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Research paper

Adaptability of *Trametes versicolor* to the lignocellulosic inhibitors furfural, HMF, phenol and levulinic acid during ethanol fermentation☆

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ABSTRACT

Ligno-cellulosic biofuels, notably ethanol produced in processes involving biological fermentation, have high potential as renewable alternatives to fossil fuels. However, ligno-cellulose pretreatment procedures generate substances that inhibit current biocatalysts. Thus, efficient methods are required for improving these organisms' tolerance or developing new biocatalysts with higher tolerance to the inhibitors. For this, greater knowledge of the mechanisms involved is needed. Therefore, we examined effects of common inhibitors (phenol, levulinic acid, HMF (hydroxymethylfurfural) and furfural) on growth, utilization of sugars (xylose, mannose and glucose) and enzyme activities of a tolerant organism, the white-rot fungus *Trametes versicolor*, during 15-day incubations. The fungus metabolized and grew in the presence of all the inhibitors (singly and together) at the applied concentration (0.2–0.6 g/L). When all inhibitors were added, no significant effect of sugar utilization was shown. However, levulinic acid added solely reduced xylose (but not xylose-degrading enzymes) and mannose utilization, but not glucose utilization. Physiological and biotechnological implications of the findings are discussed such as usage of *T. versicolor* as a detoxifying agent in ethanol production.

1. Introduction

Lignocellulosic residues have huge potential as renewable sources of biofuels (as an example ethanol) for replacing fossil fuels [1]. They are recalcitrant to hydrolysis (saccharification), which complicates their exploitation, but they can be converted to ethanol essentially in four main steps: thermo-chemical pretreatment, enzymatic hydrolysis of the liberated cellulose, fermentation of the resulting sugars by an appropriate biocatalyst, and distillation [2]. Such strategies for using lignocellulosic biomass in ethanolic fermentation are becoming cost-effective. However, pretreatment of lignocellulosic material using thermo-chemical methods (e.g. steam explosion and/or dilute-acid hydrolysis) forms a hydrolysate rich in substances that are inhibitory to many biocatalysts and enzymes. These inhibitors are byproducts of the degradation of the three main constituents of lignocellulosic material: cellulose, hemicellulose, and lignin [3–5].

The nature and concentrations of the inhibitory compounds vary greatly with the amount of solids in the reactor, pretreatment conditions (e.g. time, pH, temperature and concentrations of chemicals) and the raw material used [6]. However, they can be divided into three major groups: furaldehydes, weak acids, and phenolics. Furaldehydes in the hydrolysates include 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF), the weak acids include acetic, formic, and levulinic acids, while the phenolics include compounds such as vanillin, syringaldehyde, and coniferyl aldehyde, all of which can impair fermentation [4,5]. During the thermo-chemical pretreatments inhibitors such as furfural and HMF are derived from the dehydration of pentoses and hexoses, respectively, levulinic acid is formed via degradation of 5-HMF [6], and various phenols are generated by solubilization and hydrolytic or oxidative cleavage of lignin [2].

A major problem in industrial bio-ethanol refineries is that the most commonly used microorganism, the yeast *Saccharomyces cerevisiae*, can only ferment certain mono- and di-saccharides (such

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as glucose, fructose, maltose and sucrose) into ethanol. In addition, the presence of inhibitors in lignocellulosic liquor reduces the growth and productivity of the yeast and various other potential biocatalysts, thereby reducing ethanol yields, by inhibiting their enzymatic and physiological activities [3] [6]. Thus, to improve the process efficient methods are clearly required for fermenting pentoses and hexoses, preventing the formation of inhibitors in other ways, enhancing the tolerance of currently used biocatalysts and/or developing new biocatalysts with higher tolerance of inhibitors generated from the residues. Fortunately, a number of microorganisms including yeasts, fungi, and bacteria can naturally detoxify inhibitory compounds and have been used to pre-detoxify lignocellulosic hydrolysates before fermentation with *S. cerevisiae* for ethanol production. Notably, the filamentous soft-rot fungus *Trichoderma reesei* can reportedly degrade inhibitors in hydrolysates of willow hemicelluloses [7] including furans, acetic acid, furfural, and benzoic acid derivatives. The cited authors also found that *T. reesei* treatment can increase ethanol productivity three-fold and ethanol yields four-fold. However, growth rates of the fungus were low and it assimilated sugars from the hydrolysates.

Another organism that has shown promising potential to detoxify lignocellulosic hydrolysates is the white-rot basidiomycete fungus *Trametes versicolor*. Jönsson et al. [8] reported that laccase and lignin peroxidase of this fungus can degrade some phenolics in willow hemicellulose hydrolysates pretreated with steam and SO_2, thereby increasing ethanol productivity 2- to 3-fold. Furthermore, Kudahettige et al. [9] showed that *T. versicolor* can use hexoses and pentoses simultaneously.

