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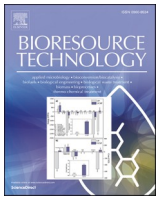

Stimulating biogas production from steam-exploded birch wood using Fenton reaction and fungal pretreatment

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HIGHLIGHTS

- For effective FR, iron-binding to wood was maximized through pH modification.
- Optimum iron dosage was identified based on maximum methane production yield.
- Up to 70 % of the methane produced in the first week for pretreated samples.
- FR and fungal pretreatment of SEBW increased methane yield to 420 mL/gVS.
- Simultaneous FR and fungal pretreatment reduced the lignin content by up to 25%.

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ABSTRACT

Delignification of steam-exploded birch wood (SEBW) was stimulated using a pretreatment method including Fenton reaction (FR) and fungi. SEBW was employed as a substrate to optimize the Fe(III) and Fe(II) dosage in FR. Maximum iron-binding to SEBW was obtained at pH 3.5. FR pretreatment increased biological methane yields from 257 mL/g vS in control to 383 and 352 mL/ g vS in samples with 0.5 mM Fe(II) and 1.0 mM Fe(III), respectively. Further enzymatic pretreatment using a commercial cellulase cocktail clearly improved methane production rate but only increased the final methane yields by 2–9 %. Finally, pretreatments with the fungi *Pleurotus ostreatus* (PO) and *Lentinula edodes* (LE), alone or in combination with FR, were carried out. SEBW pretreated with only LE and samples pretreated with PO and1 mM Fe(III) + H_2O_2 increased the methane production yield to 420 and 419 mL/g vS respectively. These pretreatments delignified SEBW up to 25 %.

1. Introduction

Lignocellulose is a part of the plant cell wall and is the most abundant biomass on earth. This abundance makes carbon-rich lignocellulose a suitable substrate for the anaerobic digestion (AD, a four-step biological process including hydrolysis, acidogenesis, acetogenesis, and methanogenesis) (Hernández-Beltrán et al., 2019). Among others, birch wood *(Betula* spp.*)* is an abundant source of lignocellulosic biomass in northern and eastern Europe, varying between 11 and 28 % of the total volume of wood growth stock in some European countries (Hynynen et al., 2010), making it industrially interesting for biofuel production. Birch wood in unused lands of Northern Europe has high productivity with a short maturing time of 15–20 years when used for biofuel production (S. Hashemi et al., 2021). Lignocellulosic materials are mainly composed of cellulose, hemicellulose, and lignin. The ratio of these components varies due to several factors, including plant family, being hard or softwood, type of species, and age (S. Hashemi et al., 2021). The rigid structure of lignocellulose makes pretreatment a crucial step in disrupting the structure of the lignocellulosic materials. Different pretreatment methods for lignocellulosic materials are extensively reviewed by (Hernández-Beltrán et al., 2019). It is reported that a hybrid pretreatment method including a thermal (e.g., steam explosion) and biological step can significantly improve the methane production yield in AD (Jacquet et al., 2015).

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A study in the Norwegian University of Life Sciences (NMBU) of birch wood could increase the accessible surface for enzymatic hydrolysis and resulted in high methane production yield during AD of birch wood (Vivekanand et al., 2013). Lamb et al. 2019, also showed that birch pretreated at 220 °C for 10 min can improve methane production yield (Lamb et al., 2019). S. Hashemi et al. (2021) employed birch pretreated at 220 °C for 10 min, as a substrate for AD. Cellulose, hemicellulose, and lignin-acting enzyme cocktails were employed to solubilize the SEBW before AD. Low methane yield of pretreated samples was linked to the lignin content of the samples indicating that lignin was inhibiting the enzymatic process (S. Hashemi et al., 2021). Ko et al. (2015) and Kim et al. (2011) suggested that thermal pretreatment of lignocellulosic materials could increase the lignin-like components that can permanently attach to enzymes and reduce their performance (Kim et al., 2011; Ko et al., 2015). Therefore, lignin degradation after steam-explosion is crucial to reduce operational costs through unwanted by-product accumulation in biogas plant where steam-exploded lignocellulose is utilized as the primary substrate.

White- and brown-rot fungi are natural lignocellulose digesters, that can digest cellulose, hemicellulose and lignin using enzyme batteries, including cellulases, hemicelluloses, lytic polysaccharide monooxygenases (LPMOs), lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Lac) (Hegnar et al., 2019; Wesenberg et al., 2003). The porosity of the native wood cell is small in comparison with the size of enzymes (>50 Å in diameter for cellulases and hemicellulases). The enzymes are therefore not able to penetrate the cell wall (Hegnar et al., 2019). However, reactive oxygen species (ROS) have a low molecular weight and can penetrate the wood cell and depolymerize the polysaccharides and lignin. This increases the porosity of the cell wall (Zhu et al., 2022). Therefore, LiP, MnP and LPMO's require H_2O_2 to carry out the initial degradation of wood cell walls, followed by facilitating their enzymatic reactions (Lee et al., 2022).

Delignifying fungi employ a non-enzymatic system called chelator-mediated Fenton reaction (FR), degrading lignin phenolic and non-phenolic components (Liu et al., 2017). Kato et al. (2014) combined the Fe(II)-based FR with fungal pretreatment using white-rot fungi for biomass pretreatment. They reported a 212 % increase in enzymatic saccharification of biomass after FR compared to unpretreated samples, resulting in threefold methane production (Kato et al., 2014). Li et al. (2016) employed dilute acid and FR in a two-step pretreatment method for degrading rice straw. They used FR (with Fe(II))at a pH of 3 and an incubation time of 12 h resulting in 39 % lignin reduction and 71.3 % glucose yield (Li et al., 2016). Almomani et al. (2019) showed that the FR pretreatment of three mixed agricultural solid wastes using Fe(II) at a pH of 3 increased the solubility of the substrates by 3–7 times while improving accumulative methane production by 23–30 % (Almomani et al., 2019). Lamb et al. (2019) revealed that a two-step pretreatment of birch wood using SE and Fe(III)-based FR could increase the methane production yield from 335 mL/gVS in SE pretreated samples to 358 mL/gVS (Lamb et al., 2019). Hegnar et al. (2019) modified an FR reaction mimicking the non-enzymatic saccharification of fungi for pretreatment of the Norwegian spruce. They showed that conducting FR in an optimized condition can significantly contribute to cellulose conversion to glucose, making it a suitable method for biomass hydrolysis for biofuel production (Hegnar et al., 2019). Fungi reduce the pH of the environment as they grow by the generation of the different acidic groups, including phenolic acids and other simple organic acids. In this way, acidic compounds chelate iron from iron oxides. Then iron-reducing compounds will reduce and solubilize iron, making them available for FR (Arantes et al., 2011). Adding iron-reducing compounds such as 2,3-dihydroxybenzoic acid (2,3-DHBA) can reduce and solubilize iron (Zhu et al., 2016). However, it has been shown that in the case of using steam-exploded lignocellulose, addition of iron-reducing compounds is not needed, probably due to the reducing potential of lignin and the presence of organic acids (Lamb et al., 2019). White-rot fungi including *Pleurotus ostreatus* (PO) and *Lentinula edodes* (LE) apply their own FR in the process of lignin degradation by generating ROS.

