11 %, and 0.7 %, respectively (Table 19). At the end of MPF treatment, Fur, Van, and Hba were almost undetectable while 15 % of glucose was still present (Table 19). In the case of MP, almost complete degradation of inhibitors and glucose occurred after 23 days of treatment. Regarding the effect of inhibitors on *B. nivea* biomass, there were no statistical differences in mycelium dry weight among Fur, Van, Syr, and the control sample (Dunnett's test, $p>0.05$, Table 19). As expected, the biomass was significantly lower with MP and MPF samples than the control (Dunnett's test, $p<0.05$, Table 19).

Table 19: Effect of Fur, Van, Hba, and Syr, as single molecules or mixes MP and MPF, on glucose consumption and biomass production during *B. nivea* growth. Control corresponds to *B. nivea* grown under the same conditions, except that the inhibitors were not present in the culture media. Values are expressed as mean values ± SE (*n=3*). The asterisks indicate a significant difference between the control and treatment samples (Dunnett's test, $p<0.05$).

	Inhibitors' concentration (g/L)	**Degradation time (days)**	**Remaining Glucose (%)**	**Dry Fungal Biomass (mg)**
Fur	1	1	86.6 ± 7.4	130±13
Van	0.5	11	0.7 ± 0.2	104±7
Hba	0.25	4	35.2 ± 3.6	91±1*
Syr	0.25	9	11.0 ± 3.8	123±6
MP	1	23	0.3 ± 0.1	85±12*
MPF	2	>23	15.1 ± 6.9	37±4*
Control	0	-	-	139±6

*Significant difference with respect to control (Dunnett's test, $p<0.05$)

HPLC analyses showed the appearance of different peaks corresponding to Van, Hba, and Syr derivatives (Figure 48). Two peaks that were detected in Hba and Syr but not in Van samples showed increasing areas as the experiment proceeded. One of them had a retention time of 1.6 min and a UV-Vis spectrum with a maximum at 270 nm that coincided with the gallic acid standard. The other peak (retention time of 1.9 min) showed a UV-Vis spectrum similar to gallic acid with a maximum at 277 nm. A peak with a longer retention time (2.2 min) and a different spectrum (maximums 232 and 280 nm) was detected only in Van chromatograms and did not appear in Hba and Syr (Figure 48). This peak does not correspond to guaiacol standard (retention time 5.5 min; maximum 276 nm), which is known to be a possible derivative of Van (Lubbers et al., 2019). On the other hand, no Fur derivatives (e.g., furoic acid and furfuryl alcohol) were detected in HPLC chromatograms (data not shown).

In view of an industrial application, an efficient detoxification step of lignocellulosic material subjected to physicochemical pretreatments requires microorganisms that are capable of transforming inhibitors into less toxic compounds. The process sustainability lays the basis on a short processing time and minimal sugar requirements to fulfil the yields required for the synthesis of the target biotechnological product. Figure 45 shows that *B. nivea* was able to degrade 1 g/L Fur in 24 h, being even faster than the kerosene fungus *A. resinae* ZN_1 that completely degraded the same concentration of Fur only after 60-70 h (Ran et al., 2014). As regards the phenolic aldehydes assayed individually, the least biodegradable compound was Van, followed by Syr and Hba (Figure 45). Even though Hba and Syr were tested at the same concentration, some discrepancies were observed. Degradation of Hba was achieved before (4 days) and with less glucose consumption (35 % residual) than Syr degradation (9 days, 11 % residual glucose). Since *B. nivea* was able to consume more glucose in the presence of Syr, it also showed higher fungal biomass at the end of the experiment (Table 19).

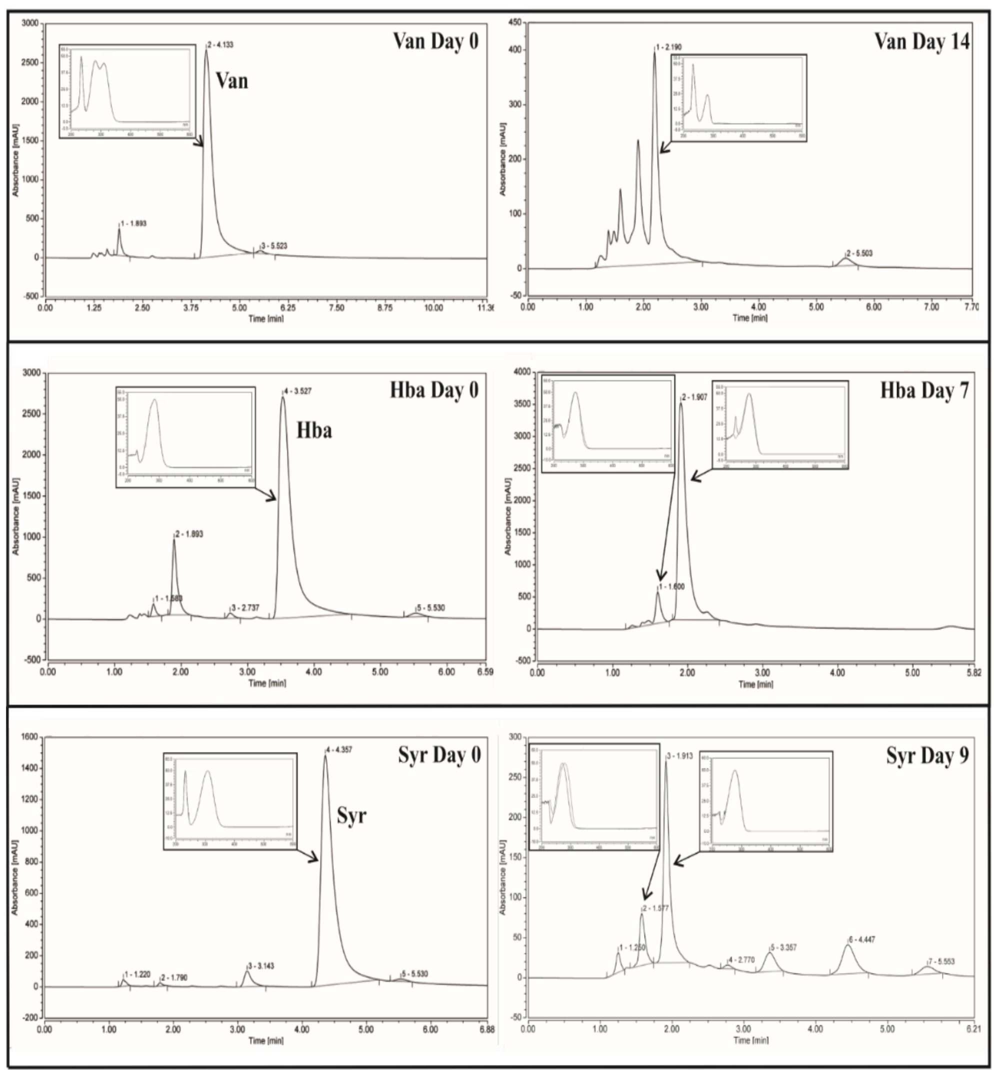


Figure 48: HPLC chromatograms showing the peaks profile detected at different time points in the supernatant of *B. nivea* cultures growing in the presence of the phenolic aldehydes as single molecules. UV-Vis spectra corresponding to the peaks marked with an arrow are shown in the insets. A wavelength of 278 nm was used to detect Van and Hba, and 309 nm was used to detect Syr.

B. nivea completely degraded Van, Hba, and Syr present in MP mix (Figure 47). However, when Fur was present in the mix, faster and preferential consumption of Fur instead of phenolic aldehydes was observed. In comparison to MP, in MPF, Van, Hba, and Syr showed a delayed transformation (Figure 47). This finding could be explained considering that Fur and phenolic aldehydes have different chemical structures, and their transformation may require the activation of different catabolic mechanisms, demanding time and resources to accomplish it (Yi et al., 2019). The peculiar preference of *B. nivea* to initiate Fur degradation first is very promising: indeed, phenolic compounds, such as Syr and Van, were reported to have a minor impact on biotechnological processes (e.g., biohydrogen production) (Monlau et al., 2014). Furthermore, using single molecule solutions or mixtures influenced the biodegradation in terms of time course profiles and maximal yields. This could be explained by the increase of the total inhibitors' concentration and a possible synergistic toxic effect (Monlau et al., 2014).

Transcriptional analysis performed by Yi et al. (2019) with *A. resinae* ZN_1 exposed to the toxic effects of Fur, Van, or Syr showed that these molecules inhibited the expression of genes encoding glucose transporter and some enzymes involved in sugar metabolism. This finding could explain the fact that these molecules were consumed before glucose (Yi et al., 2019). Likewise, as regards *B. nivea*, the toxic effect of Fur and phenolic aldehydes could have also affected the metabolization of glucose. Indeed, the glucose consumption was slower than in the control: at the end of the treatment, residual sugar content was still detected after the inhibitors were degraded (Table 19).

Even though information about furan metabolization in fungi is scarce, two degradation pathways have been proposed based on RNA-Seq data from *A. resinae* (Yi et al., 2019) and studies performed with the bacteria *C. basilensis* HMF14 (Koopman et al., 2010). Fur could be first reduced to the less toxic furfuryl alcohol and then, one or the other molecule may be oxidized to the intermediate furoic

acid. This molecule could be transformed after several enzymatic steps into 2-oxoglutarate, which is a key intermediate of the tricarboxylic acid (TCA) cycle. In the present study, furoic acid and furfuryl alcohol were not detected in HPLC analysis (data not shown). This could be due to the fast rate of Fur transformation that may have prevented these derivatives metabolites to be detected.

As regards phenolic compounds, literature data are sometimes contrasting since the studies on aromatic pathways were performed using different aromatic compounds and fungal species. Lignin-derived phenolic compounds could be transformed into seven aromatic intermediates: protocatechuic acid, catechol, hydroquinone, hydroxyquinol, gentisic acid, gallic acid, and pyrogallol. The ring of these molecules could be cleaved and converted after different enzymatic steps to pyruvate, fumarate, succinate, oxaloacetate, or acetyl-CoA, and finally channeled through the TCA cycle (Lubbers et al., 2019). For instance, *A. resinae* transformed Van, Hba, and Syr into the less toxic phenolic alcohols and then they were oxidized to phenolic acids before being metabolized through the TCA cycle. Based mainly on transcriptomic data, it was predicted that Syr could be converted to gallic acid before entering the TCA cycle, whereas Hba and Van may be converted to protocatechuic acid and then catabolized using similar enzymes (Yi et al., 2019). In the present study, results suggested that Hba and Syr were metabolized through the gallate pathway since gallic acid was formed during the degradation assays (Figure 48). Gallic acid could be transformed through different pathways (Lubbers et al., 2019). In *Aspergillus oryzae* and *P. chrysosporium*, gallic acid was transformed into progallin A, methyl gallate, and pyrogallic acid, and then enzymes such as laccase, lignin, and manganese peroxidases catalysed the ring-opening reaction. These three intermediates of gallic acid degradation were used as carbon and energy source through β-oxidation and TCA cycle pathways (Guo et al., 2014). On the other hand, the yeast *Arxula adeninivorans* was not able to completely metabolize gallic acid since the end-product was 2-hydroxymuconic acid (Sietmann et al., 2010). The aromatic ring of molecules, such as gallic acid, has

two characteristic peaks between 200-300 nm. Changes in the substituents of the benzene ring could cause a modification of the molecular polarity, and a displacement of the absorption maxima (Arizmendi-Cotero et al., 2017). As regards *B. nivea*, the presence of another peak in Hba and Syr chromatograms (retention time 1.9 min) could correspond to a molecule structurally related to gallic acid since both UV-Vis spectra were similar (Figure 48). It could be hypothesized that gallic acid and related compounds may be degraded using one of these pathways and/or accumulated in case the fungus does not harbour the enzymes to complete their metabolization. For detoxification purposes, it is worth noting that *B. nivea* was able to convert Hba and Syr into gallic acid, and most of the phenolic alcohols and acids were shown to be less toxic than the parent aldehydes because of their lower hydrophobicity (Guo et al., 2014; Hu et al., 2018). On the other hand, it was reported that filamentous fungi may catabolize Van via different pathways: i) non-oxidative decarboxylation to guaiacol, ii) oxidation of Van to protocatechuate which is followed by aromatic ring opening, and iii) oxidative decarboxylation to methoxy-p-hydroquinone (Lubbers et al., 2019). The non-oxidative degradation route of Van was described in a limited number of Ascomycota species such as *Sporotrichum thermophile* (syn *Myceliophthora thermophila)* (Topakas et al., 2003), *P. variotii* (Rahouti et al., 1989), some *Aspergillus* species and yeasts (Guiraud et al., 1992; Huang et al., 1993). Guaiacol did not appear in HPLC chromatograms of Van degradation by *B. nivea* (Figure 48). Besides, when the fungus was grown with Van, an increment in brown discoloration of the culture media was observed while the concentration of Van diminished (data not shown). Conversion of Van to methoxyhydroquinone by decarboxylating vanillate hydroxylase and further to hydroxyquinol was reported in several white-rot fungi (Buswell et al., 1981). These quinones could be the dark-colored metabolite present in those cultures where Van was degraded. For these reasons, it could be hypothesized that in *B. nivea* the catabolism of Van may proceed mainly through an oxidative pathway different from that used by Hba and Syr. Furthermore, *B.*

nivea and other fungi analysed in the present work used detoxification mechanism pathways that implied the aromatic ring cleavage since Van, Syr, and Hba were used as the sole carbon source (Figure 44). Future experiments will point out to unravel the enzymatic pathways that *B. nivea* uses to transform these phenolic compounds and their derivatives.

