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Pretreatment of Rice Hulls with Alkaline Peroxide to Enhance Enzyme Hydrolysis for Ethanol Production

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Alkaline peroxide pre-treatment was evaluated for the conversion of rice hulls to simple sugars through out enzyme hydrolysis for the subsequent ethanol production. For this purpose, the effects of peroxide concentration and reaction time were studied. Peroxide concentration showed a positive effect on degradation of rice hulls lignocellulosic structure providing a greater access to hydrolytic enzymes. Thus, better global yields (sum of the pre-treatment and hydrolysis yields) were attained when higher peroxide concentrations were used. Among the conditions tested, the highest global yield of 86.48 ± 3.07% was attained with a peroxide concentration of 7.5%, at pH 11.5, 90ºC and 1h. With the proposed pre-treatment, a global yield around 70% can be achieved in 15 minutes of incubation.

1. Introduction

The rapid depletion of fossil fuels has driven the world to utilize renewable-energy sources such as biofuel in order to reduce the total dependency on non-renewable energy sources (Tyea et al., 2012). The growing industrialization has derived in an increasing demand of fuels attempting to satisfy both the industrial and domestic demands.

Second generation bioethanol is based on raw materials rich in complex carbohydrates, resulting an interesting alternative to reduce competition with food industry. The process to obtain second generation bioethanol involves four basic steps: feedstock pretreatment, enzymatic or acid hydrolysis, sugars fermentation, and ethanol recovery (Gómez et al., 2010).

Lignocellulosic agricultural residues are promising raw materials for sugarplatform biorefinery on a large scale. As they are residues and wastes, they do not compete with primary food production. However, few biorefinery processes based on sugar-platform are cost-competitive in current markets because of the low efficiency and high cost of enzymatic conversion processes (Himmel et al., 2007).

Rice hulls, which represent 20% dry weight of the harvested rice, can serve as a low cost abundant feedstock for production of fuel (Saha and Cotta, 2007). They are considered waste materials because of their low value as animal feed due to low digestibility, peculiar size distribution, low bulk density, high ash/silica contents, and abrasive characteristics. They can be easily collected from rice-processing sites and contain about 36% cellulose and 12% hemicellulose, so they can be used after transformation for bioethanol production. For this purpose, these polymers must be hydrolysed to simple sugars, which are subsequently fermented to ethanol. However, rice husks also contain high quantities of ash (20%) and lignin (16%), which combined with hemicelluloses, results a complex structure around the cellulose, being more difficult its use as a lignocellulosic feedstock for conversion to ethanol. For this reason, pretreatments are generally applied in order to make these polymers more accessible to the enzymes to be converted into fermentable sugars (Mosier et al., 2005).

Pretreatment processes can be physical, chemical, biological or a combination of these methods (Yang et al., 2012; Yoo et al., 2011; Yu et al., 2009). Although many different types of pretreatments were tested in different conditions over the past years, advances are still needed for overall costs to become competitive.

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Pre-treatment is considered to be the most expensive step to convert lignocellulosic biomass into ethanol. Most pretreatment methods disrupt cell walls of the plant fibers to expose the sugar polymers, but do not remove much lignin (Zheng et al., 2009; Lee et al., 2010).

Alkaline and alkaline peroxide pre-treatments which belong to chemical methods are effective processes for pretreating lignocellulose materials (Xu, et al., 2010; Zhang, et al., 2011). NaOH is widely used for lignocellulose pre-treatment as it can remove partial lignin and hemicellulose in the biomass by fracturing the ester bonds thereby increasing the porosity of the biomass. Alkaline hydrogen peroxide, which is well known in the paper and cellulose industry as a bleach agent, has the great advantage of not leaving residues in the biomass, as it decomposes into oxygen and water. Besides, the formation of secondary products is practically nonexistent (Rabelo, et al., 2011). In the literature it is described that not only does alkaline hydrogen peroxide pre-treatment causes selective removal of lignin and xylan without having a large effect on cellulose, but it also decreases cellulose crystallinity and swelling of biomass, thus it decreases the recalcitrance of lignocelluloses and enhances the enzymatic digestibility of cellulose. Apart from that, it has been reported that no furfural or HMF (inhibitory sugar degradation products) were detected after this pre-treatment (Taherzadeh and Karimi, 2008). In this process the pH is one of the most important parameters for efficient application of peroxide. Depending on pH adopted during lignin oxidation, no significant changes in chemical structure might be observed since the oxidizing agent acts only in the aliphatic part of the macromolecule.
It is generally accepted that the hydroperoxide anion formed in alkaline media, is the principal active species in hydrogen peroxide bleaching systems. On the contrary, hydrogen peroxide is unstable in alkaline conditions and readily decomposes particularly in the presence of certain transition metals such as manganese, iron, and copper. The decomposition products such as hydroxyl radicals and superoxide anion radicals are thought to cause the oxidation of lignin structures, which leads to the introduction of hydrophilic (carboxyl) groups, cleavage of some interunit bonds and eventually, the dissolution of lignin and hemicelluloses.
The aim of this work was to study the efficiency of alkaline hydrogen peroxide as a pre-treatment of rice hulls to produce through out enzyme hydrolysis, fermentable sugars which could be converted into ethanol. For this purpose, the effect of H_2O_2 concentration and reaction time on the enzyme hydrolysis yield was evaluated.