The FR is an oxidation–reduction process of iron by H_2O_2. FR It is a cyclic reaction involving ferric and ferrous iron, as shown below (Shokri and Fard, 2022).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH.$

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^{+}.$

$H_2O_2 \rightarrow HO\bullet + HOO\bullet + H_2O.$

In this process, highly reactive radicals including hydroxyl (OH^{-}), hydroxyl radical (HO•), proton (H^{+}) and hydroperoxyl (HOO•) are generated that can contribute to the degradation of plant cell wall (Maamir et al., 2017). Among these, hydroxyl radicals are the most potent oxidant (E = 2.8 V versus a standard hydrogen electrode) and highly efficient in the oxidation of organic compounds (Hou et al., 2020). The cycle will proceed if the H_2O_2 is present in the system, meaning that FR efficiency is dependent on H_2O_2; however, the H_2O_2 cannot be presented in AD because of its toxic effects on methanogens and overall inhibitory role (Liu et al., 2017). ROS generated during FR are highly reactive (Almomani et al., 2019). Therefore, it is essential to keep them close to the wood biomass to mitigate the loss of ROS. For this purpose, the iron should attach to the wood structure, minimizing the ROS's distance to the plant cell wall. Optimizing the iron-binding through pH adjustment can maximize the FR efficiency due to minimum ROS loss. According to the literature, the pH range of 3–4.5 is a suitable pH range for conducting FR; however, this pH range is not necessarily the optimum condition for maximum iron-binding, and the iron-binding capacity of ferric and ferrous iron to birch wood is different. These parameters can only be identified through practical experiments. The ROS released from ferrous iron is more active than those released during ferric reduction. It is assumed that these ROS are released relatively early in the system when we have saturated H_2O_2 and ferrous iron. Therefore, one reason for using ferrous and ferric iron was to observe whether the iron type significantly affects the degradation of lignin and biogas production. PO and LE have versatile extracellular enzymes including glyoxal oxidase (GLOX) and aryl-alcohol oxidase (AAO) that can generate H_2O_2 (Daou et al., 2016; Li et al., 2019). It should be noted that H_2O_2-generating enzymes are widely distributed in both white-rot and brown-rot fungi (Xu et al., 2020). Therefore, adding the iron compound during the incubation of PO and LE may facilitate the delignification of SEBW and contribute to the excess methane yield. Moreover, integration of FR and fungal pretreatment may improve lignocellulosic biomass digestion through enzyme produced by fungi during incubation enhancing the delignification and methane production.

FR with only Fe(III) has been previously studied as a pretreatment method for SEBW prior to anaerobic digestion (Lamb et al., 2019). Hydrolysis of SEBW with Fe(II) may potentially increase the biomethane yield of SEBW due to the generation of highly active oxidants such as hydroxyl radical. To the authors' knowledge, previous studies have not investigated the optimum dosages of Fe(II) and Fe(III) for FR of SEBW. Moreover, the potential of using white-rot fungi such as PO and LE in combination with FR for delignification of birch wood has not been studied previously. Overall, this study, for the first time, has attempted to enhance methane production kinetics and yield in AD of SEBW through an optimum pretreatment method using FR and fungi. Therefore, the current work developed a step-by-step optimization process for FR condition to maximize methane production from SEBW. A novel method is employed to select the best conditions for FR through optimizing ferric and ferrous iron-binding to the SEBW structure. An enzymatic hydrolysis step was combined with different dosages of FR to improve pretreatment efficiency. This study's main objective was met by employing an optimum dosage of FR reagents together with PO and LE to stimulate the delignification and potentially extract more methane during AD of SEBW by converting carbohydrates and lignin to methane.

2. Materials & methods

2.1. Raw material

Betula pubescens (Birch wood) from Norway was used as the raw material. Birch wood (BW) dried at room temperature for 12 days was grounded to chips of 15 to 30 mm. The wood was stored in a dry environment to prevent absorbing moisture. The dry matter (DM) and volatile solids (VS) content of the wood chips after dying were respectively 81 ± 2 and 87.6 ± 0.3 % according to standard methods (APHA, 2005). Dried wood chips were further treated by the steam explosion at 220 °C for 10 min as described previously by (Vivekanand et al., 2013). Steam exploded birch wood (SEBW) was packed in vacuum bags and stored in −20 °C to avoid microbial activities. The DM, VS(dry based) of SEBW were 43.3 ± 0.1 and 86 ± 0.3 %, respectively according to standard methods (APHA, 2005). The composition of carbohydrates and lignin was determined according to the NREL/TP-510–42618 protocol (Sluiter et al., 2008). The lignin content of the SEBW was 40.3 %. The carbohydrate content of the SEBW (measured according to NREL/TP-510–42618 protocol), including glucose, xylan, arabinan, galactan, and manan were 42, 14.8, 0.8, 1.2, and 0.05 mg/g DM, respectively.

2.2. Iron binding capacity

The iron-binding capacity of SEBW at different pH was tested by soaking 1 g of SEBW (wet weight) in 20 mL buffer solution containing 0.24 mg $FeCl_3$ (Fe(III)) or $FeSO_4{\cdot}7H_2O$ (Fe(II)). The samples were incubated for two h at ambient temperature without mixing. Since SEBW has a low pH (3.2), iron-biding capacity at a pH of 3.2 was also tested by mixing SEBW and distilled water. In other experiments, Na-acetate buffer was used with molarities of 10, 20, and 50 mM. This provided three different pH ranges: 3.5, 4.5, and 5.5, respectively. Solutions without SEBW added were used as control. After incubation, the samples were centrifuged at 15000 rpm for 10 min and 2 mL of the liquid was separated for measuring iron content using Inductively coupled plasma atomic emission spectroscopy (ICP-OES). For ICP-OES analyses, 0.5 mL of the liquid sample was transferred to new tubes and mixed with 0.5 mL of concentrated HCl. The final sample was then diluted to 5 mL and analyzed by ICP-OES and the absolute amount of Fe was calculated. The difference between iron content in control samples (similar pH and solution without SEBW) and centrifuged samples were used to calculate iron-binding in the different pH solutions.

2.3. Fenton reaction

FR conducted by modifying a method previously developed by (Hegnar et al., 2019). 1 g of SEBW was suspended in 10 mL of 10 mM Na-acetate buffer in pH 3.5. According to the molar mass of the components, a 100 mM solution (in 100 mL distilled water) of ferric iron, ferrous iron, and H_2O_2 was made in different jars. Then, 0.25, 0.5, and 1 mL of solutions were added to the SEBW and buffer solution to conduct FR.

For FR with Fe(III), $FeCl_3$ was added together H_2O_2. For this purpose, $FeCl_3$ was added to the final concentration of 0.25, 0.5 and 1 mM (0.28, 0.55 and 1.1 mg Fe(III)/g SEBW). After two h incubation at ambient temperature without mixing, the H_2O_2 was added to each reaction to the same final molarity as Fe(III) (i.e., 0.25, 0.5, and 1 mM H_2O_2) to achieve a final agent ratio of 1:1. $FeSO_4{\bullet}7\ H_2O$ was used as ferrous iron (Fe(II)) reagent with 1 g of SEBW suspended in 10 mL of 10 mM Na-acetate to conduct the FR. Fe(II) was added with similar molarities as Fe(III) to serve 0.25, 0.5 and 1 mg Fe(II)/g SEBW. The H_2O_2 was added to the mixture corresponding to the amount of Fe(II) added (i.e., 0.25, 0.5, and 1 mM H_2O_2). After 3 h of incubation, the hydrogen peroxide content was measured using Quantofx Peroxide 100 strips from Macherey-Nagels (Düren, Germany), confirming consumption of H_2O_2 (levels below 10 μM). For some samples, more time was needed for the hydrogen peroxide to be reduced below 10 μM in 24 h. Thus, the incubation time of 24 h was selected for all the FRs. The reaction mixtures were incubated at 25 °C for 24 h in an Infors Minitron shaking incubator (Infors, Bottmingen, Switzerland). Nine replicates from each experiment were made. Three were used for biogas production, three for enzymatic hydrolyzation, and three for lignin and composition analysis. After incubation time, pretreated samples were stored at −25 °C before further analysis. Experiment set for the all the pretreatment methods have been provided in Table 1.

