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Ethanol Production from Residual Wood Chips of Cellulose Industry: Acid Pretreatment Investigation, Hemicellulosic Hydrolysate Fermentation, and Remaining Solid Fraction Fermentation by SSF Process

Neumara Luci Conceição Silva · Gabriel Jaime Vargas Betancur · Mariana Peñuela Vasquez · Edelvio de Barros Gomes · Nei Pereira Jr.

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Abstract Current research indicates the ethanol fuel production from lignocellulosic materials, such as residual wood chips from the cellulose industry, as new emerging technology. This work aimed at evaluating the ethanol production from hemicellulose of eucalyptus chips by diluted acid pretreatment and the subsequent fermentation of the generated hydrolysate by a flocculating strain of *Pichia stipitis*. The remaining solid fraction generated after pretreatment was subjected to enzymatic hydrolysis, which was carried out simultaneously with glucose fermentation [saccharification and fermentation (SSF) process] using a strain of *Saccharomyces cerevisiae.* The acid pretreatment was evaluated using a central composite design for sulfuric acid concentration (1.0–4.0 *v*/*v*) and solid to liquid ratio (1:2–1:4, grams to milliliter) as independent variables. A maximum xylose concentration of 50 g/L was obtained in the hemicellulosic hydrolysate. The fermentation of hemicellulosic hydrolysate and the SSF process were performed in bioreactors and the final ethanol concentrations of 15.3 g/L and 28.7 g/L were obtained, respectively.

Keywords Residual wood chips · Acid pretreatment · Hemicellulose · Cellulose · Bioethanol

Introduction

Modern society depends greatly from the non-renewable and highly polluting energy sources, such as petroleum and its derivatives, shortage of which is expected in the coming decades. As a solution to this problem, different sectors of the economy have shown interest

N. L. C. Silva · G. J. V. Betancur · E. de Barros Gomes · N. Pereira Jr. (✉)
Biochemical Engineering Department,
Federal University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro 21945-970, Brazil
e-mail: nei@eq.ufrj.br

M. P. Vasquez
Engineering Faculty–Chemical Engineering Department, University of Antioquia, Medellín, Colombia

in the use of alternative renewable fuels, which in addition to keeping them competitive in the world market also contribute to the reduction of generated pollutants [1].

One of the most promising alternatives is the ethanol produced from residual biomass, which not only will decreases the imbalance in the carbon cycle but also owns suitable fuel characteristics for combustion engines. Currently, ethanol is produced from sugarcane and corn, technologies which stand up as the most common alternatives; however, to avoid the disproportionate expansion of the areas of cultivation, the current research indicates the need for the development of biotechnological routes that allow the use of existing lignocellulosic materials, such as residual eucalyptus wood chips from the cellulose industry for second generation ethanol production [2].

These residual wood chips are composed by three main fractions: cellulose, a homopolysaccharide composed of D-glucose linked by β-1,4 bonds; hemicellulose, a heteropolysaccharide constituted, in great majority, of a mixture of D-xylose, D-mannose, D-galactose, D-glucose, and L-arabinose molecules; and lignin, a macromolecule of polymerized aromatic alcohols, which together with hemicellulose enfold the cellulose and protect it against microbial attacks. Different from cellulose, the hemicellulose structure does not present high crystallinity and therefore is more susceptible to chemical hydrolysis under milder conditions [1, 3, 4].

The ethanol production from the wood chips can be performed by fermentation of hemicellulose sugars, after solid pretreatment, and glucose from the cellulose using the simultaneous saccharification and fermentation (SSF) process.

In the utilization of lignocellulosic feedstocks, be it for the production of ethanol or other chemicals, pretreatment is understood as a process through which the cellulose molecules become more susceptible to enzymatic hydrolysis by cellulases. In the literature, frequently the term "prehydrolysis" is used as synonym of pretreatment. The increasing enzyme accessibility to the cellulose molecules is due to the removal of the hemicellulose fraction, as well as to the partial lignin removal (acid soluble lignin), promoting a sort of "opening up" of the lignocellulose matrix [5, 6]. Eriksson et al. [7] noticed a significant improvement in the performance of cellulases when the lignocellulosic biomass was subjected to pretreatments, whether chemical or thermal. Pan [6] reported a significant increase in cellulose digestibility when alkali pretreatment was performed (peroxide and hydroxides).

