Evaluation of the Conversion Potential of Maize Stover from Soil Phytoremediation to Bioethanol

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Abstract. This work aimed to evaluate the conversion potential of maize stover (MS) from phytoremediation of heavy metals contaminated soil to bioethanol. Thus, MS was submitted to an acid pretreatment with 3% (v/v) H_2SO_4, HCl, HNO_3 or CH_3COOH at 85 °C for 48 hours. An enzymatic hydrolysis step with *Accellerase* or *Ultraflo* was applied at 50 °C for 13 hours. Finally, *Saccharomyces cerevisiae* was used to ferment the glucose at 37 °C, followed by distillation to recover ethanol. The average yield in ethanol for the MS produced in the two contaminated soils was 0.51 and 0.32 $g_{ethanol}/g_{MS}$ for the MS treated with HCl and *Accellerase* and 0.39 and 0.27 $g_{ethanol}/g_{MS}$ for the MS treated with HNO_3 and *Ultraflo*, respectively. For the MS produced in the control soil, the yield was 0.37 and 0.44 $g_{ethanol}/g_{MS}$ for the treatment with HNO_3 and *Ultraflo* and HCl and *Accellerase*, respectively, being the differences in ethanol yield assigned to the different cellulose/ hemicellulose content of the MS samples. No metals were detected in the ethanol recovered. This research demonstrated the feasibility of valorization of MS from heavy metals contaminated soil phytoremediation through ethanol production, contributing to a more sustainable process of soil phytoremediation.

Keywords: Biofuel, Cadmium, Heavy Metal Contaminated Soil, Phytoremediation, Soil Decontamination, Zinc.

1 Introduction

Technological development has been driven by the high population growth over the years and by the growing demand for new goods and appliances, with the consequent increase in materials and energy consumption [1].The consequent depletion of natural resources and the intensive use of energy, mostly derived from fossil sources, caused significant negative environmental impacts that are on the basis of climate changes,

causing great concern not only by the scientists, but also by the general public that is getting increasingly aware of sustainability issues. This state of affairs has been the engine of modern society commitment to sustainable development, making it fundamental to seek new and more viable solutions for society and the environment, that reduce the dependence on fossil fuels and contribute to the GHG reduction, minimizing the potential for global warming [2]. Thus, different techniques have been developed to use renewable energy sources. For the transportation sector, the use of biofuels produced from biomass feedstock represents the most immediate and interesting alternative, as currently most renewable energy technologies for this purpose are still under development [3]. Bioethanol is presently the most widely used liquid biofuel alternative to gasoline [4].

Increasing soil contamination due to intensive agriculture and to industrial activities such as mining, which introduces considerable quantities of heavy metals into agricultural soil, has been a growing concern, as the presence of heavy metals in soil makes it unusable for food agriculture [5]. This has motivated the need to develop sustainable techniques for the extraction of its contaminants. Currently, phytoremediation is one of the most commonly used processes since it has the advantage of being a treatment performed at the contaminated site (*in situ*). Therefore, phytoremediation plays an important role in soil remediation, making them suitable again for cultivation by removing contaminants [6]. However, biomass resulting from phytoremediation, that may contain high levels of contaminants such as heavy metals, is usually composted, landfilled or sometimes submitted to thermal processes, with a significant loss of valuable resources in such process [7]. To produce second generation bioethanol, it is necessary to convert polysaccharides from lignocellulosic material. Lignocellulose is a material belonging to the wall constitution of plant cells and refers to the material composed of lignin, cellulose and hemicelluloses. Its composition varies with the type of plant, age and environment where it grows. Generally, plants have in their composition 45-25% of lignin, 40-50% cellulose and 25-30% of hemicellulose [8–10].

Maize has been reported as potentially effective in Cd, Zn, Cr, Cu and Pb removal from contaminated soil [11–13]. Thus, in the present work, maize (*Zea mays* L.) was used to perform the Cd and Zn contaminated soil phytoremediation. In order to further valorize this organic matter of lignocellulosic nature that would otherwise be discarded, and aiming to reduce the competition with the agricultural food sector, it was proposed the possibility of transforming lignocellulosic material (maize stover) resulting from phytoremediation of a soil contaminated with heavy metals (Cd and Zn) into bioethanol. Therefore, the present study investigated the feasibility of using maize stover (MS) from phytoremediation of soils contaminated with Cd and Zn for the production of bioethanol, to be used as a liquid biofuel, and compared with similar material grown in uncontaminated soil (control). It was assessed the effect of the potential presence of these heavy metals not only on alcohol yield but also on the presence of heavy metals in the biofuel produced.

