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Ethanol from laccase-detoxified lignocellulose by the thermotolerant yeast *Kluyveromyces marxianus*—Effects of steam pretreatment conditions, process configurations and substrate loadings

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ABSTRACT

In our previous work, the ability of laccase enzymes to improve the fermentation performance of the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 on steam-exploded wheat straw slurry was demonstrated. As a continuation of this study, the present research evaluates different aspects, including pretreatment conditions, process configurations and substrate loadings, with the aim to proceed towards the use of *K. marxianus* and laccases for second generation ethanol production. For it, two wheat straw slurries resulting from different steam explosion pretreatment conditions (200 °C, 2.5 min and 220 °C, 2.5 min) were employed at various substrate loadings [5–14% (w/v)] under two process configurations: SSF (simultaneous saccharification and fermentation) and PSSF (presaccharification and simultaneous saccharification and fermentation). The better performance of *K. marxianus* was observed on the slurry produced at softer conditions. Its lower inhibitors content allowed to increase the total solids loading up to 10% (w/v) in both process configurations, reaching higher ethanol concentrations (12 g/L). Moreover, laccase detoxification improved these results, particularly in SSF processes, increasing the substrate loading up to 12% (w/v) and, consequently, obtaining the highest ethanol concentration (16.7 g/L).

1. Introduction

Biofuels made from biomass offer both a reduction of greenhouse gas (GHG) emissions as well as a partially replacement of liquid fossil fuels for transportation. In recent years, some policies have been adopted for the introduction of these alternatives into the current fuel distribution systems. For instance, the European Union directive 2009/28/EC establishes a share of 10% of biofuels in the transport sector by 2020 under several binding sustainability standards [1]. To reach this target, the development towards a cost-effective lignocellulosic ethanol industry is fundamental.

Lignocellulosic ethanol performs better than conventional sugar or starch based biofuels in terms of energy balance, GHG emissions and land-use requirements. Moreover, the lignocellulosic materials are abundant, cheap and do not compete with food [2]. In this context, wheat straw is a readily available candidate for ethanol production in Europe [3].

Ethanol can be made from lignocellulosic biomass through the enzymatic hydrolysis and the subsequent fermentation by microorganisms of the carbohydrates contained in the plant cell walls. Unfortunately, due to the recalcitrant nature of lignocellulose, a pretreatment step is required to improve the saccharification and to increase the fermentable sugars yields [4]. Steam explosion, a process that combines high pressures and temperatures, is a very suitable pretreatment technology that enhances the accessibility of enzymes to cellulose by an extensive alteration of the lignocellulosic structure [4]. However, this pretreatment leads to a partial sugars and lignin degradation, forming some soluble inhibitory compounds that can affect the downstream hydrolysis and fermentation steps [5–7].

According to their chemical structure, the formed inhibitors are classified into weak acids, furan derivatives and phenols and their concentration depend on the severity of the pretreatment and the raw material used [6]. Several procedures have been assayed for the removal of these compounds in order to prevent their inhibitory action. After steam explosion, the liquid fraction is usually separated from the solid fraction, which in turn is thoroughly washed to

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obtain the water insoluble solids (WIS) fraction, used as substrate. From an economical and environmental point of view, however, it would be desirable the use of the whole slurry obtained after steam explosion as there is no need for extra equipment (filtration and washing system), the amount of wastewater generated and freshwater required is reduced and the concentration of fermentable sugars is increased [8]. For these reasons, other methods, including biological, physical and chemical treatments, have been tested for the detoxification of pretreated materials [5,9,10]. Among biological methods, a wide variety of laccases have been successfully applied on different steam-exploded materials, showing good detoxification abilities [11–16]. Laccases are multicopper-containing oxidases with phenoloxidase activity, which catalyze the oxidation of phenols generating unstable phenoxy radicals that lead to polymerization into less toxic aromatic compounds [12,13].

In terms of process configuration, simultaneous saccharification and fermentation (SSF) appears as an attractive option for lignocellulosic ethanol production [17]. During this process, the glucose released by the action of hydrolytic enzymes is converted directly to ethanol by the fermenting microorganism, minimizing the end-product inhibition of enzymes caused by cellobiose and glucose accumulation [17]. Nevertheless, the main drawback of SSF is that it is usually conducted at temperatures below the optimal for the hydrolytic enzymes. Whereas saccharification has an optimum temperature around 50 °C, most fermenting yeasts have an optimum temperature ranging from 30 to 37 °C [18]. In this case, an enzymatic presaccharification prior to simultaneous saccharification and fermentation (PSSF) has been proposed to enable hydrolytic enzymes to act at their optimal temperature, enhancing the saccharification and, consequently, the ethanol yields [19]. Furthermore, this stage promotes the liquefaction of the broth, making it more fluid and easier to handle and facilitating the mixing during the fermentation [20]. Another interesting approach to overcome this disadvantage of SSF processes is the use of thermotolerant strains that can ferment sugars at temperatures close to the optimum of the enzymatic hydrolysis [21]. In this sense, *Kluyveromyces marxianus* CECT 10875, a yeast adapted and selected by Ballesteros et al. [22], is gaining great significance due to its ability of growing and fermenting at 42 °C. Furthermore, the use of thermotolerant strains during SSF can lead to other advantages such as the reduction of cooling costs and contamination risks, the increase of saccharification yields or the continuous ethanol removal [21].