Therefore, the present work aimed at evaluating the ethanol production from hemicellulose and cellulose of residual wood chips generated by the cellulose industry. The investigation included acid pretreatment optimization using a composite experimental design procedure, fermentation of the generated hemicellulose hydrolysate in optimum conditions using a strain of *Pichia stipitis*, delignification of the remaining cellulose-containing solids followed by the simultaneous saccharification and fermentation process, using a commercial enzymatic preparation and a strain of *Saccharomyces cerevisiae*.

Materials and Methods

Raw Material

The lignocellulosic material used in this work was the residual wood chips after size screening during the cellulose production from *Eucalyptus grandis* wood manufactured by the Aracruz Celulose Industry (Brazil). These residual wood chips, free of any wood bark, had a variable size distribution characterized by values varying from 1.0–2.36 mm. The size distribution was determined using an appropriate sieve with pore diameters of 4 mm.

Considering the particle size as an influent factor in the pretreatment step [8], the solid material was milled (Mill MF 10, IKA®) to achieve an average size of 1.15 mm. According to Lee (1997) [9], the eucalyptus wood is composed by approximately 50% of cellulose, 20% of hemicellulose, and 15% of lignin, among other components.

Acid Pretreatment Optimization

To evaluate the effects of sulfuric acid concentration and solid to liquid (SL) ratio, a central composite design was performed within the levels showed in Table 1 to optimize the diluted acid pretreatment conditions for xylose concentration.

The procedure for carrying out the acid pretreatment began with analyzing the humidity of the material, which was found to be close to zero (4%). After this, the material was mixed with an appropriate volume of diluted sulfuric acid using a sprayer to ensure uniformity in the mixture. The humid solid material was transferred to conic flasks and then subjected to a thermal treatment at 121 °C in an autoclave for 45 min (excluding the heating and cooling times) [10]. After the thermal treatment, the material was cooled in an ice bath and the liquid phase was separated in a hydraulic system especially designed for this purpose [11]. The obtained liquid, denominated hemicellulosic hydrolysate, had its pH adjusted to 6 by the addition of calcium hydroxide, allowing the precipitation of sulfate ions in the form of calcium sulfate which was subsequently separated by filtration.

The hemicellulosic hydrolysate was analyzed to determine its concentration of sugars by high performance liquid chromatography (HPLC) with an HPX-87P column (BioRad®) using water as mobile phase at a flow rate of 0.6 mL/min and a separation temperature of 80 °C. For the statistical analysis of the results, the software STATISTICA 6.0 was used.

Hemicellulose Hydrolysate Fermentation

To evaluate the potential of ethanol production, using the obtained hydrolysate in the best conditions of the acid pretreatment, the fermentation was performed using the flocculating strain of *P. stipitis* CBS5774 yeast, known as a good xylose-fermenting strain [12].

The cell propagation of *P. stipitis* was performed in three steps: activation of cells in synthetic medium, as reported by Pereira Jr. (1991) [12], for 24 h; followed by the first acclimatization with a medium containing 25 *v*/*v* of the hemicellulose hydrolysate for 24 h; and the second acclimatization with medium containing 50 *v*/v of the hemicellulose hydrolysate for 48 h. The acclimated cells were centrifuged (3,000 rpm/15 min) with an appropriate volume of the propagation medium for obtaining the intended inoculum concentration. After that, the cells were aseptically resuspended in the fermentation medium.

The hemicelullose hydrolysate fermentation was performed in a 1.5-L Biostat B bioreactor (B. Braun Biotech International, Germany), using a working volume of 800 mL of the fermentation medium supplemented with a mineral salt solution (40 mL/L) [12]. The process was automatically controlled at a temperature of 30 °C, agitation of 250 rpm, specific aeration ratio of 0.03 vvm, pH 6.0, and an initial cell concentration of approximately 10 g/L. During

Table 1 Central composite experimental design for the diluted acid pretreatment

Factors	$-\alpha$	−1	0	+1	$+\alpha$
Solid to liquid ratio (g to mL)	1:4	1:4	1:3	1:2	1:2
Sulfuric acid concentration (*v*/*v*)	1.0	1.0	2.5	4.0	4.0

the fermentation, samples were withdrawn to quantify cell concentration by absorbance (600 nm) and sugars, ethanol and xylitol by HPLC, as described previously.