2.4. Fenton reactions for fungi pretreatment

10 g of SEBW were mixed with 100 mL of 10 mM Na-acetate buffer solution. Samples in triplicate were prepared by adding 1 mM Fe(III) or 0.5 mM Fe(II) and incubated for two h. After two h, the pH of the substrates was adjusted to 7 by using a 0.4 M NaOH solution. Then the mixture was stored at room temperature to reduce water content and reach around 90 % humidity. Three more replicates were prepared in the same way. Still, before adjusting pH, H_2O_2 was added, corresponding to the amount of Fe(II) and Fe(III) (i.e., 0.5 mM and 1 mM, respectively) initially added. They were incubated at 25 °C for 24 h in an Infors Minitron shaking incubator (Infors, Bottmingen, Switzerland). Then the water content of the samples was removed (using a centrifuge, 15,000 rpm, 10 min) and stored (at −20 °C) to maintain a suitable humidity level for fungi cultivation. During the cultivation, the humidity level of the samples with PO and LE was adjusted at 88–95 % and 62–80 %, respectively (Richard et al., 2020; Schimpf et al., 2019). Upon finishing the pretreatment, the samples were sterilized at 120 °C for 15 min and cooled down at room temperature to prepare them for fungal pretreatment.

2.5. Enzymatic hydrolysis

Enzymatic hydrolyzation of SEBW and samples pretreated with FR was carried out in triplicate using 50 mL screw-capped Falcon tubes, shaking at 110 rpm at 50 °C for 96 h as described previously by (Hegnar et al., 2019; Vivekanand et al., 2013). The pH of the samples was adjusted using Na-acetate buffer, pH 5.5, to a final concentration of 50 mM. The substrate concentration in the tube was 45 ± 0.3 g TS/L. For enzymatic hydrolyzation, 8 mg Cellic Ctec2 (Ctec, Novozymes, Bagsvaerd, Denmark) per g of the substrate was added. Enzyme activity was 7.8 mg/mL enzyme measured as protein using the Bradford assay (BioRad, Hercules, USA). Glucose content in the samples was measured during the experiment at 0, 3, 6, 12, 24, and 96 h by taking 0.5 mL. The samples were analyzed in high-performance liquid chromatography (HPLC) using an Agilent 1100 Series instrument (Santa Clara, USA). The instrument had an HP-X87H column (BioRad, Hercules, USA) and a Shimadzu Refractory Index Detector (Kyoto, Japan). The mobile phase was four mM H_2SO_4 with a 0.6 mL/min flow rate at 50 °C with 22 min per sample run time. After incubation, the tubes were incubated in boiled water for 5 min to terminate enzyme activity. The samples were then stored at −25 for further processing.

2.6. Fungal pretreatment

Liquid cultures containing white-rot fungi *Pleurotus ostreatus* (PO) and *Lentinula edodes* (LE) were provided by Mycelia AS (Belgium). The liquid culture was mixed by the magnet stirrer and placed on the malt agar plates (1.5 g/L) to evaluate the purity of the cultures. After seven days of incubation, the hyphae covered the plate, and no sign of contamination was observed. The suspended hyphae were observed in the liquid cultures after two weeks. The resulting cell suspension was employed to degrade the lignin in the SEBW.

Before fungal pretreatment, different samples were prepared to potentially stimulate the lignin degradation and biogas yield by fungi pretreatment by providing iron source and FR. For this purpose, 20 g of the substrate was impregnated with 1 mM $FeCl_3$ or 0.5 mM $FeSO_4$ • 7

Table 1
Design parameters utilized for experimental set-up for anaerobic digestion. In addition to the parallels for AD, three parallels from each experiment were provided for lignin and carbohydrate analysis.

	Number of parallels for AD	Substrate	$FeCl_3$	$FeSO_4{\cdot}7H_2O$	H_2O_2	Ctec	*Pleurotus ostreatus*	*Lentinula edodes*
		gr	mM	mM	mM	mg	mL	mL
SEBW	3	1	–	–	–	–	–	–
SEBW + Ctec	3	1	–	–	–	8	–	–
0.25 Fe(III) + H_2O_2	3	1	0.25	–	0.25	–	–	–
0.25 Fe(III) + H_2O_2_Ctec	3	1	0.25	–	0.25	8	–	–
0.25 Fe(II) + H_2O_2	3	1	–	0.25	0.25	–	–	–
0.25 Fe(II) + H_2O_2_Ctec	3	1	–	0.25	0.25	8	–	–
0.5 Fe(III) + H_2O_2	3	1	0.5	–	0.5	–	–	–
0.5 Fe(III) + H_2O_2_Ctec	3	1	0.5	–	0.5	8	–	–
0.5 Fe(II) + H_2O_2	3	1	–	0.5	0.5	–	–	–
0.5 Fe(II) + H_2O_2_Ctec	3	1	–	0.5	0.5	8	–	–
1 Fe(III) + H_2O_2	3	1	1	–	1	–	–	–
1 Fe(III) + H_2O_2_Ctec	3	1	1		1	8	–	–
1 Fe(II) + H_2O_2	3	1	–	1	1	–	–	–
1 Fe(II) + H_2O_2_Ctec	3	1	–	1	1	8	–	–
								–
PO	5	20	–	–	–	–	10	–
PO_Fe(II)	5	20	–	10	–	–	10	–
PO_Fe(III)	5	20	20	–	–	–	10	–
PO_Fe(II) + H_2O_2	5	20	–	10	10	–	10	–
PO_Fe(III) + H_2O_2	5	20	20	–	20	–	10	–
LE	5	20	–	–	–	–	–	10
LE_Fe(II)	5	20	–	10	–	–	–	10
LE_Fe(III)	5	20	20	–	–	–	–	10
LE_Fe(II) + H_2O_2	5	20	–	10	10	–	–	10
LE_Fe(III) + H_2O_2	5	20	20	–	20	–	–	10

H_2O described previously and incubated for 2 h without mixing. Other samples were prepared to assess the effect of FR on lignin degradation by fungi. 20 g of SEBW pretreated with FR with 1 mM $FeCl_3$ and H_2O_2 or 0.5 mM $FeSO_4 \bullet 7\ H_2O$ and H_2O_2 as described in section 3.4. All the samples were incubated in triplicate. From each batch, one sample was used for lignin analysis and determination of carbohydrates, and one sample was used for biogas production in batch reactors. After pretreatment, the carbohydrate and lignin content of samples were determined according to NREL/TP-510–42618 protocol (Sluiter et al., 2008).

Fungal pretreatment was conducted by adding 10 mL of cell suspension on 20 g (50 % v/w) of sterilized samples in 200 mL glass bowls with sterilized caps. The caps were equipped with a 0,22 μm syringe filter to enhance the airflow into the system and a self-sealing port for injecting liquid culture. Along with samples, two control cultures were colonized on malt-agar plates to assess the risk of contamination during the experiment. The substrate humidity was maintained at around 88–95 % for PO and 62–80 % for LE. The incubation was conducted at room temperature for six weeks. The initial and final conditions were considered in this experiment. Therefore, the samples for analysis were collected only at the beginning of the investigation and at the end. Before adding liquid culture, the pH was adjusted to 7 by 0.04 M NaOH. This part used samples with only SEBW and fungi as a control. After six weeks, the fungus was killed by incubating the glass bowls in a water bath at 95 °C for 15 min, and the samples were stored in −25 °C for further processing.

2.7. Inoculum

The microbial inoculum was collected from a 10-liter pilot-scale continuous stirred tank reactor (CSTR) fed by SEBW and cow manure for 120 days. The reactor was operated in mesophilic condition (40 °C). The inoculum was incubated at 40 °C for ten days to reduce endogenous biogas production. DM and vS of the inoculum were 6 % and 4.75 %. The inoculum had a pH of 7.2 and an ammonium concentration of 890 mg/L.

