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Potential of giant reed (*Arundo donax* L.) for second generation ethanol production

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ABSTRACT

Background: The production of second generation ethanol from lignocellulosic biomasses that have not had their potential fully explored as feedstock is of great importance. *Arundo donax* is one these biomasses. It is a promising grassy plant to be used as a renewable resource for the production of fuels and chemicals, because of its fast growth rate, ability to grow in different soil types and climatic conditions. The present study evaluated its use as feedstock for the production of second generation ethanol.
Results: Initially its chemical characterization was carried out, and a protocol for fractioning the biomass through diluted acid pretreatment followed by alkaline pretreatment was developed, providing a solid fraction which was undergone to enzymatic hydrolysis reaching 42 g/L of glucose, obtained in 30 h of enzymatic hydrolysis. This partially delignified material was subjected to a simultaneous saccharification and fermentation (SSF) process, resulting in an ethanol concentration of 39 g/L at 70 h.
Conclusions: The fermentability of the pretreated biomass was performed successfully through the conception of simultaneous saccharification and fermentation resulting in approximately 75 L of ethanol per ton of cellulose.

1. Introduction

Energy crops are plants which are produced with the purpose of using their biomass energetically and at the same time reduce carbon dioxide emission [1]. Giant reed, the common name of *Arundo donax* L., is one of the most promising crops for energy production because its fast growth rate, ability to grow in different soil types and climatic conditions, durable yields, and resistance to long drought period [2]. *A. donax* is a perennial rhizomatous grass native from Asia, found growing spontaneously in various regions in many countries, as Portugal, Spain and Brazil. It belongs to the *Poaceae* family and is considered one of the largest Gramineae, reaching from 2 to 8 m in height. It is commonly found in warm-temperate environment of elevated temperature and wet places. There are reports indicating that *Arundo* may reach yields up to 100 $t \cdot ha^{-1}$ of green biomass from the second or third year of cultivation under the best conditions of climate and irrigation [2], what corresponds to 3 to 37 $tDM \cdot ha^{-1}$, a bit higher of what sugar cane produces, 5 to 23 $tDM \cdot ha^{-1}$ [1]. Due to its easy adaptability to different environmental conditions, quick growth and high biomass productivity, it has recently been considered a potential feedstock for the production of fuels and chemicals [3,4].

The insertion of ethanol in the global energy matrix has been increasingly considered due to the undeniable environmental benefits that its use promotes, associated to the awareness with the reduction of energy demand from fossil sources. Therefore, technological innovations should be incorporated in order to reduce production costs of ethanol, including diversification of feedstocks. In this context, lignocellulosic biomass has been an object of intensive research all over the world because it is a renewable feedstock of carbon and energy available in great quantities.

The evolution of lignocellulosic ethanol production technology, also named as second generation ethanol, involves the search for species with high biomass productivity, cost-effective pretreatment and process conception selection [5].

Several factors can affect the efficiency of converting lignocellulosic biomass into ethanol, with particular emphasis in the need to improve the enzyme access to the biomass for the hydrolysis process. In this regard, several types of pretreatments may improve the yield of fermentable sugars to increase the efficiency of ethanol production, such as steam explosion, chemical pretreatments with acids or bases, enzymatic pretreatments, among others [5]. In this line of approach, different biomass processing technologies have been reported to increase contact surface to provide greater access for the enzymes,

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reduction of the cellulose crystallinity and recalcitrance, as well as hemicellulose and lignin removal [6]. After pretreatments, fermentable sugars are obtained through cellulose hydrolysis by the action of specific enzymes (cellulases). Usually, these cellulolytic enzymes is comprised of a mixture of endoglucanases, which cleaves the internal bindings of cellulose fiber producing cellodextrins; exoglucanases, which produce cellobiose from the reducing and non-reducing ends of the cellulose chains, and β-glucosidase, which hydrolyzes cellobiose and small oligomers releasing glucose [7].

