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Short Communication

Enhanced simultaneous saccharification and fermentation of pretreated beech wood by *in situ* treatment with the white rot fungus *Irpex lacteus* in a membrane aerated biofilm reactor

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HIGHLIGHTS

- Effect of steam and fungal pretreatment on saccharification of beech was studied.
- *I. lacteus* was the best performing white rot fungus for enhancing saccharification.
- A membrane aerated biofilm reactor allows *in situ* fungal treatment during SSF.
- *In situ* fungal treatment increased ethanol yields from 65 to 80% for SSF of beech.

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ABSTRACT

The aim of the present study was to investigate the combination of steam pretreatment and biological treatment with lignin degrading fungal strains in order to enable efficient bioprocessing of beech wood to ethanol. In a sequential process of steam and fungal pretreatment followed by enzymatic hydrolysis, *Irpex lacteus* almost doubled the glucose yield for mildly pretreated beech wood, but could not improve yields for more severely pretreated substrates. However, when simultaneous saccharification and fermentation is combined with *in situ I. lacteus* treatment, which is enabled by the application of a membrane aerated biofilm reactor, ethanol yields of optimally steam pretreated beech could be improved from 65 to 80%. Generally, *in situ* fungal treatment during bioprocessing of lignocellulose is an interesting method to harness the versatile abilities of white rot fungi.

1. Introduction

Hardwood residues are attractive lignocellulosic feedstocks for biorefineries due to their year-round availability and their high energy density. However, hardwoods show a higher lignin content and are more compact than typical herbaceous residues, which make them more recalcitrant to enzymatic sugar release (Zhu and Pan, 2010). Correspondingly, harsher pretreatment conditions are generally required for wood, leading to an increased consumption of energy and chemicals and to a higher formation of toxic enzymatic hydrolysis and fermentation inhibitors (Kim et al., 2011). It has been often proposed to use the natural ability of specialized fungi to degrade or modify lignin and thereby enabling more efficient saccharification of wood at milder pretreatment conditions (Shirkavand et al., 2016; Sindhu et al., 2016). White-rot fungi have the unique ability to preferentially decompose lignin in the presence of cellulose by expressing an array of extracellular, synergistically acting oxidative enzymes including different peroxidases and laccases (Martinez et al., 2005). The application of such fungal species has traditionally been explored to upgrade lignocellulosic materials for feed and paper applications (Martinez et al., 2009), but has also received attention as a method to improve enzymatic digestibility of lignocellulose (Alvira et al., 2010; Moreno et al., 2015). Fungal treatment has been investigated either as the sole pretreatment method or in combination with thermochemical pretreatment technologies (Shirkavand et al., 2016; Sindhu et al., 2016; Wan and Li, 2012). With fungal pretreatment alone, the lignin content of biomass is reduced by up to 30%, however also a similar amount of hemicellulose is typically consumed and sugar yields from enzymatic hydrolysis are still too low for an economic feasible process (Wan and Li, 2010; Yu et al., 2009). Fur-

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thermore, the long pretreatment times in the range of 30–90 days are a disadvantage (Sindhu et al., 2016). The combination of fungal and thermochemical pretreatment allows higher conversions yields (Shirkavand et al., 2016). Here, the fungal pretreatment can be performed either prior to thermochemical pretreatment or as the secondary treatment. For example, Sawada et al. (1995) pretreated beech wood with *P. chrysosporium* followed by steam explosion and found 13–72% higher sugar yields in enzymatic hydrolysis. Li and Chen (2014) reported a 1.3 times higher enzymatic hydrolysis yield if steam pretreated corn stalk was treated for 21 d by *P. baumii* compared to the control receiving only steam pretreatment.

