Bioconversion of agro-industrial wastes: optimization of the saccharification stage

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Abstract

Purpose: The main objective of the present work is to stablish the optimum conditions for the bioconversion of an agro-industrial waste (wheat straw) by its enzymatic saccharification.
Methods: With the purpose of obtaining sugars monomers which could be fermented to a desirable metabolite (ethanol), the lignocellulosic residue was pre-treated with alkali and hydrolysed with a cocktail of hydrolytic enzymes (cellulase, β-glucosidade and xylanase). The reducing sugars concentration released during the hydrolysis at different conditions was measured. Hydrolysis rate was estimated by a simple equation which supposes that the solid hydrolysis rate is directly proportional to the quantity of solid in the medium (first order rate).
Results: The effect of different variables (particle size, enzymes dosage, temperature, high-solids load) on the hydrolysis rate, the maximum reducing sugars concentration and the reducing sugars yield were estimated. Also hydrolysates were tested for ethanol production by *Saccharomyces cerevisiae*.
Conclusions: The optimum conditions for wheat straw saccharification were: particle size in the range of 500 to 250 μm, temperature of 50ºC and a minimum enzyme dosage of 2.5 FPU cellulase, 1.4 U β-glucosidase and 176 U xylanase per gram of dried pretreated solid. A 76% reducing sugars yield was reached by a high-solids load strategy. An ethanol concentration of 40 g/L was reached after 168 hours of the hydrolysed solid inoculation with *Saccharomyces cerevisiae*. This ethanol yield might be improved by fermentation optimization.

1. Introduction

During the last few years, the growth of the population and the increase of the industrialization have produced an increase of energy consumption. Thus, the energy crisis, coupled with severe environmental problems such as global warming and air pollution, have forced the world to look for green, non-polluting and sustainable energy resources. On the other hand, agro-industrial residues consist of many and varied wastes and their biotechnological processing would allow the obtaining of a wide range of valuable and usable products, such as transportation biofuels or chemical precursors which can be converted to high-value products [1] The use of these wastes is advantageous because these resources are abundant, cheap, renewable and non-competitive with the food chain.

Lignocellulose, the main component of agro-industrial residues, contains three major components: cellulose, hemicellulose and lignin. Cellulose is a homopolysaccharide consisting of D-glucose molecules organized in crystalline microfibrils, which are protected by an amorphous region of hemicelluloses and lignin, responsible for recalcitrant structure of plant cells [2]. As regards hemicellulose, it is a branched heteropolymer of a variety of sugars monomers, such as xylose, arabinose, mannose, glucose and galactose [3]. By selecting the right microorganism, it is possible to obtain through fermentation of these sugars a wide range of metabolites that have useful applications; however, cellulose and hemicellulose need to be previously depolymerized by hydrolysis through acid, alkali, or enzymes to their constituent monomeric sugars, which are soluble in liquid-phase media [4]. Also, a previous pretreatment step is generally applied to break down lignin and disrupt the crystalline structure of cellulose [5].

The main objective of this work is to study the effect of several variables on the enzymatic hydrolysis of a lignocellulosic residue. If proper conditions are selected, the yield of reducing sugars (RS) can be maximized and the effectiveness of the process be significantly improved. In this way, the effect of the solid particle size, the enzyme dosage and the temperature of incubation for the hydrolysis process are analysed. Also, a high-solids saccharification strategy is tested by adding a feeding stream of pretreated solid in a fed-batch mode of operation. Wheat straw has been selected as a model solid residue for this study, because it is one of the most abundant lignocellulosic agricultural residue in the world.

2. Material and methods

2.1 Solid substrate

Wheat straw was provided by IFAPA Center Rancho de la Merced (Jerez de la Frontera, Spain). The solid was milled and then sieved to collect the three following fractions: 500 μm> ϕ > 1 mm, 500 μm> ϕ > 250 μm and ϕ < 250

μm, being ϕ the particle diameter. These fractions were stored in plastic bags at room temperature until their use for the experiments.