Cellulose enzymatic hydrolysis can be carried out sequentially with subsequent fermentation, in a process design termed separate hydrolysis and fermentation (SHF), or it may occur in a simultaneously process called simultaneous saccharification and fermentation (SSF). In the SSF process, the two steps (saccharification and fermentation) are gathered, and the process contributes to cost reduction in an industrial plant. Additionally, in this process, the enzymes are less susceptible to inhibition by their hydrolysis products, because glucose is concomitantly released and fermented [5,8]. On the other hand, the optimum temperature for the activity of hydrolytic enzymes and the yeast fermentation of sugars are different, which means that the conditions are not optimal for both enzymes and yeast cells in the SSF process [9]. Other process strategies, involving fermentation of pentose (from hemicellulose) and glucose (from cellulose) concomitantly can be conceived, in a process conception named simultaneous saccharification and co-fermentation (SSCF), but this will request the use of recombinant microorganism capable to ferment efficiently both sugars.

In this study, our goal was to characterize the fractions of cellulose, hemicellulose and lignin of *A. donax*; to investigate the effect of partial delignification with dilute NaOH on the enzymatic hydrolysis of *Arundo* biomass, and to evaluate the SSF process for the production of second generation ethanol from partially delignified *A. donax* biomass.

2. Materials and methods

2.1. Feedstock and enzyme

Arundo was picked on the banks of São Gonçalo canal, in the city of Pelotas, Brazil, where the geographic coordinates were as follows: latitude 31°46′33″ south and longitude 52°21′34″west.

The *Arundo* biomass was obtained from the whole plant, milled and air dried. The drying operation was conducted for 24 h at 60° ± 3°C in a ventilated oven with the oven door closed throughout, until a final moisture content of 3%. The dried biomass was milled again, screened to select the fraction of particles with a size lower than 8 mm, homogenized in a single lot and stored at room temperature in covered plastic containers until needed.

The enzyme used in the study was a commercial cellulase (Multifect®) from *Trichoderma reesei*, with the following activities: CMCase 4360 U/mL, Avicelase 180 U/mL, 260 FPase/mL, β-glucosidase 200 U/mL, determined as described by Ghose [10].

2.2. Pretreatments

One kilogram of *Arundo* biomass was submitted to an acid pretreatment with 1.1% v/v sulfuric acid solution, at a solid:liquid ratio of 1:2.8 (g/mL), and heated in an autoclave at 121°C with an exposition time of 30 min for opening up the fibers [11]. The solid residue, termed acid cellulignin (ACCL) was repeatedly washed with water to remove the acid. The pH was adjusted to 5.0 with 2 M NaOH solution. The ACCL was dried in a forced air oven at 60°C overnight. The dried residue was weighed to evaluate the acid pretreatment. The ACCL was subjected to an alkaline pretreatment in order to reduce the lignin content and thereby improve the efficiency of enzymatic hydrolysis. Alkaline pretreatment was performed with 0.5 M NaOH solution. The reaction conditions for the alkaline pretreatment were: solid:liquid ratio of 1:20 g/mL, 120°C for 30 min, as recommended by Barcelos et al. [7]. The suspension was then filtered and the residue washed with water at least three times, and the pH adjusted to 5.0 with 0.1 M H_2SO_4 solution. The solid fraction was dried in an oven at 60°C in the same way as the original *Arundo* biomass. This fraction was termed partially delignified cellulignin (PDCL).

2.3. Enzymatic hydrolysis

Arundo original (in natura without pretreatment), ACCL and PDCL were assayed for enzymatic hydrolysis using an enzyme load of 25 FPU/g of solid suspended in citrate buffer (pH 5.0). The solid concentration used was 100 g/L, pH 5.47, 50°C for 30 h in shake flasks (200 rpm). Samples were withdrawn at predetermined times and centrifuged to quantify the amount of glucose released.