2 Materials and Methods

2.1 Sample Preparation

Maize stover (MS) was produced from the lignocellulosic parts (roots, stem and leaves) of the plants of *Zea mays* L. cultivated in three different soils: an agricultural soil, one industrial soil from Estarreja, both from the North of Portugal, and a third mining soil from Panasqueira, in the inner center of Portugal, as described in a previous research by Paulo et al. [14]. The plants were collected after 120 days of cultivation time, sun-dried, removed the attached soil and the separated parts were ground with a Fritsch grinder, to particles of about 1 x 5 mm. The stem and leaves fraction was selected for the rest of the process.

2.2 Acid Pretreatment

As previously described, the main function of pretreatment is to break down the cellulose and hemicellulose polymers present in lignin, thus increasing the accessibility to enzymes in the enzymatic hydrolysis process. In the present research an acid pretreatment was applied. Thus, about 20 g of maize stover were weighed into a 500 ml glass bottle. Subsequently, 150 ml of one of each of the acids was added to each of the flasks used – H_2SO_4, HNO_3, HCl or CH_3COOH, at 3% (v/v) concentration (Fig. 1. (a)).

The pretreatment was performed for 48 hours in a thermostatic water bath shaking at 100 rpm (Julabo, SW22) at 85 °C. Each treatment was performed in duplicates.

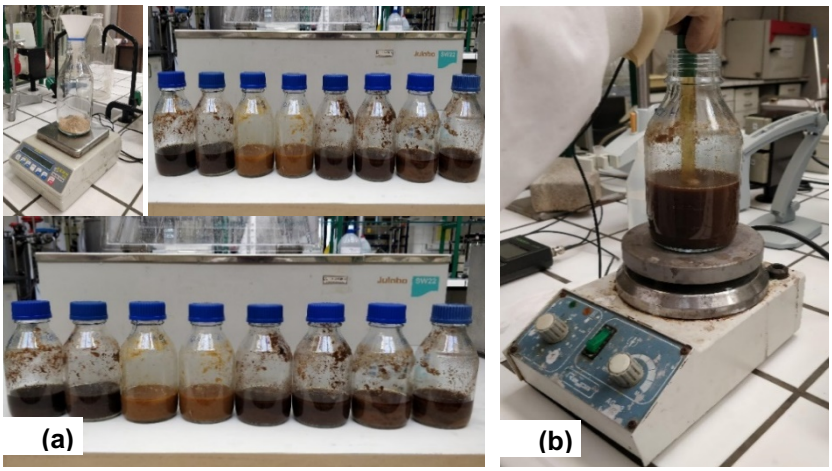

Fig. 1. (a). Top Left: Weighing maize straw in glass bottle; Top Right: Samples before pre-treatment. Bottom: Samples after pre-treatment; (b) pH regulation after pre-treatment.

2.3 Enzymatic Hydrolysis

The enzymes used in this study were kindly offered by their respective supplier. Thus, it was used *Accellerase* 1000, a liquid enzyme preparation that contains 5-10% fungal cellulase and less than 1% β-glucosidase (supplied by *Genencor*, a *Danisco* division) and *Ultraflo*, a liquid enzyme preparation that contains up to 15% endo-1,3(4)-β- glucanase (supplied by *Novozymes*).

To achieve the maximum efficiency in the enzymatic hydrolysis process, after the end of pre-treatment, the glass bottles were cooled and the pH value was measured, and adjusted to 5, through small additions of NaOH at 40% (w/v) or 0.1 M when closer to the desired pH (Fig. 1. (b)).

After pH regulation, 2 ml of *Accellerase* 1000 (*Danisco*) or *Ultraflo* (*Novozymes*) enzyme were added to each one of the bottles, stirring to homogenize. The bottles were inserted again in the water bath with agitation (Julabo, SW22) at a temperature of 50 °C for 13 hours and the hydrolysate was used for fermentation (Fig. 2).

Fig. 2. Left: Samples before the hydrolysis process; Right: Samples after hydrolysis process.

