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A cleaner delignification of urban leaf waste biomass for bioethanol production, optimized by experimental design

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Abstract: This work presents a study of the conditions leading to improved delignification of urban forest waste typical of Buenos Aires. Particularly, the leaf waste of *Platanus acerifolia* has been studied. Delignification was accomplished by acid-oxidative digestion using acetic acid and 30% hydrogen peroxide 1:1. The effect of reaction time (30–90 min), temperature (60–90 °C), and solid loading (5–15 g solid/20g liquid) on delignification and solid fraction yield were studied. The process parameters were optimized using the Box-Behnken experimental design. The highest attained lignin removal efficiency was larger than 80%. The optimized conditions of delignification, while maximizing holocellulose yield, pointed to using the minimum temperature of the examined range. Analysis of variance on the solid fraction yield and the lignin removal suggested a linear model with a negative influence of the temperature on the yield. Furthermore, a negative effect of the solid loading and low effect of temperature and time was found on the degree of delignification. Then the temperature range was extended back to 60°C, and good results both of yield and delignification were obtained. Significant delignification with good yield attained at moderate temperature. Lignin removal was visualized using confocal laser scanning microscopy. The solid structure was slightly modified as judged from scanning electron microscopy.

Keywords: Biomass delignification; acid-oxidative hydrolysis; experimental design; urban waste

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1. Introduction

The urgency of accelerating development in a sustainable direction within a context of social demand for practices in compliance with standards of minimum environmental impact is reflected in the prerequisite for existing processes intensification and reduced risks of scaling up novel cleaner energy technologies based on renewable raw materials [1] while ensuring the continuity of energy supply. In this context, biomass is fundamental to a future energy sector adaptation and effectively mitigates climate change since energy production is the main source of carbon emissions [2]. Biofuels are crucial for reducing fossil-fuel dependency and greenhouse gas emissions. Bioethanol is a promising sustainable candidate for substituting gasoline [3]. Ethanol utilization in the world is available up to 20% blend in gas fuels.

Cellulosic ethanol presents an exciting and tangible but underdeveloped economic opportunity for ethanol producers, as the fuel's greater greenhouse gas (GHG) reductions

[4]. Most of the produced bioethanol is considered a first-generation biofuel because the raw material for the process is starch or glucose, coming mainly from the arable land for food production [5]. The major negative social impact of first-generation bioethanol production can be sorted by using Lignocellulosic Biomass as starting material because of effective decoupling with food production. The limitation of the so-called second-generation bioethanol is mainly the relatively low bioavailability of glucose of the feedstock because of the presence of lignin [6], which hinders yield, and it is considered a low-value residue. Many second-generation bioethanol industrial initiatives could fail if the technology gaps are not carefully addressed. A third of the bioethanol world supply was predicted to be produced in 2020, parting from LCW [7], but currently and unfortunately, less than 2% of the market is supplied by this methodology [8]. Most pilot experiences all over the world were unsuccessful at scaling up. Unit integration and system optimization are the ultimate solutions for making the cellulosic ethanol production process [9].

Conversion of agro-industrial and urban wastes (lignocellulosic wastes – LCW) to energy is an innovative approach for waste valorization and management, simultaneously mitigating environmental pollution. Utilizing LCW has economic benefits while addressing the issue of food vs. fuel controversy, being an attractive alternative for disposal of agricultural, forestry, and urban waste [10–14]. Garden and street forest waste is an important lignocellulosic feedstock recognized as a resource [15]. Besides conventional processing methods like burial, microbial composting, and biochar production, biorefineries to produce biogas and bioethanol from garden and forestry waste have attracted global attention [16–19]. In Buenos Aires city, with streets beautifully covered by trees, this type of waste can reach hundreds of tons per day. Although they are partially treated in a city's recycling plant [20] and used for compost and pulping, specific information for optimizing this LCW valorization is relevant for intensifying and diversifying the processes.

