Effect of Biological and Chemical Pre-treatment on the Hydrolysis of Corn Leaf

Kenia Ángeles Ramírez,[a] Ainhoa Arana-Cuenca,[a] Sergio Alejandro Medina Moreno,[a] Octavio Loera-Corral,[b] Arturo Cadena Ramírez,[a] and Alejandro Téllez-Jurado [a,*]

Hydrolysis of corn leaf utilizing two treatment sequences was carried out in this study. The first treatment was chemical and involved subjecting the corn leaf to an alkaline pre-treatment and then to a smooth acid hydrolysis. The second consisted of biological delignification using the strain *Trametes* sp. 44 H88, followed by enzymatic hydrolysis using the enzymatic extract produced by *Trichoderma* sp. H88. The ligninolytic extract produced by *Trametes* sp. 44 H88 was used to detoxify the hydrolyzate. The results indicate that biological pre-treatment with delignification is more favorable and improves the subsequent hydrolysis, regardless of whether the hydrolysis is chemical or biological. The chemical treatment sequence obtained 80% conversion of monosaccharides, while the biological treatment sequence resulted in a 87% conversion rate. Finally, the use of the ligninolytic extract for the dephenolization of the hydrolyzate reduced the presence of compounds of phenolic origin by 23%.

Keywords: Delignification; Hydrolysis; Trametes

*Contact information: a: Laboratorio de Microbiología Molecular, Departamento de Biotecnología, Universidad Politécnica de Pachuca, Zempoala, Hidalgo, CP 43835, México; b: Laboratorio de Biología Molecular de Hongos Filamentosos, Universidad Autónoma Metropolitana, Iztapalapa, México D.F., CP 09340, México; *Corresponding author: alito@upp.edu.mx*

INTRODUCTION

Corn (*Zea mays* L.) is the third most important cereal crop in the world, after wheat and rice. It is utilized as food for humans and animals and, in Mexico, is the principle crop in terms of both economic and social importance because it forms a portion of the diet of the majority of Mexicans (Mendoza *et al*. 2006). The U.S. Department of Agriculture estimates that in the year 2012, worldwide production of corn reached 949.93 million tons. Calculations suggest that for each ton of cereal grain produced in the world, 1.5 tons of straws are generated (Howard *et al*. 2003), which means that on a planetary level there are 1,400 million tons of residual products available from corn cultivation alone.

Corn leaf is composed mainly of lignin (15%), hemicellulose (35%), and cellulose (45%) (Howard *et al*. 2003). To obtain the hemicellulose and cellulose and therefore the sugars they contain, which can be used in a wide variety of bio-technological processes, it is first necessary to eliminate the lignin, a biopolymer bonded covalently to the carbohydrates through ester and ether unions that provide this vegetable with support and protection against attacks by diverse organisms (Wan and Li 2012).

Various enzymatic and physical-chemical processes have been developed or combined to eliminate lignin in order to perform saccharification of the hemicellulose and cellulose. Taherzadeh and Karimi (2008) reported that pretreatment of crop residues improves the accessibility of biopolymers that are present in this waste. Some of the most