2.2. Pretreatment

An alkaline pretreatment of wheat straw was performed before the enzyme hydrolysis step to improve substrate digestibility (1 wt% NaOH, 30 ºC for 48 h in static conditions, 1 g solid/20 mL solution). After the alkaline pretreatment, the solid was filtered, washed thoroughly with distilled water until neutral pH was reached and dried in an oven at 40^{o}C for 24 h.

2.3. Enzyme hydrolysis

Enzyme hydrolysis was performed by incubating at 50^{o}C and 150 rpm, in 250 mL Erlemenyer flasks,4 g of pre-treated wheat strawand a certain volume of phosphate-citrate buffer (0.05 M, pH 5), both previously sterilized in an autoclave (121^{o}C for 20 minutes). Before incubation, 16.5 FPU of cellulase (from *Trichoderma reesei*, Celluclast®, Sigma),9.2 U β-glucosidase (from *Aspergillus niger*, Sigma) and 10 mg/L of both penicillin G and streptomycin, to avoid bacterial contamination, were added to each flask. For experiments to study the effect of the enzymes dosage on the hydrolysis yield, 1172 U of xylanase (from *Thermomyceslanuginosus*, Sigma) were also added. Samples, which were taken periodically throughout the process, were centrifuged at 10,000 rpm for 10 min, collecting the supernatant for the analysis of reducing sugars (RS) concentration by the DNS method in microtiter plate [1]. Each experiment was carried out in triplicate.

FPase (EC 3.2.1.91) activity was assayed by incubating 0.1 mL of diluted crude enzyme with 0.9 mL of citrate buffer (50 mM, pH = 4.8), containing a Whatman filter paper strip (1 x 6 cm, 50 mg), at 50^{o}C for 60 min. A unit of enzyme activity (FPU) was defined as the amount of enzyme that releases l μmol of glucose per minute under the specified conditions of pH and temperature. Also, β-Glucosidase (EC 3.2.1.21) activity was determined by incubating 0.25 mL of 4 mM p-nitrophenyl β-D-glucopyranoside (p-NPG) and0.25 mL of acetate buffer (0.1 M, pH = 5) with 0.05 mL of diluted enzymatic extract. The assay was performed at 60 ^{o}C for 10 min, after which 2 mL of 2 M Na_2CO_3 were added to stop the reaction and allow the development of the yellow colour of the p-nitrophenolate ion, which was measured at 410 nm. A unit of enzyme activity (U) was defined as l μmol 4-nitrophenol equivalent released per minute. Finally, for xylanase activity, the reaction mixture containing 0.1 mL of diluted enzymatic extract and 0.9 mL of xylan suspension (0.5 %w/w, Birchwood xylan in 0.05 M citrate buffer, pH 5.4) was incubated at 50 °C for 10 min. A unit of enzyme activity (U) was defined as the amount of enzyme that releases l μmol of xylose per minute under the specified conditions of pH and temperature. All measurements were carried out in triplicate.

2.4. Hydrolysis rate calculation

For hydrolysis rate estimation, experimental data were adjusted to a simple equation which is based on the supposition that, given the high dosage of enzymes added in the saccharification experiments, the solid hydrolysis rate is directly proportional to the quantity of solid in the medium (first order kinetics). So, the evolution of RS concentration through time would follow a first order equation $G = G_o + G_f(1 - e^{-k_h t})$(equation 1), being G_o and G_f the initial and maximum RS concentrations, respectively, being G the concentration of RS at each t instant, and k_h the hydrolysis rate constant [1].

2.5. High-solids saccharification

With the aim of reducing the liquid/solid ratio (LSR) in order to obtain a higher RS concentration in the process, after 24 h of hydrolysis, 2 g of pretreated and sterilized solid residue was added to the hydrolysed solid. This solid addition, without modifying the initial volume of buffer, causes a reduction of the LSR from 13.7 to 9.2w/w.

2.6. Ethanol fermentation

The alcoholic fermentation process started after 7 days of high-solids saccharification of wheat straw. The experiments were carried out at 37ºC and 150 rpm, using Erlemenyer flask fitted with cotton plugs. Commercial yeast *Saccharomyces cerevisiae*71B used for winemaking (Uvaferm, Canada) was employed as inoculum. Samples were taken at different times and centrifuged at 10,000 rpm for 10 min, collecting the supernatant for ethanol and RS concentration measurements. Each experiment was carried out in triplicate. Dry yeasts were previously hydrated in a ratio of 0.2 g per mL of water at 37 ºC for 20 min. Next, 150 μL of hydrated yeast were added to each hydrolysis flask.

