Using *Leiotrametes menziesii* and *Abortiporus biennis* for biological pretreatment of willow sawdust for enhancing biogas production.

Maria Alexandropoulou¹², Georgia Antonopoulou¹ *, Ioanna Ntaikou¹, Gerasimos Lyberatos¹²

¹ Institute of Chemical Engineering Sciences, Stadiou, Platani, Patras, GR 26504, Greece
² School of Chemical Engineering, National Technical University of Athens, GR 15780 Athens, Greece

*Corresponding author: geogant@chemeng.upatras.gr; tel: +302610965318, fax: +302610965318

Abstract

In this study biological pretreatment of willow sawdust (WSD) via the white rot fungi *Leiotrametes menziesii* and *Abortiporus biennis* was studied and the effect on biochemical methane potential (BMP), was evaluated. Two sets of solid state fermentation (SSF) batch experiments were performed, during which the fractionation of lignocellulosic biomass, was assessed. Scanning electron microscopy (SEM) and IR spectra were used to investigate the changes in the structural characteristics of the pretreated WSD. Samples of 15d and 30d of cultivation period (i.e. the middle and the
end of the pretreatment experiment) were used for BMP tests, in batch mesophilic reactors and the duration of the pretreatment effect, was also evaluated. In addition, combination of biological (30 d cultivation period) 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. For reasons of comparison, only alkaline pretreatment, at the same chemical concentration (20 g NaOH /100 gTS) was performed.

Keywords: delignification, write rot fungi, willow, anaerobic digestion, alkaline pretreatment
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 are included, which attack enzymatically complex carbohydrates, making thus the liberation of sugars and subsequent steps of acidogenesis, acetogenesis and methanogenesis feasible (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, that consist of cellulose, hemicellulose and lignin in different ratios (Stamatelatou et al., 2012). Cellulose and hemicellulose are the target substrates to be bio-converted, 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 have been thus proposed for the enhancement of the digestibility of such feedstocks (Hendricks and Zeeman, 2008), which could further enhance AD efficiency and biogas production.

Willow belongs to hardwood type biomass, with holocellulose and lignin content 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). It has various uses including landscaping, phytoremediation, hedges, land reclamation, slope stabilization, furniture industry etc (Isebrands and Richardson, 2014), 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). Anaerobic digestion of raw (Balasubramanya et al., 1988; Turick et al., 1991) or thermo-chemically 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 being investigated.

The present study aims to assess the effect of biological pretreatment of WSD on AD efficiency, using two different white rot fungi i.e. *L. menziesii* and *A. biennis*. *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 ligninocellulolytic enzyme system is comprised of laccase (Lac) and manganese peroxidase (MnP) (Nakagame et al., 2006), which together with lignin peroxidase (LiP) are responsible for lignin degradation (Kirk and Farrell, 1987). *A. biennis* is also reported to produce Lac and MnP with the Lac activity being actually quite high (Erden et al., 2009).
In addition, the effect of alkaline pretreatment on biologically pretreated WSD was assessed, since through alkaline pretreatment, a partial lignin removal is mainly carried out (Monlau et al., 2012) and it is well known that the lignin content of a lignocellulosic feedstock is negatively correlated to its BMP (Monlau et al., 2013).

2. Materials and methods

2.1. Feedstock

WSD was collected, in January 2015, in the region of Athens, in Attica, Greece. It was initially air-dried, chopped to a size of < 1mm diameter 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. The composition of WSD, used for the experiments, is presented in table 1.

2.2. Pretreatment

2.2.1. Microorganisms

*L. menziesii*, strain BRFM 1557 and *A. biennis*, strain BRFM 1215 were obtained from 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. 1cm² 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 were coincided for each fungus, being 3.6g/L and 2.4g/L for *L. menziesii* and *A. biennis* respectively.