There are four basic processes involved in the biochemical production and obtaining of ethanol by yeasts from cellulosic biomass: pretreatment for cellulose separation from lignin (called delignification), enzymatic hydrolysis of cellulose (called saccharification), fermentation, and distillation. The optimization of delignification and saccharification are key to improving fermentation efficiency. The pretreatment of LCW is a key stage to disrupt the recalcitrant structure of lignocellulose [10]. The delignification processes traditionally used in the extraction of cellulose for paper production and the methods that use strong acids and alkalis are not adequate to generate a bioavailable substrate for fermentation [21]. Classical dilute acid pretreatments require high temperatures and the remaining lignin still hinders access to the cellulose fibers [17]. Delignification of LCW strongly facilitates the hydrolysis of holocellulose to fermentable sugars involved in producing biofuels and other bio-based chemicals.

Moreover, at high temperatures, aliphatic acids, and furans toxic for fermenting microorganisms are formed [10,22–24]. Therefore, developing low-temperature pretreatments is important to reduce toxic molecules and energy consumption. The high oxidative mixture of hydrogen peroxide and acetic acid has been suggested as a low-temperature pretreatment with high lignin removal efficiency, leading to significant recovery of fermentable sugars [25–27]. The PoxAc delignification process [27] is based on a mixture of hydrogen peroxide in glacial acetic acid, which allows cellulose to be separated and obtaining high-quality lignin to be used in a variety of applications, such as Supercritical Water Gasification to produce hydrogen [28] and materials for 3D printing [29]. The PoxAc delignification method uses reagents that decompose into harmless compounds produces a substrate that allows better productivity of sugars through enzymatic and biological treatments. In addition, delignified LCW can be used for other purposes, such as fluid rheology modifiers [30], as reinforcement material in composites and biodegradable polymers, as strength additives in textile printing and coating products [31,32], and many more.

Platanus acerifolia is widely planted in major cities since it is a tough, durable tree that can tolerate severe pruning and smog. The plant typically grows up to 25 m and has a good shading capacity for dense leaf. This species is frequently found in many neighborhoods around the city of Buenos Aires. Hence, this work aims at examining and optimizing conditions to attain delignification of the leaf waste arising from the street sweeping of this species. For this purpose, the low-temperature acid-oxidative hydrolysis of the LCW is accomplished by digestion in a solution of glacial acetic acid and 30% hydrogen peroxide 1:1 (v/v) ratio, which was suggested as the best proportion for delignification [27].

2. Materials and Methods

2.1. Raw material pretreatment and characterization

The leaf waste was obtained from the leaves sweeping a street in the Saavedra neighborhood within Buenos Aires, where all the planted trees were *Platanus acerifolia*. The material (6kg) was separated from dust in an industrial sieve, mainly retaining dry leaves and stems. Then, the LCW was humidified, added to the same water mass, and triturated in batches of 1kg using a 2200W Turbo blender at 35000 rpm for 10 minutes. The resulting LCW was homogenized and kept in a freezer for subsequent characterization and a series of experiments. Analytical grade hydrogen peroxide, acetic acid, and sulphuric acid were used for the experiments.

The moisture of the samples was determined using a Precisa XM50 moisture analyzer (Precisa Gravimetrics AG, Dietikon, Switzerland). Standard methods determined the ash content and ethanol extractives. Lignin acid-insoluble contents of the untreated and treated samples were determined by dissolving 0.3 g of the specific substrate with 4mL of 72% v/v sulphuric acid and let to react 3 hours at room temperature (28ºC). Then, the solution was diluted to 5% v/v (1M) with distilled water and heated to 100ºC for 2.5 hours. The suspension was filtrated, dried, and weighted. The lignin content is obtained as the final dry mass referred to the initial solid dry mass. The cellulose content of the untreated sample was determined by dissolving 0.3 g of the substrate into 4mL of 72% v/v sulphuric acid. Immediately afterward, the solution was diluted to 5% v/v (1M) with distilled water, and the hydrolysis proceeded by heating to 100ºC for 2.5 hours. The suspension was filtrated, dried, and weighted. The cellulose content was determined as the difference between final and initial dry masses [33,34].