2. Material and methods

2.1 Raw material

Rice husks were provided by the Spanish company Herba Ricemills. They were milled in a blender Wonder Max selecting the particles between 1 mm and 500 μm.
The material was stored in plastic bags at room temperature until use for pre-treatment.
The biomass composition was analysed before pre-treatment. The composition of untreated rice husks was 29.8 % cellulose, 16.62 % hemicellulose and 21.6 % Klason lignin.

2.2 Analysis method

In order to evaluate the pre-treatment efficiency, reducing sugars (R.S.) were evaluated after the pre-treatment and before and after the enzyme hydrolysis. With this purpose, the dinitrosalicylic acid (DNS) method in microtiter plate was carried out using the methodology developed by Gonçalves et al (2010). Before the analysis, samples were centrifuged 10 min, at 10000 rpm and 20ºC.
The DNS reagent was prepared by dissolving 5 g of dinitrosalicylic acid in 250 mL of distilled water at 80ºC. When this solution reaches room temperature, 100 mL of NaOH 2 N and 150 g of potassium sodium tartarate-4-hydrate were added and the volume was completed with distilled water to 500 mL.
The reaction was carried out in wells of 340 μL, adding 25 μL of DNS reagent to 25 μL of sample, or distilled water (blank).
Subsequently, in order to perform the reaction, the microtiter plate (96 flat test plate of Orange Scientific, made of crystal polyester), with cap, was placed in a ThermoBlock for 10 min at 105ºC.
To stop the reaction, the plate was placed on the freezer for 4 min and 250 μL of distilled water was immediately added to each well.
The absorbance of each well was read in a microplate reader (ELx800, Biotek) with a 540 nm filter.

2.3 Alkaline peroxide pre-treatment

3 g of rice husk were slurried in 50 mL peroxide solutions at different concentrations (2.5, 5 and 7.5 % w/v), depending on the experiment, in 250 mL flasks, adjusting the pH to 11.5 with NaOH tablets. Each pre-treatment was carried out in triplicate.

Flasks were covered in aluminum foil and closed with silicone stopples. They were introduced in a water bath at 90 ºC for 2 h.

In order to determine the sole effect of peroxide, a control with 3 g of rice husk and 50 mL of water was also incubated at the same temperature.

The solid residue was collected by filtration, washed thoroughly with tap water until neutral pH of the filtrate and dried at 60 ºC overnight. Subsequently, it was weighted to determine mass loss, which corresponds to the lignin content and other solubilized compounds.

After each pretreatment, a liquid sample was taken and frozen at -70 ºC for future reducing sugars determination.

Before the analysis, samples were centrifuged and the pH was adjusted to 5 by adding 2% H_2SO_4.

Pretreatment yield was calculated as the quotient of the grams of reducing sugars measured after the pretreatment and the ones which should have been measured if all cellulose and hemicellulose contained in the original solid had been completely hydrolysed to reducing sugars.

After selecting the peroxide concentration which provided the highest hydrolysis yield, the times of reaction 1, 2 and 4 hours were evaluated.

2.4 Enzyme hydrolysis

Pretreatments were evaluated through the enzyme hydrolysis of pretreated material, analysing the reducing sugars produced after the conversion of cellulose and hemicellulose in this stage.

In this work, a mixture of six cocktails of Novozymes´cellulosic ethanol enzyme kit (NS22086, NS22083, NS22118, NS22119, NS22002 and NS22035) was used in order to ensure that all the required enzymes to hydrolyse the pretreated material were added.

Enzyme activities of each cocktail are shown in Table 1.

Table 1: Enzyme activities of the six cocktails which constitute Novozymes´cellulosic ethanol enzyme kit

Enzyme cocktail	Volume added/flask (mL)
NS22086	Cellulase complex
NS22083	Xylanase
NS22118	β-glucosidase
NS22119	Arabinase β-glucanase cellulase Hemicellulase Pectinase Xylanase
NS22002	β-glucanase Xylanase
NS22035	Glucoamylase

The washed and dried water-insoluble residues of rice husk obtained after pretreatment were hydrolyzed by the six cocktails of Novozymes´cellulosic ethanol enzyme kit. Each solid was introduced in a 100 mL sterile wide mouth flask and 29.1 mL of citrate buffer (0.05 M, pH 5), 0.3 mL of sodium azide (2%) and the six Novozymes cocktails of the kit. Volumes added of each cocktail are shown in Table 2.