Alkaline Pretreatment

In order to remove the lignin from the remaining solid fraction of the acid pretreatment, called as cellulignin, an alkaline pretreatment with sodium hydroxide (NaOH) was performed in conic flasks. In this step, the cellulignin was soaked in NaOH solution at 4 *w*/*v*, to a solid to liquid ratio of 1:20 (grams to milliliter) and subjected to a thermal treatment at 121 °C within an autoclave for 20 min (excluding the heating and cooling times). The solid material was washed, had its pH adjusted to 5 by the addition of hydrochloric acid solution (2 M) and dried in an oven at 60 °C to be used in the SSF process.

SSF Process

The SSF process was performed in a Bioflo III bioreactor (New Brunswick Scientific, USA), maintained automatically at a temperature of 37 °C, under agitation of 200 rpm, with dry weight solid content of 20 *w*/*w* and initial cell concentration of 4 g/L. The microorganism used was the commercial strain of *S. cerevisiae* (Fleischmann). Prior to the SSF process, an enzymatic prehydrolysis of 12 h was performed using a commercial cellulase preparation (Multifect-Genencor) with an enzymatic load of 30 filter paper units (FPU) per gram of solid. The commercial cellulase preparation used in this work had FPasic, β-glucosidase, and CMCasic activities of 100 FPU/U, 125 U/mL, and 5,500 U/mL, respectively. During the process, samples were withdrawn for quantification of the sugars and ethanol by HPLC. The ethanol potential (liters per ton of feedstock) was estimated by a mass balance taking into account the amount of ethanol produced related to the initial wood chips mass and extrapolating the result for 1 ton of feedstock.

Results and Discussion

During the acid pretreatment, it was observed that the low capacity of the fibers absorbed the acid solution, differently from what happens with other lignocellulosic residues [2]. This feature indicates the possibility of a high influence of the size particle on the hemicellulose fractioning and solubilization. The remaining solids from the acid pretreatment depicted a reddish color, typically from the eucalyptus lignin and tannins.

Table 2 shows the concentrations of sugars (xylose and glucose) for each experimental condition of residual wood chip acid pretreatment. It can be noticed that the highest xylose concentrations were achieved with the highest solid to liquid ratio (1:2).

From the statistical analysis of the experimental design results, it was possible to plot the Pareto chart (Fig. 1), which graphically and numerically presents the magnitude of the estimated effect of each independent variable, as well as the effect of their interactions. In this diagram, the effect is shown by bars, which only show statistical significance if they go beyond the demarcated vertical line for a significance of 95% (p level=0.05) [13]. It is possible to check that the generated linear effect (L) by the SL ratio was the only one that had statistical significance. Both linear and quadratic (Q) effects of the sulfuric acid concentration did not show statistical significance.

The analysis of variance (ANOVA), presented on Table 3, confirmed the Pareto chart results in which only the linear effect of the solid to liquid ratio showed statistical

Table 2 Responses of the central composite experimental design for the acid pretreatment performed at 121 °C for 45 min

Experiment	Temperature (°C)	Time (min)	Solid to liquid ratio (g to mL)	Acid concentration (*v*/*v*)	Glucose (g/L)	Xylose (g/L)
1	121	45	1:4	1.0	3.1	26.0
2	121	45	1:4	4.0	3.6	25.4
3	121	45	1:2	1.0	3.1	49.0
4	121	45	1:2	4.0	4.2	42.5
5	121	45	1:4	2.5	2.9	27.4
6	121	45	1:2	2.5	3.8	49.9
7	121	45	1:3	1.0	0.9	26.1
8	121	45	1:3	4.0	4.5	34.4
9 (C)	121	45	1:3	2.5	2.3	35.4
10 (C)	121	45	1:3	2.5	2.3	33.0

significance and the sum of squares was ten times higher than the second factor in importance (quadratic effect for solid to liquid ratio). Additionally, the ANOVA indicated an appropriated adjustment of the empirical model to the experimental results as confirmed by the high correlation coefficient obtained (R^2=0.93). The statistical insignificance for the lack of fit (p level>0.05) and the low pure error value corresponding to 12%, or less, of the results magnitude (between 25.4 and 49.9 g/L), confirmed that the model was appropriated for predicting the results between the evaluated interval levels in the experimental design.