To the best of the authors' knowledge, this is the first report on the degradation of lignocellulose-derivative inhibitors by *B. nivea* strains. Members of the genus *Byssochlamys* (Family Trichocomaceae) are extremotolerant fungi, adaptable to adverse environmental conditions, widespread in soils, and with a high capability to survive across a broad range of pH and temperatures, and under low-oxygen conditions (Stamps et al., 2020). Because of these features, *Byssochlamys* spp. were described to be implicated in spoilage of thermally processed or pasteurized foods, as packaged and canned fruit products (i.e., juices) (Tournas, 1994). The ability to exploit these matrices could suggest that these fungi may be tolerant towards molecules derived from thermal pretreatment of plant material (i.e., fruit), such as furans, which are formed at high temperatures and pressure due to dehydration reactions of hexose and pentose sugars (Palmqvist and Hahn-Hägerdal, 2000). Recently, the genome of *Byssochlamys* isolate BYSS01 adapted to kerosene fuel was sequenced, and several genes involved in carbon and energy metabolism, transport of molecules, and degradation of alkanes and aromatic hydrocarbons were identified. The genome sequence reflects the ability of the BYSS01 isolate to grow in the presence of fuel and this information could help to understand the adaptive mechanisms employed by *Byssochlamys* to survive in the presence of these toxic compounds (Radwan et al., 2018). *B. nivea* MUT 6321 was isolated from digestate (SFD) obtained from maize-silage based biogas production (Table 9). Digestate is the by-product of the anaerobic digestion process, which occurs at relatively high temperatures (approx. 40 °C) and it may contain several compounds resulting from the hydrolysis of plant cell wall polymers. Panuccio et

al. (2016) reported that the liquid and solid fractions of digestate can contain a total phenol concentration of 395 ± 12 mg/L and 325 ± 9 mg/L, respectively. All these findings could further explain the adaptability and ability of the selected *B. nivea* strain to grow and degrade Fur and the phenolic aldehydes Van, Hba, and Syr.

6.4 Conclusions

The present study highlights the importance to explore fungal biodiversity to discover new promising strains that can degrade phenolic and furanic inhibitors most commonly found after physicochemical pretreatment of lignocellulosic biomasses. Among the 40 fungal strains analysed, *B. nivea* MUT 6321 was the only fungus capable of growing in the presence of Fur, Van, Hba, and Syr, as single molecules or with the four inhibitors simultaneously, and as the sole carbon source. Furthermore, it showed a high performance in degrading Fur and phenolic aldehydes, as single molecules or mixes. In conclusion, the ability of *B. nivea* to degrade Fur, Van, Syr, and Hba demonstrates its promise as a detoxification agent in lignocellulosic biomass conversion.

7. Conclusions and Future Perspectives

This research aimed to identify effective biological methods to increase the yields and sustainability of the AD process. In the past, most of the efforts and studies to optimize AD have been mainly focused on Archaea and Bacteria, while very few information's are available about the potential role of fungi along the entire biogas production chain. Based on the results of this study, it can be concluded that:

- The autochthonous mycobiota inhabiting common agricultural biogas plant feedstocks and by-products represent a potential resource for the development of several biotechnological applications, including the pretreatment of lignocellulosic feedstocks. In fact, several lignocellulolytic fungi, as well as other interesting ecophysiological groups of fungi (i.e., thermophilic), were isolated. This emphasizes the importance of investigating the natural fungal biodiversity.

- The qualitative screening resulted a simple but powerful strategy to select fungal strains which can find applications in the pretreatment of maize silage and solid digestate since they have demonstrated to adapt to and to grow on these biomasses as sole nutrient source, as well as to compete with the indigenous microorganisms under non-sterile conditions.

- The screening and fungal pretreatments were developed and optimized to facilitate the future scale-up and application of the process. In fact, the fungal inocula were produced in SmF, that, compared with SSF, has a higher efficiency in the production of fungal biomass. Instead, the fungal pretreatments were carried out under SSF, which resembles the natural environment where fungi actually proliferate and mimic the conditions present during biomass storage in industrial-scale AD plants, in which the

process could be concurrently applied. However, the major innovative aspect is the development of a pretreatment technology on unsterilized biomass, which represent a fundamental advantage for future industrial application since it avoids additional energy input for sterilization.

- The application of pretreatments with selected fungi significantly affected the features (pH, TS, VS, fibers) of lignocellulosic biomasses. Interestingly, the analyses on lignocellulolytic enzymatic yields were not predictive of substrates PCWP degradation rates, but higher lignin, cellulose and hemicellulose degradation seems correlated with higher AD performance.

- The pretreatment with *C. stemonitis* MUT 6326 appears to be detrimental on the nutrient rich and easily biodegradable MS. Further experiments are needed to optimize the process on this substrate with other fungal species or with crude enzyme cocktails.

- The pretreatment with *C. cinerea* MUT 6385, *C. aegerita* MUT 5639 and *C. stemonitis* MUT 6326 appeared to be an effective tool to increase the digestibility and the AD yields of the recalcitrant SFD, opening an alternative valorisation route for this by-product.

- The fungal species used for pretreatment turned out to be one of the main factors affecting the PCWP degradation and the biogas-methane production, while the pretreatment's duration seems to have less influence. The highest cumulative biogas and methane yields were reached with biomass treated with the Ascomycota *C. stemonitis* MUT 6326, suggesting that, other than white-rot basidiomycetes (so far, the organisms predominantly investigated for lignocellulose bioconversion), it might be worth extending the research other taxonomic groups of fungi.

- The increase in biogas and methane was ascribable both to the addition of fungal biomass, which acted as an organic feedstock, and to the lignocellulose transformation due to fungal activity during pretreatments. Comparable yields were indeed obtained through co-digestion of SFD and fungal biomass (T0) and with fungal-pretreated SFD (T10, T20).

- The fungal biomass, alone or in co-digestion systems, can be considered a profitable and alternative feedstock for AD. An interesting aspect to further deepen the study is the evaluation of biogas-methane yields using fungi belonging to different species. Then, the information obtained could be applied to produce an efficient AD process for the conversion of spent fungal biomass derived from industries into renewable energy, contributing also to the reduction of waste production and disposal issues.

- The fungus *B. nivea* MUT 6321 have shown the potential to indirectly increase the performances of AD and other bioprocess by removing toxic phenolic and furanic compounds (e.g., Van, Syr, Hba and Fur) commonly found after physicochemical pretreatment of lignocellulosic biomasses. Further studies could be focused on investigation of the pathways used by *B. nivea* for the transformation of target molecules, or to deepen the analyses on other fungi that have been shown tolerance towards these inhibitors (as *P. variotii, A. niger, A. terreus, G. lucidum*).

In conclusion, this project addresses an actual territorial need exploring aspects of modern and applied mycology, providing knowledge and useful information to the scientific community and the local industrial network.

8. References

8.1 Bibliography

Adav, S.S., Ravindran, A., Sze, S.K., 2013. Proteomic analysis of temperature dependent extracellular proteins from *Aspergillus fumigatus* grown under solid-state culture condition. J. Proteome Res. 12, 2715–2731. https://doi.org/10.1021/pr4000762

Akhtar, N., Gupta, K., Goyal, D., Goyal, A., 2016. Recent advances in pretreatment technologies for efficient hydrolysis of lignocellulosic biomass. Environ. Prog. Sustain. Energy 35, 489–511. https://doi.org/10.1002/ep.12257

Akin, D.E., Rigsby, L.L., Sethuraman, A., Morrison, W.H., Gamble, G.R., Eriksson, K.E.L., 1995. Alterations in structure, chemistry, and biodegradability of grass lignocellulose treated with the white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus*. Appl. Environ. Microbiol. 61, 1591–1598. https://doi.org/10.1128/aem.61.4.1591-1598.1995

Al-Khalid, T., El-Naas, M.H., 2012. Aerobic biodegradation of phenols: A comprehensive review. Crit. Rev. Environ. Sci. Technol. 42, 1631–1690. https://doi.org/10.1080/10643389.2011.569872

Alexandropoulou, M., Antonopoulou, G., Ntaikou, I., Lyberatos, G., 2017. Fungal pretreatment of willow sawdust with *Abortiporus biennis* for anaerobic digestion: Impact of an external nitrogen source. Sustain. 9. https://doi.org/10.3390/su9010130

Alonso, V.A., Pereyra, C.M., Keller, L.A.M., Dalcero, A.M., Rosa, C.A.R., Chiacchiera, S.M., Cavaglieri, L.R., 2013. Fungi and mycotoxins in silage: An overview. J. Appl. Microbiol. 115, 637–643. https://doi.org/10.1111/jam.12178

Amon, T., Amon, B., Kryvoruchko, V., Machmüller, A., Hopfner-Sixt, K., Bodiroza, V., Hrbek, R., Friedel, J., Pötsch, E., Wagentristl, H., Schreiner, M., Zollitsch, W., 2007. Methane production through anaerobic digestion of various energy crops grown in sustainable crop rotations. Bioresour. Technol. 98, 3204–3212. https://doi.org/10.1016/j.biortech.2006.07.007

Anastasi, A., Spina, F., Romagnolo, A., Tigini, V., Prigione, V., Varese, G.C., 2012. Integrated fungal biomass and activated sludge treatment for textile wastewaters bioremediation. Bioresour. Technol. 123, 106–111. https://doi.org/10.1016/j.biortech.2012.07.026

Anastasi, A., Tigini, V., Varese, G.C., 2013. The Bioremediation Potential of Different Ecophysiological Groups of Fungi. Springer, Berlin, Heidelberg, pp. 29–49. https://doi.org/10.1007/978-3-642-33811-3_2

Anastasi, A., Varese, G.C., Marchisio, V.F., 2005. Isolation and identification of fungal communities in compost and vermicompost. Mycologia 97, 33–44. https://doi.org/10.3852/mycologia.97.1.33

Andlar, M., Rezić, T., Marđetko, N., Kracher, D., Ludwig, R., Šantek, B., 2018. Lignocellulose degradation: An overview of fungi and fungal enzymes involved in lignocellulose degradation. Eng. Life Sci. https://doi.org/10.1002/elsc.201800039

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2018.01.011

AOAC, 2000. Official methods of analysis, association of analytical chemists. 15th ed., Washington D. C. Washingt. D. C. USA. https://doi.org/10.1007/978-3-642-31241-0

Aouidi, F., Khelifi, E., Asses, N., Ayed, L., Hamdi, M., 2010. Use of cheese whey to enhance *Geotrichum candidum* biomass production in olive mill wastewater. J. Ind. Microbiol. Biotechnol. 37, 877–882. https://doi.org/10.1007/s10295-010-0752-3

Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F., Lens, P.N.L., 2014. Pretreatment methods to enhance anaerobic digestion of organic solid waste. Appl. Energy. https://doi.org/10.1016/j.apenergy.2014.02.035

Arizmendi-Cotero, D., Villanueva-Carvajal, A., Gómez-Espinoza, R.M., Dublán-García, O., Dominguez-Lopez, A., 2017. Radical scavenging activity of an inulin-gallic acid graft and its prebiotic effect on *Lactobacillus acidophilus* in vitro growth. J. Funct. Foods 29, 135–142. https://doi.org/https://doi.org/10.1016/j.jff.2016.12.014

Asgher, M., Wahab, A., Bilal, M., Nasir Iqbal, H.M., 2016. Lignocellulose degradation and production of lignin modifying enzymes by *Schizophyllum commune* IBL-06 in solid-state fermentation. Biocatal. Agric. Biotechnol. 6, 195–201. https://doi.org/10.1016/j.bcab.2016.04.003

Auxenfans, T., Crônier, D., Chabbert, B., Paës, G., 2017. Understanding the structural and chemical changes of plant biomass following steam explosion pretreatment. Biotechnol. Biofuels 10, 1–16. https://doi.org/10.1186/s13068-017-0718-z

Azman, S., Milh, H., Somers, M.H., Zhang, H., Huybrechts, I., Meers, E., Meesschaert, B., Dewil, R., Appels, L., 2020. Ultrasound-assisted digestate treatment of manure digestate for increased biogas production in small pilot scale anaerobic digesters. Renew. Energy 152, 664–673. https://doi.org/10.1016/j.renene.2020.01.096

Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257–270. https://doi.org/10.1016/0168-1656(92)90074-J

Baldrian, P., 2008. Wood-inhabiting ligninolytic basidiomycetes in soils: Ecology and constraints for applicability in bioremediation. Fungal Ecol. https://doi.org/10.1016/j.funeco.2008.02.001

Baldrian, P., 2006. Fungal laccases-occurrence and properties. FEMS Microbiol. Rev. https://doi.org/10.1111/j.1574-4976.2005.00010.x

Baldrian, P., Valášková, V., Merhautová, V., Gabriel, J., 2005. Degradation of lignocellulose by *Pleurotus ostreatus* in the presence of copper, manganese, lead and zinc. Res. Microbiol. https://doi.org/10.1016/j.resmic.2005.03.007

Baldrian, P., Voříšková, J., Dobiášová, P., Merhautová, V., Lisá, L., Valášková, V., 2011. Production of extracellular enzymes and degradation of biopolymers by saprotrophic microfungi from the upper layers of forest soil. Plant Soil 338, 111–125. https://doi.org/10.1007/s11104-010-0324-3

Balsari, P., Gioelli, F., Menardo, S., Paschetta, E., 2010. The (re)use of mechanical separated solid fraction of digested or not digested slurry in anaerobic digestion plants. 14th Ramiran Int. Conf. 4630, 2–5.