Table 2: Volume of enzyme cocktail added to each hydrolysis flask

Enzyme cocktail	Volume added/flask (mL)
NS22086	0.3
NS22083	0.06
NS22118	0.06
NS22119	0.06
NS22002	0.06
NS22035	0.06

Flasks were incubated at 50ºC, 150 rpm and 72 h. Liquid samples were withdrawn before and after enzyme hydrolysis and stored at -70ºC for future reducing sugars analysis.
Hydrolysis yield was calculated as the quotient of grams of R.S. measured after enzyme hydrolysis and the difference between the R.S. in the raw material and the ones analyzed in the pretreated solid.
Global yield was calculated as the sum of the pretreatment and hydrolysis yields.

3. Results and discussion

For each experiment, grams of solubilized compounds, yields achieved in the pretreatment and hydrolysis steps and global yields (the sum of the previous ones) will be shown.
Table 3 shows the results obtained when rice husks were pretreated for 2 h with alkaline solution (pH 11.5) at 90ºC and static conditions at different peroxide concentrations.
In order to study this sole effect of this variable, a control constituted by the solid without H_2O_2 was also incubated at the same conditions.
It can be observed in Table 3 that global yield increases as long as the concentration of peroxide used raises, obtaining the best result (77.25 ± 5.61 %) with the highest concentration of peroxide tested. These results agree with the mass of solubilized compounds released, which were higher (1.5821 g) at the highest H_2O_2 concentration (12 times higher than the control). Thus, when peroxide concentration increased from 0 to 7.5 % w/v, there was more solubilization of lignin and other compounds, decreasing cellulose crystallinity and improving its enzymatic attack together with the one of hemicellulose and pectin.

Table 3: Effect of [H_2O_2]

[H_2O_2] (%w/v)	Solubilized compounds (g)	Pretreat. Yield (%)	Hydrol. Yield (%)	Global yield (%)
0	0.1350	0.99	3.54	4.53
2.5	1.0974 ± 0.0121	2.95 ± 0.30	5.84 ± 1.47	7.89 ± 1.77
5.0	1.2006 ± 0.0764	2.51 ± 0.44	10.07 ± 3.40	12.58 ± 3.82
7.5	1.5821 ± 0.0739	6.10 ± 0.07	71.15 ± 5.57	77.25 ± 5.61

Comparing the last three columns of Table 3, it can be observed that pretreatment yields were very much lower than the ones measured in the hydrolysis stage. Thus, it was evidenced that polysaccharides were not degraded to simple sugars in the pretreatment step; the incubation of the residue with alkaline peroxide greatly increased lignocelluloses susceptibility to the next enzymes attack in the subsequent hydrolysis step, when practically all fermentable sugars were produced. Thus, probably, when peroxide concentration increased from 0 to 7.5 %w/v, lignin solubilization enhanced and cellulose crystallinity decreased, favouring its enzymatic hydrolysis, together with the one of hemicellulose and pectin.
It would be interesting to evaluate if more peroxide concentration provided higher yields, however it was not possible to implement those conditions with the system used.
The pretreatment time was also evaluated at 7.5 % H_2O_2, the concentration which provided the highest yields, showing the results obtained in Table 4. According to the data and their confidence limits, there is

not a very significance effect of time on the global yield and an efficient hydrolysis (around 70%) could be achieved with time of incubation as low as 15 min.

Table 4: Effect of the pretreatment time

Time (h)	Solubilized compounds (g)	Pretreat. Yield (%)	Hydrol. Yield (%)	Global yield (%)
0.25	1.4897 ± 0.0380	6.36 ± 0.11	69.01 ± 5.11	75.37 ± 5.00
1	1.4320 ± 0.0378	4.88 ± 0.52	81.60 ± 2.63	86.48 ± 3.07
2	1.5821 ± 0.0739	6.10 ± 0.07	71.15 ± 5.57	77.25 ± 5.61
4	1.5442 ± 0.0191	4.99 ± 0.25	76.80 ± 7.46	81.79 ± 7.51

Among the conditions tested, the highest global yield of 86.48±3.07 % was attained with a peroxide concentration of 7.5%, at pH 11.5, 90ºC and 1h. A slightly higher conversion (96 %) was achieved by Saha and Cotta (2007) by saccharified liquid and solid fractions separately after alkaline peroxide pretreatment of rice hulls at 7.5% H_2O_2, pH 11.5 and 35ºC. However, a higher reaction time was necessary in this case (24h). Thus, it is possible to reduce pretreatment time almost 24 h by increasing reaction temperature.

4. Conclusion

Alkaline peroxide pretreatment significantly improved the efficiency of enzyme hydrolysis of carbohydrates in rice hulls to fermentable sugars. Peroxide concentration showed a positive effect on enzymatic digestibility of rice hulls, attaining the global yield of 77.25 ± 5.61 % at 7.5% peroxide, pH 11.5, 90ºC and 2 h. As for the reaction time, a significant effect was not observed on the global yield. With the proposed pretreatment, an efficient hydrolysis could be attained with time of incubation as low as 15 min.

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