In this work it was attempted to develop an alternative process option that allows combining the fungal treatment with the actual biochemical conversion of the substrate to the desired product. Anticipated advantages compared to separate fungal pretreatment include the circumvention of an extra reactor for fungal treatment and the reduced sugar loss due to competition of the fermenting organisms and the fungi for the easily available sugars. The implementation of the *in situ* fungal treatment process was envisioned by application of a membrane aerated biofilm reactor (Fig. S1) that has been developed for microbial consortium based consolidated bioprocessing of lignocellulosic biomass to ethanol (Brethauer and Studer, 2014). Here, a biofilm that is aerated with oxygen diffusing through the membrane is formed by the white rot fungus that produce enzymes such as ligninases while consuming oxygen. Consequently, the upper part of the biofilm as well as the fermentation broth is oxygen depleted which allows the production of ethanol by *S. cerevisiae* in this zone. In order to select a suitable white rot fungus for this endeavor, 6 different strains were first screened for their ability to improve the enzymatic hydrolysis of steam pretreated beech wood.

2. Materials and methods

2.1. Biomass

Beech wood (*Fagus sylvatica*) from a local forest in the cantone of Berne was air-dried to a final dry matter of 94% w/w and milled through a screen to a particle size of <0.5 mm without subsequent sieving. The main components of the raw feedstock were 40.8% glucan, 19.1% xylan and 25.3% acid insoluble lignin.

2.2. Steam explosion pretreatment

In a custom made steam explosion pretreatment plant (Industrieanlagen Planungsgesellschaft (IAP), Graz, Austria) (Pielhop et al., 2016), 250 g beech wood per run was heated to the desired temperature by injection of high pressure saturated steam. After a defined incubation time, the biomass was explosively discharged into a receiving vessel. For two stage pretreatment runs, the solid and the liquid phase derived after the first pretreatment stage were separated by filtration and only the washed solids are subjected to a second steam explosion pretreatment. The applied pretreatment conditions and the corresponding severities are specified in the text.

2.3. Screening of white rot fungi

2.3.1. Microorganisms and preculture conditions

The white rot fungi *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes versicolor*, *Ganoderma applanatum*, *Heterobasidion annosum* and *Irpex lacteus* were acquired from DSMZ (Braunschweig, Germany) and maintained on malt extract agar plates at 4 °C. For precultures, 3 disks of 1 cm diameter were cut from the agar plates and added to 50 mL malt extract peptone medium (30 gL^{-1} malt extract, 3 gL^{-1} soy peptone). The 250 mL Erlenmeyer flasks were incubated in a shaking hood at 120 rpm and 28 °C for 7 days. The cultures were aseptically homogenized (Ultra-Turrax, IKA, Staufen, Germany) and used as inocula for the experiments described below.

2.3.2. Screening in shake flasks and membrane aerated biofilm reactors

For screening experiments, whole slurry pretreated beech wood, *i.e.* both the liquid and the solid phase after steam explosion pretreatment under mild conditions (180 °C, 15 min, log R_0 = 3.53), was used without any prior detoxification. For screening in shake flasks, monoseptic 30 mL cultures were grown in a shaking hood at 120 rpm for 14 days at 28 °C in 100 mL Erlenmeyer flasks containing 4% w/w pretreated beech wood (based on the original amount of beech wood that went to pretreatment), Mandels medium (2 gL^{-1} KH_2PO_4, 1.4 gL^{-1} $(NH_4)_2SO_4$, 0.3 gL^{-1} $MgSO_4{\cdot}7H_2O$, 0.4 gL^{-1} $CaCl_2{\cdot}6H_2O$, 0.3 gL^{-1} urea, 0.75 gL^{-1} peptone, 0.25 gL^{-1} yeast extract, and 1 mLL^{-1} trace element stock, pH = 5.0) and 5% v/w inoculum. For screening in membrane reactors, four commercially available stirred tank reactors with a nominal volume of 2.7 L each (Labfors 5 Bio-EtOH, Infors HT, Bottmingen, Switzerland) were equipped with a custom made stainless steel holder fitted with a tubular polydimethylsiloxane (PDMS) membrane with a diameter of 3.2 mm and a wall thickness of 0.8 mm. The membrane area to liquid volume ratio was approximately 0.42 cm^2 mL^{-1}. The air flow rate through the membrane was 0.37 $NLmin^{-1}$ and the stirring speed was 50 rpm. The pH value was set to 5.0 and was automatically controlled through dosage of 4% hydrochloric acid or 1 M sodium hydroxide.