Bansal, N., Tewari, R., Soni, R., Soni, S.K., 2012. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. Waste Manag. 32, 1341–1346. https://doi.org/10.1016/j.wasman.2012.03.006

Barelli, L., Bidini, G., de Arespacochaga, N., Pérez, L., Sisani, E., 2017. Biogas use in high temperature fuel cells: Enhancement of KOH-KI activated carbon performance toward H2S removal. Int. J. Hydrogen Energy 42, 10341–10353. https://doi.org/10.1016/j.ijhydene.2017.02.021

Bartoli, A., Cavicchioli, D., Kremmydas, D., Rozakis, S., Olper, A., 2016. The impact of different energy policy options on feedstock price and land demand for maize silage: The case of biogas in Lombardy. Energy Policy 96, 351–363. https://doi.org/10.1016/j.enpol.2016.06.018

Baruah, J., Nath, B.K., Sharma, R., Kumar, S., Deka, R.C., Baruah, D.C., Kalita, E., 2018. Recent Trends in the Pretreatment of Lignocellulosic Biomass for Value-Added Products. Front. Energy Res. 6, 141. https://doi.org/10.3389/fenrg.2018.00141

Bauer, A., Mayr, H., Hopfner-Sixt, K., Amon, T., 2009. Detailed monitoring of two biogas plants and mechanical solid-liquid separation of fermentation residues. J. Biotechnol. 142, 56–63. https://doi.org/10.1016/j.jbiotec.2009.01.016

Berrin, J.G., Navarro, D., Couturier, M., Olivé, C., Grisel, S., Haon, M., Taussac, S., Lechat, C., Courtecuisse, R., Favel, A., Coutinho, P.M., Lesage-Meessen, L., 2012. Exploring the natural fungal biodiversity of tropical and temperate forests toward improvement of biomass conversion. Appl. Environ. Microbiol. 78, 6483–6490. https://doi.org/10.1128/AEM.01651-12

Bhabhra, R., Askew, D.S., 2005. Thermotolerance and virulence of *Aspergillus fumigatus*: Role of the fungal nucleolus. Med. Mycol. https://doi.org/10.1080/13693780400029486

Bond, T., Templeton, M.R., 2011. History and future of domestic biogas plants in the developing world. Energy Sustain. Dev. 15, 347–354. https://doi.org/10.1016/j.esd.2011.09.003

Boni, M.R., D'Amato, E., Polettini, A., Pomi, R., Rossi, A., 2016. Effect of ultrasonication on anaerobic degradability of solid waste digestate. Waste Manag. 48, 209–217. https://doi.org/10.1016/j.wasman.2015.10.031

Bostedt, G., Mustonen, M., Gong, P., 2016. Increasing forest biomass supply in northern Europe – countrywide estimates and economic perspectives. Scand. J. For. Res. 31, 314–322. https://doi.org/10.1080/02827581.2015.1089930

Bouallagui, H., Touhami, Y., Ben Cheikh, R., Hamdi, M., 2005. Bioreactor performance in anaerobic digestion of fruit and vegetable wastes. Process Biochem. https://doi.org/10.1016/j.procbio.2004.03.007

Bovio, E., Garzoli, L., Poli, A., Prigione, V., Firsova, D., McCormack, G.P., Varese, G.C., 2018. The culturable mycobiota associated with three Atlantic sponges, including two new species: *Thelebolus balaustiformis* and *T. spongiae* . Fungal Syst. Evol. 1, 141–167. https://doi.org/10.3114/fuse.2018.01.07

Brémond, U., Bertrandias, A., Loisel, D., Jimenez, J., Steyer, J.P., Bernet, N., Carrere, H., 2020. 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. Renew. Energy. https://doi.org/10.1016/j.renene.2019.12.062

Bres, P., Beily, M.E., Young, B.J., Gasulla, J., Butti, M., Crespo, D., Candal, R., Komilis, D., 2018. Performance of semi-continuous anaerobic co-digestion of poultry manure with fruit and vegetable waste and analysis of digestate quality: A bench scale study. Waste Manag. 82, 276–284. https://doi.org/10.1016/j.wasman.2018.10.041

Bryant, M.P., 1979. Microbial Methane Production—Theoretical Aspects2. J. Anim. Sci. https://doi.org/10.2527/jas1979.481193x

Buswell, J.A., Eriksson, K.E., Pettersson, B., 1981. Purification and partial characterization of vanillate hydroxylase (decarboxylating) from Sporotrichum pulverulentum. J. Chromatogr. A. https://doi.org/10.1016/S0021-9673(00)81390-4

Camacho-Fernández, C., Hervás, D., Rivas-Sendra, A., Marín, M.P., Seguí-Simarro, J.M., 2018. Comparison of six different methods to calculate cell densities. Plant Methods. https://doi.org/10.1186/s13007-018-0297-4

Canas, S., Belchior, A.P., Spranger, M.I., Bruno-De-Sousa, R., 2011. HPLC method for the quantification of phenolic acids, phenolic aldehydes, coumarins and furanic derivatives in different kinds of toasted wood used for the ageing of brandies. Anal. Methods 3, 186–191. https://doi.org/10.1039/c0ay00269k

Cao, G., Ximenes, E., Nichols, N.N., Frazer, S.E., Kim, D., Cotta, M.A., Ladisch, M., 2015. Bioabatement with hemicellulase supplementation to reduce enzymatic hydrolysis inhibitors. Bioresour. Technol. 190, 412–415. https://doi.org/10.1016/j.biortech.2015.04.064

Carlsson, M., Lagerkvist, A., Morgan-Sagastume, F., 2012. The effects of substrate pre-treatment on anaerobic digestion systems: A review. Waste Manag. 32, 1634–1650. https://doi.org/10.1016/j.wasman.2012.04.016

Carrere, H., Antonopoulou, G., Affes, R., Passos, F., Battimelli, A., Lyberatos, G., Ferrer, I., 2016. Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale research to full-scale application. Bioresour. Technol. 199, 386–397. https://doi.org/10.1016/j.biortech.2015.09.007

Castelli, S., Sannazzaro, S. de, 2011. Biomasse per la produzione di energia. Produzione, gestione e processi di trasformazione. (Vol. 110). Maggioli Editore.

Castoldi, R., Bracht, A., de Morais, G.R., Baesso, M.L., Correa, R.C.G., Peralta, R.A., Moreira, R. de F.P.M., Polizeli, M. de L.T. de M., de Souza, C.G.M., Peralta, R.M., 2014. Biological pretreatment of *Eucalyptus grandis* sawdust with white-rot fungi: Study of degradation patterns and saccharification kinetics. Chem. Eng. J. 258, 240–246. https://doi.org/10.1016/j.cej.2014.07.090

Cesaro, A., Belgiorno, V., 2014. Pretreatment methods to improve anaerobic biodegradability of organic municipal solid waste fractions. Chem. Eng. J. https://doi.org/10.1016/j.cej.2013.11.055

Chang, A.J., Fan, J., Wen, X., 2012. Screening of fungi capable of highly selective degradation of lignin in rice straw. Int. Biodeterior. Biodegrad. 72, 26–30. https://doi.org/10.1016/j.ibiod.2012.04.013

Chen, H.-Z., Liu, Z.-H., 2015. Steam explosion and its combinatorial pretreatment refining technology of plant biomass to bio-based products. Biotechnol. J. 10, 866–885. https://doi.org/10.1002/biot.201400705

Chen, R., Blagodatskaya, E., Senbayram, M., Blagodatsky, S., Myachina, O., Dittert, K., Kuzyakov, Y., 2012. Decomposition of biogas residues in soil and their effects on microbial growth kinetics and enzyme activities. Biomass and Bioenergy. https://doi.org/10.1016/j.biombioe.2012.06.014

Chen, S., Zhang, X., Singh, D., Yu, H., Yang, X., 2010. Biological pretreatment of lignocellulosics: Potential, progress and challenges. Biofuels. https://doi.org/10.4155/bfs.09.13

Chen, Y., Sharma-Shivappa, R.R., Keshwani, D., Chen, C., 2007. Potential of agricultural residues and hay for bioethanol production. Appl. Biochem. Biotechnol. https://doi.org/10.1007/s12010-007-0026-3

Chinese, D., Patrizio, P., Nardin, G., 2014. Effects of changes in Italian bioenergy promotion schemes for agricultural biogas projects: Insights from a regional optimization model. Energy Policy 75, 189–205. https://doi.org/10.1016/j.enpol.2014.09.014

Chowdhury, B., Magsi, S.B., Joey Ting, H.N., Dhar, B.R., 2020. High-solids anaerobic digestion followed by ultrasonication of digestate and wet-type anaerobic digestion for enhancing methane yield from OFMSW. Processes 8, 555. https://doi.org/10.3390/PR8050555

Costa, J.C., Gonçalves, P.R., Nobre, A., Alves, M.M., 2012. Biomethanation potential of macroalgae *Ulva* spp. and *Gracilaria* spp. and in co-digestion with waste activated sludge. Bioresour. Technol. 114, 320–326. https://doi.org/10.1016/j.biortech.2012.03.011

Costa, S., Dedola, D.G., Pellizzari, S., Blo, R., Rugiero, I., Pedrini, P., Tamburini, E., 2017. Lignin biodegradation in pulp-and-paper mill wastewater by selected white rot fungi. Water (Switzerland) 9, 935. https://doi.org/10.3390/w9120935

Das, A., Ray, L., 2016. Production of crude xylanase using a isolated fungal strain *Aspergillus* sp.S6by solid state fermentation, in: Materials Today: Proceedings. Elsevier Ltd, pp. 3343–3360. https://doi.org/10.1016/j.matpr.2016.10.016

Dashtban, M., Schraft, H., & Qin, W. 2009. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int. J. Biol. Sci. 5, 578.

de Castro, R.J.S., Sato, H.H., 2015. Enzyme Production by Solid State Fermentation: General Aspects and an Analysis of the Physicochemical Characteristics of Substrates for Agro-industrial Wastes Valorization. Waste and Biomass Valorization. https://doi.org/10.1007/s12649-015-9396-x

De Marco, A., Proietti, C., Anav, A., Ciancarella, L., D'Elia, I., Fares, S., Fornasier, M.F., Fusaro, L., Gualtieri, M., Manes, F., Marchetto, A., Mircea, M., Paoletti, E., Piersanti, A., Rogora, M., Salvati, L., Salvatori, E., Screpanti, A., Vialetto, G., Vitale, M., Leonardi, C., 2019. Impacts of air pollution on human and ecosystem health, and implications for the National Emission Ceilings Directive: Insights from Italy. Environ. Int. 125, 320–333. https://doi.org/10.1016/j.envint.2019.01.064

Del Pilar Castillo, M., Andersson, A., Ander, P., Stenström, J., Torstensson, L., 2001. Establishment of the white rot fungus *Phanerochaete chrysosporium* on unsterile straw in solid substrate fermentation systems intended for degradation of pesticides. World J. Microbiol. Biotechnol. 17, 627–633. https://doi.org/10.1023/A:1012420422111

Deng, Y., Dai, B., Xu, Jiaxing, Liu, X., Xu, Jiming, 2018. Anaerobic co-digestion of rice straw and soybean straw to increase biogas production by pretreatment with *Trichoderma reesei* RUT C30. Environ. Prog. Sustain. Energy. https://doi.org/10.1002/ep.12782

Deshmukh, R., Khardenavis, A.A., Purohit, H.J., 2016. Diverse Metabolic Capacities of Fungi for Bioremediation. Indian J. Microbiol. https://doi.org/10.1007/s12088-016-0584-6

Dinuccio, E., Balsari, P., Gioelli, F., Menardo, S., 2010. Evaluation of the biogas productivity potential of some Italian agro-industrial biomasses. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2009.12.113

Dinuccio, E., Gioelli, F., Cuk, D., Rollè, L., Balsari, P., 2013. The use of co-digested solid fraction as feedstock for biogas plants. J. Agric. Eng. 44, 3–8. https://doi.org/10.4081/jae.2013.s2.e113

Dollhofer, V., Podmirseg, S.M., Callaghan, T.M., Griffith, G.W., Fliegerová, K., 2015. Anaerobic fungi and their potential for biogas production. Adv. Biochem. Eng. Biotechnol. 151, 41–61. https://doi.org/10.1007/978-3-319-21993-6_2

dos Santos, V.L., Monteiro, A. de S., Braga, D.T., Santoro, M.M., 2009. Phenol degradation by *Aureobasidium pullulans* FE13 isolated from industrial effluents. J. Hazard. Mater. 161, 1413–1420. https://doi.org/10.1016/j.jhazmat.2008.04.112

Dos Santos, V.M., Dorner, J.W., Carreira, F., 2003. Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. Mycopathologia 156, 133–138. https://doi.org/10.1023/A:1022996911563

Dutta, S. deb, Tarafder, M., Islam, R., Datta, B., 2018. Characterization of cellulolytic enzymes of Fusarium soil Isolates. Biocatal. Agric. Biotechnol. 14, 279–285. https://doi.org/10.1016/j.bcab.2018.03.011

Elbeshbishy, E., Nakhla, G., Hafez, H., 2012. Biochemical methane potential (BMP) of food waste and primary sludge: Influence of inoculum pre-incubation and inoculum source. Bioresour. Technol. 110, 18–25. https://doi.org/10.1016/j.biortech.2012.01.025

Elisashvili, V., Kachlishvili, E., Asatiani, M.D., 2018. Efficient production of lignin-modifying enzymes and phenolics removal in submerged fermentation of olive mill by-products by white-rot basidiomycetes. https://doi.org/10.1016/j.ibiod.2018.08.003

Emtiazi, G., Naghavi, N., Bordbar, A., 2001. Biodegradation of lignocellulosic waste by *Aspergillus terreus*. Biodegradation 12, 259–263. https://doi.org/10.1023/A:1013155621336

Enzmann, F., Mayer, F., Rother, M., Holtmann, D., 2018. Methanogens: biochemical background and biotechnological applications. AMB Express. https://doi.org/10.1186/s13568-017-0531-x

Ertem, F.C., Neubauer, P., Junne, S., 2017. Environmental life cycle assessment of biogas production from marine macroalgal feedstock for the substitution of energy crops. J. Clean. Prod. 140, 977–985. https://doi.org/10.1016/j.jclepro.2016.08.041

España-Gamboa, E., Vicent, T., Font, X., Dominguez-Maldonado, J., Canto-Canché, B., Alzate-Gaviria, L., 2017. Pretreatment of vinasse from the sugar refinery industry under non-sterile conditions by *Trametes versicolor* in a fluidized bed bioreactor and its effect when coupled to an UASB reactor. J. Biol. Eng. 11, 1–11. https://doi.org/10.1186/s13036-016-0042-3

Evans, T.N., Seviour, R.J., 2012. Estimating Biodiversity of Fungi in Activated Sludge Communities Using Culture-Independent Methods. Microb. Ecol. 63, 773–786. https://doi.org/10.1007/s00248-011-9984-7

Farinas, C.S., 2015. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. Renew. Sustain. Energy Rev. https://doi.org/10.1016/j.rser.2015.07.092

Feldman, D., Kowbel, D.J., Glass, N.L., Yarden, O., Hadar, Y., 2015. Detoxification of 5-hydroxymethylfurfural by the *Pleurotus ostreatus* lignolytic enzymes aryl alcohol oxidase and dehydrogenase. Biotechnol. Biofuels 8, 1–11. https://doi.org/10.1186/s13068-015-0244-9

Ferreira, J.A., Mahboubi, A., Lennartsson, P.R., Taherzadeh, M.J., 2016. Waste biorefineries using filamentous ascomycetes fungi: Present status and future prospects. Bioresour. Technol. 215, 334–345. https://doi.org/10.1016/j.biortech.2016.03.018

Fliegerová, K., Mrázek, J., Hoffmann, K., Zábranská, J., Voigt, K., 2010. Diversity of Anaerobic Fungi within Cow Manure Determined by ITS1 Analysis, Folia Microbiol, 55(4), 319-325.