### 2.2.2. Experimental set up

#### 2.2.2.1 Biochemical pretreatment

For the pretreatment of WSD, two sets of 12 identical solid-state 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 5 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. 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, 2 cultures were removed and forwarded for analysis.
2.2.2.2 Alkaline pretreatment

The biologically pretreated samples, after 30 d of cultivation, were also pretreated with an alkaline solution of NaOH at 80 °C for 24 h, at the concentration of 1% w/v, corresponding to a chemical loading of 20 gNaOH /100 g TS. Experiments, in which only alkaline treatment (20 gNaOH /100 g TS at 80 °C for 24 h) were carried out in raw WSD, for comparison purposes. For the alkaline pretreatment methods used, the mass/volume ratio of solid (g TS) to aquatic solution (prepared with deionised water) (mL) was 5:100 (solids load 5% w/v).

2.3. Biochemical Methane Potential (BMP) tests

BMP experiments were carried out in duplicate at 35°C in serum bottles of 160mL, according to the modified protocol of Owen and Chynoweth (1993). BMP experiments were performed for a) biologically pretreated samples after 15 d and 30 d of cultivation, b) for alkaline pretreated WSD samples, and c) for combined alkaline / biologically pretreated samples after 30d of cultivation. Anaerobic sludge from the anaerobic digester of Metamorphosis, Athens wastewater treatment plant, operating at steady state at an HRT of 15 d, was used as inoculum. The main characteristics of the sludge were: pH: 8.14, dissolved chemical oxygen demand, (dCOD): 0.19 g/L, TSS: 2.56 g/L and VSS: 1.2 g/L. For the biologically pretreated samples, 20 mL mixed anaerobic culture, 80 mL water and appropriate amounts of samples were added, in order to acquire the
desirable VS content of 2g VS / L. For the alkaline pretreated samples (raw WSD or biologically pretreated WSD), 20 mL mixed anaerobic culture were seeded with 4mL of the whole pretreatment slurry (mixed liquor of the alkaline pretreated feedstocks at a solids load of 5% w/v), so as the final VS content in the vial being 2g VS / L and deionised water to a final volume of 100 mL. The microbial culture was supplemented with 10 mL/L of a (NH₄)₂HPO₄ (7.21 g/L) solution, 10 mL/L of a FeSO₄.7H₂O (0.7g/L) solution and 10 mL/L of a trace metals solution (Skiadas and Lyberatos, 1998). Control experiments for checking the methanogenic biomass activity were carried out using glucose. Blank experiments were also carried out in order to determine the background gas productivity of the inoculum. The content of the vials was gassed with a mixture of N₂/CO₂ (80/20) in order to secure anaerobic conditions. The vials were sealed with butyl rubber stoppers and aluminum crimps and methane production was monitored as a function of time according to Owen and Chynoweth (1993).

2.4. Analytical methods

Raw samples were air-dried and then used for ethanol extraction (exhausted extraction for 24 h) (Sluiter et al., 2008a) prior to the compositional analysis. Carbohydrate and lignin content were determined according to the National Renewable Energy Laboratory (NREL)’s standard laboratory analytical procedure (LAP) for the determination of structural carbohydrates in biomass (Sluiter et al., 2008b). Then, the extractive free
biomass (0.3 g sample) was used to determine the structural carbohydrates with a two-step acid hydrolysis method. After initial hydrolysis at 30°C with 3 mL of 72% (w/w) sulfuric acid, the samples were diluted with distilled water to a total volume of 84 mL and autoclaved (at 121°C) for 1 h in pressure tubes. Detection and quantification of sugar monomers (glucose, xylose and arabinose) were performed with an HPLC-RI with an Aminex HPX-87H column (Biorad) at 60°C and a Cation H micro-guard cartridge (biorad Laboratories) using H₂SO₄ 0.006 N as an eluent at a flow rate of 0.6 mL/min. Acid soluble and insoluble (Klason) lignin contents were calculated according to NREL’s standard laboratory analytical procedure (Sluiter et al., 2008b) respectively. For the characterization of the biologically pretreated samples, the biomass was suspended in 80 mL deionized water for 1 h at 30°C and the liquid and solid fractions were separated, through filtering with 0.7μm filters. For the characterization of the alkaline pretreated samples, only separation of liquid and solid fractions was made via filtration.