After fermentation, the amount of solid biomass was measured after homogenization, filtration and washing and part of the solids were subjected to enzymatic hydrolysis.

2.3.3. Enzymatic hydrolysis

The washed solids isolated after fungal fermentation were subjected to enzymatic hydrolysis at a solid loading of 2% biomass based on the original amount of unpretreated biomass. Thereby comparative total sugar yields are obtained because it is accounted for any sugars that are lost during the pretreatment steps. A cellulase loading of 15 filter paper units (FPU) per g of raw beech wood was applied using Accelerase 1500 (Genencor). The pH was maintained at 5.0 using 0.05 M sodium citrate buffer and 0.01 gL^{-1} sodium azide prevented microbial growth in the hydrolysis mixtures. Enzymatic hydrolysis reactions were performed in 100 mL Erlenmeyer flasks closed with screw caps that were incubated in a shaking hood at 40 °C and 150 rpm for 3 days. Sugars in the supernatant were quantified by HPLC.

2.4. Simultaneous saccharification and fermentation with in situ treatment by white rot fungi

For simultaneous saccharification and fermentation (SSF) of steam pretreated beech wood with *in situ* treatment by white rot fungi, the above described membrane aerated bioreactor and similar conditions were applied (Section 2.3.3.). The reactor containing 4% w/w steam pretreated beech wood and Mandels medium was inoculated with 5% fungal preculture and incubated at 28 °C for at least 48 h until a visible biofilm has formed on the membrane. Then, *S. cerevisiae* inoculum from an overnight culture on YP medium was added to a final OD_{600} of 0.5 together with Accelerase 1500 (15 $FPU/g_{biomass}$) to start ethanol production. SSF controls without fungal treatment were performed under similar conditions in the same reactors but without a membrane.

2.5. Analytical methods

Glucose, xylose and ethanol concentrations in liquid samples were analyzed by HPLC and ethanol in the off-gas of the membrane by GC (Brethauer and Studer, 2014).

3. Results and discussion

3.1. Screening of white rot fungi for their ability to enhance the enzymatic digestibility of steam pretreated beech wood

The aim of this study was to develop methods to improve the conversion of lignin rich steam pretreated beech wood to ethanol by applying lignin degrading white rot fungi. To this end, we first tested 6 different white rot fungi for their ability to enhance glucose yields in enzymatic hydrolysis of mildly steam pretreated beech wood (180 °C, 15 min, log R_0 = 3.53). The strains were grown for 14 days on whole slurry pretreated beech wood either in shake flasks or in membrane aerated biofilm reactors. Then, the isolated and washed fungal treated biomass was subjected to enzymatic hydrolysis.

When using shake flasks as fungal pretreatment reactors, all strains decreased the glucose and xylose yields in the subsequent enzymatic hydrolysis reaction (supplementary information, Fig. S2). However, we also tested the fungal strains in a membrane aerated bioreactor. In the subsequent enzymatic hydrolysis, higher glucose yields could be reached than in the controls that received only the steam pretreatment but xylose yields were negatively impacted (Fig. 1). The best performing fungus *I. lacteus* approximately doubled the enzymatic glucose yields and was selected for further experiments.

Besides the differences between the form of fungal growth (biofilm vs. suspended pellets) in the two screening systems that are known to influence gene expression and enzyme production (Qureshi et al., 2005), also the different availability of oxygen could explain the different results. In shake flasks, showing oxygen mass transfer coefficients (k_La) in the range of 50–150 h^{-1}, the liquid reaction phase is well aerated (Meier et al., 2016). For the membrane aerated bioreactor, we estimated a k_La of 1–3 h^{-1} and only the first layer of the biofilm directly on the membrane is oxygenated. Thus, it is possible, that the fungi under the fully aerated conditions are more metabolically active and also degraded hemicellulose and cellulose to such an extent that the resulting net sugar yields are lower than in the untreated controls.