common processes for the pre-treatment of lignocellulosic waste to eliminate lignin include using alkaline media (Mosier *et al.* 2005; Kumar *et al.* 2009), lime (Chang *et al.* 1998), steam-explosion in an alkaline medium (Ballesteros *et al.* 2006), dilution in acidic media (Esteghlalian *et al.* 1997; Mosier *et al.* 2005), the organosolv method (Sidiras and Koukios 2004), hydrothermal treatments (Garrote 1999; Ingram *et al.* 2009), and the use of peroxide (Gould 2004). Other delignification methodologies developed entail the use of fungi in the class basidiomycetes that eliminate the lignin in various types of vegetable residues. Among the most common are those used with wheat chaff (Hatakka 1983; Okano *et al.* 2005; Patel *et al.* 2006), sugar cane bagasse (Dias *et al.* 2009), corn silage (Oleskowicz-Popiel *et al.* 2008), corn stover (Quintanar *et al.* 2012), wheat straw (Müller and Trösch 1986), water lilies (Mukherjee and Nandi 2004), and several forest residues such as poplar (Reid 1985), oak (Kamei *et al.* 2012), pine (Mendoça *et al.* 2002), and eucalyptus wood (Del Río *et al.* 2002). Alkaline pretreatments are best used for solubilization of lignin plant residues, and it is common to use NaOH, $Ca(OH)_2$, or ammonia. It has been described that the lignocellulosic residues pretreated with alkali increase accessibility of the enzymes to biopolymers like cellulose and hemicellulose, so that the yield of total sugars in the process of hydrolysis increases considerably (Kassimn and El-Shahed 1986). Silverstein *et al*. (2007) found that pretreatment with 2% NaOH for 90 min and 120 ºC solubilizes to 65% of the lignin present in cotton waste and improves the conversion of cellulose by 61%. Similarly, Zhao *et al.* (2007) found that pretreatment with NaOH enhances the enzymatic conversion of cellulose of crofton weed stem. Moreover, biological pretreatments involve the use of basidiomycetes fungi for their ability to produce ligninolytic enzymes (Keller *et al*. 2003). The main benefit for biological pretreatments are an increase in yield of monosaccharides during hydrolysis process; the pretreatments greatly improve the hydrolysis of lignin (Isroi *et al*. 2011). A decrease of 35% of the lignin present has been reported in corn straw after a 30-day pretreatment using *Trametes versicolor* (Yu *et al*. 2010). In wheat straw, the biological pretreatment using *Pleurotus ostreatus* caused a decrease of 39% lignin (Zadrazil and Puniya 1994). Prior treatment of lignocellulosic residues can favor later chemical-biological hydrolysis of such materials, thus considerably increasing yields of fermentable sugars. In the present study we compare alkali pretreatment and biological delignification (using *Trametes* sp. 44) and their effects on the chemical and enzymatic hydrolysis of corn leaf to produce carbohydrates.

EXPERIMENTAL

Materials

Organisms and Culture media

The study utilized the fungi *Trametes* sp. 44 and *Trichoderma* sp. H88. The first was isolated from the sub-tropical zone called the Huasteca Hidalguense in the state of Hidalgo, México (Cruz *et al.* 2012); the second was provided by Dr. Aldo González Becerra of the *Centro de Biología Molecular Severo Ochoa* in Spain. Both were cultured in plates with potato dextrose agar (PDA) and incubated at 30 °C for 7 days. The fungi were stored at 4 °C until use, and re-cultured monthly to maintain their viability.

Preparation of the Substrate

The corn leaf was provided by corn producers in the Valle del Mezquital state of Hidalgo, México. It was washed in cold water and dried in an oven at 60 °C for 24 h. Once

dried, the leaf was subjected to a trituration process using a hammer mill. Finally, it was sifted to select for particle size no. 8 (2.38 mm).

Methods

Alkaline pre-treatment

The conditions employed were as follows: room temperature (25 °C) for 12, 24, and 48 h; to reflux for 1, 3, and 4 h; at 121 °C with 1 atm of pressure for 5, 10, and 20 min. In each assay, 15 g of corn leaf was used and 100 mL of NaOH solution (2% and 4%, w/v) was added.

Biological pre-treatment

For the biological pre-treatment, the methodology described by Quintanar *et al*. (2012) was utilized. This involved the use of particle size 8 corn leaf and the basidiomycete fungus *Trametes* sp. 44.

Production of ligninolytic enzymes for dephenolization

The ligninolytic enzymes were produced by *Trametes* sp. 44 strain. The pre-inoculum originated from a petri dish containing PDA that was inoculated with the fungus and grown for 10 days. Agar cubes approximately 0.5 cm^3 were cut with a sterile knife, added to a flask containing 100 mL of sterile water, and incubated for one complete night at 150 rpm. Then, 10 % of the supernatant was added to 100 mL of Kirk medium (Kirk *et al.* 1986) and incubated for 4 days at 32 °C.