The solid fractions were washed with water, air-dried and characterized as described above for the raw samples, but without performing an extraction process, prior to the characterization. Since for the purpose of chemical compositional analysis, only the solid fraction obtained after pretreatment was used, the solid material recovery due to the loss of weight has been taken into account (figure 1). Thus, in order to express the lignocellulosic content of the pretreated biomass per 100 g of initial TS, the loss of solid
material (g TS /100 gTS\textsubscript{initial}) was multiplied by the values of lignocellulosic content expressed per kg of pretreated TS. The liquid fractions were used for soluble carbohydrates’ content determination, according to Joseffson (1983).

The measurements of total solids (TS), volatile solids (VS), TSS, VSS and dCOD were carried out according to Standard Methods (APHA, 1995). Raw and extractive-free samples were also used to determine Total Kjeldahl Nitrogen (TKN) according to Standard Methods (APHA, 1995). The crude protein content was determined by multiplying TKN by a factor of 6.25.

SEM images were captured using a Zeiss SUPRA 35VP, after coating the samples with a homogeneous Au layer by ion sputtering.

The chemical changes of the structure of raw and pretreated WSD samples were investigated using the Attenuated Total Reflection (ATR) (MIRacle of PIKE technologies accessorize) in an IR BRUKER EQUINOX55 spectrometer. ATR Infrared Spectroscopy is a rapid technique and much easier to use than typical FTIR spectroscopy, by preparing small pellets with KBr. The great advantage is that ATR is a nondestructive method and samples are measured without any pre-treatment. All spectra were taken at a spectral resolution of 4 cm\textsuperscript{-1}. Samples and background were scanned with 100 scans.

3. Results and discussion

3.1 Composition of feedstock used
From table 1, the composition of the WSD used is presented, and it is obvious that cellulose and lignin are the main components representing 35.6 ± 0.9 % and 28.7 ± 0.2% of the dried biomass weight respectively, while hemicellulose represents 21.5 ± 0.9 %. These values are in accordance with other research studies (Jurado et al., 2013).

Table 2: The main characteristics of WSD used in this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS (%)</td>
<td>94.5 ± 1.1</td>
</tr>
<tr>
<td>VS (g/100gTS)</td>
<td>94.1 ± 1.2</td>
</tr>
<tr>
<td>Cellulose (g/100gTS)</td>
<td>35.6 ± 0.9</td>
</tr>
<tr>
<td>Hemicellulose (g/100gTS)</td>
<td>21.5 ± 0.9</td>
</tr>
<tr>
<td>Lignin (g/100gTS)</td>
<td>28.7 ± 0.2</td>
</tr>
<tr>
<td>Extractives (g/100gTS)</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>Ash, (g/100gTS)</td>
<td>5.9 ± 1.6</td>
</tr>
<tr>
<td>Proteins(g/100gTS)</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

3.2 The effect of biological pretreatment on the lignocellulosic content of WSD

Figure 1 summarizes the effect of fungal pretreatment on biomass fractionation in terms of cellulose, hemicellulose and lignin, during the SSF experiments with  L. menziesii and A. biennis, respectively. The values in figure 1 are expressed per kg of initial TS, taking into account that the solid material recovery of  biomass (gTS solid pretreated biomass/g TS initial biomass) after fungal pretreatment was below 100% (figure 1).