2.8. Biomethane potential

100 mL gas sealed medical syringes in triplicated were used to investigate the biogas production potential (BMP) of pretreated substrates as described previously by (Østgaard et al., 2017). For this purpose, 30 mL of inoculum was poured into the syringe, and then 1 g of pretreated sample was added. After defrizzing the samples at room temperature, samples (e.g., including separated liquid, watery samples of pretreatment steps such as FR and/or by the enzyme, and the fungi-SEBW mixtures) were transferred directly to the syringes to avoid losing soluble volatiles. Three syringes were filled with only inoculum to act as a negative control. The control biogas was subtracted from all other samples to exclude endogenous biogas production. Three control syringes containing 1 mL boiled enzyme were made to exclude the biogas produced from protein in the enzyme. The biogas from these controls was subtracted from the enzymatic pretreated samples. For fungal pretreated samples, five parallels were employed mainly due to the valium of pretreated samples. After preparing the batch test, the syringes were sealed and caped by an airtight on/off valve. The biogas was released regularly and measured by volume marks on the syringe. Gas composition was analyzed by a SRI 8610C gas chromatograph (GC) (SRI Instruments, USA). The gas volume was measured at 40 °C and at atmospheric pressure. Then the gas volume was converted to the corresponding volume at room temperature (25 °C) using the ideal gas law ($V_2 = V_1{*}0.952$), where V_2 is the gas volume at room temperature, V_1 is the gas volume at 40 °C and 0.952 is the ratio of Kelvin-based temperatures. Proceed samples were kept at 40 °C for 40 days. Content of syringes was frequently mixed by shaking.

2.9. Composition analyses

Analysis of the dry matter (DM), volatile solids (VS) and ash content were determined using standard methods (APHA, 2005). Lignin and monosaccharide composition of SEBW and pretreated samples were analyzed according to NREL/ TP-510–42618 (Sluiter et al., 2008). For lignin content, the measurements have been run in triplicates. Klason lignin was calculated gravimetrically, while soluble lignin was measured

in UV/Vis spectrophotometer with a wavelength of 205 nm. After two steps of acid hydrolyses, high-performance liquid chromatography (HPLC) was utilized to quantify the carbohydrates in the samples. The HPLC was equipped with an Aminex HPX-87P column (BIORAD, 300 nm × 7.8 nm, 9 μm particle size), a carbo-P guard column (BIORAD), and a refractive index (RI) detector (Perkin Elmer). RI detector was operated at 50 °C and an oven temperature of 85 °C. The injection volume was 30 30 μl with 0.25 mL/min of flow rate for the mobile phase.

3. Results and discussion

3.1. pH selection for iron binding

In this study, it has been investigated if the efficiency of the FR is a function of iron compounds bound to the SEBW substrate, potentially linking FR activity to the iron-binding capacity of the SEBW (Hegnar et al., 2019). Iron binding to the wood structure is important for maximizing the FR activity inside the wood (Arantes et al., 2011). Arantes et al. (2009) has shown that Fe(II) has very poor binding capacity to the wood cell wall matrix in comparison with Fe(III) (Arantes et al., 2009). However, due to the disruption in the wood structure after the steam explosion pretreatment, the Fe(II) binding to the SEBW substrate might be improved. To investigate the Fe(II) and Fe(III) binding to the SEBW 1 g gr of the substrate was incubated at pH levels of 3.2, 3.5, 4.5, and 5.5. Previous research has shown that a pH between 3 and 4 is suitable for conducting FR for lignocellulosic materials using Fe(III) (Huang et al., 2020). Iron concentration in liquid was measured using ICP-OES and the results of the iron-binding are provided in Fig. 1. For all conditions, Fe (II) had a higher binding capacity than the Fe(III). Fe(III) had a poor binding capacity in water (pH 3.2), while the Fe(II) binding capacity in the water was 50-fold higher. In general, the binding capacity of Fe(III) in the buffered solution improved significantly. The maximum Iron binding took place in the samples when the 10-mM Na-ac buffer solution was employed at a pH of 3.5. Hence, this buffer solution and pH level were selected for conducting all the experiments in this study.

3.2. Selection of iron dosage

Reactive radicals generated during FR may contribute to degradation of polysaccharides and lignin (Almomani et al., 2019). These reactions can potentially convert woody biomass to soluble sugars and lignin fragments that will be utilized as substrates in the AD process for methane production. One of the objectives of this study was to obtain a suitable iron dosage for FR for fungus pretreatment of SEBW. For this purpose, three different dosages of the Fe(III) and Fe(II) (i.e., 0.25, 0.5, and 1 mM) were added to the SEBW and buffer solution at a pH of 3.5 and incubated together with H_2O_2. In addition to chemical hydrolysis of SEBW using FR, parallel experiments were conducted using FR in combination with a cellulase cocktail (Cellic Ctec2) to mimic how fungi may degrade wood. All the experiments were conducted in triplicate. After treatment all the samples were digestated anaerobically and the accumulated biomethane on days 5 and 40 have been provided in Fig. 2. The accumulated methane after ten days constituted for all the samples over 75 % of the total biomethane produced during the entire period, except for SEBW without further pretreatment (68 %) (data is not presented in here). The biomethane production kinetic in 8 samples pretreated with FR reagents and cellulases on day 5 was higher from 63 to 85 % in all the samples meaning that the cellulase addition had significant effect on the kinetic of methane production rather that the yield.

Maximum biogas production from control (only SEBW) without adding enzyme cocktail was 256 mL/ g vS This is very similar to the values previously reported from batch anaerobic digestion of SEBW using similar steam explosion conditions (S. Hashemi et al., 2021). After enzymatic pretreatment of the SEBW, this value increased to 278 mL/ g vS It should be noted that all the experiments which included iron reagents, either with or without cellulases, improved the biomethane production yield. The highest biomethane production was achieved when 0.5 mM Fe(II) was employed as the FR reagent. For this condition, a biomethane production after 40 days of 383 and 393 mL/ g vS was achieved for samples without and with cellulases, respectively. Apart from the additional methane production yield, all the samples containing cellulase had higher biomethane production kinetic, specifically in the first week of the incubation. The samples with 1 mM Fe(III) had maximum accumulative biomethane production after 40 days of 352 mL/ g vS and 384 mL/ g vS for samples without and after further pretreatment with cellulases, respectively.

Overall, methane production in experiments with Fe(II) as the FR reagent yielded higher methane production (especially in lower dosages) compared to corresponding experiments with Fe(III). There can be two potential hypotheses involved in this phenomenon. First, highly active hydroxyl radicals from Fe(II) oxidation during FR can potentially enhance the hydrolyzation of lignocellulosic materials (Du et al., 2020),

