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Research article

Fungal pretreatment of willow sawdust and its combination with alkaline treatment for enhancing biogas production

Maria Alexandropoulou [a, b], Georgia Antonopoulou [a, *], Efsevia Fragkou [c], Ioanna Ntaikou [a], Gerasimos Lyberatos [a, b]

[a] Institute of Chemical Engineering Sciences, Stadiou, Platani, Patras GR 26504, Greece
[b] School of Chemical Engineering, National Technical University of Athens, GR 15780 Athens, Greece
[c] Department of Chemical Engineering, University of Patras, Karatheodori 1, Patras GR 26500, Greece

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ABSTRACT

In this study fungal pretreatment of willow sawdust (WSD) via the white rot fungi *Leiotrametes menziesii* and *Abortiporus biennis* was studied and the effect on fractionation of lignocellulosic biomass and biochemical methane potential (BMP), was evaluated. Scanning electron microscopy (SEM) and IR spectroscopy were used to investigate the changes in the structural characteristics of the pretreated WSD. Fungal pretreatment results revealed that *A. biennis* is more attractive, since it resulted in higher lignin degradation and lower holocellulose uptake. Samples of the 14th and 30th d of cultivation (i.e. the middle and the end of the pretreatment experiment) with both fungi were used for BMP tests and the effect of pretreatment duration was also evaluated. BMP increase by 31 and 43% was obtained due to the cultivation of WSD with *A. biennis*, for 14 and 30 d, respectively. In addition, combination of biological (after 30 d of cultivation) with alkaline (NaOH 20 g/100 gTS) pretreatment was performed, in order to assess the effect of the chemical agent on biologically pretreated WSD, in terms of lignocellulosic content and BMP. Combination of alkaline with fungal pretreatment led to high lignin degradation for both fungi, while the cellulose and hemicellulose removal efficiencies were higher for combined alkaline and *L. menziesii* pretreatment. The maximum BMP was observed for the combined alkaline and *A. biennis* pretreatment and was 12.5 and 50.1% higher than the respective alkaline and fungal pretreatment alone and 115% higher than the respective BMP of raw WSD.

1. Introduction

Anaerobic digestion (AD) is considered an efficient, cost effective and competitive process for the production of renewable energy from various wastes and residues. During AD, different microbial species are implicated in a synergetic way for the complete degradation of organic substrates and recovery of energy in the form of methane (Narihiro et al., 2015). Among them, fibrolytic species, which attack enzymatically complex carbohydrates, making thus the liberation of sugars and subsequent steps of acidogenesis, acetogenesis and methanogenesis feasible, are included (Ali Shah et al., 2014). Lignocellulosic biomass includes different types of wastes and residues of plant origin, such as softwood, hardwood, yard trimmings, food and paper industry wastes, agro-industrial residues etc. It consists of cellulose, hemicellulose and lignin in varying ratios (Stamatelatou et al., 2012). Cellulose and hemicellulose are the target substrates of bioconversion, whereas lignin, being actually a barrier for the efficient exploitation of lignocellulosic feedstocks, is hardly metabolized by most microbial species (Sawatdeenarunat et al., 2015).

Different types of pretreatment, physical, chemical and biological, have been proposed for the enhancement of the digestibility of such feedstocks, in the effort to enhance AD efficiency and biogas production (Hendriks and Zeeman, 2008; Carrere et al., 2016). The objectives of an efficient pretreatment method should be feedstock delignification, sugar solubilization and cellulose crystallinity reduction, with delignification being the main goal for the enhancement of methane production (Monlau et al., 2012a). Among the different pretreatment strategies proposed so far, biological and alkaline pretreatment methods are considered most promising for enhancing the AD of lignocellulosics, since both

* Corresponding author.
E-mail address: geogant@chemeng.upatras.gr (G. Antonopoulou).

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methods are quite effective in the breakdown of lignin (Carrere et al., 2016).

Biological pretreatment methods present certain advantages over physicochemical, since they are carried out under mild conditions, with low energy demands and without the release of toxic compounds. However, their main drawback is the prolonged treatment times required, which vary from at least one to a few weeks. Among biological pretreatment methods, fungal pretreatment by white-rot fungi is the most effective for selective delignification (degradation of lignin over cellulose). The efficiency of delignification depends significantly on the production of lignolytic enzymes, such as lacasse, lignin peroxidase and manganese peroxidase. *Phanerochaete chrysosporium, Pleurotus ostreatus, Trametes versicolor, Flammulina velutipes and Ceriporiopsis subvermispora* are some of the selective lignin-degrading fungi, which have been used to reduce the recalcitrance of lignocellulosic biomass (Sindhu et al., 2016) and to enhance methane generation (Lalak et al., 2016; Amirta et al., 2006; Zhao et al., 2014). In the present study, fungal pretreatment of lignocellulosic biomass using *Leiotrametes menziesii* and *Abortiporus biennis* is considered.

L. menziesii (=*Trametes menziesii* according to Welti et al. (2012)) belongs to the genus Trametes, which includes some of the most efficient lignin-degrading species. Their lignocellulolytic enzyme system is comprised of laccase and manganese peroxidase (Nakagame et al., 2006), which together with lignin peroxidase are responsible for lignin degradation. The fungus *A. biennis* is also reported to produce laccase and manganese peroxidase with the laccase activity being actually quite high (Erden et al., 2009).