Acid hydrolysis

For the acid hydrolysis of the corn leaf, sulfuric acid (H_2SO_4) at concentrations of 2% and 4% (v/v) was utilized. To perform chemical hydrolysis, the residue that resulted from the chemical pre-treatment with NaOH and biological pre-treatment with the *Trametes* sp. 44 fungi was the substrate. Next, 100 mL of acid solution, at the concentrations mentioned above (2 and 4%), was added to the residue of pre-treated corn leaf utilizing conditions of the reflux reaction at 122 °C and ambient temperature. Each sample was incubated at ambient temperature for 24 h.

Enzymatic hydrolysis

As a first step, an enzyme extract obtained from growth of *Trichoderma* sp. H88 on the corn leaf previously washed with hot water and with a particle size of 4.7 mm was used. The substrate was inoculated with 1 x 10^6 spores/g of dry material and adjusting the initial moisture to 75%. After 5 days of fungal culture, 30 mL of sterile water was added per gram of substrate and stirred for 30 min at room temperature. The supernatant obtained was filtered, lyophilized, and stored in a desiccator until use.

Enzymatic hydrolysis was carried out using the residues obtained from the process of chemical and biological pre-treatment. For every 0.5 g of residue, 50 mL of acetate buffer solution was added, and the enzyme extract containing 63 xylanase activity unit (AU) and 5.44 AU of cellulase (the unit of activity was defined as the amount of protein which hydrolyzes one microgram of cellulose or xylan by minute). The residue whit enzyme extract was incubated at 28 °C for 72 h.

Determination of total phenolic compounds

The colorimetric Folin-Ciocalteu (Singleton *et al*. 1998) method was used to determine the concentration of phenol. The method involves placing 30 μL of the sample and 470 μL of distilled water in a 5-mL test tube and then adding 250 μL of 1 N Folin-Ciocalteu reagent and 1.25 mL of sodium carbonate (Na_2CO_3). The mixture was incubated for 40 min at ambient temperature (25 ºC) and read at 725 nm on a spectrophotometer. To estimate total phenolic content, the data was compared to a standard curve of gallic acid that was prepared at a concentration of 1.0 g/L.

Determination of total carbohydrates

This analysis was carried out using the phenol-sulfuric method described by Dubois *et al.* (1956). Briefly, 10 μL of sample was mixed with 990 μL of distilled water in a 10 mL test tube. Next, 600 μL of phenol at a concentration of 5 % (w/V) was added, followed by 3.6 mL of concentrated sulfuric acid. The contents of the tube were agitated, allowed to react at ambient temperature (25 ºC) for 30 min, and read on a spectrophotometer at 490 nm with a quartz cell. To estimate total sugar (TS) content, the data was compared to a standard curve of glucose that was prepared at a concentration of 1.0 g/L.

Determination of reducing sugars

The modified Miller method (Miller *et al*. 1960) was used to determine the concentration of reducing sugars. Briefly, 500 μL of the extract was mixed with 750 μL of DNS (3,5-dinitrosalicylic acid) reagent and heated to a boil for 10 min. It was left to cool to room temperature and then read at 640 nm using a spectrophotometer. To estimate the content of reducing sugars, the data was compared to a standard curve of glucose that was prepared at a concentration of 1.0 g/L.

Determination of simple sugars

To determine the concentration of simple sugars, an HPLC (Grace-Alltech Deerfield, IL) was utilized with a 5 μm Prevail Carbohydrate ES column (Grace-Alltech, Deerfield, IL) and an ELSD 800 detector (Grace-Alltech, Deerfield, IL) at an average pressure of 1200 psi. The mobile phase was a water:acetonitrile (30:70) mixture run at a flow velocity of 1 mL/min. Glucose, xylose, and arabinose standards at known concentrations were used to quantify the sugars.

Lignin determination

Lignin was determined in accordance with the norm TAPPI T222 om-88 (1988). Quantification of acid insoluble lignin was determined from hydrolysis of 3 mL 24 N sulfuric acid and 0.3 g of corn leaf. The mixture was incubated at 30 ° C for 1 h, subsequently added 84 mL of distilled water, and filtered using glass fiber pore size M (medium). The acid insoluble lignin was determined by dry weight.

Data Analysis

To determine differences between treatments, all experimental data were analyzed using SPSS v.17 (IBM) software using a one-factor ANOVA.