Ethanol concentration was analysed by an enzymatic simplified procedure for the determination of ethanol in alcoholic beverages (R-Biophram). Ethanol yield (Y_E) was calculated as the percentage of theoretical yield using equation 2, being 1.111 the conversion factor from cellulose to glucose and 0.511 the conversion factor from glucose to ethanol.

$$Y_E = \frac{ethanol\ produced\ (g/L) \cdot 100}{initial\ weigh\ of\ residue\ in\ the\ medium\ (g/L) \cdot cellulose\ (\%) \cdot 1.111 \cdot 0.511} \qquad (equation\ 2)$$

3. Results and discussion

3.1. Effect of the solid particle size

Enzymatic hydrolysis of agro-industrial wastes is a multi-step and heterogeneous solid-liquid process, involving, among others steps, the transport of soluble enzymes from the bulk liquid to the solid-liquid interface and the diffusive transport of the products (soluble sugars) out of the pores into the bulk phase. Therefore, particle size reduction of the solid wastes is necessary to increase the available superficial area of the solid and to decrease cellulose crystallinity. Also the particle size reduction before the pre-treatment and hydrolysis steps diminishes the medium viscosity, favouring the mix and decreasing the mass transfer limitations. For these reasons, different solid particle size fractions were assayed to evaluate its hydrolysis rates. Regardless of the particle size range assayed in the saccharification experiments, the observed temporal evolution of reducing sugars concentration is similar. In a first stage, the RS concentration increases rapidly and secondly continues to increase, but more slowly. As an example, results obtained for the smaller particle sizes assayed are included in Figure 1. As it can be observed, hydrolysis rate is higher at the beginning of the process, reaching a RS concentration of 21.09 g/L after 45 h, while after 168 h the final RS concentration is 23.77 g/L.

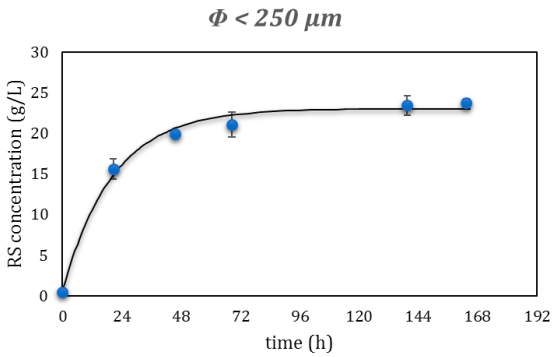

Figure 1. Reducing sugars concentration evolution during the hydrolysis of solid particles smaller than 250 μm.

Adjustment of experimental data of RS to equation 1 allowed the estimation of the kinetic constant k_h and the fitted maximum RS concentration which could be reached G_f. Values obtained for the different size fractions assayed are included in Table 1. Also RS yield (Y_G) was calculated as the ratio between fitted and theoretical maximum concentration of RS.As it can be observed, the same hydrolysis rate constants are obtained for particle sizes in the ranges $1 > \phi > 500$ μm and $500 > \phi > 250$ μm. However, for $\phi < 250$ μm the hydrolysis rate constant shows double value (0.049 h^{-1}), meaning that for the smallest particles, the hydrolysis rate is higher. With regard to the theoretical maximum reducing sugars which could be reached, the highest value was reached for the intermedium size.

According to the results obtained, the smallest particle size fraction (ϕ<250 μm) would be the optimum in order to reach a high hydrolysis rate. However, after the alkaline pre-treatment this solid fraction was completely compacted and it was necessary to disintegrate it by a second milling step. Besides, a higher buffer volume (77 mL) than for the other two solid particle sizes fractions was necessary for the hydrolysis step, as a consequence of the viscosity of the medium. In addition, lower RS yield are reached compared to the other two sizes. Therefore, from a practical point of view, a particle size in the range of $500 > \phi > 250$ μm is recommended for the hydrolysis as no additional milling step is necessary after the pretreatment and a final high reducing sugars yields is reached.