The oxidative hydrolysis was carried out in batch mode in 100mL Erlenmeyer, with orbital shaking. A solution (20 mL) containing equivalent volumes of glacial acetic acid and 30% hydrogen peroxide was contacted with different masses of LCW (5 to 15 g). The orbital shaker allowed regulating temperature and time. The temperature was modified within the 60-90°C range, while digestion was varied between 30 and 90 minutes. For reference, one sample was treated with a dilute solution of sulfuric acid (0.1M) under the conditions (90min, 90°C, 5g) that were found as optimal for biomass pretreatment aimed at subsequent enzymatic saccharification for bioethanol production [35]. The treated samples were filtered from the acid-oxidant liquor with a cheesecloth. Moisture and lignin content of the obtained solid samples were determined to get the solid fraction yield and lignin removal efficiency, as expressed in Eqs. 1 and 2, were m_0^{dry} and m_f^{dry} are the initial and final mass of solid samples on a dry and ash-free basis, and $\%Lignin$ indicates the fraction of insoluble lignin in the dry and ash-free basis.

$$\text{Solid fraction yield} = \frac{m_f^{dry}}{m_0^{dry}} \quad (1)$$

$$\text{Lignin removal} = \frac{\%Lignin_0 m_0^{dry} - \%Lignin_f m_f^{dry}}{\%Lignin_0 m_0^{dry}} \quad (2)$$

For data analysis, the so-called severity factor, Ro, defined as in Eq. (3) was considered, where "t" represents the digestion time in minutes, "T" is the treatment temperature in °C, and "T_{ref}" is 100 °C, a reference temperature [12,22].

$$\text{Severity factor } \text{Ro} = \text{t} \cdot exp\left(\frac{\text{T} - \text{T}_{\text{ref}}}{14.75}\right) \quad (3)$$

2.2. Confocal Laser Scanning Microscopy (CLSM)

Variations of the lignin content between treated and untreated samples were assessed by CLSM, discriminating the leaves and stems portions of the LCW. Under the conditions used to observe the samples, lignin exhibits autofluorescence and can be easily distinguished by this technique from other components without contrast reagents [36–38]. The images were obtained using an Olympus FV300/BX61 confocal microscope (Olympus, Japan). The samples were first dried, fixed on glass slides, and covered with a thin glass lid. They were first observed under an optical microscope (UPLFL 10X). After establishing the proper focus, multiple stacks of 1024p x 1024p pictures were taken with laser excitation at 405nm and fluorescence emission detection at 550nm, characteristic of lignin, in scanning mode [36]. Magnification was at 10X as well as an objective lens (UPLFL 10X). Images were analyzed using the ImageJ open-source software (https://imagej.nih.gov/). The stack was averaged over the Z-axis and converted to a 0-255 grayscale (8-bit). Mean intensity was calculated from these images averaging all pixels of the best-focused image, as suggested by Hernández-Hernández et al. (2016, 2014) [38,39].

2.3. Scanning electron microscopy (SEM)

Changes in surface morphology of untreated and delignified samples were examined under scanning electron microscope Carl Zeiss NTS-Supra 40 (Carl Zeiss AG, Oberkochen, Germany). Before analysis, the samples were fixed on the sample plates using carbon tape as a non-conducting adhesive. Then, the samples were subjected to gold sputtering to increase their conductivity before taking the micrographs.