From figure 1, it can be seen that the loss of biomass increased with pretreatment time and it was different for the two fungi. For example, the percentage material recovery
after 30 d of cultivation with *L. menziesii* was 67 %, while after treatment with *A. biennis* was 82.4%. In addition, *L. menziesii* was found to be more efficient in delignification than *A. biennis*, after 30 d of cultivation. Thus, the percentage removal of lignin for *L. menziesii* was 30.5% (from 28.7 ± 0.2 to 19.9 ± 0.2 g/100gTS) while for *A. biennis* was 17% (from 28.7 ± 0.2 to 23.8 ± 0.6 g/100gTS). Although, *L. menziesii* resulted in higher lignin degradation, cellulose and hemicellulose uptake was relatively higher than the respective for *A. biennis*, implying that the second microorganism seems to be more efficient for biological pretreatment of WSD. Thus, the cellulose and hemicellulose uptake by *L. menziesii* was 26.6 and 42.3% (from 35.6 ± 0.9 to 26.1 ± 1.1 and 21.5 ± 0.9 to 12.4 ± 0.6 g/100gTS) while for *A. biennis* was just 7.6 and 19.5%, respectively (from 35.6 ± 0.9 to 32.9 ± 1.7 and 21.5 ± 0.9 to 17.3 ± 1.2 g/100gTS) (after 30d of cultivation). From figure 1, it can be observed that for both microorganisms and especially for *L. menziesii*, pretreatment until 22 d effected lignin and carbohydrates degradation, since all fractions decreased with time. However, cultivation periods of 22 and 30 d seem to lead to similar results, implying that the optimum pretreatment time could be in the range of 22 d, for both fungi.
Figure 1: The effect of biological pretreatment on solid material recovery (gTS /100 gTS _initial_) as well as on fractionation of WSD in terms of cellulose, hemicellulose and lignin, during pretreatment with _L. menziesii_ (a) and _A. biennis_ (b), respectively. Samples were taken after 5, 14, 22 and 30 d of cultivation.

The morphology of the untreated and pretreated WSD with both fungi was investigated using SEM. Selected SEM images were taken after 14 and 30 d of pretreatment with _L. menziesii_ and _A. biennis_. It is obvious that raw WSD (figure 2a) had an intact surface structure, with acute edges due to the trimming process, while after 14 d of pretreatment mycelium growth was apparent, with hyphae starting to colonize on the substrate (figure 2b and 2d). A cultivation time of 30d, as anticipated, led to higher mycelium growth, since almost the whole surface of the substrate was covered by the mycelia (figures 2c and 2e).
Figure 2: Selected SEM images of the raw (a), and biologically pretreated WSD with *L. menziesii* (b, c) and *A. biennis* (d, e), respectively after 14 (b, d) and 30 d (c, e) of cultivation.

### 3.3 The effect of alkaline pretreatment on the lignocellulosic content of WSD

The pretreated samples with *L. menziesii* and *A. biennis*, after 30d of SSF, were successively pretreated with 20 gNaOH /100 g TS at 80 °C for 24 h, so as to assess the effect of pretreatment methods’ combination, on the fractionation of WSD. For comparison purposes, addition of only 20 gNaOH /100 g TS at 80 °C for 24 h on WSD was carried out. Figure 3 summarizes the effect of alkaline pretreatment on biomass fractionation in terms of cellulose, hemicellulose and lignin. It should be noticed that the values in figure 3 are expressed per kg of initial TS, taking into account the solid material recovery of biomass (gTS solid pretreated biomass/g TS initial biomass) after alkaline pretreatment, which is also presented in the same figure.
Figure 3: The effect of alkaline pretreatment on solid material recovery (g TS /100gTS initial) and on fractionation in terms of cellulose, hemicellulose and lignin of WSD as well as of the pretreated WSD with *L. menziesii* (menz_NaOH) and *A. biennis* (bien_NaOH), respectively.

It can be seen that the loss of biomass decreased significantly with the addition of alkali and it was similar for both pretreated biomasses. The percentage lignin removal for WSD was 59.5% (from 28.7 ± 0.2 to 11.6 ± 0.0 g/100 gTS) due to combination of alkaline with biological pretreatment with *L. menziesii* and 54% (from 28.7 ± 0.2 to 13.1 ± 0.0 g/100 gTS), due to the combined chemical and fungal pretreatment with *A. biennis*. Lignin removal was also observed during only NaOH pretreatment (from 28.7 ± 0.2 to 19.6 ± 0.2 g/100 gTS). The fact that alkaline pretreatment influences lignin removal is also confirmed by other studies (Monlau et al., 2013). Regarding holocelluloses, the respective fractions of hemicellulose and cellulose removal were
51.1 and 73.5% (from 35.6 ± 0.9 to 17.4 ± 0.1 and from 26.1 ± 1.1 to 6.9 ± 0.6 g/100 gTS) for WSD pretreated with *L. menziesii* and NaOH and 29.2 and 49.8% (from 35.6 ± 0.9 to 25.2 ± 0.5 and 26.1 ± 1.1 to 13.1 ± 0.0 g/100 gTS) for WSD pretreated with *A. biennis* and NaOH, respectively. As anticipated, the lignin and holocelluloses removal efficiency was higher for combined alkaline and biological pretreatment with *L. menziesii* than with *A. biennis*, which could be attributed to higher lignin degradation and higher cellulose and hemicellulose uptake efficiencies for *L. menziesii*. Cellulose was remained unaffected due to alkaline pretreatment, while NaOH pretreatment led to hemicellulose solubilization by 39.2% (from 26.1 ± 1.1 to 15.87 ± 0.2 g/100 gTS).

