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Detoxification of furanic and phenolic lignocellulose derived inhibitors of yeast using laccase immobilized on bacterial cellulosic nanofibers

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ABSTRACT

Biotransformation of lignocellulose by microbial fermentation is usually preceded by thermo-chemical pretreatments followed by enzymatic hydrolysis of cellulose. Derivatives formed during the pretreatment of the lignocellulosic biomass inhibit enzymatic hydrolysis as well as microbial fermentation. Pretreated lignocellulose hydrolysate contains many derivatives of either furanic or phenolic inhibitory derivatives. In the present study, laccase was used to detoxify three different types of lignocellulosic derivatives that are highly toxic to microbial fermentation due to their low hydrophilic nature, namely furfural, acetosyringone, and coniferyl aldehyde. A minimal inhibitory concentration (MIC) test was carried out with *Saccharomyces cerevisiae*. The MIC of furfural, acetosyringone, and coniferyl aldehyde was 12 mM, 24 mM, and 1.5 mM, respectively. Laccase was immobilized on to cellulose nanofiber produced by *Gluconacetobacter xylinus*. Immobilized laccase showed a better pH and thermal stability than free laccase. Reuse of immobilized laccase retains 85% of its enzyme activity after 16 recycles. Immobilized laccase completely degraded the three lignocellulose inhibitory derivatives after 36 h of incubation at 40 °C. Finally, the degradation was confirmed by ultraviolet visible spectroscopy (UV–VIS spectrum), high performance liquid chromatography and liquid chromatography mass spectrometry. Interestingly, it was found that the effect of enzymatic degradation depends on the structural variation of the lignocellulosic derivatives as laccase alone detoxified the furfural and coniferyl aldehyde, whereas a redox mediator HOBt was needed for the detoxification of ketone based lignin derivative acetosyringone.

1. Introduction

Bioethanol is an emerging modern alternative to fossil fuels because it is produced from renewable biomass, generates no net CO_2, and is compatible with the current fuel distribution infrastructure [1]. This bioethanol can be produced from lignocellulosic biomass. The carbohydrates contained in lignocellulose are polymeric compounds such as cellulose and hemicelluloses, which are covered with lignin. The amount of lignin content makes it recalcitrant to the hydrolysis (saccharification) of polysaccharides and requires several steps before polysaccharides are converted to bioethanol. The conversion of lignocellulosic biomass to ethanol is carried out in four main steps: thermo-chemical pretreatment, enzymatic hydrolysis of the cellulose, yeast-based fermentation of the resulting sugars, and distillation. Pretreatment is carried out under severe conditions to break and/or remove the lignin, depolymerize cellulose, and hemicellulose to make the biomass more amenable to hydrolytic enzymes [2,3].

In addition to sugars, these methods also lead to the formation of many inhibitory compounds. In addition, the pretreatment conditions and raw material used greatly affect the nature and concentration of the final inhibitory compounds. The presence of different inhibitory compounds can also give rise to synergistic inhibitory effects [4]. Most yeast strains, including industrial

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate; AS, acetosyringone; BC, bacterial cellulose; BC-NFs, bacterial cellulose nanofibers; CA, coniferyl aldehyde; FF, furfural; FESEM, field emission scanning electron microscopy; FTIR, fourier transform infrared; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; LAC, Laccase; LC-ESI, MS-Liquid chromatography electrospray ionization mass spectrometry; MIC, minimal inhibitory concentration; $NaIO_4$, sodium periodate.

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strains, are susceptible to these lignocellulosic inhibitors even at low concentrations [5]. Some *Saccharomyces cerevisiae* yeast strains may be relatively resistant to inhibitors, but detoxification may still be necessary in order to achieve maximum productivity in the fermentation process. The major challenge for a sustainable biofuel industry is to circumvent the impact of lignocellulosic inhibitory compounds [6].

The inhibitory compounds are differentiated in to three major categories, namely furaldehydes, weak acids, and phenolics. Various physio-chemical methods are available for the detoxification of inhibitory compounds, whereas these methods add high production costs, complicate the biomass to ethanol process, and generate additional waste products. An alternative is the use of biological or biochemical mediated detoxification because little waste is generated, among other advantages. Biological detoxification is the use of microorganisms or their enzymes, which act on the toxic inhibitory compounds present in the lignocellulosic hydrolysate and convert in to less toxic metabolites [7,8].

Laccase is one of the lignin degrading enzymes that have specificity to many lignocellulosic toxic derivatives. It can be applied to various phenolic and non-phenolic (with redox mediator) compounds as well as to certain furan aldehydes. Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) belong to the blue-copper family of oxidases. They are glycoproteins, which are ubiquitous in nature. They mainly catalyze the polymerization or depolymerization processes of lignin formation in plants [9]. However, it is known that laccases are among the main enzymes involved in delignification processes due to white rot fungi. Laccases are widely used in the textile, dyeing, and printing industries for processes related to the decolorization of dyes and in the pulp and paper industries for the delignification of woody biomass specifically in the bleaching process [10].