Particle size range (μm)	Solid mass (g)	Buffer volume (mL)	k_h (h^{-1})	G_f (g/L)	Y_G	r^2
$\phi < 250$	4	77	0.049	23.03	74.3	0.98
$500 > \phi > 250$	4	55	0.025	25.98	59.9	0.97
$1{,}000 > \phi > 500$	4	65	0.026	21.95	59.8	0.99

Table 1. Hydrolysis rate constant k_h, theoretical maximum reducing sugars concentration which could be reached (G_f) and reducing sugars yield Y_G for different particle sizes.

3.2. Effect of enzymes dosage

For these experiments, 4 g of pre-treated wheat straw at the recommended particle size defined in Section 3.1 ($500 > \phi > 250$ μm) were incubated in 55 mL of phosphate-citrate buffer at pH 5 and 50ºC. Several enzymes dosages were assayed, corresponding 100% enzymes dosage to 16.5 FPU cellulase, 92 U β-glucosidase and 1172 U xylanase. The percentage of enzymes dosage was reduced to 80, 60 and 20 %by decreasing proportionately the amount of each enzyme in the mixture. As it is well known, higher enzyme dosages in saccharification contribute to a high process cost.

For this reason, the quantity of enzymes to be added should be reduced as much as possible. Experimental data were adjusted to equation 1, and the results obtained are shown in Figure 2.

As it can be observed in Fig. 2, as the enzymes percentage increases, the hydrolysis process is shorter and the hydrolysis rate increases. For 100% enzymes dosage, the process is completed in about 48 hours, reaching a RS concentration of 38.18 g/L. In this case, the highest hydrolysis rate and theoretical maximum RS concentration are obtained. The duration of the process increased when the amount of enzyme is reduced and, for 20% enzyme dosage, stationary phase is not reached even after 192 hours. For 20% enzyme dosage, G_f is much higher than the last experimental value measured at 192 h. Theoretically, if no deactivation of the enzymes happened this value would be achieved after a hydrolysis time sufficiently long. However, these deactivation phenomena always occur after a certain reaction time and probably G_f could not be reached in practice.

The RS yields calculated for 100, 80 and 60% enzymes dosages were 70.5, 65.1 and 60.9%, respectively. The same relationship between sugars yield and enzymes dosage has been previously reported [6]; it was found that as cellulase activity units increase the hydrolysis yield also increases. Therefore, if the ultimate goal is to obtain the maximum sugars concentration in the shortest possible time, a 100% enzymes dosage is recommended. However, from an economical point of view, 60% dosage is more adequate as the hydrolysis rate is not decisive in the process and the final RS concentration differs with respect to 100% dosage only in 5g/L.