Font-Palma, C., 2019. Methods for the Treatment of Cattle Manure—A Review. C 5, 27. https://doi.org/10.3390/c5020027

Franke-Whittle, I.H., Walter, A., Ebner, C., Insam, H., 2014. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. Waste Manag. 34, 2080–2089. https://doi.org/10.1016/j.wasman.2014.07.020

Fritsche, U.R., Sims, R.E.H., Monti, A., 2010. Direct and indirect land-use competition issues for energy crops and their sustainable production - an overview. Biofuels, Bioprod. Biorefining. https://doi.org/10.1002/bbb.258

Ganesh, R., Torrijos, M., Sousbie, P., Lugardon, A., Steyer, J.P., Delgenes, J.P., 2014. Single-phase and two-phase anaerobic digestion of fruit and vegetable waste: Comparison of start-up, reactor stability and process performance. Waste Manag. 34, 875–885. https://doi.org/10.1016/j.wasman.2014.02.023

Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F., Xi, Y., 2008. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour. Technol. 99, 7623–7629. https://doi.org/10.1016/j.biortech.2008.02.005

Garcia-Casals, X., Ferroukhi, R., Parajuli, B., 2019. Measuring the socio-economic footprint of the energy transition. Energy Transitions 3, 105–118. https://doi.org/10.1007/s41825-019-00018-6

Garoma, T., Pappaterra, D., 2018. An investigation of ultrasound effect on digestate solubilization and methane yield. Waste Manag. 71, 728–733. https://doi.org/10.1016/j.wasman.2017.03.021

Garon, D., Sage, L., Wouessidjewe, D., Seigle-Murandi, F., 2004. Enhanced degradation of fluorene in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. Chemosphere 56, 159–166. https://doi.org/10.1016/j.chemosphere.2004.02.019

Ge, X., Matsumoto, T., Keith, L., Li, Y., 2015. Fungal pretreatment of albizia chips for enhanced biogas production by solid-state anaerobic digestion. Energy and Fuels 29, 200–204. https://doi.org/10.1021/ef501922t

Gerardi, M.H., 2003. The Microbiology of Anaerobic Digesters, The Microbiology of Anaerobic Digesters. John Wiley & Sons, Inc. https://doi.org/10.1002/0471468967

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268. https://doi.org/10.1351/pac198759020257

Gioelli, F., Dinuccio, E., Balsari, P., 2011. Residual biogas potential from the storage tanks of non-separated digestate and digested liquid fraction. Bioresour. Technol. 102, 10248–10251. https://doi.org/10.1016/j.biortech.2011.08.076

Gömöri, C., Nacsa-Farkas, E., Kerekes, E.B., Vidács, A., Bencsik, O., Kocsubé, S., Khaled, J.M., Alharbi, N.S., Vágvölgyi, C., Krisch, J., 2018. Effect of essential oil vapours on aflatoxin production of *Aspergillus parasiticus*. World Mycotoxin J. https://doi.org/10.3920/WMJ2017.2260

Granier, C., Bessagnet, B., Bond, T., D'Angiola, A., van der Gon, H.D., Frost, G.J., Heil, A., Kaiser, J.W., Kinne, S., Klimont, Z., Kloster, S., Lamarque, J.F., Liousse, C., Masui, T., Meleux, F., Mieville, A., Ohara, T., Raut, J.C., Riahi, K., Schultz, M.G., Smith, S.J., Thompson, A., van Aardenne, J., van der Werf, G.R., van Vuuren, D.P., 2011. Evolution of anthropogenic and biomass burning emissions of air pollutants at global and regional scales during the 1980-2010 period. Clim. Change 109, 163–190. https://doi.org/10.1007/s10584-011-0154-1

Guarnieri, M.T., Ann Franden, M., Johnson, C.W., Beckham, G.T., 2017. Conversion and assimilation of furfural and 5-(hydroxymethyl)furfural by *Pseudomonas putida* KT2440. Metab. Eng. Commun. 4, 22–28. https://doi.org/10.1016/j.meteno.2017.02.001

Guilayn, F., Jimenez, J., Rouez, M., Crest, M., Patureau, D., 2019. Digestate mechanical separation: Efficiency profiles based on anaerobic digestion feedstock and equipment choice. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2018.11.090

Guiraud, P., Steiman, R., Seigle-Murandi, F., Benoit-Guyod, J.L., 1992. Metabolism of vanillic acid by Micromycetes. World J. Microbiol. Biotechnol. https://doi.org/10.1007/BF01201877

Guo, D., Zhang, Z., Liu, D., Zheng, H., Chen, H., Chen, K., 2014. A comparative study on the degradation of gallic acid by *Aspergillus oryzae* and *Phanerochaete chrysosporium*. Water Sci. Technol. 70, 175–181. https://doi.org/10.2166/wst.2014.213

Gutiérrez-Soto, G., Medina-González, G.E., García-Zambrano, E.A., Treviño-Ramírez, J.E., Hernández-Luna, C.E., n.d. Selection and Characterization of a Native Pycnoporus sanguineus Strain as a Lignocellulolytic Extract Producer from Submerged Cultures of Various Agroindustrial Wastes.

Hamill, P.G., Stevenson, A., McMullan, P.E., Williams, J.P., Lewis, A.D.R., S, S., Stevenson, K.E., Farnsworth, K.D., Khroustalyova, G., Takemoto, J.Y., Quinn, J.P., Rapoport, A., Hallsworth, J.E., 2020. Microbial lag phase can be indicative of, or independent from, cellular stress. Sci. Rep. 10, 5948. https://doi.org/10.1038/s41598-020-62552-4

Hansen, T.L., Sommer, S.G., Gabriel, S., Christensen, T.H., 2006. Methane Production during Storage of Anaerobically Digested Municipal Organic Waste. J. Environ. Qual. 35, 830–836. https://doi.org/10.2134/jeq2005.0239

Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: Exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro2519

Hart, T.D., De Leij, F.A.A.M., Kinsey, G., Kelley, J., Lynch, J.M., 2002. Strategies for the isolation of cellulolytic fungi for composting of wheat straw. World J. Microbiol. Biotechnol. 18, 471–480. https://doi.org/10.1023/A:1015519005814

He, J., Liu, X., Xia, J., Xu, J., Xiong, P., Qiu, Z., 2020. One-step utilization of non-detoxified pretreated lignocellulose for enhanced cellulolytic enzyme production using recombinant *Trichoderma reesei* RUT C30 carrying alcohol dehydrogenase and nicotinate phosphoribosyltransferase. https://doi.org/10.1016/j.biortech.2020.123458

He, Y., Zhang, J., Bao, J., 2016. Acceleration of biodetoxification on dilute acid pretreated lignocellulose feedstock by aeration and the consequent ethanol fermentation evaluation. Biotechnol. Biofuels 9, 1–13. https://doi.org/10.1186/s13068-016-0438-9

Hegde, G., Pullammanappallil, P., 2007. Comparison of thermophilic and mesophilic one-stage, batch, high-solids anaerobic digestion. Environ. Technol. 28, 361–369. https://doi.org/10.1080/09593332808618797

Helal, G.A., 2005. Bioconversion of Straw into Improved Fodder: Fungal Flora Decomposing Rice Straw. Mycobiology 33, 150. https://doi.org/10.4489/myco.2005.33.3.150

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18. https://doi.org/10.1016/j.biortech.2008.05.027

Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C., Lundell, T., 2010. New and classic families of secreted fungal heme peroxidases. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-010-2633-0

Holm-Nielsen, J.B., Al Seadi, T., Oleskowicz-Popiel, P., 2009. The future of anaerobic digestion and biogas utilization. Bioresour. Technol. 100, 5478–5484. https://doi.org/10.1016/j.biortech.2008.12.046

Hom-Diaz, A., Baldi, F., Blánquez, P., Lombardi, L., Martín-González, L., Vicent, T., 2016. Exhausted Fungal Biomass as a Feedstock for Increasing Methane Production During the Anaerobic Digestion of Organic Wastes. Waste and Biomass Valorization 7, 307–315. https://doi.org/10.1007/s12649-015-9450-8

Höök, M., Tang, X., 2013. Depletion of fossil fuels and anthropogenic climate change-A review. Energy Policy 52, 797–809. https://doi.org/10.1016/j.enpol.2012.10.046

Hu, M., Wang, J., Gao, Q., Bao, J., 2018. Converting lignin derived phenolic aldehydes into microbial lipid by *Trichosporon cutaneum*. J. Biotechnol. 281, 81–86. https://doi.org/10.1016/j.jbiotec.2018.06.341

Huang, Z., Dostal, L., Rosazza, J.P.N., 1993. Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rhodotorula rubra*. J. Biol. Chem.

Hübner, T., Mumme, J., 2015. Integration of pyrolysis and anaerobic digestion - Use of aqueous liquor from digestate pyrolysis for biogas production. Bioresour. Technol. 183, 86–92. https://doi.org/10.1016/j.biortech.2015.02.037

Hutnan, M., 2016. Maize Silage as Substrate for Biogas Production, in: Advances in Silage Production and Utilization. https://doi.org/10.5772/64378

Isikhuemhen, O.S., Mikiashvili, N.A., Kelkar, V., 2009. Application of solid waste from anaerobic digestion of poultry litter in *Agrocybe aegerita* cultivation: Mushroom production, lignocellulolytic enzymes activity and substrate utilization. Biodegradation. https://doi.org/10.1007/s10532-008-9226-y

Ja'afaru, M.I., 2013. Screening of Fungi Isolated from Environmental Samples for Xylanase and Cellulase Production. ISRN Microbiol. 2013, 1–7. https://doi.org/10.1155/2013/283423

Jabasingh, S.A., ValliNachiyar, C., 2011. Optimization of cellulase production by *Aspergillus nidulans*: Application in the biosoftening of cotton fibers. World J. Microbiol. Biotechnol. 27, 85–97. https://doi.org/10.1007/s11274-010-0431-6

Jasko, J., Skripsts, E., Dubrovskis, V., 2012. Novel substrates for biogas production : *Paxillus involutus* and *Phaeolus schweinitzii* mushroom biomass. Agricultural Engineering, 8-12.

Jiang, D., Ge, X., Zhang, Q., Li, Y., 2016. Comparison of liquid hot water and alkaline pretreatments of giant reed for improved enzymatic digestibility and biogas energy production. Bioresour. Technol. 216, 60–68. https://doi.org/10.1016/j.biortech.2016.05.052

Jing, D., Li, P., Stagnitti, F., Xiong, X., 2007. Optimization of laccase production from *Trametes versicolor* by solid fermentation. Can. J. Microbiol. 53, 245–251. https://doi.org/10.1139/W06-121

Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 199, 103–112. https://doi.org/10.1016/j.biortech.2015.10.009

Kainthola, J., Kalamdhad, A.S., Goud, V. V., Goel, R., 2019a. Fungal pretreatment and associated kinetics of rice straw hydrolysis to accelerate methane yield from anaerobic digestion. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2019.121368

Kainthola, J., Shariq, M., Kalamdhad, A.S., Goud, V. V., 2019b. Enhanced methane potential of rice straw with microwave assisted pretreatment and its kinetic analysis. J. Environ. Manage. https://doi.org/10.1016/j.jenvman.2018.11.052

Kar, S., Sona Gauri, S., Das, A., Jana, A., Maity, C., Mandal, A., Das Mohapatra, P.K., Pati, B.R., Mondal, K.C., 2013. Process optimization of xylanase production using cheap solid substrate by *Trichoderma reesei* SAF3 and study on the alteration of behavioral properties of enzyme obtained from SSF and SmF. Bioprocess Biosyst. Eng. 36, 57–68. https://doi.org/10.1007/s00449-012-0761-x

Kataki, S., Hazarika, S., Baruah, D.C., 2017. Investigation on by-products of bioenergy systems (anaerobic digestion and gasification) as potential crop nutrient using FTIR, XRD, SEM analysis and phyto-toxicity test. J. Environ. Manage. 196, 201–216. https://doi.org/10.1016/j.jenvman.2017.02.058

Kausar, H., Sariah, M., Mohd Saud, H., Zahangir Alam, M., Razi Ismail, M., 2010. Development of compatible lignocellulolytic fungal consortium for rapid composting of rice straw. Int. Biodeterior. Biodegrad. 64, 594–600. https://doi.org/10.1016/j.ibiod.2010.06.012

Kim, D.H., Kim, S.H., Kwon, S.W., Lee, J.K., Hong, S.B., 2013. Fungal diversity of rice straw for meju fermentation. J. Microbiol. Biotechnol. 23, 1654–1663. https://doi.org/10.4014/jmb.1307.07071

Kirk, T.K., Farrell, R.L., 1987. Enzymatic "Combustion": The Microbial Degradation of Lignin. Annu. Rev. Microbiol. https://doi.org/10.1146/annurev.mi.41.100187.002341

Koch, K., Hafner, S.D., Weinrich, S., Astals, S., Holliger, C., 2020. Power and Limitations of Biochemical Methane Potential (BMP) Tests. Front. Energy Res. https://doi.org/10.3389/fenrg.2020.00063

Komarkova, E., Paca, J., Klapkova, E., Stiborova, M., Soccol, C.R., Sobotka, M., 2003. Physiological changes of *Candida tropicalis* population degrading phenol in fed batch reactor. Brazilian Arch. Biol. Technol. 46, 537–543. https://doi.org/10.1590/S1516-89132003000400007

Kong, W., Fu, X., Wang, L., Alhujaily, A., Zhang, J., Ma, F., Zhang, X., Yu, H., 2017. A novel and efficient fungal delignification strategy based on versatile peroxidase for lignocellulose bioconversion. Biotechnol. Biofuels 10, 218. https://doi.org/10.1186/s13068-017-0906-x

Koopman, F., Wierckx, N., De Winde, J.H., Ruijssenaars, H.J., 2010. Identification and characterization of the furfural and 5-(hydroxymethyl)furfural degradation pathways of *Cupriavidus basilensis* HMF14. Proc. Natl. Acad. Sci. U. S. A. 107, 4919–4924. https://doi.org/10.1073/pnas.0913039107

Kowalczyk, J.E., Peng, M., Pawlowski, M., Lipzen, A., Ng, V., Singan, V., Wang, M., Grigoriev, I. V., Mäkelä, M.R., 2019. The white-rot basidiomycete *Dichomitus squalens* shows highly specific transcriptional response to lignocellulose-related aromatic compounds. Front. Bioeng. Biotechnol. 7. https://doi.org/10.3389/fbioe.2019.00229

Kratzeisen, M., Starcevic, N., Martinov, M., Maurer, C., Müller, J., 2010. Applicability of biogas digestate as solid fuel. Fuel 89, 2544–2548. https://doi.org/10.1016/j.fuel.2010.02.008

