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Research paper

Furfural and 5-hydroxymethyl-furfural degradation using recombinant manganese peroxidase

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ABSTRACT

Biomass pretreatment-derived degradation compounds, such as furfural and 5-hydroxymethyl-furfural (HMF), inhibit the growth of fermentation microorganisms that utilize biomass to produce fuels and chemicals. Here we report that recombinant manganese peroxidase (rMnP) produced from the yeast *Pichia pastoris* can degrade furfural and HMF making them less toxic to microorganisms. Treatment with rMnP or manganese(III) acetate reduced furfural and HMF concentrations in a dose-dependent manner. Furfural disappearance was accompanied by malonate disappearance and accumulation of four distinct degradation products. Furfural was more readily degraded by rMnP and manganese(III) acetate than HMF. Growth assays using *Saccharomyces cerevisiae* indicated that rMnP treatment reduced the toxicity of furfural and HMF. This work provides an avenue to use rMnP to increase the growth of fermentation microorganisms that are inhibited by toxic compounds derived from pretreatment of biomass.

1. Introduction

Lignocellulosic biomass, composed of cellulose, hemicellulose, and lignin, is an abundant low cost material that can be converted via biochemical platforms to liquid fuels. The carbohydrates from both cellulose and hemicellulose, hexose and pentose sugars, respectively, need to be utilized to achieve economical yields [1]. When biomass is in its native state, the conversion of cellulose to fermentable sugars is low (< 20%) due to the crystallinity and water insoluble properties of cellulose, the protective lignin sheath, and the hemicellulose content. Pretreatments are used to increase the enzyme-accessible surface area of cellulose by disruption of cell wall components. In dilute acid-based pretreatments this includes hydrolysis of the hemicellulose and, potentially, the formation of unwanted inhibitory degradation products [2]. It is well documented that as pretreatment severity increases, the accessibility of the cellulose for hydrolysis increases; however, the amount of hemicellulose sugars degraded through condensation and dehydration reactions also increases. Dilute acid pretreatments typically yield a water insoluble material enriched in cellulose and a water soluble fraction composed of the hydrolyzed hemicellulose sugars and lignin degradation products. Hemicellulose-derived sugar concentrations are typically 30–60 g/L in the water soluble fraction. However, biofuel/bioproduct fermentations typically operate at 100–300 g sugar/L to achieve the highest possible ethanol concentration, thus concentration of the prehydrolysate liquor may be required. In this process, the inhibitory degradation products are co-concentrated with sugars, resulting in inhibitory effects on the growth of the yeast used for biofuel production [1,3,4].

The inhibitory compounds formed during pretreatment are dependent on the type of lignocellulosic biomass and the pretreatment conditions such as pH, temperature, pressure, time, and catalyst [5]. There are three main categories of inhibitors produced during pretreatment; furan derivatives, weak acids, and phenolics. The furan derivatives are 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (furfural), and they are formed from the dehydration of the hexose and pentose sugars, respectively [6,7]. A postulated mechanism for the growth inhibition by furans is that they inhibit production of enzymes used in glycolysis and ethanol production: alcohol dehydrogenase, pyruvate dehydrogenase, and aldehyde dehydrogenase. Furfural has also been shown to damage vacuole and mitochondrial membranes, chromatin,

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and actin, and induce the accumulation of reactive oxygen species in *Saccharomyces cerevisiae,* which is a commonly used fermentation microorganism [5,6,8,9].

The removal of inhibitors has the potential to increase growth rates, conversion rates, and final yields of biofuel. Pretreatment hydrolysates can be detoxified by biological, physical, or chemical methods [7]. Biological detoxification can use enzymes or whole cells. The soft-rot fungus *Trichoderma reesei* degraded inhibitors in steam-pretreated willow hydrolysate resulting in a four-fold increase in ethanol yield [10]. An improvement in fermentation of dilute acid pretreated spruce hydrolysate following treatment with *T. reesei* was also observed [11]. Furfural and HMF were degraded by whole cell *Amorphotheca resinae* ZN1 treatment [12], and a recombinant Z*ymomonas* capable of furfural and HMF degradation increased ethanol productivity from corn stover hydrolysate [13]. The purified enzyme furfural reductase has been used to degrade furfural to furfuryl alcohol and detoxify pretreatment hydrolysate [14]. While the use of MnP expressing whole cells was described in recent reviews on biological treatment for delignification and detoxification [15,16], the use of the purified enzyme MnP has not been described. Enzyme preparations, rather than actively growing cells, may be easier to employ in commercial biofuels production.

White rot fungi, such as *T. reesei and Phanerochaete chrysosporium*, are natural degraders of both hardwood and softwood. Their capacity to degrade wood is tied to their ability to secrete two heme peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP) [17,18]. The catalytic cycle of MnP uses H_2O_2 as a substrate to oxidize the Mn(II) to Mn(III). The generated Mn(III) is chelated to an organic acid for stability [19,20]. The Mn(III)-organic acid complex is a strong oxidizer, and it is known to oxidize phenols and dyes, decarboxylate or demethoxylate aromatic substrates, and oxidize model phenolic lignin compounds and dimers [19,21]. MnP is thus known as a phenol oxidizing enzyme. A possible mechanism for the oxidation of phenolic substrates involves a one electron or hydrogen abstraction leading to the formation of a radical [19,22]. MnP has also been shown to degrade pollutants such as chlorophenols, dinitrotoluenes, and methoxy benzenes [23–25]. However, there are no reports of MnP degradation of furfural and HMF, specifically.