RESULTS AND DISCUSSION

Effect of the Alkaline Pre-treatment on Corn Leaf

The main effect of the alkaline pre-treatment of lignocellulosic residues is its impact on the removal of lignin. Alkaline conditions favor the rupture of ester bonds that bind the lignin to the hemicellulose (Cheng *et al.* 2010). Also, it makes the components of the biomass more readily available for the subsequent hydrolysis treatments (Binod *et al.* 2010). Given that initial lignin content in the corn leaf was 17.8% (2.67 g lignin/15 g corn leaf), the objective of the alkaline treatment was to reduce the concentration of this compound to facilitate the hydrolysis of the resulting residue. The pre-treatments tested showed different levels of delignification, but the best pre-treatment was at room temperature (25 ºC) with 4% NaOH followed by pre-treatment under pressure (121 °C and 1.5 atm) and finally refluxed pretreatment. At the end of pretreatment, delignification of 60, 45, and 32%, respectively was observed (Table 1). Similar processes have been described, but for other substrates; rice stalks, with results of 23 to 36% delignification (Binod *et al.* 2010; Cheng *et al.* 2010), sugar cane bagasse, with 36 to 75.5% delignification (Aguiar 2001; Zhao *et al.* 2009), and corn stover, with 55% delignification (Zhao and Xia 2009).

In contrast to the procedures reported by these authors, most of whom used temperatures from 55 to 121 °C, the treatment in the present study was performed at approximately 25 °C, which required much less energy to achieve 60% delignification. It is likely that the reduced energy requirement for chemical delignification of corn leaf is related to the silica content in its structure, as the amount of ash present in the corn leaf was 2.2%, which is below the ash content reported for barley straw (4 to 8%), rice stalks (16 to 30%), and sugar cane bagasse (2 to 8%) (Salaber and Maza 1972). This lower proportion of ash may indicate a lower silica content, which makes the delignification process more efficient.

Lignin degradation releases sugars that are associated with hemicellulose. The amount of sugars present in the hydrolyzed was determined and may be indicative of the efficiency of the alkaline pre-treatment. This effect was observed in the treatments performed. Of the alkali pre-treatments employed (reflux, room temperature, and pressure), the best results with respect to the release of total sugars were attained with 4% NaOH still the best pretreatment pressure (5.9 g/L), which was slightly better than the reflux pre-treatment (5.8 g/L) and the pre-treatment at room temperature (5.7 g/L) (Fig. 1). However, no significant differences were observed among these three treatments.

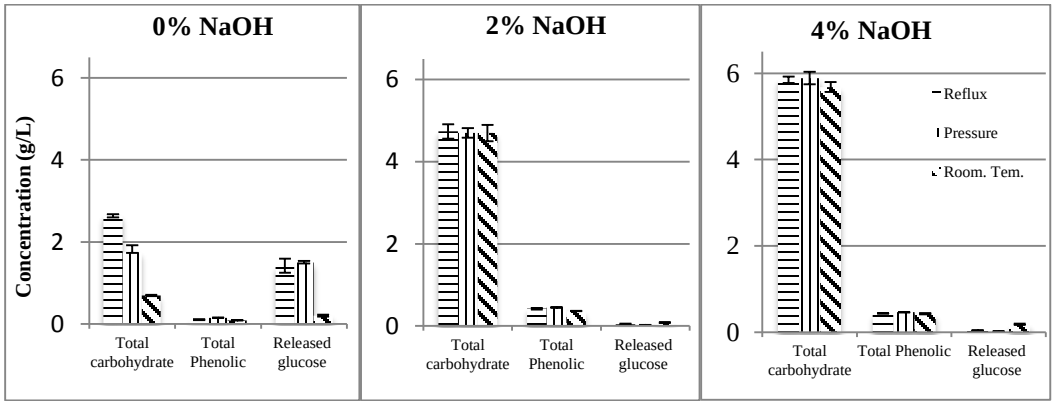

Fig. 1. Alkali pretreatment of corn leaf and its effect on the release of sugars

The presence of phenolic compounds in the hydrolyzed, probably result from solubilization of the lignin, may also be indicative of the delignification process. In treatments with 2% and 4% of alkali, the concentration of phenol is very similar and negligible in the control (without alkali). Moreover, it was observed that alkaline pretreatment promotes hydrolysis of the hemicellulose because the presence of glucose (derived from cellulose) was negligible for treatments 2 and 4% of alkali (Fig. 1). Other authors have found that the combined effect of temperature and pressure may favor release of sugars (Aziz *et al.* 2002).