In figure 4, selected SEM images where the effect of alkaline pretreatment on WSD after 30 d of cultivation with *L. menziesii* (4a) and *A. biennis* (4b), are presented.

![SEM Images](image)

Figure 4: Selected SEM images of the combined alkaline and biological pretreatment with *L. menziesii* (a) and *A. biennis* (b), respectively after 30 d of cultivation.

It can be seen that the fibers are somewhat separated and exposed, compared to the raw samples (figure 2a). A large amount of mass seems to have been removed from the
initial connected structure, while pinholes and gaps are also visible in the treated samples, leading to the speculation that the surface area and the porosity, have also increased.

3.4 The effect of pretreatment on the structural and chemical changes of WSD biomass through IR spectra

IR spectroscopy has been widely used for the investigation of the structural and chemical changes of lignocellulosic feedstocks during various chemical pretreatments (Alemdar et al., 2008). Representative ATR spectra were obtained in this study, in order to determine the effect of pretreatment on the chemical and structural characteristics of WSD. In figure 5, the IR spectra of raw WSD, biologically pretreated WSD with L. menziesii (a) and A. biennis (b), respectively after 14 and 30 d of cultivation, as well as combination of alkaline pretreatment with biological treatments after 30d of cultivation, are presented in a spectral range of 800 to 1800 cm\(^{-1}\).
Figure 5: Representative IR spectra of the raw, biologically pretreated WSD with *L. menziesii* (a) and *A. biennis* (b) after 14 and 30 d of cultivation and the combination of alkaline and biological pretreatment.

As a result of the pretreatments, a decrease of the wide Raman bands in the region 1180-1300 and 1550 -1700 cm\(^{-1}\) corresponding to aromatic skeletal vibrations in lignin, as well as a decrease of the Raman peak centered at ~1735 cm\(^{-1}\) related to carbonyl C=O stretching in lignin are observable.

### 3.5 The effect of pretreatment on BMP experiments

The production of methane from raw WSD is presented in figure 6. The calculated methane production, after subtraction of the methane produced from blank experiments was 19.00 ± 0.86 mL. From figure 6, it is obvious that a significant portion of the produced methane evolved (95%) within the first 30 d. The biological methane potential of WSD was calculated as 95.5 ± 4.3 L CH\(_4\)/kg TS and is similar with the respective of other studies using willow as substrate (Jurado et al., 2013).
A theoretical methane potential (L CH\(_4\)/ kg VS) can be calculated according to the elemental composition of each degradable compounds of the substrate C\(_a\)H\(_b\)O\(_c\)N\(_d\)S\(_e\) (Monlau et al., 2012):

\[
Y_{CH_4}^{theoretical} (L/gsubstance) = \frac{22.4(4a+b-2c-3d-2e)}{8(12a+b+16c+14d+16e)}
\]  