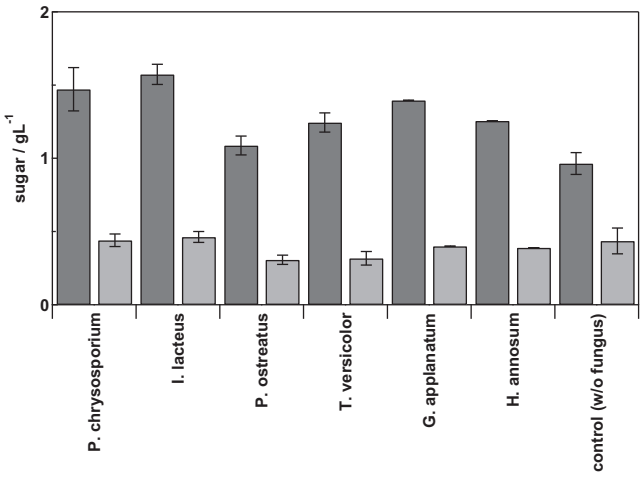

Fig. 1. Sugar yields in enzymatic hydrolysis of beech wood after combined steam and fungal pretreatment. Prior to enzymatic hydrolysis, beech wood was pretreated by steam explosion under mild conditions (180 °C, 15 min) followed by fungal treatment for 14 days in the membrane aerated biofilm reactor. Shown are the resulting glucose (dark grey) and xylose (light grey) yields in enzymatic hydrolysis after treatment with the different white rot fungi.

3.2. Influence of the steam pretreatment conditions on the ability of *I. lacteus* to improve sugar yields in the subsequent enzymatic hydrolysis

At the pretreatment conditions chosen for the fungal screening, the glucose yield of around 15% is still much too low for a reasonable process. Thus, we grew *I. lacteus* in the membrane aerated biofilm reactors on whole slurry beech wood pretreated at different severities and subjected the resulting solid material to enzymatic hydrolysis (Table 1). It could be shown that with increasing pretreatment severity the positive influence of the fungal treatment decreases. At a steam pretreatment temperature of 220 °C, the enzymatic hydrolysis yields were even lower when the material was additionally treated with *I. lacteus*.

3.3. SSF of washed and whole slurry two stage steam pretreated beech wood with in situ treatment by I. lacteus

As an alternative to the above described sequential steam and fungal pretreatment process followed by enzymatic hydrolysis, a SSF process employing commercial cellulases was investigated, where the biomass was treated *in situ* by *I. lacteus*. To this end, only *I. lacteus* was grown on the substrate in the membrane aerated bioreactor until a visible biofilm was formed. Then, yeast inoculum was added together with commercial cellulase. In the first run, washed steam pretreated beech wood (230 °C, 15 min, log R_0 = 5.0) was used as substrate and the resulting ethanol profile is shown in Fig. 2. At the beginning of the fermentation, higher ethanol concentrations could be found in the *I. lacteus* supported process, e.g. after 3 days the ethanol yield was 26% higher than in the control culture. However, after about 4 days, the ethanol concentration began to decrease steadily indicating a consumption of ethanol by the involved microorganisms. Up to now, it remained unclear which microorganism was responsible for the ethanol decomposition. It is possible, that only *I. lacteus* consumed ethanol in the aerobic part of the biofilm because the yeast cells consumed all available glucose. Alternatively, also the yeast cells could be responsible because the oxygen consumption rate of *I. lacteus* might have dropped in the later stage of the fermentation resulting in the availability of oxygen in the liquid phase of the fermentation broth.

Thus, it was tested whether the presence of xylan derivatives (xylan, soluble xylooligmers or xylose) that can only be metabolized by *I. lacteus* can circumvent the consumption of ethanol. We hypothesized that xylan derivatives are preferred over ethanol as a substrate for the fungus and that their availability renders the metabolic activity of *I. lacteus* high enough to prevent oxygen entering the liquid phase. Thus, a SSF experiment was performed using whole slurry two stage steam pretreated beech wood as feedstock. In the first pretreatment stage (180 °C, 44 min, log

Table 1
Influence of steam pretreatment conditions on glucose yields in enzymatic hydrolysis of beech wood after combined steam and *I. lacteus* pretreatment. Prior to enzymatic hydrolysis, beech wood was pretreated by steam explosion under 3 different conditions followed by treatment with *I. lacteus* for 14 days in the membrane aerated biofilm reactor. Shown are the resulting glucose yields in enzymatic hydrolysis of beech wood.