MnP has been shown to degrade non-phenolic lignin model compounds by generating a peroxyl radical from an unsaturated fatty acid, such as linoleic acid, through lipid peroxidation [19,26]. The peroxyl radical (ROO·) is a stronger oxidizing agent than the typically considered Mn(III) organic acid complexes. Highly reactive radicals may also arise as a result of Mn(III)-organic acid decomposition. These radicals can degrade bonds in a number of different structures, including a non-phenolic β-O-4–linked lignin, a lignin dimer representing the arylglycerol-2-aryl ether substructure of lignin, polyethylene, phenanthrene, and water insoluble milled pine wood [27–30]. MnP degradation of these non-phenolic substrates suggests that furfural and HMF may also react with peroxyl radicals generated by Mn(III) organic acid complexes.

Here we report on the use of recombinant manganese peroxidase (rMnP) for the detoxification of furfural and HMF. The inhibitory effects of these two compounds, prior to and after exposure to rMnP under different reaction conditions, on the yeast *S. cerevisiae* were determined using a growth assay. The effect of rMnP treatment dose and the addition of linoleic acid in a Tween-20 emulsion on detoxification were evaluated. Enzyme-free experiments using Mn(III) acetate were included to verify that rMnP-generated Mn(III) is capable of modifying furfural and HMF.

2. Materials and methods

2.1. Chemicals

All chemicals were of reagent grade. HMF, linoleic acid, Tween-20, and manganese(III) acetate dihydrate were purchased from Sigma-Aldrich (St Louis, MO, USA). Furfural was obtained from TCI America (Portland, OR, USA). D-glucose was purchased from Mallinckrodt Chemicals (St Louis, MO, USA). Yeast extract and peptone were purchased from Fisher Scientific (Hampton, NH, USA). *Saccharomyces cerevisiae* was obtained from the laboratory of Dr. Alan Bakalinsky, Oregon State University.

2.2. Production and concentration of rMnP

rMnP was produced and concentrated from *Pichia pastoris* αMnP1-1 bioreactor cultivations [31–33]. The yeast cells and heme particulates were removed by high-speed (10,000 x *g*) centrifugation. Acetone precipitation and dialysis were used to concentrate the rMnP and remove salts. Freeze-drying was used to remove water for long-term storage at − 20 °C. Activity of rMnP was measured by monitoring the oxidation of 2,6-dimethoxyphenol (2,6-DMP) at 469 nm [20]. Total protein was determined using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA), which is based on the Bradford colorimetric method. Bovine serum albumin was used as a standard protein. The final specific activity was 1.6 U/mg protein. rMnP was reconstituted in the appropriate buffer or distilled deionzed water just prior to initiating experiments.

2.3. rMnP degradation of inhibitor

Furfural (1 g/L) and HMF (1 g/L) were treated with rMnP at 30 °C in sterile 20 mL glass vials containing the reagents sodium malonate (50 mM, pH 4.5), $MnSO_4{\cdot}H_2O$ (0.04 mM), H_2O_2 (0.1 M), and rMnP (100 U/L or 200 U/L). Controls contained either inactive (boiled) rMnP, no rMnP, or no rMnP and no reagents. In some cases reaction mixtures also contained 2 mM linoleic acid, which was added prior to the reaction in a 0.2% (wt/wt) Tween 20 emulsion. Reactions were initiated by the addition of H_2O_2 to otherwise complete temperature equilibrated reaction mixtures. Assays were performed in triplicate.

Degradation of furfural and HMF were monitored using absorbance measurements with a Thermo-Electron Genesys 6 scanning UV–vis (190–1100 nm) spectrophotometer.The spectrophotometer was zeroed with the reaction mixture without inhibitor and H_2O_2. Sacrificial samples of 100 μL were removed from reaction mixtures at 0, 3, 9, 24, and 100 h. Mn(III) generated from the catalytic cycle of active rMnP interferes with absorbance readings for determining inhibitor concentrations. Therefore, prior to recording the absorbance, 10 μL of 10 mg/mL catalase was added to the sacrificial sample and allowed to incubate at 30° C for 10 min to degrade H_2O_2, and thus limit further production of Mn(III). After the 10 min incubation with catalase, 10 μL of 0.1 mM 2,6-DMP was added to the catalase-treated samples to reduce existing Mn(III) to Mn(II). The Mn(III)-free samples were then diluted 200-fold and the absorbances at 278 nm for furfural or 286 nm for HMF were recorded. Standard deviations of the means of triplicate samples were determined.

2.4. Manganese(III) acetate degradation of inhibitor

Manganese(III) acetate was weighed into scintillation vials (20 mL), followed by addition of 10 mL of 50 mM sodium malonate buffer (pH 4.5) containing 1 g/L furfural or HMF. Vials were incubated in 30 or 37 °C water baths with intermittent mixing. Furfural and HMF concentrations in reaction mixtures following 24 h incubation were determined using a Shimadzu HPLC (Koyota, Japan) equipped with a system controller (CBM-20A), solvent delivery modules (LC-20AD), an autosampler (SIL-20AHT), a column oven (CTO-20A), and a UV/VIS photodiode array detector (SPD-M20A). The injection volume was 20 μL, the stationary phase was an SB-C18 reversed phase column (250 mm × 4.6 mm)(Agilent, Santa Clara, CA, USA), the column temperature was 30 °C, and the detection wavelength was 278 nm. The flow rate was maintained at 0.5 mL/min with a methanol gradient.