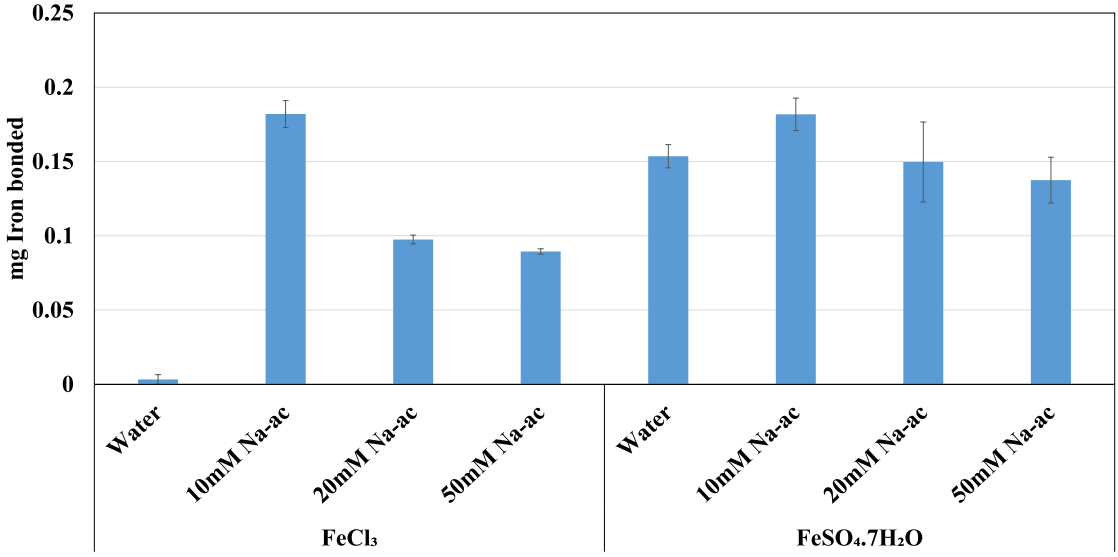

Fig. 1. Iron binding capacity of $FeCl_3$ and $FeSO_4{\cdot}7H_2O$ The binding capacity is presented as the total iron remaining in the liquid samples. The analysis has been calibrated with distilled water as a baseline. The maximum amount of iron in all the samples was 0.25 mg. The difference between iron concentration in the samples with SEBW and without SEBW has been reported as iron-binding capacity of SEBW.

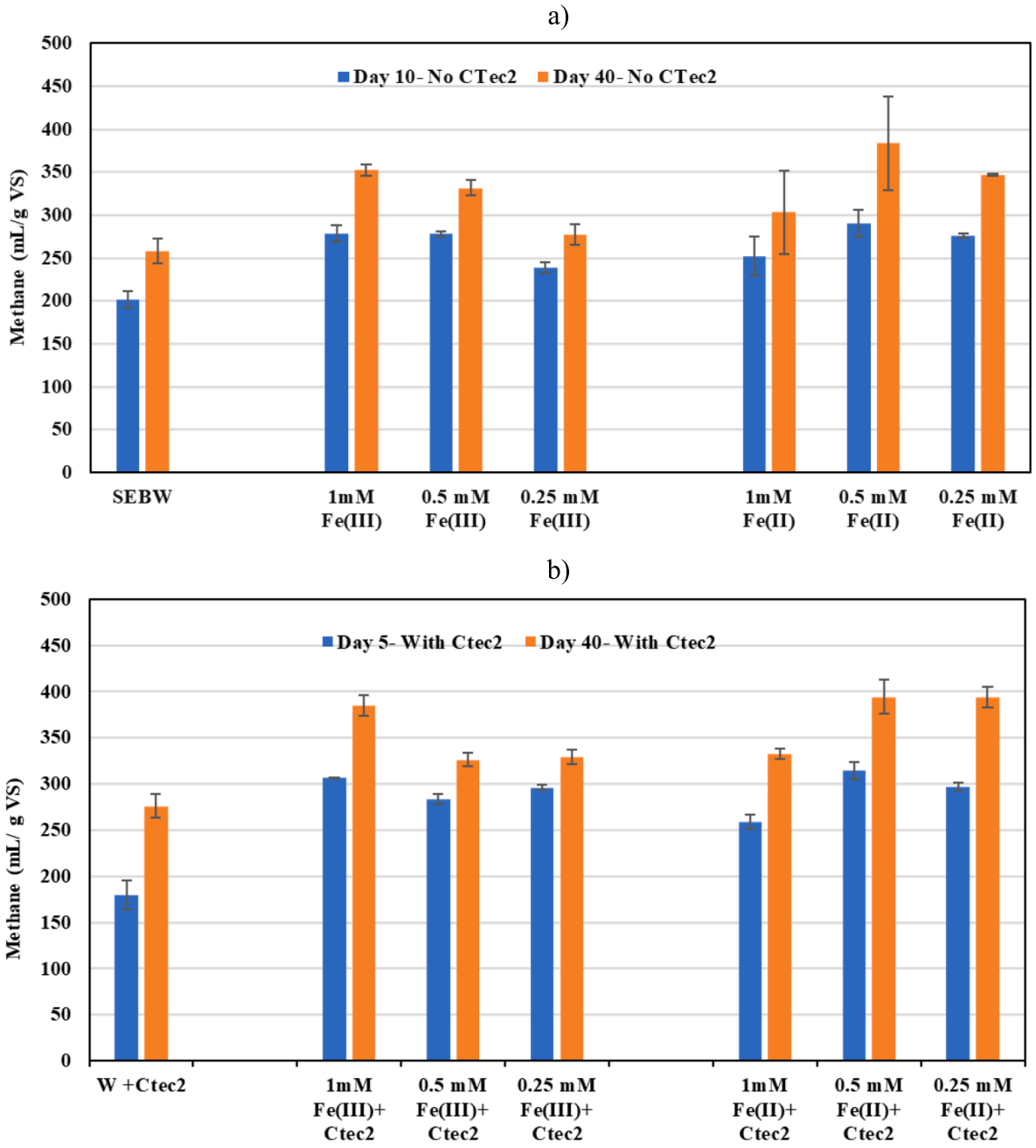

Fig. 2. Accumulative methane production of experiments pretreated with only Fenton-like reactions in different dosages (a), and samples pretreated with Fenton-like reaction as well as enzyme Ctec (b) in day 5 and day 40.

and second, the sulfur content of $FeSO_4$, in very low dosages, can potentially contribute to biomethane yield improvement through stimulating the electron transfer among syntrophic microorganisms by generating iron-sulfate proteins (e.g., ferredoxin) (Wagner et al., 2017). However, these hypotheses cannot be addressed with the current results.

The maximum methane yield of the experiments with 1 mM Fe(III) and 0.5 mM Fe(II) were 36 and 49 % higher than that in control samples with only SEBW. Moreover, the biomethane production of FR reagent candidates with the highest biomethane production after one additional enzymatic hydrolysis step was increased by 9 % and 2 % for 1 mM Fe(III) and 0.5 mM Fe(II), respectively. Since the accumulative biomethane after additional enzymatic pretreatment did not increase significantly after 40 days of incubation, this may suggest that the Fenton reactions can potentially act as the chemical equivalent of cellulose- and hemicellulose-acting enzyme, increasing the biomethane production rate of SEBW (Niu et al., 2022). Regardless of the amount of methane produced after 40 days of incubation, the most important effect of enzyme pretreatment was on the methane production kinetics, so that the amount of methane produced in the first 5 days was significantly higher (63–85 %) in the samples pretreated with enzyme. In a full-scale biogas production plant, this can allow a significant improvement in annual biogas production yield, without having to increase the size of the anaerobic digester.

Fig. 3 summarizes the average methane production yields and lignin content in experiments with different dosages of iron reagents. As shown in Fig. 3.a, methane production for SEBW is 257 mL/g vS while this value increased to 275 mL/g vS when using cellulase enzymes. Pretreating SEBW using 0.25 and 0.5 mM Fe(II) increased methane