Kudahettige Nilsson, R.L., Holmgren, M., Madavi, B., Nilsson, R.T., Sellstedt, A., 2016. Adaptability of *Trametes versicolor* to the lignocellulosic inhibitors furfural, HMF, phenol and levulinic acid during ethanol fermentation. Biomass and Bioenergy 90, 95–100. https://doi.org/10.1016/j.biombioe.2016.03.030

Kumar, V., Sharma, A., Kohli, S.K., Bali, S., Sharma, M., Kumar, R., Bhardwaj, R., Thukral, A.K., 2019. Differential distribution of polyphenols in plants using multivariate techniques. Biotechnol. Res. Innov. 3, 1–21. https://doi.org/10.1016/j.biori.2019.03.001

Kumar, V., Yadav, S.K., Kumar, J., Ahluwalia, V., 2020. A critical review on current strategies and trends employed for removal of inhibitors and toxic materials generated during biomass pretreatment. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2019.122633

Kurakov, A. V., Lavrent'ev, R.B., Nechitaĭlo, T.I., Golyshin, P.N., Zviagintsev, D.G., 2008. Diversity of facultatively anaerobic microscopic mycelial fungi in soils. Mikrobiologiia 77, 103–112. https://doi.org/10.1134/s002626170801013x

Kushwaha, J.P., Srivastava, V.C., Mall, I.D., 2013. Sequential batch reactor for dairy wastewater treatment: Parametric optimization; Kinetics and waste sludge disposal. J. Environ. Chem. Eng. 1, 1036–1043. https://doi.org/10.1016/j.jece.2013.08.018

Lafferty, K.D., 2009. The ecology of climate change and infectious diseases. Ecology 90, 888–900. https://doi.org/10.1890/08-0079.1

Lalak, J., Kasprzycka, A., Martyniak, D., Tys, J., 2016. Effect of biological pretreatment of Agropyron elongatum "BAMAR" on biogas production by anaerobic digestion. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2015.10.022

Lawal, A.T., 2017. Polycyclic aromatic hydrocarbons. A review. Cogent Environ. Sci. https://doi.org/10.1080/23311843.2017.1339841

Lee, S., Jang, Y., Lee, Y.M., Lee, J., Lee, H., Kim, G.H., Kim, J.J., 2011. Rice straw-decomposing fungi and their cellulolytic and xylanolytic enzymes. J. Microbiol. Biotechnol. 21, 1322–1329. https://doi.org/10.4014/jmb.1107.07022

Levin, L., Herrmann, C., Papinutti, V.L., 2008. Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. Biochem. Eng. J. 39, 207–214. https://doi.org/10.1016/j.bej.2007.09.004

Li, Y., Park, S.Y., Zhu, J., 2011. Solid-state anaerobic digestion for methane production from organic waste. Renew. Sustain. Energy Rev. 15, 821–826.

Liers, C., Arnstadt, T., Ullrich, R., Hofrichter, M., 2011. Patterns of lignin degradation and oxidative enzyme secretion by different wood- and litter-colonizing basidiomycetes and ascomycetes grown on beech-wood. FEMS Microbiol. Ecol. 78, 91–102. https://doi.org/10.1111/j.1574-6941.2011.01144.x

Liew, L.N., Shi, J., Li, Y., 2012. Methane production from solid-state anaerobic digestion of lignocellulosic biomass. Biomass and Bioenergy. https://doi.org/10.1016/j.biombioe.2012.09.014

Liguori, R., Faraco, V., 2016. Biological processes for advancing lignocellulosic waste biorefinery by advocating circular economy. Bioresour. Technol. 215, 13–20. https://doi.org/10.1016/j.biortech.2016.04.054

Lindner, J., Zielonka, S., Oechsner, H., Lemmer, A., 2015. Effects of mechanical treatment of digestate after anaerobic digestion on the degree of degradation. Bioresour. Technol. 178, 194–200. https://doi.org/10.1016/j.biortech.2014.09.117

Liu, S., Li, X., Wu, S., He, J., Pang, C., Deng, Y., Dong, R., 2014a. Fungal Pretreatment by *Phanerochaete chrysosporium* for Enhancement of Biogas Production from Corn Stover Silage. Appl. Biochem. Biotechnol. 174, 1907–1918. https://doi.org/10.1007/s12010-014-1185-7

Liu, S., Wu, S., Pang, C., Li, W., Dong, R., 2014b. Microbial pretreatment of corn stovers by solid-state cultivation of *Phanerochaete chrysosporium* for biogas production. Appl. Biochem. Biotechnol. 172, 1365–1376. https://doi.org/10.1007/s12010-013-0604-5

Liu, S., Xu, F., Ge, X., Li, Y., 2016. Comparison between ensilage and fungal pretreatment for storage of giant reed and subsequent methane production. Bioresour. Technol. 209, 246–253. https://doi.org/10.1016/j.biortech.2016.02.129

Liu, T., Sun, L., Müller, B., Schnürer, A., 2017. Importance of inoculum source and initial community structure for biogas production from agricultural substrates. Bioresour. Technol. 245, 768–777. https://doi.org/10.1016/j.biortech.2017.08.213

Liu, Y., Liu, X., Ren, N., Feng, Y., Xue, L., Yang, L., 2019. Effect of pyrochar and hydrochar on water evaporation in clayey soil under greenhouse cultivation. Int. J. Environ. Res. Public Health 16, 2580. https://doi.org/10.3390/ijerph16142580

Logan, M., Visvanathan, C., 2019. Management strategies for anaerobic digestate of organic fraction of municipal solid waste: Current status and future prospects. Waste Manag. Res. 37, 27–39. https://doi.org/10.1177/0734242X18816793

López-Bellido, L., Wery, J., López-Bellido, R.J., 2014. Energy crops: Prospects in the context of sustainable agriculture. Eur. J. Agron. https://doi.org/10.1016/j.eja.2014.07.001

López, M.J., Moreno, J., Nichols, N.N., Dien, B.S., Bothast, R.J., 2004. Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. Appl. Microbiol. Biotechnol. 64, 125–131. https://doi.org/10.1007/s00253-003-1401-9

López, M.J., Suárez-Estrella, F., Vargas-García, M.C., López-González, J.A., Verstichel, S., Debeer, L., Wierinck, I., Moreno, J., 2013. Biodelignification of agricultural and forest wastes: Effect on anaerobic digestion. Biomass and Bioenergy 58, 343–349. https://doi.org/10.1016/j.biombioe.2013.10.021

Lubbers, R.J.M., Dilokpimol, A., Visser, J., Mäkelä, M.R., Hildén, K.S., de Vries, R.P., 2019. A comparison between the homocyclic aromatic metabolic pathways from plant-derived compounds by bacteria and fungi. Biotechnol. Adv. 37, 107396. https://doi.org/https://doi.org/10.1016/j.biotechadv.2019.05.002

Lukehurst, C. T., Frost, P., & Al Seadi, T. (2010). Utilisation of digestate from biogas plants as biofertiliser. IEA bioenergy, 2010, 1-36. http://task37.ieabioenergy.com/files/daten-redaktion/download/publi-task37/Digestate_Brochure_Revised_12-2010.pdf

Lundell, T.K., Mäkelä, M.R., Hildén, K., 2010. Lignin-modifying enzymes in filamentous basidiomycetes - Ecological, functional and phylogenetic review. J. Basic Microbiol. https://doi.org/10.1002/jobm.200900338

Majeau, J.A., Brar, S.K., Tyagi, R.D., 2010. Laccases for removal of recalcitrant and emerging pollutants. Bioresour. Technol. 101, 2331–2350. https://doi.org/10.1016/j.biortech.2009.10.087

Makhuvele, R., Ncube, I., Jansen van Rensburg, E.L., La Grange, D.C., 2017. Isolation of fungi from dung of wild herbivores for application in bioethanol production. Brazilian J. Microbiol. 48, 648–655. https://doi.org/10.1016/j.bjm.2016.11.013

Malandrino, O., Sica, D., Testa, M., Supino, S., 2017. Policies and measures for sustainable management of solar panel end-of-life in Italy. Sustain. https://doi.org/10.3390/su9040481

Mao, C., Feng, Y., Wang, X., Ren, G., 2015. Review on research achievements of biogas from anaerobic digestion. Renew. Sustain. Energy Rev. https://doi.org/10.1016/j.rser.2015.02.032

Martínez, A.T., 2002. Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme Microb. Technol. https://doi.org/10.1016/S0141-0229(01)00521-X

Martínková, L., Kotik, M., Marková, E., Homolka, L., 2016. Biodegradation of phenolic compounds by Basidiomycota and its phenol oxidases: A review. Chemosphere 149, 373–382. https://doi.org/10.1016/j.chemosphere.2016.01.022

McPhillips, K., Waters, D.M., Parlet, C., Walsh, D.J., Arendt, E.K., Murray, P.G., 2014. Purification and characterisation of a β-1,4-xylanase from *Remersonia thermophila* CBS 540.69 and its application in bread making. Appl. Biochem. Biotechnol. 172, 1747–1762. https://doi.org/10.1007/s12010-013-0640-1

Menardo, S., Balsari, P., Dinuccio, E., Gioelli, F., 2011a. Thermal pre-treatment of solid fraction from mechanically-separated raw and digested slurry to increase methane yield. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2010.09.067

Menardo, S., Gioelli, F., Balsari, P., 2011b. The methane yield of digestate: Effect of organic loading rate, hydraulic retention time, and plant feeding. Bioresour. Technol. 102, 2348–2351. https://doi.org/10.1016/j.biortech.2010.10.094

Merlin Christy, P., Gopinath, L.R., Divya, D., 2014. A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. Renew. Sustain. Energy Rev. 34, 167–173. https://doi.org/10.1016/j.rser.2014.03.010

Meyer, W., Mitchell, T.G., 1995. Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: Strain variation in *Cryptococcus neoformans*. Electrophoresis 16, 1648–1656. https://doi.org/10.1002/elps.11501601273

Miao, Y., Li, J., Xiao, Z., Shen, Q., Zhang, R., 2015. Characterization and identification of the xylanolytic enzymes from *Aspergillus fumigatus* Z5. BMC Microbiol. 15, 1–12. https://doi.org/10.1186/s12866-015-0463-z

Misra, J.K., Pandey, S., Gupta, A.K., Deshmukh, S.K., 2014. Coprophilous fungi-a review and selected bibliography, in: Fungi From Different Substrates. https://doi.org/10.1201/b17646

Möller, K., Müller, T., 2012. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. Eng. Life Sci. https://doi.org/10.1002/elsc.201100085

Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J.P., Carrère, H., 2014. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. Biotechnol. Adv. 32, 934–951. https://doi.org/10.1016/j.biotechadv.2014.04.007

Monlau, F., Sambusiti, C., Ficara, E., Aboulkas, A., Barakat, A., Carrère, H., 2015. New opportunities for agricultural digestate valorization: Current situation and perspectives. Energy Environ. Sci. 8, 2600–2621. https://doi.org/10.1039/c5ee01633a

More, T.T., Yan, S., Tyagi, R.D., Surampalli, R.Y., 2010. Potential use of filamentous fungi for wastewater sludge treatment. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2010.05.033

Musatti, A., Ficara, E., Mapelli, C., Sambusiti, C., Rollini, M., 2017. Use of solid digestate for lignocellulolytic enzymes production through submerged fungal fermentation. J. Environ. Manage. 199, 1–6. https://doi.org/10.1016/j.jenvman.2017.05.022

Mussatto, S.I., Fernandes, M., Milagres, A.M.F., Roberto, I.C., 2008. Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enzyme Microb. Technol. 43, 124–129. https://doi.org/10.1016/j.enzmictec.2007.11.006

Mustafa, A.M., Poulsen, T.G., Sheng, K., 2016. Fungal pretreatment of rice straw with Pleurotus ostreatus and Trichoderma reesei to enhance methane production under solid-state anaerobic digestion. Appl. Energy 180, 661–671. https://doi.org/10.1016/j.apenergy.2016.07.135

Mustafa, A.M., Poulsen, T.G., Xia, Y., Sheng, K., 2017. Combinations of fungal and milling pretreatments for enhancing rice straw biogas production during solid-state anaerobic digestion. Bioresour. Technol. 224, 174–182. https://doi.org/10.1016/j.biortech.2016.11.028

Muthangya, M., Mshandete, A.M., Kivaisi, A.K., 2009. Two-stage fungal pre-treatment for improved biogas production from sisal leaf decortication residues. Int. J. Mol. Sci. 10, 4805–4815. https://doi.org/10.3390/ijms10114805

Mutschlechner, M., Illmer, P., Wagner, A.O., 2015. Biological pre-treatment: Enhancing biogas production using the highly cellulolytic fungus *Trichoderma viride*. Waste Manag. 43, 98–107. https://doi.org/10.1016/j.wasman.2015.05.011

Nakasaki, K., Mimoto, H., Tran, Q.N.M., Oinuma, A., 2015. Composting of food waste subjected to hydrothermal pretreatment and inoculated with Paecilomyces sp. FA13. Bioresour. Technol. 180, 40–46. https://doi.org/10.1016/j.biortech.2014.12.094

Narkhede, M.K., 2013. Screening of potential lignin degrading fungus from natural habitat. Enzymol. Biotechnol. Prospect. Lignolytic Syst. White Rot Fungi 1–40.