Clearly, it is essential to determine the physiological mechanisms responsible for both inhibitors' effects on biocatalysts currently used in bio-ethanol refineries and their detoxification by other biocatalysts. Thus, the aims of the study presented here were to further characterize the ability of *T. versicolor* to produce ethanol from mixtures of sugars typically generated from lignocellulosic residues at Domsjö paper pulp factory, Sweden (largely glucose, mannose and xylose [9,10]) in the presence of typical inhibitors. More specifically, we compared the biomass production, ethanol production, decomposition of inhibitors and sugar utilization efficiencies of the fungus in the presence of phenol, levulinic acid, HMF and furfural at selected concentrations under hypoxic conditions. We also analyzed activities of relevant enzymes — alcohol dehydrogenase (ADH), acetaldehyde dehydrogenase (ALDH), pyruvate dehydrogenase complex (PDC), xylose reductase (XR), xylitol dehydrogenase (XDH), laccase, peroxidase, manganese1 peroxidase (Mn1P) and phenol oxidase) — of *T. versicolor* cultures in media containing hexose, xylose and the inhibitors. Physiological and biotechnological implications of the results are discussed.

2. Materials and methods

2.1. Organisms, media and culture conditions

T. versicolor (CBS 109428) culture was obtained from the CBS culture collection (The Netherlands) and stock cultures were grown in 300 mL bottles containing 100 mL of MBMC medium according to Kudahettige et al. [9], for 3 weeks. Fungal hyphae corresponding to 6 g/L fresh weight (FW) taken from a stock culture were transferred to a MBMC medium including only glycerol (1.2 g/L) as carbon source for 7 days before each inhibitor experiment.

2.2. Growth experiment

Culture grown on the glycerol-containing medium was diluted to 6 g/L (FW), then 1 mL portions were pelleted and added to 8 mL of MBMC media containing sugars (20 g/L glucose, 8 g/L mannose and 8 g/L xylose) and the inhibitors to be tested in 50-ml glass bottles. The inhibitors — phenol, HMF, furfural and levulinic acid — were each added separately to four sets of bottles, at concentrations of 0.4, 0.3, 0.2 and 0.6 g/L, respectively. They were all also added to another set of bottles [at the listed concentrations] to evaluate their combined effects. No inhibitors were added to a set of controls. The bottles were then sealed with a rubber septum (SubaSeal, William Freeman Ltd, South Yorkshire, UK) and incubated with shaking (150 rpm on a KS 250 IKA Labortechnik shaker) at 27 °C under hypoxic conditions. Triplicates of the 8-mL cultures were harvested after 2, 5, 10 and 15 days incubation for FW measurements and biochemical analyses of the hyphae and media as described below.

2.3. Sugar assays

Sets of three random replicates (8 mL each) of the media of cultures subjected to each treatment were collected after 15 days of cultivation to determine their residual xylose, mannose and glucose contents according to Kudahettige et al. [9].

2.4. Inhibitor assays

Levulinic acid, HMF and furfural, were analyzed by HPLC using an Ultimate 3000 instrument (Dionex) equipped with an Aminex HPX-7H column and accompanying guard column from Bio-Rad Laboratories, 19 mM sulfuric acid as the mobile phase and both an RI detector and a UV detector. Phenol were analyzed using the same HLPC instrument equipped with a Reprosil-Pur C18-AQ column (Dr. Maisch, Ammerbruch, Germany), the UV detector, and a linear gradient of aqueous phosphoric acid and acetonitrile as the mobile phase. The detector was calibrated using known amounts of phenol at the Mo Research laboratories, Sweden.

2.5. Ethanol assay

After 2, 5, 10, and 15 days of each treatment ethanol contents of supernatants obtained from triplicate cultures were measured using gas chromatography, as previously described by Holmgren and Sellstedt [10], and ethanol (1%–10%) as a standard.

2.6. Preparation of samples for enzyme assays

To prepare samples for the assays described below triplicate 8 mL cultures were homogenized under liquid nitrogen using a mortar and pestle. Extracts were then incubated in mixtures described below, in 96-well micro-plates (with four standards per plate), and the enzymatic reactions were monitored using a SPECTRA max ®190 spectrophotometer [11]. Vblank and Vmax assays were performed for each sample in three replicates. Samples were randomly distributed to avoid local artifacts, and the acquired data were analyzed with SOFTmax ® PRO software using path length analysis.

2.6.1. Enzyme activity assays - XR, XDH and ADH activities

XR,XDH and ADH activites of triplicate 8 mL samples of fungal cultures grown under each treatment determined after 2, 5, 10 and 15 h of incubation. Crude protein was extracted from 50 mg of homogenized tissue by adding 400 μL of ice-cold disintegration buffer (pH 7) containing 0.1 M triethanolamine, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 0.5 mM EDTA. The extracts were then centrifuged at 16,000 × g (4 °C for 15 min). Enzyme activities of the resulting supernatants were determined following Eliasson et al. [12], by monitoring the absorbance at 340 nm of mixtures of 10 μL extract, 190 μL of reaction buffer and substrate. The reaction buffers consisted of: 100 mM