2.4. Ethanol fermentation

The fermentability of the enzymatic hydrolyzate was performed using the conception of SSF, however a step of pre-hydrolysis prior to SSF was conceived for increasing the glycolytic flux in the beginning of the fermentation step. The experiment was carried out in a bioreactor Bioflo 310, New Brunswick Scientific, Edison, NJ with a solid load of 250 g/L and a working volume of 1 L. The PDCL and the buffer containing nutrients for supplementation were sterilized at 0.5 atm for 15 min.

The prehydrolysis was carried out with 100 g of PDCL in a sodium citrate buffered, with an enzyme load of 25 FPU/g solids and with supplements for the SSF process: urea (1.25 g/L), KH_2PO_4 (1.1 g/L), yeast extract (2 g/L) and a salt solutions (40 mL/L), as recommended by Maeda et al. [12]. The temperature and stirring rate were maintained at 50°C and 200 rpm, respectively, for 12 h. Two samples were collected during the pre-hydrolysis step (6 h and 12 h) for the determination of sugars released. Thereafter, the temperature was adjusted to 37°C and the medium was inoculated with cells of a commercial strain of *Saccharomyces cerevisiae* at a concentration of 6 g dw/L for the beginning of simultaneous saccharification and fermentation process. As the medium became liquefied, additional 50 g of PDCL were fed to the medium with intervals of 12 h, up to a load of 250 g/L.

The kinetic profiles of substrate consumption and ethanol production were monitored during 78 h. The efficiency of converting cellulose to ethanol was calculated by [Equation 1].

$$FECE(\%) = \frac{P}{(CC \times 1.11) \times 0.511} \times 100 \qquad \text{[Equation 1]}$$

where:

FECE	fermentation efficiency of converting cellulose to ethanol (%)
P	final ethanol concentration (g/L)
CC	concentration of cellulose in the PDCL
0.511	conversion factor theoretical maximum substrate in ethanol
1.11	conversion factor related to the conversion of cellulose to glucose.

2.5. Analysis of biomass chemical composition, sugars and ethanol

The contents of cellulose, hemicellulose, lignin of *Arundo* (in natura), ACCL and PDCL were determined as described by Ververis et al. [13]. Ash and moisture were determined for integral *Arundo* biomass, according to standard AOAC [14].

The concentrations of glucose, cellobiose, xylose, arabinose and ethanol were determined by high performance liquid chromatography

(HPLC), using a HPX87P column (Bio Rad Laboratories, Munich, Germany) maintained at 65°C and with a differential refractive index detector (Waters).

3. Results and discussion

3.1. Characterization of different fractions of Arundo biomass

The chemical composition of integral *Arundo* biomass and its pretreated fractions, in terms of their main components, are summarized in Table 1.

Integral *Arundo* contains roughly 31% cellulose, 35% hemicellulose and 6.1% ashes. Barcelos et al. [7] have found 7.9% ashes for sorghum bagasse, and have compared it to rice and wheat straw that contain 17 and 11% ashes, respectively. The ashes lower content of integral *Arundo* may be considered an advantage, because during the pretreatment the biomass-containing salts solubilize in the hemicellulose and cellulose hydrolysates. This increase in the concentration of ions leads to an increase in the osmotic pressure in the medium, hindering the fermentability of the generated hydrolysates.

The acid pretreatment has removed 28.6% (w/w) of the initial weight of the integral *Arundo* (Table 2) due to the removal of 62.5% of hemicellulose, which has lowered its content to 18.5% from its initial composition of 35.3%. The fraction object of this study – cellulose – has been concentrated from 31 to 72%, when both pretreatments were used, increasing its percentage in the solids by 2.3 fold.