The main disadvantage of applying the enzymes at a large scale is the production cost. This could be reduced by reusing the enzymes by binding them to an effective carrier support. Many synthetic carriers are available for the immobilization of enzymes, whereas the carrier molecules themselves are chemically synthesized. An alternative is the use of naturally available biomolecules for the immobilization process [11]. Among the various biopolymers, cellulose has a promising future potential for the immobilization of enzymes. It is the most abundant polymer available on the planet, as well as the most rigid biopolymer that is not easily soluble in many organic solvents.

Cellulose is a polysaccharide with a β-1, 4-glycosidic linkage, formed after condensation polymerization of long chains of anhydroglucose units. It is obtained from many sources such as woody trees and agricultural and municipal residues, although the latter is not of high purity. It contains various hemicelluloses residues and the least amount of lignin content [12]. However, the production of cellulose from microorganisms has many advantages over the production of cellulose from biomass. Most bacteria are used for the production of cellulose in many industries, but some algae and fungi produce cellulose similar to that produced by bacteria; cellulose produced by bacteria, algae, and fungi may be referred to as microbial cellulose. Considering other microbial sources, bacterial cellulose is easy to produce with simple medium and the right growth conditions. Overall, when compared to the cellulose derived from other sources, bacterial cellulose (BC) has better purity, mechanical properties, and biocompatibility. The mechanical properties of BC include its high crystallinity and elastic modulus (EM). The main bacteria involved in the production of bacterial cellulose are *Gluconacetobacter xylinum,* and *Sarcina ventriculi* [13]. In this study, cellulose was produced from *Gluconacetobacter xylinus* and it was used for the immobilization of laccase from *Trametes versicolor*. The bacterial cellulose nanomembrane immobilized laccase (BC-NFs-LAC) was used for the detoxification of three different types of lignocellulosic inhibitors, namely furfural (FF), acetosyringone (AS), and coniferyl aldehyde (CA) which are mainly present in the lignocellulosic hydrolysate after pretreatment. Furfural is a furan with aldehyde and acetosyringone is a ketone containing phenolic derivative, while coniferyl aldehyde is a phenolic compound with an aldehyde derivative. These compounds were selected among various lignocellulosic inhibitory compounds, as the predicted *log P* was higher than other derivatives of this type. High *log p* implies a low hydrophilicity, and low hydrophilic derivative compounds are potent inhibitors to the growth of yeasts [14].

2. Materials and methods

2.1. Chemicals and biochemicals

Laccase (EC 1.10.3.2: *p*-diphenol: dioxygen oxidoreductase; 20 U mg^{-1}) from *Trametes versicolor*, 1-hydroxybenzotriazole (HOBt), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), ethyl acetate, sodium periodate, furfural, acetrosyringone, and coniferyl aldehyde were obtained from Sigma–Aldrich Chem. Co., St. Louis, MO, USA. All other chemicals were of analytical grade and were used as received, without further purification.

2.2. Production of cellulose from Gluconacetobacter xylinus

Gluconacetobacter xylinus – 17012 was obtained from Korean Agricultural Culture Collection (KACC) and maintained in the medium as described by Matsaoka et al. Cellulose layers were produced from *Gluconacetobacter xylinus* using the modified protocol of Matsaoka et al. [15]. A standard medium which consisted of 2% peptone, 0.5% yeast extract, 0.5% D-glucose, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.2% ethanol (pH6.0) was used for cellulose production. The cells were grown in a conical flask and incubated at 30 °C for 2 days without shaking. The cellulose was formed as a mat like structure in the medium and it was collected for further processing.

2.3. Activation of bacterial cellulose nanomembrane (BC–NFs)

The cellulose produced from *Gluconacetobacter xylinus* was boiled in water for 5 min to remove the absorbed medium compounds. BC nanofibers (BC–NFs) were then washed with sterilized water and dried at 50 °C for further usage. The dried BC-NFs were cut in to pieces of around 5 mg each and oxidized for further immobilization.

2.4. Immobilization of laccase onto oxidized BC–NFs

For immobilization of laccase, aldehyde groups were generated onto the BC-NFs. In order to generate the aldehyde groups, 5 mg of BC-NFs were oxidized with 4.2 mg mL^{-1} of $NaIO_4$ solution (pH 6.1) at 30 °C in a shaking water bath for 8 h [16].

The oxidized BC-NFs were then immersed in 30 mL of sodium phosphate buffer (0.05 M) containing 5 mg of laccase (20 U mg^{-1}) and incubated at 25 °C under constant shaking for 2 h at 150 rpm. The reaction mixture was then cooled to 4 °C and incubation was carried out for an additional 10 h. The samples were extracted and washed with distilled water to remove non-bound enzymes. The non-reacted free aldehyde groups in the laccase immobilized BC-NFs were then covered with a Tris-HCl buffer (100 mM; pH 7.5) for 90 min. Finally, the resultant laccase immobilized BC-NMs were stored in a sodium phosphate buffer (100 mM; pH 4.5) at 4 °C.

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