Figure 2 presents the response surface curves for the experimental design results, which clearly shows that the SL ratio of 1:2 was the best within the evaluated range. Although the response surface curves have not presented an absolute maximum, this condition can be regarded as the best due to operational unfeasibility of working with SL ratio higher than 1:2 since there is an enormous technical difficulty in extracting the liquid phase using higher SL ratios. Thus, the best pretreatment condition within the experimental design to maximize the xylose concentration, as predicted by the empirical model from the

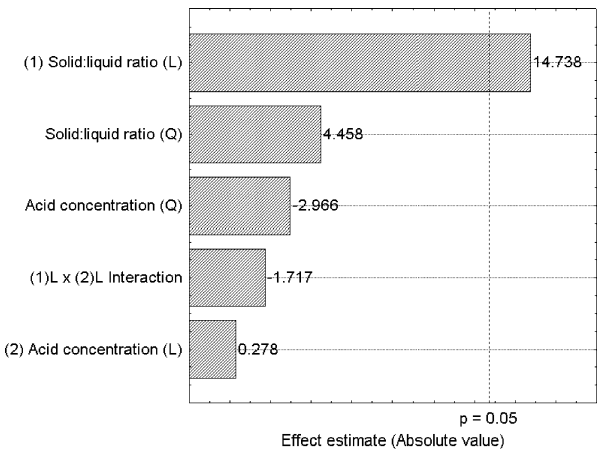

Fig. 1 Pareto chart of standardized effects for optimizing xylose concentration (grams per liter)

Table 3 ANOVA for the effects of the solid to liquid ratio and acid concentration in the diluted acid pretreatment

Factors	Sum of squares	Degrees of freedom	Mean square	Fisher value	*p* level
(1) Solid to liquid ratio (L)	651.88	651.88	217.20	0.0431	
Solid to liquid ratio (Q)	59.64	1	59.64	19.87	0.1405
(2) Acid concentration (L)	0.23	1	0.23	0.08	0.8274
Acid concentration (Q)	26.41	1	26.41	8.80	0.2070
(1)L×(2)L interaction	8.85	1	8.85	2.95	0.3357
Lack of fit	49.75	3	16.58	5.53	0.3008
Pure error	3.00	1	3.00		
Total sum of squares	788.61	9			

STATISTICA software, was an SL ratio of 1:2 and an acid concentration of 1.2 *v*/*v* which resulted in a xylose concentration of 50 g/L.

The fermentation kinetics of the hemicellulosic hydrolysate obtained in the optimum pretreatment conditions is presented in Fig. 3, showing the consumption of the substrate, cell, and ethanol concentrations as a function of time. Regarding the uptake of fermentable sugars, it can be noticed that glucose, which was present in a very low concentration, was totally consumed in the first 3 h of fermentation. Xylose, the major sugar, was totally consumed in 40 h of fermentation. The ethanol concentration reached 15.3 g/L, which corresponds with a volumetric productivity of 0.32 g/Lh and an ethanol yield on substrate consumed ($Y_{P/S}$) by 0.32 g/g, which are in agreement with results reported in the literature [14, 15]. Additionally, since the fermentative process was inoculated with a high inoculum size (10 g/L), little variation in the cell concentration was observed, resulting in a cell yield on substrate consumed ($Y_{X/S}$) by 0.015 g/g, indicating that in such conditions the strain

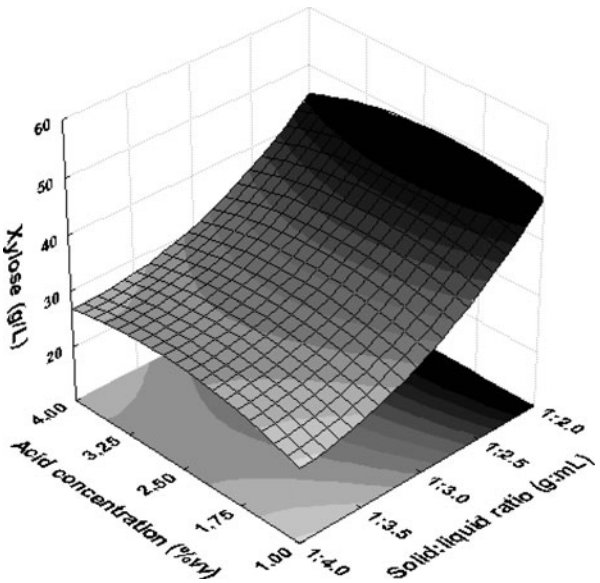

Fig. 2 Response surface plots of experimental design for optimizing xylose concentration (grams per liter)