3. Experimental design

The acid-oxidative hydrolysis of the LCW is a multivariable process in which the variables interact with one another. Therefore, experimental design techniques present a more balanced alternative to the *ceteris paribus* (i.e., one variable at a time) approach to obtain information and improve the conditions for the delignification stage with good holocellulose yield [40–42]. The Statistical Design of Experiments (DoE) combined with the Response Surface Methodology (RSM) analysis covers only a fraction of the experimental space. The DoE-RSM method allows drawing effective conclusions while estimating the interactions between the different variables. Methods for experimental design can be broadly divided into two categories:

i) One includes designs used to explore a potentially large number of input variables to discover those that are statistically significant and estimate their magnitude. Plackett-Burman (PBSD) and Factorial Fractional designs are examples of these methods [42].

ii) The other category includes methods for optimizing a process given a reduced number of variables selected previously. A typical scenario is Central Composite (CC) or Box Behnken Design (BBD) and a RSM analysis on linear or quadratic models. Moreover, RSM analysis allows fine-tuning of any process variables, an issue to be considered when an industrial scale is aimed [42]. The objectives of the

RSM are to confirm observed or assumed effects in the variable selection stage and quantify the most appropriate values for the parameters under study to optimize the response. Finally, the predicted optimum must be verified after model building and optimization.

3.1. Box Behnken Design of Experiment

Box Behnken DoE aims to find the effects of factors, estimate the curvature or quadratic effects, and determine the most appropriate values for the parameters under study to find the response surface and minimize the required set of experiments. The influences of solid loading, acid-oxidative hydrolysis temperature, and time on delignification efficiency and solid fraction yield were evaluated using full factorial design in Minitab 17 software. Mathematical matrices will be constructed within the ranges of the chosen variables. The three independent variables have three levels (-1, 0, and +1; **Table 1**) and three central points, leading to 15 combinations (**Table 2**). The severity factor calculated from the time and temperature conditions is included in **Table 2**. Apart from the combinations arising from the Box & Behnken Design, the hydrolysis conditions with sulfuric acid were carried out for comparison. The experiments carried out to extend the analysis of the temperature influence are indicated in **Table 2**.

Table 1: Levels of factors tested in the Box and Behnken Design of Experiments for optimizing LCW delignification.

Factors	**Levels**		
	-1	0	1
Time (min)	30	60	90
Temperature (°C)	70	80	90
Solid Loading (g/ 20mL liquid)	5	10	15

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Table 2: Factor combinations considered for the Box & Behnken Design. 210

Run	Time (min)	Temperature (°C)	Solid Loading (g/20mL liquid)	Log (Ro)
1	90	80	15	1.37
2	30	80	15	0.89
3	90	70	10	1.07
4	60	90	5	1.48
5	90	80	5	1.37
6	60	70	15	0.89
7	90	90	10	1.66
8	60	80	10	1.19
9	60	80	10	1.19
10	30	70	10	0.59
11	60	70	5	0.89
12	30	80	5	0.89
13	30	90	10	1.18
14	60	90	15	1.48
15	60	80	10	1.19
S1	90	90	5	1.66
E1	60	60	5	0.60

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4. Results and discussion

4.1. Effect of the acid-oxidative hydrolysis on the LCW characteristics

The proximal composition of the *Platanus acerifolia* leaf waste was studied to evaluate their potential for biofuel or biochemical production. LCW moisture was evaluated every time it was used for experiments to assess the exact solid dry mass. Moisture was always around 70% w/w. The dry leaf waste contained a minor amount of ash (3.2% w/w) and ethanol extractives (4% w/w), significant insoluble lignin (44% w/w), cellulose (12% w/w), and hemicellulose (36% w/w). After the hydrolysis, moisture and lignin content were determined to get the solid fraction yield and % delignification. **Figure 1** illustrates a photograph of the untreated sample and samples treated with dilute sulphuric acid (Run #S1, **Table 2**) and the acid-oxidative hydrolysis (Runs #6, #7, #11, and #E1, **Table 2**). There is a significant difference in the color of the sample, which correlates with the lignin content. Still, the appearance of the pre-treated leaf waste is similar to the untreated sample.