With the alkaline pre-treatment, the overall reductions in the hemicellulose content and in the lignin removal were roughly 85% and 75%, respectively. This partial delignification is essential for the subsequent enzymatic hydrolysis, because it improves the accessibility of the enzymes to cellulose [7]. Furthermore, lignin limits the rate and the extent of enzymatic hydrolysis by acting as a "shield", preventing the digestible parts of the substrate to be hydrolyzed [12].

Although acid pretreatment followed by alkaline pretreatment resulted in a low mass yield (64.7%), which was expected, because the removal of hemicellulose, lignin and extractives cause weight loss (Table 2), delignification showed to be essential to increase the yield of enzymatic hydrolysis. Delignification allows a swelling of the fibers and breaks the structural association between lignin and carbohydrates [7].

Another factor that directly affects the yield of enzymatic hydrolysis is the concentration of acid and base used in the pretreatment. Thus, acid and alkaline pretreatments should be used in a lesser possible degree of severity (combination of temperature, time of exposition and concentration of the pretreatment agent). Experiments evaluating levels of alkaline pretreatment on sugar cane bagasse indicated that the amount of sodium hydroxide used for delignification can be reduced to 0.25 M or even 0.125 M without a significant loss in the yield of the enzymatic hydrolysis [15].

3.2. Enzymatic hydrolysis

Integral *Arundo*, ACCL and PDCL were hydrolyzed for 24 h, using an enzyme load of 25 FPU/g of solid (Fig. 1). The yield of glucose and the rate of hydrolysis was increased by the acid pretreatment and alkaline pretreatment.

Table 1
Percent composition of integral *Arundo donax* and its pretreated fractions.

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Integral *Arundo*	31.10 ± 1.03	35.27 ± 2.80	18.49 ± 0.10
ACCL	42.49 ± 1.88	18.53 ± 1.42	24.75 ± 0.32
PDCL	72.17 ± 5.90	11.53 ± 1.61	10.05 ± 0.20

ACCL: acid cellulignin; PDCL: partially delignified cellulignin.

Table 2
Mass yield after acid and alkaline pretreatment of *Arundo donax*.

Pretreatment	Initial mass (g)	Final mass (g)	Mass yield (%)
Acid	1000.0 (integral *Arundo*)	713.6 (ACCL)	71.4
Acid + alkaline	500.0 (ACCL)	337.0 (PDCL)	64.7

The highest glucose concentration 42.0 g/L was obtained with PDCL with 30 h of hydrolysis, whereas with the untreated biomass (integral *Arundo*) and ACCL, the maximum glucose concentrations obtained were only 6.8 and 13.9 g/L, respectively. The highest cellulose conversion value of 52% for the hydrolysis of PDCL may appear relatively low, but this can be ascribed to the inhibition of the cellulolytic complex by the end products of the hydrolysis. Barcelos et al. [7] obtained maximum glucose concentration of 40.4 g/L from sugar cane bagasse using enzymatic hydrolysis with commercial cellulase which corresponded also to an efficiency of cellulose conversion of 50%.

More recently, De Bari et al. [16] evaluated the hydrolizability of concentrated solid suspensions of steam pretreated *A. donax* with solid loads varying from 20 to 200 g/L. It was clearly shown that high solid loads reduce the enzymatic yields due to the increasing resistance the mass transfer. Enzymatic hydrolysis at a high solid load (200 g/L) resulted in a glucose concentration of 65 g/L, corresponding to an enzymatic yield of 57%, whereas at a low solid load (20 g/L) the glucose concentration was only 8 g/L, however the enzymatic yield was 84%. In both cases the highest glucose concentrations were obtained with reaction time longer than 100 h.

Several factors can interfere with the cellulose hydrolysis, as the specific structure of the cellulose arrangements, presence of residual hemicellulose, cellulose crystallinity, contact area of enzyme/substrate and lignin content. Partial removal of lignin is essential, because it minimizes unproductive linkages formed with cellulolytic enzymes, reducing the rate of hydrolysis. The alkaline pretreatment possibly causes a cleavage in the complex lignin/carbohydrate improving hydrolysis. As a result, there is a formation of pores and swelling of the biomass, facilitating hydrolysis and subsequent liquefaction of the material [7,17,18,19].