2.4 Fermentation

Commercial *Saccharomyces cerevisiae*, currently used in the bakery process, was used for the fermentation process. 40 g of yeast was diluted in 200 ml of deionized water and after homogenization, 25 ml of inoculum was added to each of the bottles. The mixture was stirred and the bottles were covered with porous stopper, placed in the thermostatic water bath with agitation at 60 rpm at 37 °C for 11 days (Fig. 3. (a)). After fermentation, the samples were filtered under vacuum, to separate the liquid phase from the sludge. Ethanol was recovered from the filtered fermentation broth in a rotary evaporator (Buchi, R-210) at 120 mbar and 60 °C (Fig. 3. (b)).

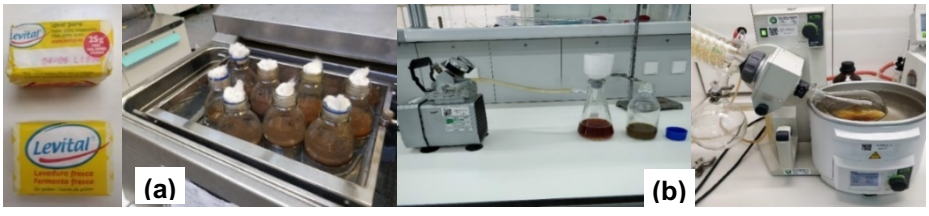

Fig. 3. (a) Applied yeast and fermentation in a thermostatic bath with slow shaking; (b) Vacuum filtration and ethanol recovery using a rotary evaporator.

2.5 3,5-Dinitrosalicylic Acid Method (DNS) for Sugar Quantification

The reducing sugars content in the samples collected during the different stages of ethanol production was evaluated according to the methodology described by Miller [15], in which the DNS is reduced to 3-amino, 5-nitrosalicylic acid with the simultaneous oxidation of the free carbonyl group present in reducing sugars.

The concentration of reducing sugars was determined reading the absorbance at λ = 500 nm using a UV-Vis spectrophotometer (Shimadzu, UV-1700 Pharma Spec; Shimadzu, V260) (Fig.4.) and a calibration curve previously prepared.

Fig. 4. UV-Vis spectrophotometer (left) and the standards used to prepare the calibration curve (right).

3 Results and Discussion

3.1 Identification of the Most Promising Acids for Biomass Pretreatment

For the samples collected from the supernatant of maize stover from non-contaminated soil, after the pre-treatment and enzymatic hydrolysis, the amount of sugar released was evaluated using the DNS method, as previously described. Fig. 5.a) shows the total reducing sugars concentration for each combination acid + enzyme, which allowed to identify the combinations that were the most effective in the conversion of polysaccharides to simple sugars. Fig. 5.a) shows that the liquor produced from the pretreatment with acetic acid, is the one that has the lowest concentration of sugars for both enzymes, which shows that it is the least effective in pretreatment and therefore promotes lower conversion of sugars. This conclusion is consistent with the fact that acetic acid is the weakest one, and therefore was not able to effectively break the lignin molecules. These results are consistent with those obtained by Gundupalli and Bhattacharyya [16] who tested the effects of sulphuric, hydrochloric and nitric acids on coconut coir, and concluded that nitric acid yielded the highest reducing sugar amount.

3.2 Optimization of Pretreatment and Enzymatic Hydrolysis Conditions

After analyzing the results obtained in the preliminary tests (Fig. 5. (a)) and verifying the most effective combinations of acid and enzyme, the same steps described above were performed applying the two most effective combinations to biomass collected

from all of the three soils. The tests applying the best operating conditions to biomass from non-contaminated soil were performed again to serve as a control, simultaneously to the other maize stover samples from phytoremediation of contaminated soils from Estarreja and Panasqueira. The results of total reducing sugar concentration obtained in these experiments are shown in Fig. 5. (b). The results obtained for both biomasses under the same processing conditions (Fig. 5.b), show the presence of different concentrations of contaminants in the soil can influence the production of reducing sugars, probably because the presence of Zn and Cd in the soil affects the composition of the plant parts that were used in the study [14].