The study also found that the concentration of carbohydrates present in the alkaline hydrolyzed varied widely as a function of the pre-treatment. The carbohydrates analyzed were glucose, xylose, and arabinose; *i.e.*, those found in the highest proportions in this type of residue (Quintanar *et al.* 2012). Once again, the treatments with 4% NaOH achieved the best results. The amounts of glucose detected were 0.72 g/L, 0.09 g/L, and 0.01 g/L for the room temperature, reflux, and pressure treatments, respectively. The findings show that temperature had a marked effect on the release of glucose, since application of a higher temperature caused greater degradation of this carbohydrate. These results are similar to those obtained by Yang and Montgomery (2007), who found almost complete degradation of the glucose in an aqueous solution that was heated to 100 °C for 30 min.

In the case of xylose, the study detected 3.6, 2.9, and 0.8 g/L for the reflux, pressure, and room temperature pre-treatments, respectively. The presence of the largest amount of xylose is probably due to the action of NaOH on the lignin and to the bonds between this biopolymer and the hemicellulose that release the xylose present in this bond, as observed by López-Miranda *et al*. (2009). Hence, the large production of xylose can be attributed to the increased hydrolysis of hemicellulose present in the corn leaf. Finally, for the carbohydrate arabinose, the following values were detected 0.36 g/L, 0.22 g/L, and 0.19 g/L for the reflux, pressure, and room temperature pre-treatments, respectively. Observations showed that the treatment at room temperature favored the release of glucose, so that it was not degraded by the combined action of temperature and alkali. For xylose and arabinose, the reflux treatment proved to be the most favorable, suggesting the possibility that temperature combined with the alkaline medium favored lignin degradation. The presence of oligosaccharides was not detected in the reflux pre-treatment because they were completely hydrolyzed, as observed by Pedersen and Meyer (2010), who determined that applying heat favors the formation of monosaccharides. For the pre-treatment under pressure, were detected 14% of oligosaccharides, this concentration is increased to 17% at room temperature. According to Biswas *et al*. (2005), hemicellulose is solubilized at alkaline pH, but heat is necessary for long periods to achieve complete hydrolysis.

Acid Hydrolysis of Corn Leaf Obtained with Alkaline Pre-treatment

Once the residues had been subjected to the alkaline pre-treatment, they were filtered to separate the high carbohydrate content liquid and the cellulose fibers with hemicellulose. The cellulose fibers were then subjected to the process of acid hydrolysis. The reaction conditions were pressure, reflux, and room temperature, utilizing sulfuric acid at 2% and 4%. The best results were obtained with 4% sulfuric acid because they were the conditions where the higher levels of TS were obtained. The TS maximum concentration of 5.2 g/L was obtained with pressure at 20 min and 4% H_2SO_4. Addition of 2% sulfuric acid under the same reaction conditions resulted in 4.1 g/L TS. With pretreatment at room temperature, the maximum amount of TS was 1.6 g/L after 72 h with 4% H_2SO_4, and for

the reflux pre-treatment, TS was 4.2 g/L after 4 h with 4% H_2SO_4. It is important to note that under these testing conditions, it is necessary to apply heat to accelerate the acid hydrolysis of the residual cellulose and hemicellulose. Acid hydrolysis of lignocellulosic residues is a common procedure. Lopez *et al*. (2008), for example, performed hydrolysis of sunflower stems with 4% sulfuric acid, while Herrera *et al*. (2004) carried out hydrolysis of sorghum residues using sulfuric acid and temperatures as high as 100 °C. Thus, the pH of hydrolysis is a factor that must be considered when developing hydrolysis systems since an acidic medium promotes the rupturing of bonds that bind the monomers of the cellulose and hemicellulose (Vázquez *et al*. 2006).