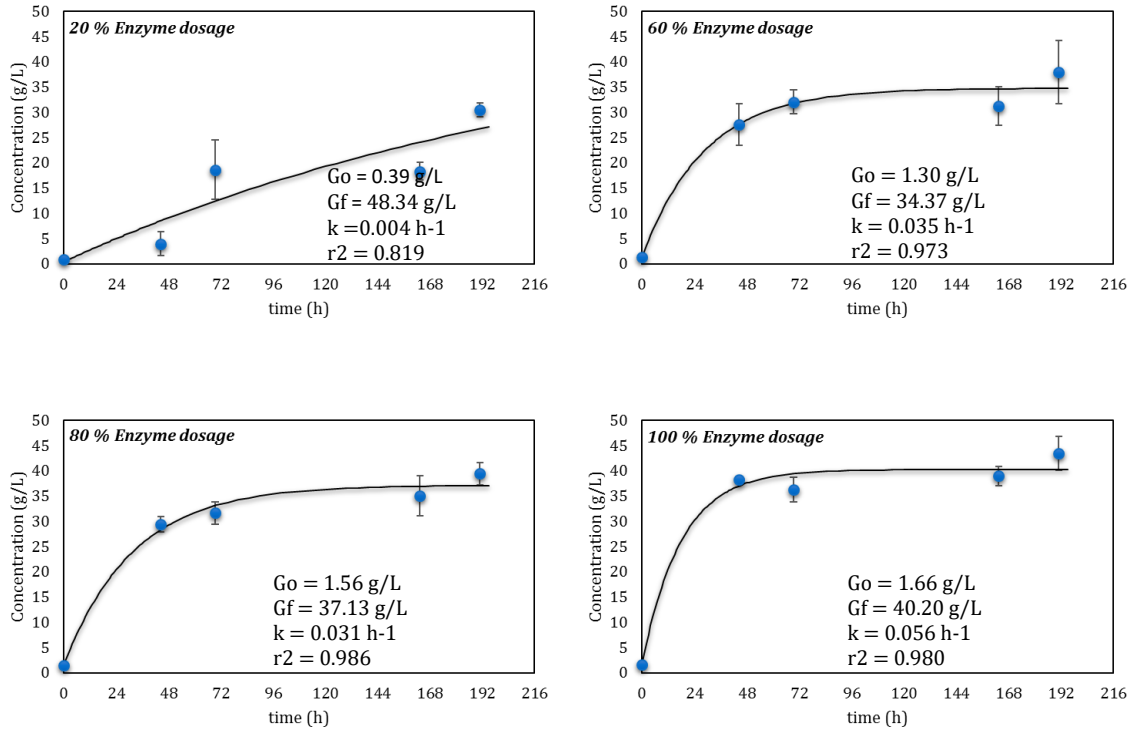

Figure 2. Reducing sugars concentration evolution during the hydrolysis with different enzymes dosages.

3.3. Effect of the temperature

As the cellulase enzyme employed (Celluclast®) has an optimum temperature of operation in the range of 45-50°C, the hydrolysis of the lignocellulosic residue was performed at 45ºC. For this experiment, the same conditions as those described in Section 3.2 were established using a 100% enzymes dosage. The kinetic parameters obtained for both experiments are shown in Table 2.

Temperature (ºC)	k_h (h^{-1})	G_f (g/L)	Y_G	r^2
45	0.015	44.38	102.3	0.95
50	0.056	40.20	92.6	0.98

Table 2. Hydrolysis rate constant (k_h), fitted maximum reducing sugars concentration which could be reached (G_f) and reducing sugars yield (Y_G) for different temperatures.

For the highest temperature assayed (50 ºC), a RS concentration of 38 g/L were reached after 44 h of process; however, at 45ºC, the same RS concentration was reached after 164 h of hydrolysis. This fact is reflected in the higher value of k_h at 50ºC (3.5 times higher). Regarding to the G_f values at the two temperatures assayed, the highest value is reached at 45ºC.Similar results have been reported for the hydrolysis of sugar beet [7]. After testing 50, 45 and 37ºC,

the study concludes that 50ºC is the optimum temperature to develop the process for 72 hours. Therefore, we also estimate that the optimum temperature for wheat straw hydrolysis is 50ºC,in spite of the final reducing sugars concentration is slightly lower than that for 45ºC, the hydrolysis rate is much higher.

3.4. High-solids saccharification and ethanol fermentation

For these experiments 4 g of pretreated wheat straw in 55 mL of citrate buffer were hydrolysed with a 100% enzyme dosage at 50ºC for 24 hours. At this moment, 2 g of pretreated wheat straw were added and, after 7 days of incubation, the hydrolysed solid was inoculated with the yeast *Saccharomyces cerevisiae*. These experiments were planned with the goal of increasing the RS concentration at the end of the hydrolysis. As the initial moisture content of pretreated wheat straw is very low, when it is mixed with the buffer to adjust the LSR at the desired value, the solid absorbs practically all the liquid. Later, after 24 hours of hydrolysis, the solid is liquefied as a consequence of the enzymes activity, and this behaviour allows the addition of a new amount of pretreated solid. The RS and ethanol concentration measured during the fermentation are shown in Figure 3.