The feasibility of *K. marxianus* for ethanol production has been successfully reported on various steam-exploded materials, using WIS fraction as substrate [23–25]. However, some of these studies have also showed the restriction of the yeast when using the whole slurry due to the presence of inhibitory compounds [25]. Recently, this restriction has been overcome thanks to the use of laccases, observing growth and ethanol production on steam-exploded wheat straw slurry [16]. In order to proceed towards the use of *K. marxianus* and laccases for ethanol production, an optimization study about pretreatment conditions, process configurations and substrate loadings was carried out. Thus, the whole slurries obtained by steam explosion of wheat straw at two severity conditions were subjected for SSF and PSSF processes at increasing substrate loadings. To evaluate these aspects, inhibitors content, cell viability and both glucose consumption and ethanol production were investigated.

2. Materials and methods

2.1. Raw material and steam explosion pretreatment

Wheat straw, supplied by Ecocarburantes de Castilla y León (Salamanca, Spain), was used as raw material. It presented the following composition (% dry weight): cellulose, 40.5; hemicellulose, 26.1 (xylan, 22.7; arabinan 2.1; and galactan, 1.3); lignin, 18.1; ashes, 5.1; and extractives, 14.6.

Prior to steam explosion, wheat straw was milled, using a laboratory hammer mill, in order to obtain a chip size between 2 and 10 mm. Then, the raw material was pretreated in a 10 L reactor at two conditions: 220 °C, 2.5 min and 200 °C, 2.5 min. For analytical purpose, one portion of recovered slurry was vacuum filtered with the aim of obtaining a liquid fraction or prehydrolysate and a solid fraction. To obtain the WIS fraction, the solid fraction was thoroughly washed with distilled water until the filtrate was clean. The remaining slurry was used as substrate for the different assays.

Chemical composition of both raw and pretreated material (WIS) was determined using the standard Laboratory Analytical Procedures for biomass analysis (LAP-002, LAP-003, LAP-004, LAP-017 and LAP-019) provided by the National Renewable Energies Laboratory [26]. Dry weight (DW) of slurry and WIS were determined by drying the samples at 105 °C for 24 h (LAP-001).

Liquid fraction was also analyzed in terms of sugars and degradation compounds. In the case of sugars quantification, a mild acid hydrolysis [4% (v/v) H_2SO_4, 120 °C for 30 min] was required in order to convert the oligomers into monomers.

2.2. Enzymes

Pycnoporus cinnabarinus laccase (60 IU/mL of laccase activity; 7–8 mg/mL of protein content), from Beldem (Belgium), was used for detoxification. Activity was measured by oxidation of 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its cation radical (ε_{436} = 29,300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 5) at 24 °C.

For saccharification, a mixture of NS50013 and NS50010, both produced by Novozymes (Denmark), was employed. NS50013 (60 FPU/mL of cellulase activity; 140 mg/mL of protein content) is a cellulase preparation produced by *Trichoderma* spp. that presents low β-glucosidase activity; therefore supplementation with NS50010 (810 IU/mL of β-glucosidase activity; 188 mg/mL of protein content), produced by *Aspergillus niger* and that mainly presents β-glucosidase activity, is typically applied in the biochemical transformation processes of lignocellulosic biomass into ethanol. The overall cellulase activity was determined using filter paper (Whatman No. 1 filter paper strips) and β-glucosidase activity was measured using cellobiose as substrate. Both enzymatic activities were followed by the release of reducing sugars [27], defining one unit of enzyme activity as the amount of enzyme that transforms 1 μmol of substrate per minute.

In addition to the activity, total protein content from all enzymatic preparations was analyzed by BCA protein assay kit (Pierce Ref. 23225), using bovine serum albumin as standard.

2.3. Microorganism and growth conditions

The fermentative yeast used in this study was *K. marxianus* CECT 10875, a thermotolerant strain selected by Ballesteros et al. [22]. Active cultures for inoculation were obtained in 100-mL flasks with 50 mL of growth medium containing 30 g/L glucose, 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4 \cdot 7H_2O$. After 16 h on a rotary shaker at 150 rpm and 42 °C, the preculture was centrifuged at 9000 rpm for 10 min. Supernatant was discarded and cells were washed once with distilled water and then diluted to obtain the desired inoculum size.

2.4. Laccase detoxification

The slurries obtained after pretreatment at 220 °C, 2.5 min and 200 °C, 2.5 min were subjected to different laccase detoxification