This study also analyzed the principle carbohydrates present in the syrup obtained after acid hydrolysis (4% sulfuric acid). The highest proportion found was xylose under the pre-treatment at pressure was 3.3 g/L, followed by the reflux treatment with 2.5 g/L and at room temperature; the presence of xylose was negligible. These result are consistent with that described by Agbogbo and Wenger (2007), who reported that the acid medium solubilized the hemicellulose rich in xylose, thus increasing the porosity of the material. The second most common carbohydrate was glucose, for which the highest amount detected was under the pre-treatment at pressure with 4% H_2SO_4; this reached a concentration of 0.4 g/L after 10 min of pre-treatment. Earlier studies have shown that the combination of high temperature, acid medium, and prolonged treatment times may affect glucose yields, since these conditions can degrade this carbohydrate (Dien *et al*. 2006). This might explain, at least in part, the low yield of this sugar. Finally, the amount of arabinose present in the syrup was 0.11 g/L in both the pressure and reflux treatments. This finding concurs with that reported by Lenihan *et al*. (2010), who affirmed that arabinose is a minor element in lignocellulosic residues and, moreover, highly sensitive to the presence of heat. The treatment at room temperature did not favor hydrolysis of the cellulose and hemicellulose, clearly indicating that temperature plays a very important role in the acid hydrolysis of these biopolymers.

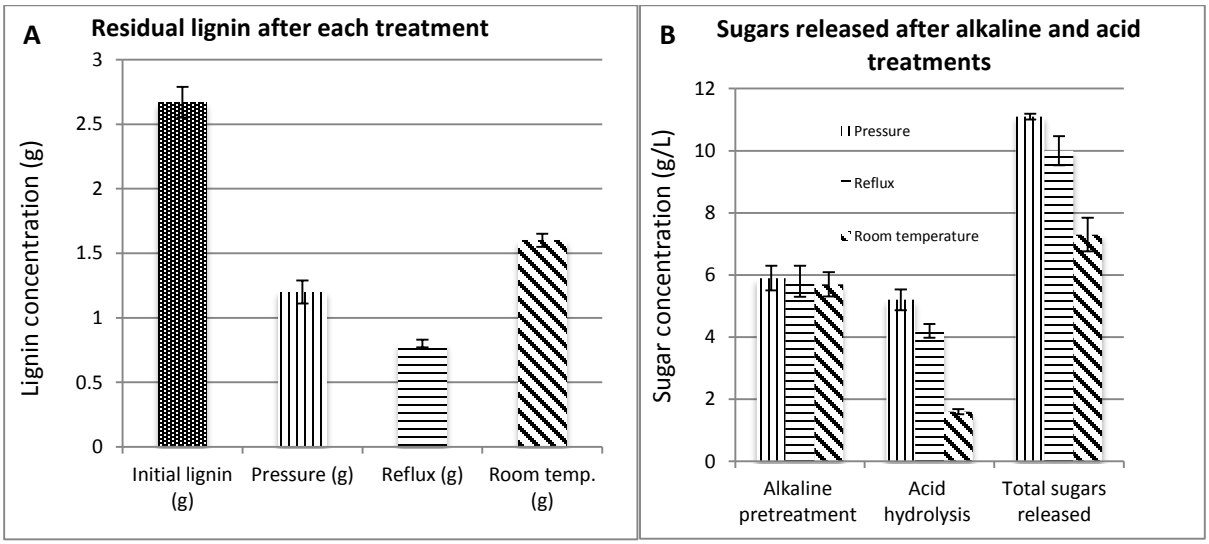

Fig. 2. Residual lignin after pressure, reflux and room temperature treatments representing 45, 32, and 60% delignification of corn leaf respectively (**Fig. 2A**). Sugars released during alkaline pre-treatment (4% NaOH) and acid treatment (4% H_2SO_4) under pressure, reflux and room temperature conditions (**Fig. 2B**).

The results showed that the best chemical hydrolysis train was using pressure with an 80% conversion with initial biomass of 15 g (Fig. 2A). As a next step, the possible combined effect of a chemical-biological train treatment in order to improve the conversion rate of biomass carbohydrate was studied.