Pretreatment conditions and severity	Glucose yield, control [g/L]	Glucose yield, with fungal treatment [g/L]
180 °C, 15 min, 3.53	0.96 ± 0.08	1.62 ± 0.08 (+68%)
210 °C, 10 min, 4.25	4.51	5.56 (+23%)
220 °C, 5 min, 4.25	6.63 ± 0.06	5.87±0.13 (−13%)

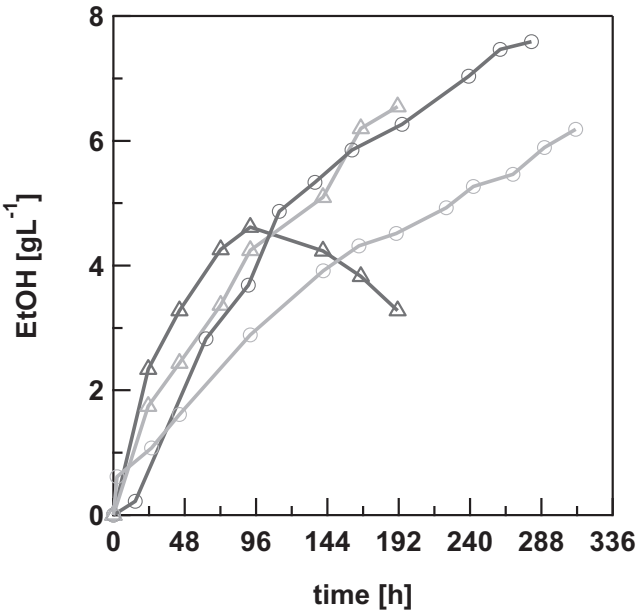

Fig. 2. Effect of *in situ* treatment with *I. lacteus* on SSF of washed and whole slurry pretreated beech wood. Washed steam pretreated beech wood (230 °C, 15 min) was converted to ethanol in the membrane aerated biofilm reactor with (triangles, dark grey) and without (triangles, light grey) the presence of *I. lacteus*. Alternatively, beech wood was pretreated in two stages (stage 1: 180 °C, 44 min; stage 2: 230 °C, 8 min) to increase the availability of xylan derivatives. For the SSF experiment, the two liquid phases and the solids were recombined. Shown are the ethanol yields with (circles, dark grey) and without (circles, light grey) the presence of *I. lacteus*.

R_0 = 4.0), most of the xylan is solubilized. In the second stage, only the solids were then pretreated again at 230 °C for 8 min (log R_0 = 4.75). By the two stage pretreatment, the virtually complete xylan degradation, that occurred during the one stage pretreatment process, could be avoided. For the SSF experiment, the two liquid phases and the solids were recombined. As shown in Fig. 2, it was possible under these conditions to circumvent the ethanol consumption and the final ethanol yield could be increased from 65 to 80% by the presence of *I. lacteus*, corresponding to an improvement of 33%. Part of the yield improvement might also be explained by the detoxification of the pretreatment slurry by *I. lacteus*. Besides phenolic compounds, especially xylooligomers and xylose have been shown to inhibit enzymatic hydrolysis (Zhai et al., 2016). Thus the consumption of the latter presumably also improved the ethanol yields.

4. Conclusions

It could be shown that the *in situ* treatment with *I. lacteus* enables increased SSF yields from lignocellulosic material that is pretreated under conditions that were optimized for maximal glucose yields by enzymatic hydrolysis. Advantages compared to separate fungal pretreatment include the circumvention of an extra reactor for fungal treatment and the reduced sugar loss due to competition of the yeast and the fungi for the easily available sugars. Overall, *in situ* fungal treatment is a promising method to harness the versatile abilities of white rot fungi such as lignin degradation or pretreatment slurry detoxification that merits further research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.03.050.

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