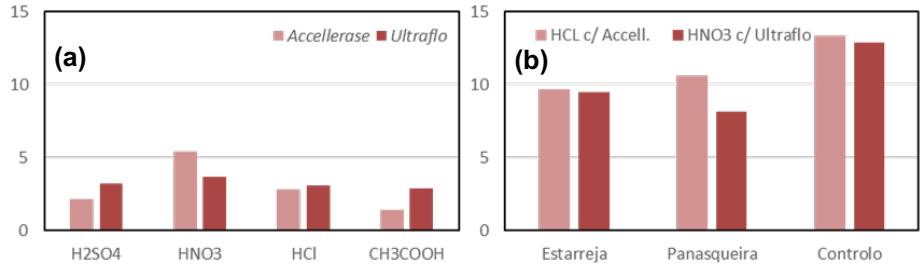

Fig. 5. Reducing sugar concentration a) after pretreatment followed by enzymatic hydrolysis of biomass from control soil, in the preliminary experiments; b) using the two best combinations of acid pretreatment followed by enzymatic hydrolysis applied to all three biomass samples.

3.3 Results After Fermentation

Samples were collected at two different stages of fermentation (6th day and end), and the total reducing sugars concentration were read using the spectrophotometer. Fig. 6 shows the results obtained for total reducing sugars concentration during the different stages of ethanol production.

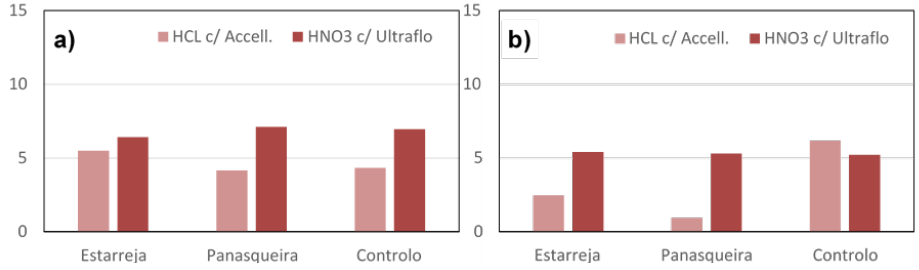

Fig. 6. Concentration of total reducing sugars (a) on the 6th day of fermentation; (b) at the end of fermentation.

Through the analysis of the total reducing sugar concentration along the fermentation process (Fig. 6), it was concluded that at all stages, the pretreatment with HNO_3 and the enzymatic hydrolysis with *Ultraflo* provided a rather constant amount of reducing sugars. For the treatment with HCl and *Accellerase* 1000, it is concluded that there was a high consumption of the sugars present in the liquor, which may indicate that this

combination contributes to higher consumption of glucose by the yeasts in the fermentation process, probably due to the presence of lower amount of inhibitors that resulted from the HNO_3 pretreatment.

It was also concluded that the final concentration of reducing sugars for the biomass from control soil is higher. It was hypothesized that:1) the contaminants retained by biomass during phytoremediation may have a positive effect on the conversion of sugars, or 2) the contaminants present in soil affected the biomass composition, contributing to increase the contents in glucose of the corresponding biomass. The second hypothesis was further confirmed by the compositional analysis of the three biomasses [17], that showed that the cellulose content was 27.1, 32.5 and 24.0%, and the hemicellulose content was 32.0, 31.3 and 23.5%, respectively, for the biomasses from control, Panasqueira and Estarreja soils. For the same biomasses, the lignin content was 20.9, 18.6 and 23.6%, and the ash content was 13.9, 9.0 and 21.8%, respectively.

Table 1 summarizes the results of the sugar and ethanol concentration and recovery obtained for the biomass from contaminated and control soil.

Table 1. Summary of total reducing sugar and ethanol concentration for biomass from contaminated and control soil.

Soil	Pretreatment + enzyme	Reducing sugar concentration after hydrolysis (g/l)	Reducing sugar concentration remaining after fermentation (g/l)	Volume of ethanol solution recovered (ml)	Volume of ethanol (ml)
Estarreja	HCl + *Accellerase*	9.74	2.47	338.75	8.05
	HNO_3 + *Ultraflo*	9.47	5.40	288.22	5.13
Panasqueira	HCl + *Accellerase*	10.61	0.94	330.50	6.22
	HNO_3 + *Ultraflo*	8.15	5.30	253.04	4.25
Control	HCl + *Accellerase*	13.38	6.17	355.24	7.02
	HNO_3 + *Ultraflo*	12.91	5.20	293.00	5.79

Biomass from the control soil yielded the highest amount of total sugars, of 13.4 g/L when the pretreatment was performed using HCl followed by an enzymatic hydrolysis with *Accellerase* 1000. However, the yield in ethanol was also lower for this biomass, probably due to the fact that it contained more hemicellulose that release C5 sugars, that are non-fermentable by *Saccharomyces cerevisiae*. The biomass from Estarreja soil, which is a Petrochemical site, yielded the highest amount of ethanol, probably because the biggest fraction of released sugars was glucose.