Zhang and Lynd [20] reported that hydrolysis of biomass with high crystallinity is slower because amorphous cellulose is more amenable to be converted to glucose than crystalline cellulose. This trend, however, has not been fully elucidated in other studies. Hendriks and Zeeman [21] concluded that the pore size of the substrate relatively to the size of enzymes is the main limiting factor in the enzymatic hydrolysis of lignocellulosic biomass.

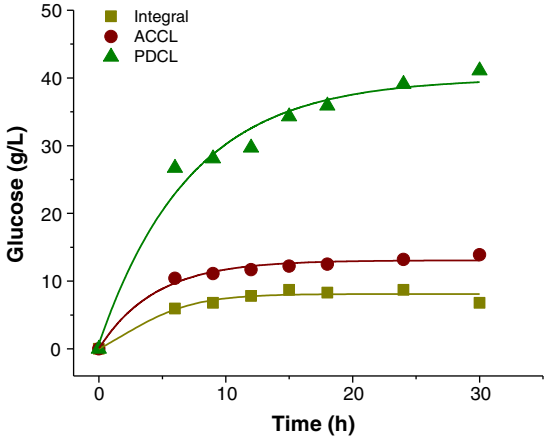

Fig. 1. Cellulose enzymatic hydrolysis of different fractions of *Arundo donax*. ACCL: acid cellulignin; PDCL: partially delignified cellulignin.

Santos et al. [22] observed that in the hydrolysis of delignified sugarcane bagasse, the concentration of cellobiose increased during the first 10 h, after which it remained constant up to 24 h and then declined slowly by the action of β-glucosidase. This is likely to be due to insufficient amount of β-glucosidase in the enzymatic preparation, as certain amount of cellobiose remained without being hydrolysed. It is accepted that cellobiose formed on delignified cellulignin is greater than that coming from the non-delignified bagasse and the amount of cellulose is higher in the PDCL. Olofsson et al. [6] point out that there is often the need for supplemental β-glucosidase in the commercial cellulase preparations to prevent end product inhibition by cellobiose.

The commercial enzymatic preparation used in the present investigation also displayed xylanolytic activity since residual xylan in the partially delignified cellulignin was hydrolysed, resulting in a building up of xylose in the medium (Fig. 2). This feature is of interest to explore in the future the conception of SSCF of *Arundo* hemicellulose hydrolysates, including additionally the xylose from the acid pretreatment.

An important factor that may also affect the efficiency of hydrolysis is the enzyme load. Barcelos et al. [7] tested enzyme loads ranging from 25 FPU/g to 150 FPU/g for the hydrolysis of alkaline pretreated sugarcane bagasse, and reported that no significant increase in the yield of enzymatic hydrolysis with increasing enzymatic load was found, after 24 h of hydrolysis. This article and another one reported by Betancur and Pereira [11] served as reference to determine the enzyme load for the present study with *Arundo*. The rationalization of the enzyme load is of economic importance for the process, considering that enzymes are costly, incorporating economic impact in the process.

3.3. Simultaneous saccharification and fermentation of Arundo cellulose fraction

The solid concentration is a limiting factor for ethanol production in SSF process [9]. In this study, SSF was conducted with PDCL in a final solid load of 250 g/L (Fig. 3). The bioreactor was fed with PDCL gradually, starting with 100 g/L, followed by three additional feedings (50 g) to achieve the final solid load. According to Maeda et al. [23], fed batch system presents several advantages, among them, the levels of fermentation inhibitors can be kept low, and concerning fermentation systems fed with solids, this mode of operation facilitates agitation of the medium reducing problems of momentum, heat and mass transfer. This is because the cellulose