Nasir, I.M., Mohd Ghazi, T.I., Omar, R., 2012. Anaerobic digestion technology in livestock manure treatment for biogas production: A review. Eng. Life Sci. https://doi.org/10.1002/elsc.201100150

Nayan, N., van Erven, G., Kabel, M.A., Sonnenberg, A.S.M., Hendriks, W.H., Cone, J.W., 2019. Evaluation of fungal degradation of wheat straw cell wall using different analytical methods from ruminant nutrition perspective. J. Sci. Food Agric. https://doi.org/10.1002/jsfa.9634

Nichawee Wipusaree, 2011. Purification and characterization of a xylanase from the endophytic fungus *Alternaria alternata* isolated from the Thai medicinal plant, *Croton oblongifoli*us Roxb. African J. Microbiol. Res. 5, 5697–5712. https://doi.org/10.5897/ajmr11.1037

Nicholls, R.J., Cazenave, A., 2010. Sea-level rise and its impact on coastal zones. Science 328(5985), 1517-1520. https://doi.org/10.1126/science.1185782

Nichols, N.N., Sharma, L.N., Mowery, R.A., Chambliss, C.K., van Walsum, G.P., Dien, B.S., Iten, L.B., 2008. Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate. Enzyme Microb. Technol. 42, 624–630. https://doi.org/10.1016/j.enzmictec.2008.02.008

Niku-Paavola, M.L., Karhunen, E., Salola, P., Raunio, V., 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. 254, 877–883. https://doi.org/10.1042/bj2540877

Nuchdang, S., Vatanyoopaisarn, S., Phalakornkule, C., 2015. Effectiveness of fungal treatment by *Coprinopsis cinerea* and *Polyporus tricholoma* on degradation and methane yields of lignocellulosic grass. Int. Biodeterior. Biodegrad. 104, 38–45. https://doi.org/10.1016/j.ibiod.2015.05.015

Olanbiwoninu, A.A., Odunfa, S.A., 2016. Production of Cellulase and Xylanase by *Aspergillus terreus* KJ829487 Using Cassava Peels as Subtrates. Adv. Microbiol. 06, 502–511. https://doi.org/10.4236/aim.2016.67050

Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html

Opatokun, S.A., Kan, T., Al Shoaibi, A., Srinivasakannan, C., Strezov, V., 2016. Characterization of Food Waste and Its Digestate as Feedstock for Thermochemical Processing. Energy and Fuels. https://doi.org/10.1021/acs.energyfuels.5b02183

Oren, L., Ezrati, S., Cohen, D., Sharon, A., 2003. Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. Appl. Environ. Microbiol. 69, 1695–1701. https://doi.org/10.1128/AEM.69.3.1695-1701.2003

Overland, J., Dunlea, E., Box, J.E., Corell, R., Forsius, M., Kattsov, V., Olsen, M.S., Pawlak, J., Reiersen, L.O., Wang, M., 2019. The urgency of Arctic change. Polar Sci. https://doi.org/10.1016/j.polar.2018.11.008

Paavola, T., Rintala, J., 2008. Effects of storage on characteristics and hygienic quality of digestates from four co-digestion concepts of manure and biowaste. Bioresour. Technol. 99, 7041–7050. https://doi.org/10.1016/j.biortech.2008.01.005

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour. Technol. 74, 25–33. https://doi.org/10.1016/S0960-8524(99)00161-3

Panagos, P., Van Liedekerke, M., Yigini, Y., Montanarella, L., 2013. Contaminated sites in Europe: Review of the current situation based on data collected through a European network. J. Environ. Public Health. https://doi.org/10.1155/2013/158764

Panuccio, M.R., Attinà, E., Basile, C., Mallamaci, C., Muscolo, A., 2016. Use of Recalcitrant Agriculture Wastes to Produce Biogas and Feasible Biofertilizer. Waste and Biomass Valorization 7, 267–280. https://doi.org/10.1007/s12649-015-9445-5

Panwar, N.L., Kaushik, S.C., Kothari, S., 2011. Role of renewable energy sources in environmental protection: A review. Renew. Sustain. Energy Rev. https://doi.org/10.1016/j.rser.2010.11.037

Parmar, K.R., Ross, A.B., 2019. Integration of hydrothermal carbonisation with anaerobic digestion; Opportunities for valorisation of digestate. Energies 12, 1586. https://doi.org/10.3390/en12091586

Pawlak-Kruczek, H., Niedzwiecki, L., Sieradzka, M., Mlonka-Mędrala, A., Baranowski, M., Serafin-Tkaczuk, M., Magdziarz, A., 2020. Hydrothermal carbonization of agricultural and municipal solid waste digestates – Structure and energetic properties of the solid products. Fuel 275, 117837. https://doi.org/10.1016/j.fuel.2020.117837

Peay, K.G., Kennedy, P.G., Talbot, J.M., 2016. Dimensions of biodiversity in the Earth mycobiome. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro.2016.59

Perera, A.T.D., Nik, V.M., Chen, D., Scartezzini, J.L., Hong, T., 2020. Quantifying the impacts of climate change and extreme climate events on energy systems. Nat. Energy 5, 150–159. https://doi.org/10.1038/s41560-020-0558-0

Perera, F., 2018. Pollution from fossil-fuel combustion is the leading environmental threat to global pediatric health and equity: Solutions exist. Int. J. Environ. Res. Public Health. https://doi.org/10.3390/ijerph15010016

Peterson, R., Grinyer, J., Nevalainen, H., 2011. Secretome of the coprophilous fungus *Doratomyces stemonitis* C8, isolated from koala feces. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.00252-11

Phutela, U.G., Sahni, N., Sooch, S.S., 2011. Fungal degradation of paddy straw for enhancing biogas production. Indian J. Sci. Technol. https://doi.org/10.17485/ijst/2011/v4i6/30087

Picco, A.M., Angelini, P., Ciccarone, C., Franceschini, A., Ragazzi, A., Rodolfi, M., Varese, G.C., Zotti, M., 2011. Biodiversity of emerging pathogenic and invasive fungi in plants, animals and humans in Italy. Plant Biosyst. https://doi.org/10.1080/11263504.2011.633118

Pitt, J.I., Hocking, A.D., 2009. Fungi and food spoilage, Fungi and Food Spoilage. Springer US. https://doi.org/10.1007/978-0-387-92207-2

Pitt, J.I., Hocking, A.D., 1977. Influence of solute and hydrogen ion concentration on the water relations of some xerophilic fungi. J. Gen. Microbiol. 101, 35–40. https://doi.org/10.1099/00221287-101-1-35

Poli, A., Lazzari, A., Prigione, V., Voyron, S., Spadaro, D., Varese, G.C., 2016. Influence of plant genotype on the cultivable fungi associated to tomato rhizosphere and roots in different soils. Fungal Biol. 120, 862–872. https://doi.org/10.1016/j.funbio.2016.03.008

Radwan, O., Gunasekera, T.S., Ruiz, O.N., 2018. Draft genome sequence of *Byssochlamys* sp. isolate BYSS01, a filamentous fungus adapted to the fuel environment. Genome Announc. 6, 4–5. https://doi.org/10.1128/genomeA.00164-18

Rahouti, M., Seigle-Murandi, F., Steiman, R., Eriksson, K.E., 1989. Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum.* Appl. Environ. Microbiol. https://doi.org/10.1128/aem.55.9.2391-2398.1989

Ramarajan, R., Manohar, C.S., 2017. Biological pretreatment and bioconversion of agricultural wastes, using ligninolytic and cellulolytic fungal consortia. Bioremediat. J. 21, 89–99. https://doi.org/10.1080/10889868.2017.1282937

Ran, H., Zhang, J., Gao, Q., Lin, Z., Bao, J., 2014. Analysis of biodegradation performance of furfural and 5- hydroxymethylfurfural by Amorphotheca resinae ZN1. Biotechnol. Biofuels 7, 1–12. https://doi.org/10.1186/1754-6834-7-51

Reid, I.D., 1989. Optimization of solid-state fermentation for selective delignification of aspen wood with *Phlebia tremellosa*. Enzyme Microb. Technol. https://doi.org/10.1016/0141-0229(89)90053-7

Rincón, B., Borja, R., González, J.M., Portillo, M.C., Sáiz-Jiménez, C., 2008. Influence of organic loading rate and hydraulic retention time on the performance, stability and microbial communities of one-stage anaerobic digestion of two-phase olive mill solid residue. Biochem. Eng. J. 40, 253–261. https://doi.org/10.1016/j.bej.2007.12.019

Rouches, E., Herpoël-Gimbert, I., Steyer, J.P., Carrere, H., 2016. Improvement of anaerobic degradation by white-rot fungi pretreatment of lignocellulosic biomass: A review. Renew. Sustain. Energy Rev. 59, 179–198. https://doi.org/10.1016/j.rser.2015.12.317

Rougeron, A., Giraud, S., Alastruey-Izquierdo, A., Cano-Lira, J., Rainer, J., Mouhajir, A., Le Gal, S., Nevez, G., Meyer, W., Bouchara, J.P., 2018. Ecology of *Scedosporium* Species: Present Knowledge and Future Research. Mycopathologia 183, 185–200. https://doi.org/10.1007/s11046-017-0200-2

Ruan, Z., Hollinshead, W., Isaguirre, C., Tang, Y.J., Liao, W., Liu, Y., 2015. Effects of inhibitory compounds in lignocellulosic hydrolysates on *Mortierella isabellina* growth and carbon utilization. Bioresour. Technol. 183, 18–24. https://doi.org/10.1016/j.biortech.2015.02.026

Rumbold, K., van Buijsen, H.J.J., Overkamp, K.M., van Groenestijn, J.W., Punt, P.J., Werf, M.J.V.D., 2009. Microbial production host selection for converting second-generation feedstocks into bioproducts. Microb. Cell Fact. 8, 1–11. https://doi.org/10.1186/1475-2859-8-64

Sambusiti, C., Monlau, F., Ficara, E., Musatti, A., Rollini, M., Barakat, A., Malpei, F., 2015. Comparison of various post-treatments for recovering methane from agricultural digestate. Fuel Process. Technol. 137, 359–365. https://doi.org/10.1016/j.fuproc.2015.04.028

Saqib, A.A.N., Hassan, M., Khan, N.F., Baig, S., 2010. Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF). Process Biochem. 45, 641–646. https://doi.org/10.1016/j.procbio.2009.12.011

Sawatdeenarunat, C., Surendra, K.C., Takara, D., Oechsner, H., Khanal, S.K., 2015. Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities. Bioresour. Technol. 178, 178–186. https://doi.org/10.1016/j.biortech.2014.09.103

Scarlat, N., Dallemand, J.F., Fahl, F., 2018. Biogas: Developments and perspectives in Europe. Renew. Energy. https://doi.org/10.1016/j.renene.2018.03.006

Schmoll, M., Schuster, A., 2010. Biology and biotechnology of *Trichoderma.* Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-010-2632-1

Schnürer, J., Clarholm, M., Boström, S., Rosswall, T., 1986. Effects of moisture on soil microorganisms and nematodes: A field experiment. Microb. Ecol. 12, 217–230. https://doi.org/10.1007/BF02011206

Sharma, H.K., Xu, C., Qin, W., 2019. Biological Pretreatment of Lignocellulosic Biomass for Biofuels and Bioproducts: An Overview. Waste and Biomass Valorization. https://doi.org/10.1007/s12649-017-0059-y

Sherief, A.A., El-Tanash, A.B., Atia, N., 2010. Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran. Res. J. Microbiol. 5, 199–211. https://doi.org/10.3923/jm.2010.199.211

Shirkavand, E., Baroutian, S., Gapes, D.J., Young, B.R., 2016. Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment - A review. Renew. Sustain. Energy Rev. 54, 217–234. https://doi.org/10.1016/j.rser.2015.10.003

Shortall, O.K., 2013. “Marginal land” for energy crops: Exploring definitions and embedded assumptions. Energy Policy 62, 19–27. https://doi.org/10.1016/j.enpol.2013.07.048

Shraddha, Shekher, R., Sehgal, S., Kamthania, M., Kumar, A., 2011. Laccase: Microbial sources, production, purification, and potential biotechnological applications. Enzyme Res. https://doi.org/10.4061/2011/217861

Shrestha, P., Ibáñez, A.B., Bauer, S., Glassman, S.I., Szaro, T.M., Bruns, T.D., Taylor, J.W., 2015. Fungi isolated from *Miscanthus* and sugarcane: Biomass conversion, fungal enzymes, and hydrolysis of plant cell wall polymers. Biotechnol. Biofuels 8, 1–14. https://doi.org/10.1186/s13068-015-0221-3

Shrestha, P., Szaro, T.M., Bruns, T.D., Taylor, J.W., 2011. Systematic search for cultivatable fungi that best deconstruct cell walls of *Miscanthus* and sugarcane in the field. Appl. Environ. Microbiol. 77, 5490–5504. https://doi.org/10.1128/AEM.02996-10

Shrestha, S., Fonoll, X., Khanal, S.K., Raskin, L., 2017. Biological strategies for enhanced hydrolysis of lignocellulosic biomass during anaerobic digestion: Current status and future perspectives. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2017.08.089

Sietmann, R., Uebe, R., Böer, E., Bode, R., Kunze, G., Schauer, F., 2010. Novel metabolic routes during the oxidation of hydroxylated aromatic acids by the yeast *Arxula adeninivorans*. J. Appl. Microbiol. 108, 789–799. https://doi.org/10.1111/j.1365-2672.2009.04474.x

Simonazzi, M., Pezzolesi, L., Guerrini, F., Vanucci, S., Samorì, C., Pistocchi, R., 2019. Use of waste carbon dioxide and pre-treated liquid digestate from biogas process for *Phaeodactylum tricornutum* cultivation in photobioreactors and open ponds. Bioresour. Technol. 292, 121921. https://doi.org/10.1016/j.biortech.2019.121921

Sindhu, R., Binod, P., Pandey, A., 2016. Biological pretreatment of lignocellulosic biomass - An overview. Bioresour. Technol. 199, 76–82. https://doi.org/10.1016/j.biortech.2015.08.030

Singh, B., Poças-Fonseca, M.J., Johri, B.N., Satyanarayana, T., 2016. Thermophilic molds: Biology and applications. Crit. Rev. Microbiol. https://doi.org/10.3109/1040841X.2015.1122572

Singh, P., Sulaiman, O., Hashim, R., Peng, L.C., Singh, R.P., 2013. Evaluating biopulping as an alternative application on oil palm trunk using the white-rot fungus *Trametes versicolor*. Int. Biodeterior. Biodegrad. 82, 96–103. https://doi.org/10.1016/j.ibiod.2012.12.016

Singh, S., Harms, H., Schlosser, D., 2014. Screening of ecologically diverse fungi for their potential to pretreat lignocellulosic bioenergy feedstock. Appl. Microbiol. Biotechnol. 98, 3355–3370. https://doi.org/10.1007/s00253-014-5563-4

Soetaert, W., Vandamme, E.J., 2010. Industrial biotechnology: sustainable growth and economic success. John Wiley & Sons.