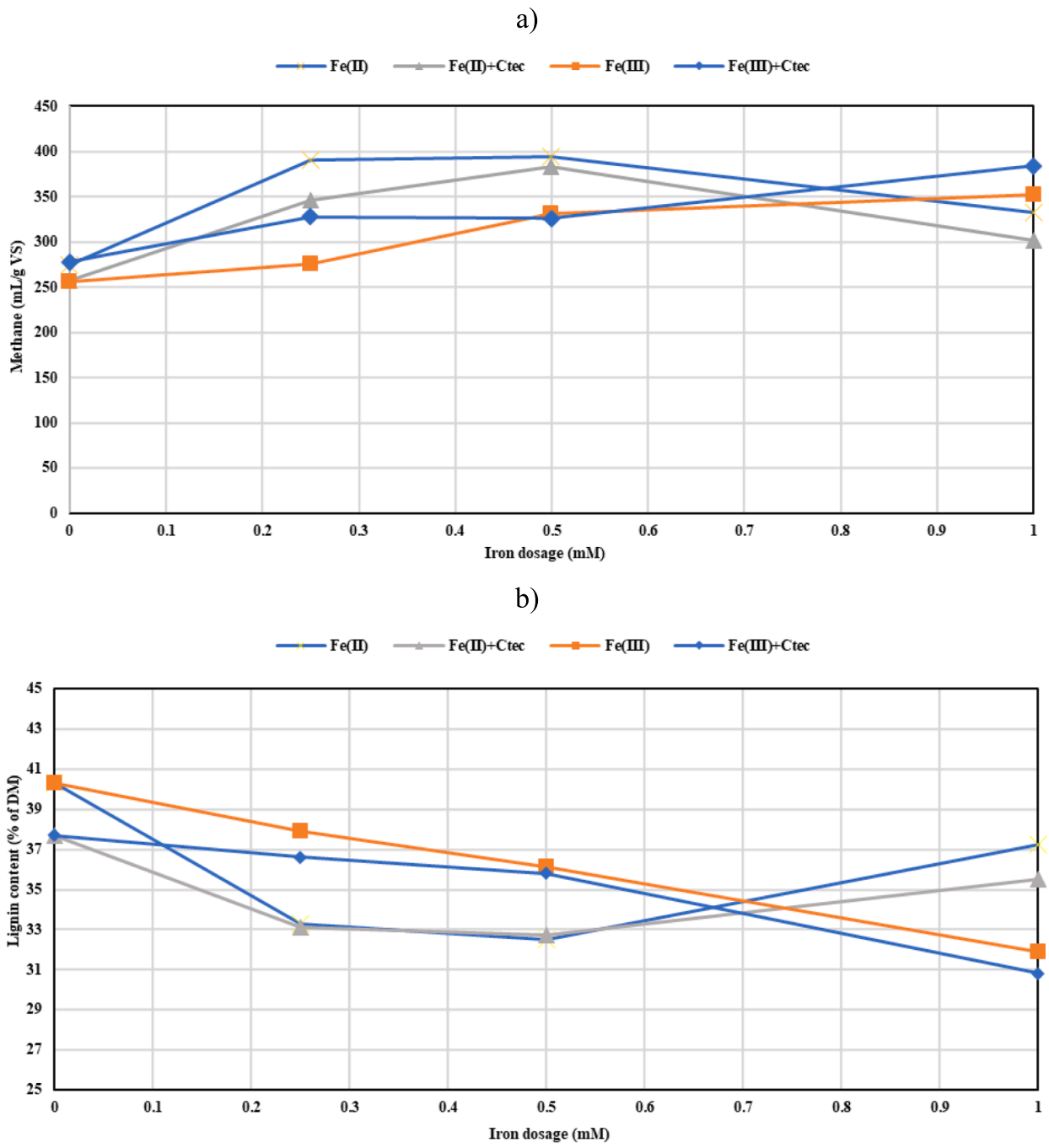

Fig. 3. Correlation of iron dosage with methane production (a), and lignin degradation (b). Average methane production yield and lignin content have been presented with 95 % confidence level.

production to 346 and 383 mL/g vS respectively. By increasing Fe(II) dosage to 1 mM, the methane yield dropped to 302 mL/g vS Additional pretreatment with enzyme shows similar trends where the highest methane yield using Fe(II) (394 mL/g VS) was achieved using 0.5 mM Fe (II). For ferric iron, methane production yield is closely correlated with Fe(III) dosages; by increasing the Fe(III) dosages from 0 to 1 mM, methane production was increased. The highest methane production was 352 mL/g vS via 1 mM Fe(III) as an FR reagent. Further enzymatic pretreatment showed a similar trend and increased the final methane yield to 384 mL/g vS using 1 mM Fe(III).

Fig. 3.b shows the average lignin content of the SEBW and pretreated samples with and without enzymatic saccharification. The lignin content of the substrate increased from 21 % (of dry matter) in non-pretreated samples to over 40 in SEBW due to pseudo-lignin formation. Enzymatic pretreatment reduced the lignin content of SEBW to 37.7 %. Moreover, employing Fe(II) for FR reduced the lignin content to a minimum of 32.7 and 32.5 % using 0.5 mM Fe(II)with and without enzyme, respectively. The lignin content was in the range 35.5–37.2 for all experiments using 1 mM Fe(II) as the FR reagent. As for Fe(III), the lignin content was reduced to a minimum of 31.9 % using 1 mM Fe(III). Further pretreatment of SEBW using 1 mM Fe(III) and enzyme reduced the lignin content to the lowest amount of 30.8 %. The FR using Fe(III) had the highest delignification potential among all the pred-treatment presented in this study.

Methane produced from SEBW in the original samples without further pretreatment was 257 mL/g vS Vivekanand et al. (2013)

reported a maximum methane production of 369 mL/gvS from SEBW (pretreated at 220 °C for 10 min). They suggested that SE could also positively affect enzymatic saccharification (Vivekanand et al., 2013). The lower methane production here, might be linked to the type of inoculum and also the original substrate that has bee used for this study (may contain some barks, diluting the carbohydrate content). Mulat et al. (2018) reported methane production improvement during AD of SEBW pretreated at 210 °C for 10 min and lignocellulose hydrolysis microorganisms. They reported a maximum methane production yield of 196 mL/gvS (Mulat et al., 2018). Costa et al. (2019) employed several hydrolysis enzymes and H_2O_2 together with the SEBW to assess the methane production yield. They reported a methane yield improvement from 110 mL/gvS in samples with only birch wood to over 200 mL/gvS in samples with enzyme and H_2O_2 (Costa et al., 2019). Matsakas et al. (2020) employed a combined organosol-SE for the pretreatment of birch wood. They reported a maximum methane production yield of 327.2 mL/g vS with high-speed methane production kinetics in thermophilic conditions (over 90 % of total methane generated in 120 h) (Matsakas et al., 2020). Hashemi et al. (2021) pretreated SEBW with lignin-degrading enzymes. The maximum methane production yield (345 mL/gVS) was achieved by enzymatic hydrolysis of the SEBW at room temperature. Over 40 % of the lignin was degraded in a similar pretreatment condition (S. Hashemi et al., 2021). It has previously reported by Lamb et al. (2019) that FR using 1 mM Fe(III) has led to a maximum biomethane production yield of 358 mL/ g vS using SEBW, which was lower than that achieved by Fe(III) and Ctec that has been reported in this study (Lamb et al., 2019). The highest methane production yield of 394 and 384 mL/gvS achived when 0.5 mM Fe(II) and 1 mM Fe(II0 was employed together with enzyme.

Looking at Fig. 3.a and 3.b, the biogas production seems to improve as the lignin content is reduced. Thus, the more delignification, the more methane has been produced supporting the idea that the degraded lignin may contribute to methane production in AD (Mulat and Horn, 2018).

3.3. Fungal pretreatment

SEBW was employed as the main substrate to cultivate two different with-rot fungi (*Pleurotus ostreatus*, PO, and *Lentinula edodes*, LE) to increase biomethane production and potentially reduce the lignin content. Before the fungi pretreatment, the SEBW was mixed with ten mM Na-ac, and 1 mM Fe(III) and 0.5 mM Fe(II) were added to the mixture and incubated for 2 hr. For some of the samples, the FR was conducted prior to the fungal pretreatment, as explained in section 3.4. After incubation of each sample, the liquid was removed to avoid loos of sugars and stored at −20 °C. The separated liquid was mixed with a fungi-SEBW mixture before introducing samples to AD.