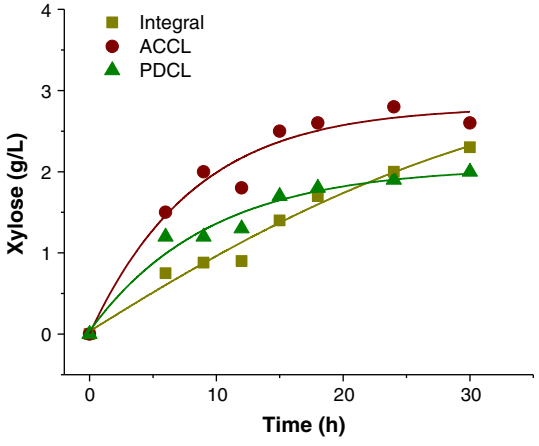

Fig. 2. Xylan enzymatic hydrolysis of different fractions of *Arundo donax*. ACCL: acid cellulignin; PDCL: partially delignified cellulignin.

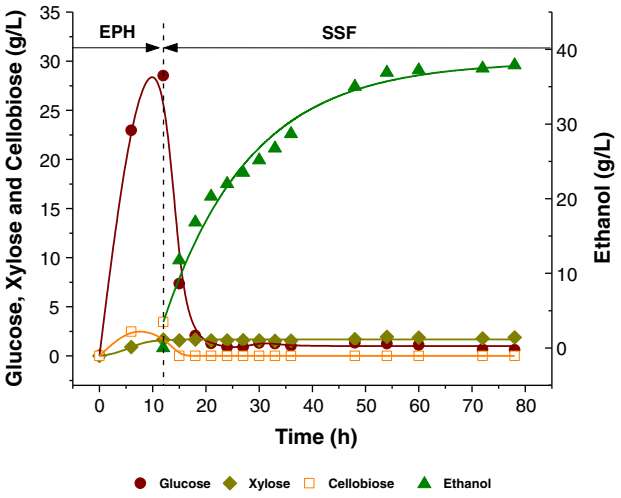

Fig. 3. SSF of partially delignified cellulignin of *Arundo donax* with a commercial strain of *S. cerevisiae*. EPH: enzymatic pre-hydrolysis.

containing-solid suspension is liquefied as the cellulase preparation acts, allowing additional solid loads.

Prior to the addition of yeast to the SSF fermentation, prehydrolysis of 12 h was carried out, resulting in a concentration of glucose of 28 g/L. After prehydrolysis, the system was inoculated with commercial yeast, which promptly converted glucose released into ethanol. In this initial fermentation phase the rate of conversion of glucose is high due to the higher initial availability of glucose. In a second step, when the glucose concentration decreases, the ethanol production is regulated by the activity of the enzymes and the rate of ethanol production starts to decrease due to the high yeast cell population, which consumes straightway all glucose releases by the enzymatic hydrolysis.

This way, the enzymatic reaction rate does not meet the fermentation rate; however the concentration of sugars (cellobiose and glucose) remained at low concentrations indicating that the enzymes added in the system remained active during the fermentation, and the glucose released (final product of the enzymatic hydrolysis) was fermented simultaneously to ethanol. Concerning the presence of xylose, a slight increase in its concentration was observed in the prehydrolysis step, after which it remained constant, showing the inability of the yeast in consuming this pentose, as expected.

The final ethanol concentration was 39.0 g/L and the volumetric productivity was 0.56 g/L.h, which can be considered very promising results since no optimization studies have been carried out yet.

Scordia et al. [24] working with two different yeast strains, *Scheffersomyces stipitis*, which is a native xylose and cellobiose fermenter, and *Saccharomyces carlsbergensis*, which does not ferment xylose or cellobiose, were used along with commercial cellulolytic enzymes in SSF process. Diluted oxalic acid pretreated giant reed was used to evaluate the fermentability of both streams containing sugars derived from hemicellulose and cellulose. The strain of *S. carlsbergensis* attained a maximum ethanol concentration of 15.9 g/L after 48 h, while *S. stipitis*, at the same conditions, took 96 h to achieve a similar ethanol concentration.