Song, K., Yeerken, S., Li, L., Sun, J., Wang, Q., 2019. Improving Post-Anaerobic Digestion of Full-Scale Anaerobic Digestate Using Free Ammonia Treatment. ACS Sustain. Chem. Eng. 7, 7171–7176. https://doi.org/10.1021/acssuschemeng.9b00152

Song, L., Yu, H., Ma, F., Zhang, X., 2013. Biological Pretreatment under Non-sterile Conditions for Enzymatic Hydrolysis of Corn Stover. BioResources 8, 3802–3816. https://doi.org/10.15376/biores.8.3.3802-3816

Songulashvili, G., Spindler, D., Jimenéz-Tobón, G.A., Jaspers, C., Kerns, G., Penninckx, M.J., 2015. Production of a high level of laccase by submerged fermentation at 120-L scale of *Cerrena unicolor* C-139 grown on wheat bran. Comptes Rendus - Biol. 338, 121–125. https://doi.org/10.1016/j.crvi.2014.12.001

Spina, F., Tigini, V., Romagnolo, A., Varese, G.C., 2018. Bioremediation of landfill leachate with fungi: Autochthonous vs. allochthonous strains. Life. https://doi.org/10.3390/life8030027

Sridevi, A., Sandhya, A., Ramanjaneyulu, G., Narasimha, G., Devi, P.S., 2016. Biocatalytic activity of *Aspergillus niger* xylanase in paper pulp biobleaching. 3 Biotech 6, 1–7. https://doi.org/10.1007/s13205-016-0480-0

Srilekha Yadav, K., Naseeruddin, S., Sai Prashanthi, G., Sateesh, L., Venkateswar Rao, L., 2011. Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis*. Bioresour. Technol. 102, 6473–6478. https://doi.org/10.1016/j.biortech.2011.03.019

Stajich, J.E., Berbee, M.L., Blackwell, M., Hibbett, D.S., James, T.Y., Spatafora, J.W., Taylor, J.W., 2009. The Fungi. Curr. Biol. https://doi.org/10.1016/j.cub.2009.07.004

Stamps, B.W., Bojanowski, C.L., Drake, C.A., Nunn, H.S., Lloyd, P.F., Floyd, J.G., Emmerich, K.A., Neal, A.R., Crookes-Goodson, W.J., Stevenson, B.S., 2020. In situ Linkage of Fungal and Bacterial Proliferation to Microbiologically Influenced Corrosion in B20 Biodiesel Storage Tanks. Front. Microbiol. 11, 1–13. https://doi.org/10.3389/fmicb.2020.00167

Stefaniuk, M., Oleszczuk, P., 2015. Characterization of biochars produced from residues from biogas production. J. Anal. Appl. Pyrolysis 115, 157–165. https://doi.org/10.1016/j.jaap.2015.07.011

Stepanova, E. V., Koroleva, O. V., Vasilchenko, L.G., Karapetyan, K.N., Landesman, E.O., Yavmetdinov, I.S., Kozlov, Y.P., Rabinovich, M.L., 2003. Fungal Decomposition of Oat Straw during Liquid and Solid-State Fermentation. Appl. Biochem. Microbiol. 39, 65–74. https://doi.org/10.1023/A:1021702211169

Stoumpou, V., Novakovic, J., Kontogianni, N., Barampouti, E.M., Mai, S., Moustakas, K., Malamis, D., Loizidou, M., 2020. Assessing straw digestate as feedstock for bioethanol production. Renew. Energy 153, 261–269. https://doi.org/10.1016/j.renene.2020.02.021

Sun, Shaoni, Sun, Shaolong, Cao, X., Sun, R., 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. Bioresour. Technol. 199, 49–58. https://doi.org/10.1016/j.biortech.2015.08.061

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review, International Journal of Molecular Sciences. https://doi.org/10.3390/ijms9091621

Tian, X. fei, Fang, Z., Guo, F., 2012. Impact and prospective of fungal pre-treatment of lignocellulosic biomass for enzymatic hydrolysis. Biofuels, Bioprod. Biorefining. https://doi.org/10.1002/bbb.346

Tigini, V., Bevione, F., Prigione, V., Poli, A., Ranieri, L., Spennati, F., Munz, G., Varese, G.C., 2018. Tannery mixed liquors from an ecotoxicological and mycological point of view: Risks vs potential biodegradation application. Sci. Total Environ. 627, 835–843. https://doi.org/10.1016/j.scitotenv.2018.01.240

Tigini, V., Franchino, M., Bona, F., Varese, G.C., 2016. Is digestate safe? A study on its ecotoxicity and environmental risk on a pig manure. Sci. Total Environ. 551–552, 127–132. https://doi.org/10.1016/j.scitotenv.2016.02.004

Tišma, M., Planinić, M., Bucić-Kojić, A., Panjičko, M., Zupančič, G.D., Zelić, B., 2018. Corn silage fungal-based solid-state pretreatment for enhanced biogas production in anaerobic co-digestion with cow manure. Bioresour. Technol. 253, 220–226. https://doi.org/10.1016/j.biortech.2018.01.037

Topakas, E., Kalogeris, E., Kekos, D., Macris, B.J., Christakopoulos, P., 2003. Bioconversion of ferulic acid into vanillic acid by the thermophilic fungus *Sporotrichum thermophile*. LWT - Food Sci. Technol. https://doi.org/10.1016/S0023-6438(03)00060-4

Tournas, V., 1994. Heat-resistant fungi of importance to the food and beverage industry. Crit. Rev. Microbiol. 20, 243–263. https://doi.org/10.3109/10408419409113558

Traversi, D., Villa, S., Acri, M., Pietrangeli, B., Degan, R., Gilli, G., 2011. The role of different methanogen groups evaluated by real-time qPCR as high-efficiency bioindicators of wet anaerobic co-digestion of organic waste. AMB Express 1, 1–7. https://doi.org/10.1186/2191-0855-1-28

Valentine, J., Clifton-Brown, J., Hastings, A., Robson, P., Allison, G., Smith, P., 2012. Food vs. fuel: The use of land for lignocellulosic "next generation" energy crops that minimize competition with primary food production. GCB Bioenergy 4, 1–19. https://doi.org/10.1111/j.1757-1707.2011.01111.x

Vamvakaki, A.N., Kandarakis, I., Kaminarides, S., Komaitis, M., Papanikolaou, S., 2010. Cheese whey as a renewable substrate for microbial lipid and biomass production by Zygomycetes. Eng. Life Sci. 10, 348–360. https://doi.org/10.1002/elsc.201000063

van Asselt, E.D., Azambuja, W., Moretti, A., Kastelein, P., de Rijk, T.C., Stratakou, I., van der Fels-Klerx, H.J., 2012. A Dutch field survey on fungal infection and mycotoxin concentrations in maize. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 29, 1556–1565. https://doi.org/10.1080/19440049.2012.689997

Van, D.P., Fujiwara, T., Tho, B.L., Toan, P.P.S., Minh, G.H., 2020. A review of anaerobic digestion systems for biodegradable waste: Configurations, operating parameters, and current trends. Environ. Eng. Res. https://doi.org/10.4491/eer.2018.334

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. J. Dairy Sci. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Vasco-Correa, J., Ge, X., Li, Y., 2016. Fungal pretreatment of non-sterile *Miscanthus* for enhanced enzymatic hydrolysis. Bioresour. Technol. 203, 118–123. https://doi.org/10.1016/j.biortech.2015.12.018

Vasco-Correa, J., Khanal, S., Manandhar, A., Shah, A., 2018. Anaerobic digestion for bioenergy production: Global status, environmental and techno-economic implications, and government policies. Bioresour. Technol. 247, 1015–1026. https://doi.org/10.1016/j.biortech.2017.09.004

Vasco-Correa, J., Li, Y., 2015. Solid-state anaerobic digestion of fungal pretreated *Miscanthus sinensis* harvested in two different seasons. Bioresour. Technol. 185, 211–217. https://doi.org/10.1016/j.biortech.2015.02.099

Verein Deutscher Ingenieure, 2006. Fermentation of organic materials Characterisation of the substrate, sampling, collection of material data, fermentation tests, VDI-Gesellschaft Energietechnik.

Wagner, A.O., Schwarzenauer, T., Illmer, P., 2013. Improvement of methane generation capacity by aerobic pre-treatment of organic waste with a cellulolytic *Trichoderma viride* culture. J. Environ. Manage. 129, 357–360. https://doi.org/10.1016/j.jenvman.2013.07.030

Wan, C., Li, Y., 2011. Effectiveness of microbial pretreatment by *Ceriporiopsis subvermispora* on different biomass feedstocks. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2011.05.026

Wang, P., Wang, H., Qiu, Y., Ren, L., Jiang, B., 2018. Microbial characteristics in anaerobic digestion process of food waste for methane production–A review. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2017.06.152

Wang, X., Gao, Q., Bao, J., 2015. Transcriptional analysis of *Amorphotheca resinae* ZN1 on biological degradation of furfural and 5-hydroxymethylfurfural derived from lignocellulose pretreatment. Biotechnol. Biofuels 8, 1–13. https://doi.org/10.1186/s13068-015-0323-y

Watkiss, P., Downing, T., Handley, C., Butterfield, R., (2005). The Impacts and Costs of Climate Change Final Report September 2005 AEA Technology Environment 2 Stockholm Environment Institute, Oxford Commissioned by European Commission DG Environment.

Wei, S., 2016. The application of biotechnology on the enhancing of biogas production from lignocellulosic waste. Appl. Microbiol. Biotechnol. 100, 9821–9836. https://doi.org/10.1007/s00253-016-7926-5

Weiland, P., 2010. Biogas production: Current state and perspectives. Appl. Microbiol. Biotechnol. 85, 849–860. https://doi.org/10.1007/s00253-009-2246-7

Woo, H.L., Hazen, T.C., Simmons, B.A., DeAngelis, K.M., 2014. Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils. Syst. Appl. Microbiol. 37, 60–67. https://doi.org/10.1016/j.syapm.2013.10.001

Wu, P.C., Su, H.J.J., Ho, H.M., 2000. A comparison of sampling media for environmental viable fungi collected in a hospital environment. Environ. Res. 82, 253–257. https://doi.org/10.1006/enrs.1999.4017

Wuebbles, D.J., Jain, A.K., 2001. Concerns about climate change and the role of fossil fuel use, Fuel Processing Technology , 71(1-3), 99-119.

Xu, J., Mustafa, A.M., Lin, H., Choe, U.Y., Sheng, K., 2018. Effect of hydrochar on anaerobic digestion of dead pig carcass after hydrothermal pretreatment. Waste Manag. 78, 849–856. https://doi.org/10.1016/j.wasman.2018.07.003

Xu, X., Xu, Z., Shi, S., Lin, M., 2017. Lignocellulose degradation patterns, structural changes, and enzyme secretion by *Inonotus obliquus* on straw biomass under submerged fermentation. Bioresour. Technol. 241, 415–423. https://doi.org/10.1016/j.biortech.2017.05.087

Yadav, D., Barbora, L., Bora, D., Mitra, S., Rangan, L., Mahanta, P., 2017. An assessment of duckweed as a potential lignocellulosic feedstock for biogas production. Int. Biodeterior. Biodegrad. 119, 253–259. https://doi.org/10.1016/j.ibiod.2016.09.007

Yen, H.W., Brune, D.E., 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresour. Technol. 98, 130–134. https://doi.org/10.1016/j.biortech.2005.11.010

Yi, X., Gao, Q., Zhang, L., Wang, X., He, Y., Hu, F., Zhang, J., Zou, G., Yang, S., Zhou, Z., Bao, J., 2019. Heterozygous diploid structure of *Amorphotheca resinae* ZN1 contributes efficient biodetoxification on solid pretreated corn stover. Biotechnol Biofuels 12, 126. https://doi.org/10.1186/s13068-019-1466-z

Yilmaz, A.G., Hossain, I., Perera, B.J.C., 2014. Effect of climate change and variability on extreme rainfall intensity-frequency-duration relationships: A case study of Melbourne. Hydrol. Earth Syst. Sci. 18, 4065–4076. https://doi.org/10.5194/hess-18-4065-2014

Yoon, L.W., Ang, T.N., Ngoh, G.C., Chua, A.S.M., 2014. Fungal solid-state fermentation and various methods of enhancement in cellulase production. Biomass and Bioenergy. https://doi.org/10.1016/j.biombioe.2014.05.013

Young, D., Dollhofer, V., Callaghan, T.M., Reitberger, S., Lebuhn, M., Benz, J.P., 2018. Isolation, identification and characterization of lignocellulolytic aerobic and anaerobic fungi in one- and two-phase biogas plants. Bioresour. Technol. 268, 470–479. https://doi.org/10.1016/j.biortech.2018.07.103

Yu, Y., Feng, Y., Xu, C., Liu, J., Li, D., 2011. Onsite bio-detoxification of steam-exploded corn stover for cellulosic ethanol production. Bioresour. Technol. 102, 5123–5128. https://doi.org/10.1016/j.biortech.2011.01.067

Zabed, H.M., Akter, S., Yun, J., Zhang, G., Awad, F.N., Qi, X., Sahu, J.N., 2019. Recent advances in biological pretreatment of microalgae and lignocellulosic biomass for biofuel production. Renew. Sustain. Energy Rev. https://doi.org/10.1016/j.rser.2019.01.048

Zegada-Lizarazu, W., Elbersen, H.W., Cosentino, S.L., Zatta, A., Alexopoulou, E., Monti, A., 2010. Agronomic aspects of future energy crops in Europe. Biofuels, Bioprod. Biorefining. https://doi.org/10.1002/bbb.242

Zeng, G., Yu, M., Chen, Y., Huang, D., Zhang, J., Huang, H., Jiang, R., Yu, Z., 2010. Effects of inoculation with *Phanerochaete chrysosporium* at various time points on enzyme activities during agricultural waste composting. Bioresour. Technol. 101, 222–227. https://doi.org/10.1016/j.biortech.2009.08.013

Zézé, A., Sulistyowati, E., Ophel-Keller, K., Barker, S., Smith, S., 1997. Intersporal genetic variation of *Gigaspora margarita*, a vesicular arbuscular mycorrhizal fungus, revealed by M13 minisatellite-primed PCR. Appl. Environ. Microbiol. 63, 676–678. https://doi.org/10.1128/aem.63.2.676-678.1997

Zhao, J., Ge, X., Vasco-Correa, J., Li, Y., 2014. Fungal pretreatment of unsterilized yard trimmings for enhanced methane production by solid-state anaerobic digestion. Bioresour. Technol. 158, 248–252. https://doi.org/10.1016/j.biortech.2014.02.029

Zheng, Y., Yu, X., Zeng, J., Chen, S., 2012. Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw. Biotechnol. Biofuels 5, 1–10. https://doi.org/10.1186/1754-6834-5-50

Zhi, Z., Wang, H., 2014. White-rot fungal pretreatment of wheat straw with *Phanerochaete chrysosporium* for biohydrogen production: Simultaneous saccharification and fermentation. Bioprocess Biosyst. Eng. https://doi.org/10.1007/s00449-013-1117-x

Zhong, Y., Liu, Z., Isaguirre, C., Liu, Y., Liao, W., 2016. Fungal fermentation on anaerobic digestate for lipid-based biofuel production. Biotechnol. Biofuels 9, 1–11. https://doi.org/10.1186/s13068-016-0654-3

Zhou, S., Raouche, S., Grisel, S., Navarro, D., Sigoillot, J.C., Herpoël-Gimbert, I., 2015. Solid-state fermentation in multi-well plates to assess pretreatment efficiency of rot fungi on lignocellulose biomass. Microb. Biotechnol. 8, 940–949. https://doi.org/10.1111/1751-7915.12307

Ziganshin, A.M., Liebetrau, J., Pröter, J., Kleinsteuber, S., 2013. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Appl. Microbiol. Biotechnol. 97, 5161–5174. https://doi.org/10.1007/s00253-013-4867-0

Zmitrovich, I., Wasser, S., Ţura, D., 2014. Wood-Inhabiting Fungi, in: Fungi From Different Substrates. https://doi.org/10.1201/b17646-3

8.2 Sitography

COM, 2007. Communication From The Commission To The Council, The European Parliament, The European Economic And Social Committee And The Committee Of The Regions. Limiting Global Climate Change To 2 Degrees Celsius. The Way Ahead For 2020 And Beyond. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2007:0002:FIN:EN:PDF (accessed 23.07.21).