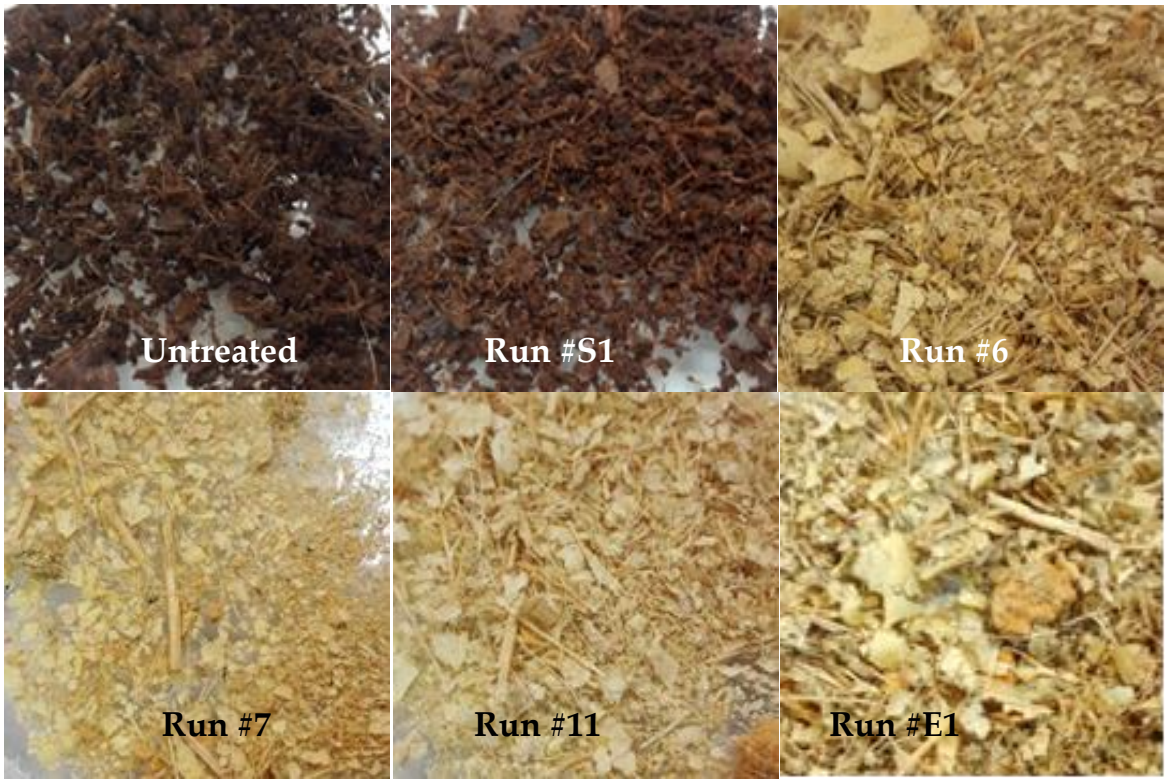

Figure 1: Photograph of the untreated LCW, the LCW pre-treated with a dilute sulphuric acid solution (Run #S1-Table 2), and the LCW pre-treated by the acid-oxidative hydrolysis at conditions of Run #6, #7, #11, and E1.

Attaining a high solid fraction with the acid-oxidative hydrolysis pretreatment since the carbohydrates usable for bioprocess remain in the solid. The solid fraction decreased as the severity increased (**Figure 2**), mainly related to the temperature effect. The yield has an almost linear negative dependence on the severity factor, particularly for the lowest solid loading. On the contrary, the effect of the severity factor on the delignification efficiency (**Figure 3**) is slightly positive, and an influence of the solid loading is perceived. Despite significant variations, the attained delignification was always larger than 60% except for one condition corresponding to the highest solid loading examined. The highest delignification degree was attained for the lowest examined solid loading, reaching a value of 85.2% for Run #4 of **Table 2**, although solid yield for this condition was less than 50%.