Biological Pre-treatment of Corn Leaf

The corn leaf was subjected to a fermentation process using the basidiomycete fungus *Trametes* sp. 44 to biologically reduce the lignin present in this residue, following the method of Quintanar *et al*. (2012). After culturing the fungus for 13 days on the corn leaf, observations showed a decrease of 35% in the amount of lignin present. The product obtained was then used in chemical hydrolysis with H_2SO_4 and enzymatic hydrolysis.

Acid Hydrolysis of Corn Leaf Obtained from Biological Pre-Treatment

The residue obtained from the biological pre-treatment was subjected to acid hydrolysis using 2% and 4% H_2SO_4 under the following three conditions: reflux, at pressure (121 °C and 1.5 atm), and at room temperature. The best conditions of hydrolysis was achieved in all treatments with 4% H_2SO_4. The best treatment was at pressure with a yield of 8.1 g/L TS. Reflux treatment was next detected 6.0 g/L TS, the treatment at room temperature released 4.9 g/L TS (Table 1). Chromatographic analysis indicated that the most common carbohydrate was xylose at 5.9, 4.5, and 3.6 g/L for pressure, reflux, and room temperature treatments, respectively. This is followed by arabinose, with 1.5, 1.3, and 1.1 g/L for pressure, reflux, and room temperature treatments, respectively. Finally, the least common carbohydrate was glucose, with 0.7, 0.6, and 0.5 g/L for pressure, reflux, and ambient temperature treatments, respectively. It is highly probable that prior culture of the fungus on the substrate globally affected the lignin present in the corn leaf, such that the acid gains greater access to the lignocellulosic matrix, thus favoring hydrolysis of the vegetable residue and generating the higher yields obtained with biological delignification.

Production of Hydrolytic Enzymes

The residues that resulted from the alkaline pre-treatment were used to cultivate *Trichoderma* sp. H88 for producing hydrolytic enzymes that were subsequently used for the enzymatic hydrolysis of corn leaf residues. This fungus is considered an excellent producer of hydrolytic enzymes (Manjarres *et al.* 2011), but is not capable of degrading lignin (Howard *et al.* 2003). For this reason, the substrate previously delignified by chemical means was used with substrate. The highest production of cellulases and xylanases was attained at 8 days of culture with 5.44 and 63 AU/mL, respectively. The obtained extract was placed at 4 °C and utilized in the enzymatic hydrolysis of the corn leaf.

Enzymatic Hydrolysis of Corn Leaf

For enzymatic hydrolysis, only the residue from the treatment at pressure with 4% H_2SO_4 was utilized because it was the best treatment where the TS yield was observed. Three different temperatures were tested (28, 37, and 50 °C) with three different enzymes concentrations (5.44 and 63; 10.88 and 126; 16.32 and 189, cellulolytic and xylanolytic AU, respectively). Results showed that the highest amount of TS (4.1 g/L) was detected after 48 h of incubation at 50 °C (Table 1). Temperature is thus a factor of great importance, for it increases the rate of enzyme catalysis, thereby raising its efficiency (Talebnia *et al.* 2009). Another study has reported that temperatures close to 50 °C favor hydrolysis of

lignocellulosic residues (Kumar *et al.* 2009). Also, it has been reported that many cellulases present good catalytic activity at temperatures 45 to 55 °C, with an optimal temperature of 50 °C (Binod *et al.* 2009). Lu *et al*. (2009) obtained similar results when evaluating cellulose hydrolysis at temperatures of 30, 40, and 50 °C, and obtained the best results at 50 °C. The effect of enzyme concentration on hydrolysis was also analyzed, and observations showed that the highest release of TS (4.3 g/L) was achieved with 126 AU xylanolytic and 10.88 AU cellulolytic at a temperature of 50 °C. With a reduction of 50 % in the enzyme concentration (63 AU xylanolytic and 5.44, AU cellulolytic); after 48 h of incubation, no significant difference was observed with respect to the release of sugars. It has been reported that the degree of hydrolysis depends on the concentration of the enzymes utilized (Mojovic *et al.* 2006), so this must be taken into account when designing hydrolysis processes for vegetable residues.