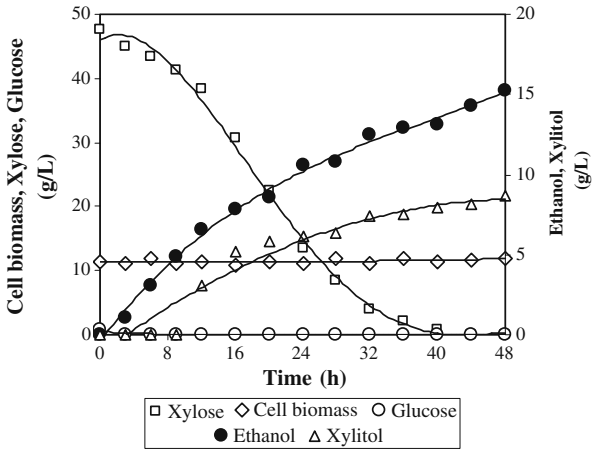

Fig. 3 Kinetics of the hemicellulosic hydrolysate fermentation in bioreactor by *P. stipitis*

metabolism favored the production of ethanol and xylitol in detriment of cell growth. The xylitol yield on xylose consumed was 0.18 g/g, and the presence of this polyalcohol is surely due to the low specific aeration rate used (0.03 vvm). It is well-known that xylose metabolism in yeasts and filamentous fungi proceeds via a two-step reaction in which xylose is first reduced to xylitol by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-dependent xylose reductase, followed by oxidation of xylitol to xylulose by NAD^+-dependent xylitol dehydrogenase. It is evident that under anaerobic conditions there will be an overproduction of NADH, resulting in a redox imbalance, which blocks the metabolic activity, since it cannot be re-oxidized in the absence of oxygen; however, in xylose-fermenting yeasts, such as cells of *P. stipitis*, these two first enzymes of xylose catabolism display dual coenzyme specificity. Thus, the reduction equivalents produced in the second reaction can be used for the initial step of xylose metabolism, therefore reducing the overproduction of NADH and consequently alleviating the cell redox imbalance.

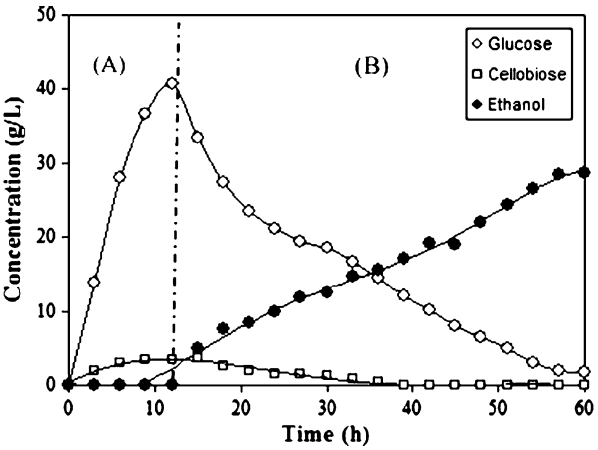

Fig. 4 Kinetics of the SSF process by commercial enzymes and *S. cerevisiae*. **a** Enzymatic prehydrolysis, **b** SSF process

Nonetheless, oxygen-limited conditions will be necessary since this dual specificity does not occur in the same extent, and the secretion of xylitol will occur anyway [1].

The kinetics of the SSF process is shown in Fig. 4, in which the ethanol concentration began to be stable (28.7 g/L) after 55 h, reaching a volumetric productivity value of 0.52 g/Lh. Additionally, the enzymatic prehydrolysis time (12 h) was not sufficiently long to incur in the inhibition of the cellulolytic enzymes by their hydrolysis products (glucose and cellobiose). The productivity achieved in the SSF process was also higher than those reported in the literature [16–18].

The cell concentration values cited in the literature [4, 16] (4.5 and 5.0 g/L) are not far from the ones used in this work (4 g/L), emphasizing the importance of the balance that has to exist between the enzyme load and cell concentration to increase the efficiency and to reduce the process costs.

Conclusion

Based on the results obtained by experimental design for the acid pretreatment of residual wood chips from the cellulose industry, it was possible to conclude that the concentration of xylose is directly related to the content of solids and is not influenced by the sulfuric acid concentration. Additionally, it was shown that the hemicellulosic hydrolysate and the hydrolysate from enzymatic hydrolysis of cellulose were promptly fermented by *P. stipitis* and *S. cerevisiae*, achieving ethanol concentrations of 15.3 and 28.7 g/L, respectively, corresponding to 100 L/ton of eucalyptus wood chips processed under the conditions evaluated in this work. Therefore, the hemicellulose, as much as the cellulose of the residual wood chips from the cellulose industry, presents a potential alternative source of carbohydrates for second generation ethanol production.

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