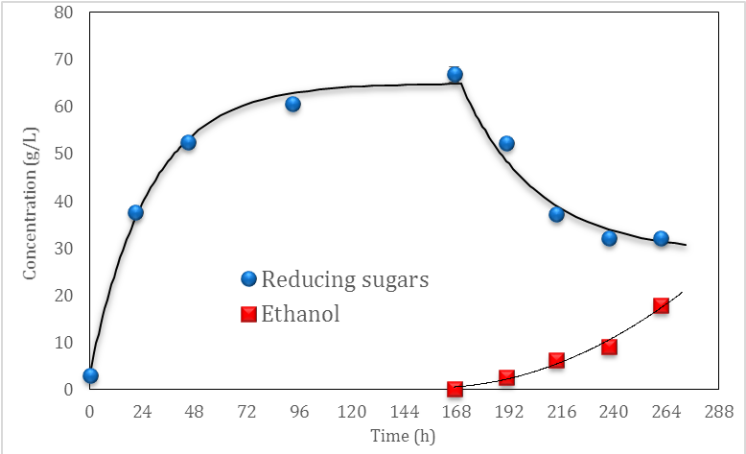

Figure 3. Reducing sugars concentration and ethanol concentration evolution during the hydrolysis and fermentation stages.

As it can be seen, the RS concentration increases during the hydrolysis stage, reaching 52.52 g/L after 47 h and 67 g/L at the end of the stage. From the adjustment of experimental data to equation 1, the following kinetic parameters were obtained: k_h = 0.037 h^{-1} and G_f = 64.99 g/L (r^2 = 0.996). If those values are compared with the corresponding ones when no extra solid is added (k_h = 0.056 h^{-1}, G_f = 40.20 g/L, r^2 = 0.980), in spite of the hydrolysis rate decreases, the final maximum RS concentration expected increases significantly. As a consequence, this high-solids saccharification strategy supposes an RS yield of 99.8 %. The same behaviour (decrease in the hydrolysis rate and increase in the maximum RS reached) has been reported for beet bagasse by Liu *et al* [8]. Those authors suggest that an increase in the solid content may result in an increase in the amount of glucose released and also in the ethanol produced.

After the yeast inoculation, the RS decreases continuously reaching 32 g/L after 96 h of fermentation, while ethanol concentration increases up to the maximum value of approximately 20 g/L after 168 h of inoculation, which implies that all consumed sugars have been fermented to ethanol. Similar values of ethanol concentration (37.1 g/L) has been reported by Alvira *et al*., when the water-insoluble solids fraction from steam-exploded wheat straw was used as substrate for *Saccharomyces cerevisiae* fermentation, operating at high substrate loadings [9].

It can also be observed in figure 3 that not all the sugars were consumed at the end of the fermentation process. This fact is probably due to chemical composition of wheat straw [10], since pentoses derived from hemicellulose hydrolysis cannot be fermented naturally by *Saccharomyces cerevisiae*, which metabolizes hexose sugars, mainly glucose.

4. Conclusions

The choice of a suitable solid particle size distribution is important since it will affect the rheological properties of the solid/liquid mixture and hence the final concentration of sugars. Also, particle size reduction causes a decrease in cellulose crystallinity and medium viscosity, improving mixing and reducing resistance to the mass transfer between liquid and solids. According to the results obtained, the smallest particle size fraction (ϕ<250 μm) would be the optimum in order to reach the highest hydrolysis rate. However, after the alkaline pre-treatment this solid fraction was completely compacted and it was necessary to be disintegrated by a second milling step. Therefore, from a practical point of view, a particle size in the range of 500 > ϕ > 250 μm is recommended for the hydrolysis.

On the other hand, as the cost of the enzymes represents a high percentage of the total cost of the process, several enzymes dosages have been tested. From results obtained it can be concluded that enzymes dosage can be reduced to 60% (2.5 FPU cellulase, 1.4 U β-glucosidase and176 U xylanase per gram of dried pretreated solid) as the final RS concentration differs from 100% dosage only in 5g/L. Concerning the effect of temperature, the optimum for

wheat straw hydrolysis is 50ºC. In spite of the final reducing sugars concentration is slightly lower than that for 45ºC, the hydrolysis rate is much higher.

Finally, the development of a high-solids saccharification strategy by adding a certain amount of pretreated solid once the hydrolysis has started, allows obtaining a high reducing sugars yield (practically total hydrolysis). Moreover, the hydrolysed solid medium was tested as substrate for ethanol production by *Saccharomyces cerevisiae*, reaching 24 g/L after 168 hours of inoculation

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