More recently, Scordia et al. [25] continued the previous work using experimental design to optimize the ethanol fermentation of diluted oxalic acid pretreated giant reed by the SSF process, using a commercial cellulase cocktail and a strain of the yeast *S. stipitis*. Enzymatic hydrolysis and ethanol production reached 95% of glucan conversion and 18 g/L (75% of the maximum theoretical ethanol yield) respectively.

Miscanthus, another potentially important energy crop was also evaluated for ethanol production [26]. As a lignocellulosic material, Miscanthus has to comply with the common procedures for its utilization, namely fractionation, which includes pretreatment, enzymatic hydrolysis and fermentation. The fermentability of its hemicellulose hydrolysate by a strain of *S. stipitis* produced 12.0 g/L of ethanol after 96 h incubation.

As it turns out the low ethanol concentrations and the long fermentation times reported in literature indicate that there is much room for improvements in the utilization of these recently reported energy crops (*A. donax* and *Miscanthus x giganteus*). The results herein achieved showed to be very interesting and stimulate us to go on exploiting the use of these new biomass for the production of ethanol and other chemical within the context of biorefinery.

From the global mass balance, considering all stages of the process, it is possible to estimate that 1 kg of integral *Arundo* (3% humidity), after acid and alkaline pretreatments, generated 480 g of ALCL, containing 72% cellulose. Considering an enzymatic hydrolysis efficiency of 52%, obtained with *Arundo* PDCL, the fermentation efficiency of converting cellulose to ethanol was approximately 40%, which can be considered a promising result taking into account that no optimization strategy was adopted. The target herein was to evaluate the potential of using Giant Reed as feedstock for the production of second generation ethanol.

It is also possible to estimate a ratio of approximately 75 L (60 kg) of ethanol per ton of dry *Arundo* biomass. Maeda et al. [23], working with sugarcane bagasse in an optimized situation, reached a ratio of 110 L/ton, considering only the cellulosic fraction, as well. Ask et al. [27], working with enzymatic hydrolysis and fermentation of *A. donax*, have found low concentrations of ethanol (lesser than 20 g/L), which approximately half of that were obtained in the present work. Using the hemicellulose fraction arisen from the oxalic acid pretreated *Arundo*, Scordia et al. [24] obtained 26.0 g/L xylose, 5.0 g/L glucose and 2.4 g/L arabinose. This hemicellulose hydrolysate was fermented yielding 8.2 g/L ethanol, after 48 h. It is worth noting that, the conversion of the hemicellulose fraction can be an important strategy to improve the overall yield of ethanol from lignocellulosic materials.

4. Concluding remarks

The lignocellulosic materials have been object of intensive researches all over the world because they are renewable feedstocks of carbon and energy available in great quantities in many countries, which will probably lead to significant realignments in key sectors of these countries' economies (liquid fuels, food/fodder, chemical supplies, cellulose and paper etc.). The production of second generation ethanol from lignocellulosic biomass that has not had their potential fully explored is of great importance. *A. donax* is strong candidate to be used as a renewable feedstock source in the biochemical platform within the concept of biorefinery because it was prone to enzymatic hydrolysis, whose hydrolysate displayed promptly fermentability. It contains 31% cellulose, 35% hemicellulose and 18% lignin. Partial delignification of *Arundo* increases the efficiency of its enzymatic hydrolysis, because it enables the rapid liquefaction and homogenization of the medium favoring the action of yeast. From the process investigated herein, it was possible to estimate a ratio of 75 L of ethanol per ton of *Arundo* biomass. However, the possibility of obtaining higher ratios of enzymatic hydrolysate fermentation and inclusion of the hemicellulose derived sugars may be strategic for future studies.

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