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Development and optimization of single and combined detoxification processes to improve the fermentability of lignocellulose hydrolyzates

Daniel Ludwig [a], Manfred Amann [b], Thomas Hirth [c,d], Steffen Rupp [d], Susanne Zibek [d,*]

[a] Evonik Industries AG Rodenbacher Chaussee 4, 63457 Hanau-Wolfgang, Germany
[b] Wacker Chemie AG Consortium für Elektrochemische Industrie, Zielstattstraße 20, 81379 München, Germany
[c] Institute of Interfacial Engineering, University of Stuttgart, Nobelstraße 12, 70569 Stuttgart, Germany
[d] Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Nobelstraße 12, 70569 Stuttgart, Germany

HIGHLIGHTS

- Immobilized laccase for the detoxification of the xylan rich fraction (XRF).
- *In situ* product removal of low molecular lignins.
- Combined detoxification method of immobilized laccase and anion exchanger.

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ABSTRACT

In this work, an enzyme catalyzed detoxification process of lignocellulose hydrolyzates with immobilized laccase from *Trametes versicolor* was developed and optimized. Further, the immobilized laccase significantly reduced the amount of toxic phenolic compounds in the xylan rich fraction (XRF) by polymerization within 1 h. The insoluble products precipitated onto the carrier surface and could be reversible resolubilized by an aqueous ethanol solution. Consequently, an *in situ* product removal could be realized. The reusability of the immobilized laccase could be additionally shown. The reaction kinetics could be described by a reversible Michaelis Menten equation giving the prerequisite for scaling up the process. In a second step, the organic acids, hydroxymethylfurfural and phenolic acids could be further removed by employing an anion exchanger. Both, the laccase and the laccase + anion exchanger treatment enhanced successfully the fermentability of an organosolv wheat straw fraction.

1. Introduction

Lignocellulose is the most abundant and low cost renewable raw material for the production of bio-based chemicals, biofuels or bioenergy. It consists mainly of cellulose, hemicellulose and lignin and can be derived from agriculture residues, from forest products or from special fast growing biomass plants like miscanthus or switchgrass. Due to its high polysaccharide content (60–75%), it can be used to generate fermentable sugars without competing with food and feed industry. Whereas the hydrolysis of the structural carbohydrates cellulose and hemicellulose can be enzymatically catalyzed by cellulases and hemicellulases, lignocellulose itself is very resistant towards enzymatic hydrolysis. Thus chemical or physical pretreatment processes have to be applied to increase the fiber accessibility resulting in a hemicellulose-rich hydrolyzate, which can be used for further fermentation processes. Unfortunately, the pretreatment generates also soluble sugars and lignin degradation products, which can affect the enzymatic hydrolysis negatively as well as the fermentation process (Klinke et al., 2004). The nature and concentration of these toxic compounds depend on the raw material, the applied pretreatment process and the severity of the pretreatment. They can be divided into furan derivatives (furfural and 5-hydroxymethylfurfural from pentose and hexose sugars), organic acids (mainly acetic acid, formic acid and levulinic acid) and phenolic compounds (soluble low molecular compounds derived from lignin). Some investigations about the inhibition mechanisms were carried out, but due to this complex system, reliable predictions about the inhibitory effect on certain fermenting microorganisms cannot be made up to now (Klinke et al., 2004).

The adaption of the production strains to achieve higher productivities can be one strategy to use these xylan rich fractions (XRF) (Parekh et al., 1986). Additionally, the XRF can be detoxified by several methods (Klinke et al., 2004). They can be divided into chemical, physical or biological processes. Biological methods

* Corresponding author. Tel.: +49 711 970 4167; fax: +49 711 970 4200.
E-mail address: susanne.zibek@igb.fhg.de (S. Zibek).

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include the usage of special microorganisms that remove lignocellulose degradation products (Yu et al., 2011). Biological methods normally take few days to sufficiently detoxify the XRF. Besides microorganisms, lignolytic enzymes such as peroxidases and laccases can be employed to oxidize side groups of phenolic compounds (Jurado and Prieto, 2009). The oxidation products can further polymerize to non-toxic, often insoluble substances.

For industrial enzyme processes, immobilization can be applied to efficiently reuse the catalyst and to reduce process costs (Hilterhaus et al., 2008). Some studies were carried out with immobilized laccase to decolorize waste water streams (Kunamneni and Ghazi, 2008), to treat waste water effluents of olive mills (D'Annibale et al., 1999) or simply to oxidize model compounds like cresols, chlorophenols and other phenol derivatives (Hublik and Schinner, 2000; Salis and Pisanoa, 2009). As carriers for the immobilization of laccase, different functionalized materials were employed. For example pre-silanized CPC silica beads (Champagne and Ramsay, 2010), activated agarose beads (Reyes and Pickard, 1999), Eupergit© particles (Hublik and Schinner, 2000), epoxy-functionalized divinylbenzene particles (Kunamneni and Ghazi, 2008) or magnetic particles (Zhu and Kaskel, 2007) were used.