After two weeks, the mycelium covered the surface of the substrates (Fig. 3, right). Overall, it was observed that the samples including PO were covered by mycelium faster (especially in samples including Fe(II)) than those with LE. After six weeks, the mycelium in all the samples had a compact soft-fluffy texture in white with a thickness of approximately 1.5 mm. No visible sign of contamination was observed on the surface of the control plates. Some changes in the appearance of the SEBW were observed during the pretreatment using PO and LE. In the original samples of SEBW, wood fibers were visible in tiny sizes, while in fungal pretreated samples, the fibers were seemed degraded.

The average biomethane potential of pretreated SEBW using fungi and FR have been provided in Fig. 4. Except for the LE_Fe(III)_ H_2O_2 that produced only 2 % higher methane from the control, all the experiments pretreated by fungi had an increase in biomethane production yield compared to control from 24 to 64 %. AD of LE-pretreated SEBW gave the highest methane production of 420 mL/ g vS (64 % improvement compared to experiments with only SEBW), the highest methane production yield ever reported from the AD of SEBW. The PO_Fe(III) + H_2O_2 had the second-highest methane production (419 mL/ g vS 63 % higher), followed by PO_Fe(II) + H_2O_2 (396 mL/ g vS 54 % higher). For PO, a clear positive trend can be observed in the methane production yield of samples by including FR treatment. It has previously been claimed that *Pleurotus ostreatus* can utilize H_2O_2 to produce active radicals (Wang et al., 2018). Moreover, H_2O_2 is an essential component in activating some of the fungal enzymes including LiP and MnP (Andlar et al., 2018; Dashtban et al., 2010). These peroxidases may have degraded part of the lignin fraction and thus contributed to the improved biomethane production after adding H_2O_2. In contrast, adding iron and H_2O_2 to LE inhibited the biomethane production, even though the growth of mycelium could be observed on the substrate. Hatvani and Mécs (2010)

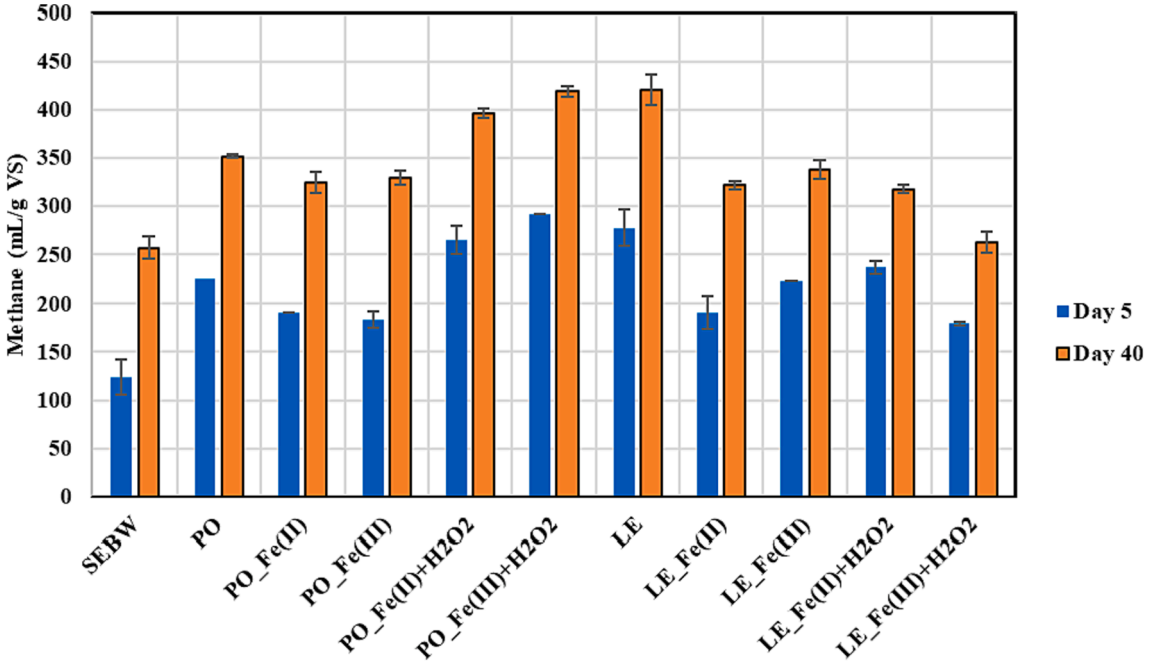

Fig. 4. Accumulated methane production of experiments pretreated with only fungi (*Pleurotus ostreatus*, PO, and *Lentinula edodes*, LE), experiment with addition of iron, and experiments pretreated with both iron and Fenton reaction in day 5 and day 40.

showed that the iron content in the samples could have a negative effect on the enzymatic lignin degradation by LE (Hatvani and Mécs, 2003). Ferric and ferrous addition prior to fungal pretreatment with either PO or LE did not increase the methane production yield compared to experiments with only PO or LE.

This study observed a tangible difference between FR using ferric and ferrous iron. It was shown that ferrous iron's binding capacity to SEBW structure was higher than ferric iron. The ferrous iron can be employed in a broader pH range. In contrast, the ferric iron capacity was susceptible to the pH and the buffer solution used in the experiments. According to the experimental results, a pH of 3.5 using 10 mM Na-acetate buffer solution was the best operational condition for conducting the FR. The most effective iron reagent for FR was selected according to the samples' maximum methane production yield and dignification level. It was shown that the highest methane yield and lowest lignin content were achieved using 0.5 mM Fe(II) and 1 mM for Fe(III). Combined FR and fungal pretreatments improved methane yields up to 420 mL/g vS however, the relative long pretreatment process is in this case a disadvantage. The results also indicated that the delignification by FR and enzyme processes was more successful than using fungi and FR. Overall, selecting the optimum pretreatment process among the proposed approaches depends on the implementation of the process at full scale including potential applications of produced fungal biomass.

3.4. Lignin content and theoretical methane production

The lignin and carbohydrate content of all the experiments have been reported in Table 2. The lignin content of birch wood increased after the steam explosion from 20 % (of DM) to just above 40 %. Likely, the lignin and some of the sugar degradation products have polymerized during pretreatment to form a lignin-like structure (known as pseudo-lignin) that will be measured as in Klason lignin (Duverger et al., 2020; Vivekanand et al., 2013). Employing FR as a pretreatment method could reduce the lignin content of the SEBW by up to 25 % (FR, 1 mM Fe (III)). As expected, further treatment with the cellulase cocktail did not significantly affect the content of lignin. Prousek (2007) reported that H_2O_2-derived active radicals could damage the enzymes; therefore, residues of active radicals may contribute to the inactivation of the lignin degrading enzymes (Prousek, 2007). Moreover, it has been suggested that high lignin content can inhibit the enzymatic hydrolysis since the enzymes (e.g., cellulases) will be permanently attached to the lignin-like components (Li et al., 2018). Table 2 shows that the samples pretreated with FR and enzyme have higher methane production yield while the lignin content has been reduced in these samples. The enzyme has actively degraded cellulose, increasing the glucose content in the samples. Therefore, the authors did not identify enzyme deactivation during the pretreatment. The FR was conducted before the enzymatic hydrolysis to mitigate the enzyme deactivation. Optimizing the iron-biding and more prolonged FR incubation minimized the concentration of free active radicals, reducing the risk of enzyme deactivation. LE, PO_Fe(II) + H_2O_2 and PO_Fe(III) + H_2O_2 had the highest delignification among fungal pretreated simples and reduced the lignin content by 12.5 to 15 %. However, samples pretreated bye PO in general had lower average lignin content than those pretreated by LE.