COM, 2011 – 112 final. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions - A Roadmap for moving to a competitive low carbon economy in 2050 — European Environment Agency https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2011:0112:FIN:EN:PDF (accessed 23.07.21).

COM, 2011 - 885 final. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions - Energy Roadmap 2050 - European Environment Agency. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52011DC0885&from=EN (accessed 23.07.21).

COM, 2019 - 640 final. Communication from the Commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions - The European Green Deal - European Environment Agency. https://ec.europa.eu/info/sites/info/files/european-green-deal-communication_en.pdf (accessed 23.07.21).

Decree Law 134, art. 52, comma 2 bis of the 7/08/2012. Conversione in legge, con modificazioni, del decreto-legge 22 giugno 2012, n. 83, recante misure urgenti per la crescita del Paese. (12G0152). https://www.tuttocamere.it/files/Archivio/2012_134.pdf (accessed 23.07.21).

Decree Law 5046 of the 25/02/2016. Criteri e norme tecniche generali per la disciplina regionale dell'utilizzazione agronomica degli effluenti di allevamento e delle acque reflue di cui all'art. 113 del Decreto legislativo 3 aprile 2006 n. 152, nonché per la produzione e l'utilizzazione agronomica del digestato di cui all'art. 52, comma 2-bis del decreto legge 22 giugno 2012, n. 83, convertito in legge 7 agosto 2012 n. 134. https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/9780 (accessed 23.07.21).

Decree of the Minister of Economic Development of 6 July 2012. Incentivi per energia da fonti rinnovabili elettriche non fotovoltaiche. https://www.mise.gov.it/images/stories/normativa/DM_6_luglio_2012_sf.pdf (accessed 23.07.21).

Directive 2009/28/EC. Directive 2009/28/EC of the European Parliament and of the Council of 23 April 2009 on the promotion of the use of energy from renewable sources and amending and subsequently repealing Directives 2001/77/EC and 2003/30/EC. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009L0028&from=en (accessed 23.07.21).

Directive 2018/2001. Directive (EU) 2018/2001 of the European Parliament and of the Council of 11 December 2018 on the promotion of the use of energy from renewable sources. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018L2001&from=EN (accessed 23.07.21).

Directive 2015/1513. Directive (EU) 2015/1513 of the European Parliament and of the Council of 9 September 2015 amending Directive 98/70/EC relating to the quality of petrol and diesel fuels and amending Directive 2009/28/EC on the promotion of the use of energy from renewable sources https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015L1513&from=EN (accessed 23.07.21).

DM 23/06/2016. Incentivazione dell'energia elettrica prodotta da fonti rinnovabili diverse dal fotovoltaico.https://www.gse.it/documenti_site/Documenti%20GSE/Servizi%20per%20te/FER%20ELETTRICHE/NORMATIVE/DM%2023%20giugno%202016.PDF (accessed 23.07.21).

EAA, 2017. European Environment Agency (EEA) Report No 3/2017. Renewable energy in Europe 2017 — Recent growth and knock-on effects — European Environment Agency https://www.eea.europa.eu/publications/renewable-energy-in-europe (accessed 23.07.21).

EBA, 2016. Annual Report. European Biogas Association. https://www.eba.europa.eu/sites/default/documents/files/documents/10180/1879387/4c08e80f-ea87-4643-90cb-a2a02230c0a2/EBA%20Annual%20Report%202016.pdf (accessed 23.07.21).

EBA, 2017. Statistical Report. European Biogas Association. https://www.europeanbiogas.eu/eba-statistical-report-2017-published-soon/ (accessed 23.07.21).

EBA, 2020. Statistical Report. European Biogas Association. https://www.europeanbiogas.eu/eba-statistical-report-2020/ (accessed 23.07.21).

EU Regulation EC1772/2002. Commission Regulation (EC) No 1772/2002 of 3 October 2002 fixing the maximum export refund on oats in connection with the invitation to tender issued in Regulation (EC) No 1582/2002. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002R1772&from=EN (accessed 23.07.21).

European Union Nitrate Directive (91/676/EEC). Council Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31991L0676&from=EN (accessed 23.07.21).

EUROSTAT, 2021 - Renewable energy statistics - Statistics Explained. https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Renewable_energy_statistics (accessed 5.25.21).

IEA, 2020. Global energy-related CO2 emissions, 1900-2020 – Charts – Data & Statistics – IEA. https://www.iea.org/data-and-statistics/charts/global-energy-related-co2-emissions-1900-2020 (accessed 23.07.21).

JEC, 2014. Joint Research Centre (JRC) Publications Repository - EU renewable energy targets in 2020: Revised analysis of scenarios for transport fuels. JEC Biofuels Programme. Institute for Energy and Transport. https://publications.jrc.ec.europa.eu/repository/handle/JRC89393 (accessed 23.07.21).

Law 99/23 July 2009. Disposizioni per lo sviluppo e l'internazionalizzazione delle imprese, nonché in materia di ener-gia. Gazzetta Ufficiale n. 176 del 31 luglio 2009 - Supplemento ordinario n. 136. https://www.gazzettaufficiale.it/eli/id/2009/07/31/009G0111/sg (accessed 23.07.21).

Other Web Sources:

https://www.e-education.psu.edu/egee439/node/727

http://www.fabbiogas.eu/en/home/

http://www.lippsilos.com

9. List of Publications

Articles in Peer-Reviewed International Journal

- Spina F., Spini G., Poli A., Romagnolo A., **Zanellati A.**, Bentivegna N.G., El-Azhari N., Regnier T., Blieux A.L., Echairi A., Prigione V., Puglisi E., Varese G.C., 2018, Screening of anionic biosurfactants production among fungi and bacteria, Chemical Engineering Transactions, 64, 493-498 (https://doi.org/10.3303/CET1864083)

- **Zanellati, A**., Spina, F., Rollé, L., Varese, G. C., & Dinuccio, E. (2020). Fungal pretreatments on non-sterile solid digestate to enhance methane yield and the sustainability of anaerobic digestion. Sustainability, 12(20), 8549. (https://doi.org/10.3390/su12208549).

- **Zanellati, A**., Spina, F., Poli, A., Rollé, L., Varese, G. C., & Dinuccio, E. (2021). Fungal pretreatment of non-sterile maize silage and solid digestate with a *Cephalotrichum stemonitis* strain selected from agricultural biogas plants to enhance anaerobic digestion. Biomass and Bioenergy, 144, 105934. (https://doi.org/10.1016/j.biombioe.2020.105934).

- **Zanellati, A**., Spina, F., Bonaterra, M., Dinuccio, E., Varese, G. C., & Scarpeci, T. E. (2021). Screening and evaluation of phenols and furans degrading fungi for the biological pretreatment of lignocellulosic biomass. International Biodeterioration & Biodegradation, 161, 105246. (https://doi.org/10.1016/j.ibiod.2021.105246)

Poster, Abstract and Oral Presentation in National and International Congresses

- Spina F., Spini G., Poli A., Romagnolo A., **Zanellati A.**, Bentivegna N.G., El-Azhari N., Regnier T., Blieux A.L., Echairi A., Prigione V., Puglisi E., Varese G.C. (2017) – Biological Remediation of Soil Contaminated by Hydrocarbons. In "9th International Conference on Environmental Engineering and Sustainability". 6-9 September 2017, Bologna, Italy. (Conference paper in volume)

- Spina F., Spini G., Poli A., Romagnolo A., **Zanellati A.**, Bentivegna N.G., El-Azhari N., Regnier T., Blieux A.L., Echairi A., Prigione V., Puglisi E., Varese G.C. (2017) - Bioremediation of Contaminated Land by Autochtonous Fungi: Life-Biorest Strategy. In "Bioremediation of Contaminated Land by Autocthonous Fungi: Life Biorest strategy". 25-29 June, Prague, Czech Republic. (Oral presentation)

- Romagnolo A., Spina F., Galvagno I., **Zanellati A.**, Dinuccio E., Varese G.C. (2018) - Valorization of Non-Sterile Lignocellulosic Waste by Means of Filamentous Fungi Pretreatment to Enhance Biogas Production. In "International Forum on Industrial Biotechnology and Bioeconomy - 8th edition" 27-28th September 2018, Torino, Italy. (Poster)

- Spina F., Spini G., Poli A., Romagnolo A., **Zanellati A.**, Bentivegna N.G., El-Azhari N., Regnier T., Blieux A.L., Echairi A., Prigione V., Puglisi E., Varese G.C. (2017) – Biological Remediation of Soil Contaminated by Hydrocarbons. In "International Forum on Industrial Biotechnology and Bioeconomy (IFIB)- 8th edition" 27-28th September 2018, Torino, Italy. (Poster)

- **Zanellati A**., Spina F., Romagnolo A., Rollé L., Dinuccio E., Varese G.C. (2019) - Fungal pretreatment on solid fraction of digestate to enhance its reuse as a feedstock for anaerobic digestion plants. In "15th edition of the International Conference on Renewable Resources & Biorefineries". 3-5 June 2019, Toulouse, France. (Abstract and Oral Presentation)

- **Zanellati A.,** Spina F., Poli A., Rollé L., Dinuccio E., Varese G.C. (2019) - Biotechnological Potential of Fungi Isolated from Agricultural Biogas Plants Feedstocks. In "XXXVIII European Culture Collections' Organisation (ECCO) Annual Meeting". 12-14 June 2019, Torino, Italy. (Poster)

- Spina F., Florio Furno M., Poli A., Prigione V., **Zanellati A.**, Puglisi E., Re I., Bava A., Beltrametti F., Gaggero E., Malandrino M., Fabbri D., Calze P., Varese G.C. (2020) - Fungal-based technologies to face (and solve) soil pollution. In "115° Congresso della Società Botanica Italiana; 9-11 September 2020, online. (Meeting abstract in volume)

- **Zanellati A.,** Spina F., Rodriguez F., Martin M., Dinuccio E., Varese G.C., Scarpeci T.E. (2020) - Phenolic Aldehydes and Furfural Degrading Fungi for the Biological Pretreatment of Lignocellulosic Biomass. In "Congreso Conjunto de la Sociedad Argentina de Investigación Bioquímica y Biología Molecular y Sociedad Argentina de Microbiología General - SAIB-SAMIGE 2020", 2 November 2020, online. (Oral Presentation)

- **Zanellati A.,** Spina F., Poli A., Rollé L., Dinuccio E., Varese G.C. (2020) - Incremento delle Rese e della Sostenibilità del Processo di Digestione Anaerobica Mediante l'utilizzo dei Funghi In "13th National Conference on Biodiversity – Biodiversity 2020". 7-9 September 2021. (Abstract and Oral Presentation)

10. Acknowledgments

Throughout the PhD project and the writing of this thesis I have received a great deal of support and assistance.

First and foremost, I would like to express my sincere gratitude to my PhD tutor, Prof. Giovanna Cristina Varese, for allowing me to take part of the *MUT* lab team, for the continuous support at every stage of the research project, for her unwavering patience and for all of the opportunities I was given to further my research. I will always be grateful for introducing me into the fascinating world of mycology and for her belief in me.

I would like to extend my thanks to Dr. Federica Spina, Dr. Anna Poli, Dr. Iolanda Perugini, Prof. Valeria Prigione and the other *MUT* colleagues, for their expert guidance, the encouragement and the precious assistance extended to me during the years. I hope that next years will be as joyful and constructive as those passed together. Special thanks go to my laboratory partner "in crime" Matteo Florio Furno, for the great help and for all the fun we have had together.

I am deeply grateful to Prof. Elio Dinuccio for following me both during the biomass sampling, the experimentation concerning biomethanation and the writing of articles and thesis. Besides, his expertise was invaluable in formulating the research questions and methodology and for the interpretations of the results.

I would like to offer my special thanks to Prof. Telma Eleonora Scarpeci. Thanks to her I was able to travel and discover the wild and marvellous Argentina, and the beautiful people who populate it. Despite everything, by joining forces we managed to obtain excellent results and the publication of a beautiful article. Gracias.

I would like to thank Dr. Luca Rollé and Dr. Mariana Bonaterra for their technical support on my study concerning biomethanation and HPLC, respectively. The technical and scientific support of JRU MIRRI-IT is also greatly acknowledged.

I thank the students Andrea Osella and Giulia Blanchi, who helped the experimental work during their university internship.

I would also like to thank the biogas plants owners, in particular the Agricultural Society Coppo & Garrione S.S., for allowing the sampling of biomass used in the experimental work.

I also thank my dear friends Matteo, Simone and Andrea because they have always supported me throughout my journey.

Thanks also to my other special friends, the “Music” and the “Nature”: life would be meaningless without you.

Finally, I express my gratitude to my parents, Letizia and Luigi, to my brother Denis, and to my beloved Eleonora, for providing me with unfailing support and continuous encouragement throughout my years. This accomplishment would not have been possible without them. Thank you with all my heart.

There is a pleasure in the pathless woods,
there is a rapture on the lonely shore,
there is society, where none intrudes,
by the deep sea, and music in its roar:
I love not Man the less, but Nature more.

George Gordon Byron
Childe Harold's Pilgrimage
(1812-1818)