Eq. (1) leads to theoretical methane potentials of 415 mL CH\(_4\)/g cellulose (C\(_6\)H\(_{10}\)O\(_5\))\(_n\), 424 mL CH\(_4\)/g xylan (C\(_5\)H\(_8\)O\(_4\))\(_n\) and 420 mL CH\(_4\)/g proteins (C\(_{14}\)H\(_{12}\)O\(_7\)N\(_2\))\(_n\). Since WSD is composed of 37.8%, 22.8% and 0.7% cellulose, hemicellulose and proteins, respectively, per VS a methane potential of 256.8 L/kg VS can be theoretically expected. The biodegradability (BD) of WSD before pretreatment can be determined as follows:

\[
BD = \frac{\text{experimental yield L/kg VS}}{\text{theoretical yield L/kg VS}} \times 100\% = \frac{101.5}{256.8} \times 100\% = 39.52\%
\]
In figure 7, the BMP of raw and pretreated feedstocks, is presented. Specifically, in figure 7a, the effect of biological pretreatment of WSD, after 14 and 30 d of SSF with both fungi is presented, while in figure 7b the effect of alkaline pretreatment on raw or biologically pretreated samples, is presented. It should be noticed that the values in figure 7 are expressed per kg of initial TS, taking into account the solid material recovery of biomass (gTS solid pretreated biomass/g TS initial biomass) after pretreatments.

Figure 7: The effect of fungal pretreatment (a) after 14 and 30 d of cultivation with *L. menziesii* (menz) and *A. biennis* (bien) on WSD as well as the respective of alkaline pretreatment (b) on WSD and biologically pretreated WSD with *L. menziesii* (menz _NaOH_) and *A. biennis* (bien _NaOH_).

From figure 7a, it can be seen that the methane potential after biological pretreatment with *L. menziesii* decreased compared to the respective of WSD. Specifically, WSD pretreatment for 14 and 30 d with *L. menziesii* resulted in a 8.9 and 34.6% reduction of
the methane yield. This was not observed in the case of biological pretreatment with *A. biennis*, which resulted in a BMP increase of 31 and 43%, after cultivation for 14 and 30 d respectively. The reduction of BMP due to pretreatment could be attributed to the high holocelluloses uptake of *L. menziesii*. The higher the time of cultivation the higher the percentage of cellulose and hemicellulose uptake observed and the lower the BMP measured (26.6% cellulose uptake and 42.3% hemicellulose uptake (after 30d of cultivation)). *L. menziesii* seems to degrade a high portion of the polymeric carbohydrates contained in the WSD, which could not converted to methane by the mixed anaerobic culture.

From figure 7b, it is obvious that alkaline pretreatment enhanced by 91% the methane yield of WSD (182.4 ± 0.2 L CH₄/ kg TS), which could be attributed to the lignin reduction observed, and it is well known that the lignin content of a lignocellulosic feedstock is negatively correlated to its BMP (Monlau et al., 2013). Combination of alkaline treatment with the biological pretreatment enhanced the BMP of WSD (142.2 ± 0.3 L CH₄/ kg TS for the WSD pretreated with *L. menziesii* and 198.7 ± 0.3 L CH₄/ kg TS for the biomass pretreated with *A. biennis*, respectively). These values indicate that the main contribution for the BMP increase, was due to alkaline treatment, especially in the case of the fungal pretreatment with *L. menziesii*. However, maximum BMP was observed for the combined pretreatment with *A. biennis* and NaOH and was 108% and 9% higher than the respective of raw and alkaline treated WSD.
4. Conclusions

Biological pretreatment results revealed that *L. menziesii* resulted to higher lignin degradation with high cellulose and hemicellulose uptake than the respective of *A. biennis*, implying that the second microorganism seems to be more efficient for biological pretreatment of WSD. Observation of the SEM images and IR spectra, corroborates these arguments. Combination of alkaline with fungal pretreatment, after 30 d of cultivation resulted to a very high lignin and holocellulose removal especially for combined alkaline and biological pretreatment with *L. menziesii*. The BMP of WSD used in this study was 95.5 ± 4.3 L CH₄/ kg TS and its BD was 39.52%. Biological pretreatment with *A. biennis*, resulted in a BMP increase by 31 and 43%, for 14 and 30 d cultivation, respectively. Combination of alkaline treatment with the biological pretreatment enhanced the BMP of WSD and the maximum BMP was observed for the combined alkaline pretreatment with biological (with *A. biennis*) and was 108% and 9% higher than the respective of raw and alkaline treated WSD.

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