The immobilization of laccase to detoxify the XRF is a new approach, which has not been optimized so far. So, only little information is hitherto available for process design and optimization of laccase catalyzed detoxification of the XRF. Thus, the aim of this work was the development of an enzyme based on the detoxification method with immobilized laccase. Additionally, the reusability of the enzyme carrier should be evaluated. The process should be optimized and process models should be developed, prerequisites for its scaling up. Because laccase removes mainly the phenolic compounds, an anion exchanger was also used as a second treatment to detoxify the XRF. Both treatments were compared according to their ability to remove lignocellulose degradation products and to enhance the fermentability of an organosolv wheat straw fraction (OWSF).

2. Methods

2.1. Raw material and organosolv pretreatment

All pretreatment experiments were carried out at the Fraunhofer Institute for Chemical Technology (Pfinztal, Germany). Wheat straw was chopped to 1 cm fibers by using a mill (SM 100, Retsch GmbH, Haan, Germany). Then 700 g DM (dry matter) wheat straw (8% w/w DM) were pretreated by an ethanol organosolv process (50% w/w ethanol in water) for 120 min at 220 °C in a 13 L reactor. Solid liquid separation was done by filtration with a polypropylene fleece.

The fibers were washed two times with 50% ethanol solution and two times with tap water at a liquid to solid ratio of 5:1. The fibers were finally dried in a fume hood at room temperature to a moisture content of 10–12% w/w.

The ethanol was removed from the mother liquor by vacuum evaporation to precipitate organosolv lignin. Lignin was separated and the mother liquor (concentration factor = 2) was vacuum filtered, giving the organosolv wheat straw fraction (OWSF) and solid organosolv lignin. This OWSF was used for the detoxification experiments. The concentrations of the lignocellulose degradation products are 3.45 g/L phenolic compounds, 2.16 g/L acetic acid, 1.93 g/L formic acid, 0.35 g/L ethanol, 0.10 g/L HMF, 0.01 g/L furfural, and 0.18 g/L levulinic acid.

2.2. Laccase, enzyme carriers and immobilization

Laccase from *Trametes versicolor* was provided as a dry powder by Wacker Chemie AG (Munich, Germany). The powder was stored at 4 °C. The laccase was dissolved in 50 mM acetate buffer at pH 4.75 for the use as free enzyme. For immobilization, the laccase was dissolved in the immobilization buffer (20 mM K_2HPO_4, pH 8.0). The immobilization of laccase on the Sepabeads® EC-EP and EC-EA carriers was done according to the manual of the manufacturer (Resindion S.r.l.). The EC-EA activation buffer consisted of 200 mM glutardialdehyde in 20 mM phosphate buffer (pH 8.0). EC-EP is an active epoxide carrier, so there was no need for an additional activation step. For stabilization of the immobilized laccase, 1 M phosphate buffer at pH 7.0 was used as storage buffer (Note: although pH optima of laccase was in the range of pH 4.5–5.0, the immobilized laccase and the carrier was more stable at pH 7). Storage temperature was 4 °C.

2.3. Microorganism and growth conditions

Pichia stipitis was used to evaluate the effect of the different detoxification methods. The cultures were cultivated on agar plates (2% agar, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L xylose). Mineral medium (pH 5.0), according to Nigam (2001), was used to cultivate the preculture (20 g/L xylose, 60 mL media in 100 mL Erlenmeyer flasks, 120 rpm, amplitude 1.9 cm). The main culture was inoculated with the preculture giving a biomass concentration of 0.2 g/L. The main culture (300 mL in 500 mL Erlenmeyer flasks, 120 rpm, amplitude 1.9 cm) contained either detoxified or not detoxified OWSF (dilution factor 2) supplemented with minerals. As reference, mineral medium without OWSF was used. The xylose content of all cultures was adjusted to 50 g/L xylose. The cultivation temperature was 30 °C and the fermentation lasted for 128 h.

2.4. Analytical methods

2.4.1. Laccase activity

Laccase activity was measured with ABTS (2.2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) at 439 nm The assay was performed using acetate buffer (50 mM, pH 4.75) at a final ABTS concentration of 4 mM (Niku-Paavola et al., 1988).

Laccase activity of the immobilized laccase was measured in a 100 mL Erlenmeyer flask. 10–20 mg of carrier was incubated with 20 mL of 5 mM ABTS in 50 mM acetate buffer (pH 4.75). The reaction mixture was stirred with a magnetic stirrer at 200 rpm. A sample was withdrawn every 30 s and filtered with a 0.2 μm syringe filter. After filtering, the supernatant was inactivated at 95 °C for 5 min. Then the absorbance was measured at 439 nm.

2.4.2. Folin Ciocalteu (FC) assay

The total phenol content (phenolic compounds) was measured according to a modified method from Cicco et al. (2009). 25 μL of sample were mixed with 25 μL of Folin reagent (Sigma 47641). After 2 min incubation, 200 μL 5% w/v $NaCO_3$ solution was added. The reaction mixture was incubated at 40 °C for 20 min. Then, 200 μL were pipetted into a 96 well plate and the absorbance was measured at 740 nm (Synergy 2, Biotek). The total phenol content was calculated as vanillin equivalents. The conversion of phenolic compounds was defined as:

$$X = 1 - \frac{c(t)}{c_{\text{initial}}} \quad (1)$$

2.4.3. Determination of sugars, by-products, and degradation products in liquid fraction process samples

The determination of sugars, by-products, and degradation products in liquid fraction process samples was carried out according to Sluiter et al. (2008). Aminex HPX87 H was used as the separation column. The flow rate was set to 0.6 mL/min and