Theoretical Biomethane potential (TBMP) of pretreated and control samples was calculated using 415 and 727 mL CH_4/g for carbohydrates and lignin as proposed by (Møller et al., 2004). Biomethane production potential (BMP) of LE pretreated SEBW was the highest BMP among all the experiments (420 mL/ g VS). Considering the composition of the substrates (provided in Table 2), the maximum TBMP of LE was 521 mL/ g vS From that, around 300 mL/ g vS (57 % of TBMP) could be produced from carbohydrates. BMP of the LE experiment was 80 % of the theoretical biomethane. The difference between lignin content in BW and SEBW was 20 %, giving TBMP of around 145 mL/g. Since steam explosion potentially can disrupt the structure of lignin and make it available for AD, the lignin-like components because of steam explosion may potentially contribute to biomethane production. In particular, the formed pseudo lignin may be converted to methane during AD (Vivekanand et al., 2013). Lignin degradation during anaerobic conditions is known to be difficult (Mulat and Horn, 2018), yet several studies have reported anaerobic lignin degradation (Billings et al., 2015). Lignin in hardwood can decomposed into degradable components, including guaiacyl and syringyl (Sheng et al., 2021). During the decomposition of

Table 2
Lignin and carbohydrate content of the original and pre0treated samples. The theoretical biomethane production (TBMP) from lignin ($TBMP_{Lig}$) and from carbohydrates ($TBMP_{CH}$) is provided. Total TBMP is summation of $TBMP_{LIG}$ and $TBMP_{CH}$. BMP is accumulative methane produced in day 40.

	Lignin[a]	Ara[a]	Gal[a]	Glu[a]	Xyl[a]	Theoretical Methane from Lignin [b]	Theoretical Methane CH [b]	Total Theoretical Methane [b]
BW	20	1	2	36	17	145	232	377
SEBW	40	1	1	42	15	293	244	537
SEBW + Ctec	38	1	1	44	10	274	233	507
0.25 Fe(III) + H_2O_2	38	1	1	41	12	275	227	502
0.25 Fe(III) + H_2O_2_Ctec	37	1	1	41	11	266	224	490
0.25 Fe(II) + H_2O_2	33	1	1	42	14	242	240	482
0.25 Fe(II) + H_2O_2_Ctec	33	1	1	43	13	241	239	480
0.5 Fe(III) + H_2O_2	36	1	1	41	13	262	234	496
0.5 Fe(III) + H_2O_2_Ctec	36	1	1	43	10	260	231	491
0.5 Fe(II) + H_2O_2	33	1	1	39	13	237	224	460
0.5 Fe(II) + H_2O_2_Ctec	33	1	1	44	11	238	238	476
1 Fe(III) + H_2O_2	32	1	1	44	12	232	239	471
1 Fe(III) + H_2O_2_Ctec	31	1	1	43	10	224	228	452
1 Fe(II) + H_2O_2	37	1	1	42	13	271	235	506
1 Fe(II) + H_2O_2_Ctec	36	1	1	45	9	258	231	489
PO	35	1	1	45	12	253	243	496
PO_Fe(II)	35	1	1	43	11	252	234	486
PO_Fe(III)	35	1	1	45	13	255	245	500
PO_Fe(II) + H_2O_2	34	1	1	49	12	249	260	508
PO_Fe(III) + H_2O_2	35	1	1	50	11	255	264	520
LE	34	1	1	51	10	247	263	510
LE_Fe(II)	37	1	1	46	11	269	243	512
LE_Fe(III)	36	1	1	43	11	261	230	490
LE_Fe(II) + H_2O_2	36	1	1	40	11	261	216	477
LE_Fe(III) + H_2O_2	38	1	1	46	11	274	242	516

a) Presented as percentage of dry matter content.
b) Calculated using values of 415 and 727 mL CH_4/g of carbohydrates and lignin, respectively (Møller et al., 2004).

guaiacyl and syringyl, vanillin and syringaldehyde will be produced, which happens to have high methane production potential. It is shown that white-rot fungi are capable of converting lignin to the source of guaiacyl and syringyl (Dong et al., 2013). Therefore, this might be reasonable to conclude that some part of lignin-like components contributes to methane production leading to high methane yield in samples pretreated by only PO and LE and those with PO-FR. The other potential explanation for such a high methane production from SEBW is that the initial inoculum was adapted for AD of SEBW for 120 days. The inoculum was rich in lignocellulose hydrolysis microorganisms (data is not provided here), making it suitable culture for degrading lignin.

It should be noted that the PO and LE are very sensitive and cannot compete for food with other fungi in an open environment. To scale up the proposed approach, it is essential to consider the following factors: 1) the birch wood after the steam explosion is already sterilized and can be used for the cultivation of fungi, saving energy for sterilization of substrate, and 2) a six-week pretreatment is a long time in industrial-scale therefore, the pretreatment step should be integrated with other steps such as substrate storage. Many white-rot fungi including PO and LE are edible mushroom can be used as a source for food production and lignin degradation. Considering this feature of the pretreatment technique, new possibilities in circular economy in food production can be envisioned. A techno-economical assessment of the proposed approaches would be worth investigating in the future.

The SEBW substrate lacks important nutrients for growth of microorganisms. Even though the slow hydrolysis of SEBW can be addressed using the pretreatment approaches, the SEBW is not a suitable substrate to be use as the only substrate in continuous flow AD and a co-digestion approach will be needed (Almomani, 2020). The methane production data generated in the study is based on batch tests using a microbial inoculum rich in nutrients. Real large-scale applications would require continuous AD reactors and a co-substrate like manure to provide nutrients for the process. Thus, this study should be followed up using CSTR reactors to demonstrate this approach in a continuous system. Additionally, applications of the produced digestate should be investigated like the use as organic fertilizer and/or soil amendment. Using a six-week fungal pretreatment is a long time at industrial-scale and would need to be integrated with other steps such as substrate storage to be economical feasible. Many white-rot fungi including PO and LE are edible mushrooms and should be considered as a value-added product from the pretreatment/storage step. This approach could be integrated in existing biogas plants by adding a separate line for pretreatment and storage of woody biomass and co-digest this with the other substrates. Applying this technology to existing biogas plants will limit investment costs and open up for using abundant wood biomass as a substrate for methane production.

4. Conclusions

Steam explosion significantly increased the Fe(II) binding to the wood structure. The need for adding iron-reducing components (e.g., 2,3-dihydroxybenzoic acid) in samples with Fe(III) was eliminated. The optimum dosages of Fenton reagents were identified based on the methane yields observed after 40 days. Since FR with 1 mM Fe(III) and 0.5 mM Fe(II) resulted in highest methane yields with 352 and 383 mL/g vS respectively, these dosages were selected for combined FR/fungal pretreatments. Samples pretreated with LE alone and PO_FR both gave a methane yield of around 420 mL/ g vS surpassing previously reported yields from this substrate.

CRediT authorship contribution statement

Seyedbehnam Hashemi: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Linn Solli:** Supervision, Conceptualization, Methodology, Validation, Writing – review & editing. **Roald Aasen:** Methodology, Writing – review & editing. **Jacob J. Lamb:** Writing – review & editing. **Svein Jarle Horn:** Supervision, Conceptualization, Validation, Writing – original draft. **Kristian M. Lien:** Supervision, Validation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Seyedbehnam Hashemi reports financial support, administrative support, article publishing charges, equipment, drugs, or supplies, travel, and writing assistance were provided by Norwegian University of Science and Technology. Svein Jarle Horn reports financial support and writing assistance were provided by Norwegian University of Life Sciences. Jacob J. Lamb reports statistical analysis and writing assistance were provided by Norwegian University of Science and Technology. Kristian M. Lien reports administrative support, equipment, drugs, or supplies, and writing assistance were provided by Norwegian University of Science and Technology. Seyedbehnam Hashemi reports a relationship with ENERSENSE strategic research program initiative that includes: funding grants and travel reimbursement. One of the co-authors works at the Norwegian institute for bioeconomy research (NIBIO).

Data availability

Data will be made available on request.

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