Hydrochloric acid pretreatment followed by enzymatic hydrolysis with *Accellerase* yielded the best results in our study. Consequently, no acetic acid and no sulfuric acid were yielding the best results (data not shown). The pretreatment with hydrochloric acid followed by enzymatic hydrolysis with *Accellerase* and pretreatment with nitric acid followed by enzymatic hydrolysis with *Ultraflo* yielded the best results in ethanol production from maize stover, compared with the pretreatment with any of the sulfuric

or acetic acids. Sulfuric acid is a strong acid that probably originates fermentation inhibitory compounds, while acetic acid is a week acid that does not sufficiently damage the lignin molecules, thus impeding further polysaccharides hydrolysis.

Maize stover from contaminated soil yielded more ethanol while pre-treated with hydrochloric acid followed by hydrolysis with *Accellerase* - and it was 32-36% higher - than when the pre-treatment was performed with HNO_3 and the enzymatic hydrolysis with *Ultraflo.* On the other hand, maize stover from contaminated soil yielded 12% higher and 11% lower ethanol concentration, as compared to the control soil, for the same conditions.

In this work, the maize stover pretreated with HNO_3 followed by enzymatic hydrolysis with *Ultraflo* yielded on average 290, 210 and 255 L ethanol per 1 ton dry biomass, respectively for the control, Panasqueira and Estarreja soil. from control soil yielded 290 or 350. Interestingly, when HCl was used in the pretreatment and *Accellerase* in the enzymatic hydrolysis, the yield was significantly higher, of 350, 310 and 405 L ethanol per 1 ton dry-biomass, respectively for the control, Panasqueira and Estarreja soil.

Table 2 lists the ethanol yields expected for various biomass feedstock.

Table 2. Biomass and its potential ethanol yield.

Biomass feedstock	Potential ethanol yield (L/ ton dry biomass)	Reference
Bagasse	437	[18]
Barley	410	[19]
Corn grain	470	[18]
Corn stover	428	[18]
Cotton gin trash	215	[18]
Forest thinning	309	[18]
Hardwood sawdust	382	[18]
Mixed paper	440	[18]
Oat	410	[19]
Rice straw	416	[18]
Sweet sorgum	80	[20]
Wheat	430	[19]

Comparing the data in Table 2 with the estimated average yield of the biomass in the current work, it is observed that under the best conditions tested, the ethanol yields obtained are quite close to those found in the literature, particularly for the biomass grown in Estarreja soil. Thus, it is concluded that the conversion of maize stover from contaminated soil phytoremediation is of potential interest for bioethanol production.

Concerning the potential leaching of the heavy metals to the bioethanol produced in the current work, in none of the samples of ethanol recovered from our assays, zinc or cadmium was detected, making it suitable for biofuel or for sanitary uses.

Although the ethanol yield achieved is interesting, it could be increased if a pentose fermenting yeast had been used.

4 Conclusion

In this work, maize stover from plants used in the heavy metal contaminated soil phytoremediation was used as a lignocellulosic biomass feedstock for bioethanol production. The biomass was submitted to acid pretreatment, enzymatic hydrolysis followed by fermentation with *Saccharomyces cerevisiae yeast.* To conclude, the yield in ethanol using hydrochloric acid and *Accellerase* 1000 or nitric acid and *Ultraflo* was of 7.0 ml and 5.8 ml ethanol per 20 g biomass. Based on the results obtained, the yield in alcohol for maize stover produced in both contaminated soils was 0.5, 0.39, 0.32 and 0.27 g ethanol per 1 g of maize stover and for the maize stover produced in non-contaminated soil it was 0.44 and 0.37 g ethanol per 1 g of maize stover when hydrochloric acid and nitric acids were used, respectively. It would probably be recommended to use another yeast that could also ferment the C5 sugars to yield an higher amount of ethanol, particularly when converting the maize stover from the control and the Estarreja soils, as the remaining unfermented sugar concentration was still high at the end of the fermentation. Therefore, it can be concluded that ethanol production from biomass of maize plants from phytoremediation of heavy metal contaminated soils is technically feasible, allowing to further valorise an underexploited feedstock for bioethanol production.

Funding

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