Alkaline pretreatment, through the use of alkaline solutions of NaOH, $Ca(OH)_2$, or ammonia, has been found to be efficient for partial lignin removal and AD (Antonopoulou et al., 2015a, b; Carrere et al., 2016). During alkaline pretreatment, apart from lignin breakdown, an enhancement of the solubilization of hemicellulose to its oligomers is also carried out, while cellulose is affected to a smaller degree (Stamatelatou et al., 2014). In addition, the combination of different pretreatment methods might be considered as an alternative pretreatment strategy, with promising results in fractionation of lignocellulosics (Sindhu et al., 2016). The combination of fungal pretreatment with steam explosion (Sawada et al., 1995), liquid hot water (Wang et al., 2012) or acid pretreatment (Ma et al., 2010) has been reported to result in improved enzymatic saccharification of lignocellulosic biomass, when compared with a single step treatment. To date, the combination of fungal with another pretreatment strategy, aiming at enhancing the final methane yields via AD, has not been considered.

Willow is a hardwood-type biomass, with holocellulose (cellulose and hemicellulose) and lignin contents ranging from 63.7% w/w to 64.5% w/w and 24.5% w/w to 28.8% w/w on a dry mass basis, respectively (Lavoie et al., 2010; Ray et al., 2012; Jurado et al., 2013). It has various uses including landscaping, phytoremediation, hedges, land reclamation, slope stabilization, furniture industry etc, and has also been considered lately as a promising feedstock for second generation biofuels, due to its abundance and relatively fast growth rates (Phillips et al., 2014). The anaerobic digestion of raw (Balasubramanya et al., 1988; Turick et al., 1991) or thermochemically pretreated willow biomass (Uellendahl et al., 2008; Horn et al., 2011; Estevez et al., 2012; Jurado et al., 2013) has already been proposed, leading to promising methane yields. However, to our knowledge, it is the first time that the effect of biological pretreatment of WSD is investigated.

The present study aims at the assessment of the effect of biological pretreatment of WSD on the fractionation of lignocellulosic content and AD efficiency, using two different white rot fungi i.e. *L. menziesii* and *A. biennis*. The effect of pretreatment time was evaluated in terms of lignocellulosic content and biochemical methane potential (BMP). Additionally, for the first time, the combination of fungal with alkaline pretreatment was studied and its effect on the lignocellulosic content and AD efficiency of WSD, was assessed.

2. Materials and methods

2.1. Feedstock

WSD was collected, in January 2015, in the region of Athens, Greece. It was initially air dried, chopped to a size of < 1 mm with a house blender (izzy X3, E560T3, Titanium), milled with a lab grinder (IKA A11 basic) and the final product was collected as powder after passing through a sieve with a pore size of 0.7 mm. Finally, it was air-dried at ambient temperature before being used for the experiments.

2.2. Pretreatment methods used

2.2.1. Fungal pretreatment

2.2.1.1. Microorganisms. *L. menziesii*, strain BRFM 1557 and *A. biennis*, strain BRFM 1215 were kindly provided by the CIRM- CF fungal collection (Banque de Ressources Fongiques de Marseille) on malt agar slants and were maintained at 4 °C. Inoculation cultures were prepared by transferring a ca. 1 cm^2 culture piece on malt agar plates and incubation at 27 ± 0.5 °C for 2 weeks. Subsequently, mycelia from three agar plates were homogenized with 90 mL of sterilized, deionized water in a stainless steel homogenizer, under aseptic conditions and aliquots of the suspensions were used for the inoculation of WSD. Aliquots of the suspensions were also used for total suspended solids (TSS) and volatile suspended solids (VSS) determinations. TSS and VSS values coincided for each fungus, and were 3.6 g/L and 2.4 g/L for *L. menziesii* and *A. biennis* respectively.

2.2.1.2. Experimental set up. For the pretreatment of WSD, two sets of 12 identical solid-state fermentation (SSF) batch cultures were prepared with 6 g sterilized air-dried WSD in 250 mL Erlenmeyer flasks, without any nutrients addition. Inoculation was performed by transferring aseptically 8 mL of mycelia suspension, corresponding to 4.8 mg of fungus/g WSD and 3.2 mg of fungus/g WSD for *L. menziesii* and *A. biennis,* respectively. Two Erlenmeyer flasks with 6 g sterilized air-dried WSD without inocula addition were also prepared and used as blanks. Sterilized deionized water was added to both cultures and blanks in order to achieve 80% humidity, since based on earlier studies the initial moisture content of 70–80% was reported as optimal for lignin degradation and ligninase activities of most white rot fungi (Reid, 1989). Subsequently, all flasks were plugged with hydrophobic cotton and were incubated at 27 ± 0.5 °C. In each sampling at 5, 14, 22 and 30 d, two cultures were removed and forwarded for analysis. Samples of the 14th and 30th d of cultivation period with both fungi, were used for assessing their methane potential, through Biochemical Methane Potential (BMP) tests, in batch reactors. The dry matter loss as well as the lignin, cellulose or hemicellulose loss is given by the Equation (1).

$$\text{Ci loss} = \frac{Ci_{initial} - Ci_{final}}{Ci_{initial}} \quad (1)$$

where $Ci_{initial}$ and Ci_{final} are the TS content concentration when referred to dry mass loss, or the concentration of lignin, cellulose or hemicellulose before and after fungal pretreatment, respectively. It should be noted that the concentration of lignocellulosics after pretreatment are expressed per kg of initial TS, taken into account the dry mass loss during pretreatment.