Biological delignification generated a decrease of 35% of the lignin present in the corn leaf. The resulting residue was subjected to acid hydrolysis (4% H_2SO_4) and the TS released were evaluated. Comparing the result of chemical pretreatment, it was observed that biological pretreatment improved total sugar release by 9%. The treatment train: biological pretreatment, acid hydrolysis and enzymatic hydrolysis, conversion rate of 87% of TS released was obtained (Table 1). A variety of hydrolysis methods have been used, including pre-treatments to eliminate lignin. Zhao *et al*. (2007) obtained a conversion rate of 60% utilizing corn stover with an alkaline pre-treatment (NaOH) and a mixture of commercial cellulolytic enzymes. It has been suggested that biological pre-treatment improves the yield in the saccharification process (Gao *et al.* 2012), as described by Du *et al*. (2011), who studied the effect of biological pre-treatment with *Irpex lacteus* on the subsequent enzymatic hydrolysis of corn leaf and obtained 315.5 mg/g after 28 days of treatment. Meanwhile, López-Miranda *et al*. (2009) used pine sawdust pretreated with NaOH, followed by enzymatic hydrolysis with cellulases, to obtain a conversion rate of 48%.

Table 1. Yields Obtained with Biological Pretreatment-Acid Hydrolysis-Enzymatic Hydrolysis Treatment Sequence

Treatment	Initial biomass (g)	Initial Lignin (%)	Biological delignification (%)	Biomass residual (g/L)	Total released sugars (g/L)		Total (g/L)	% Hydrolysis
					Acid hydrolysis	Enzymatic hydrolysis		
Pressure	15	17.8	35	14	8.1	4.1	12.2	87
Reflux	15	17.8	35	14	6.4			
Room temperature	15	17.8	35	14	5.2			

Detoxification of the Hydrolyzate

Severe chemical treatments can produce inhibitory compounds that will later interfere with production processes, whether of metabolites or of biomass. For this reason, the hydrolyzed must be treated to eliminate such inhibitory molecules (Lenihan *et al.* 2010). During the chemical treatment process, the study detected that the resulting hydrolyzed presented a final concentration of 0.117 g/L of phenolic compounds that were probably produced during the alkaline and acid pre-treatments. As a result, the

concentration of the phenolic compounds that result from the degradation of lignin, furfural, and hydroxymethylfurfural from the cellulose and hemicellulose must be eliminated, or at least reduced, in the hydrolyzed before any future application. Diverse detoxification methods have been tested, including ion-exchange resins to treat hydrolyzed from sugar cane bagasse that improved the ensuing fermentative process (Viñals *et al.* 2006). Activated carbon has also been tested as a means of eliminating phenolic compounds from the hydrolyzed of rice stalks and wheat straw (Martínez *et al.* 2002). Another process involves the use of electrodialysis to eliminate toxic compounds from the hydrolyzed of sugar cane bagasse (Cheng *et al.* 2008). One alternative is to utilize enzymatic extracts with ligninolytic activity, due to their capacity to degrade lignin and compounds of phenolic origin (Alvira *et al.* 2010). In this study, the enzymatic extract produced by the white rot basidiomycete fungus *Trametes* sp. 44 was used to reduce the possible toxic effect of the hydrolyzed. This extract was generated after 4 days of culture of the fungus in Kirk medium, when it presented an average of 8 AU of MnP/mL, and 79 AU of laccase/mL. Three different temperatures were tested for a 48-h incubation of the enzymatic extract and the hydrolyzed; the incubation at 50 °C showed a reduction in the concentration of phenolic compounds from 0.117 to 0.09 g/L, which represents a decrease of 23 % in concentration (Fig. 3A). Subsequently, three different concentrations of enzymes were tested (1, 2, and 4 mL) and showed that higher enzyme concentrations led to greater decreases in the major phenolic compounds in the hydrolyzed (Fig. 3B). The efficiency of the reduction of toxic compounds was probably due to the presence of phenol oxidase (MnP) and polyphenol oxidase (laccase) enzymes in the enzymatic extract that, because of their catalytic characteristics of lignin degradation, can degrade compounds of phenolic origin (Alvira *et al.* 2009) and contribute to the decrease in toxicity of the hydrolyzed.

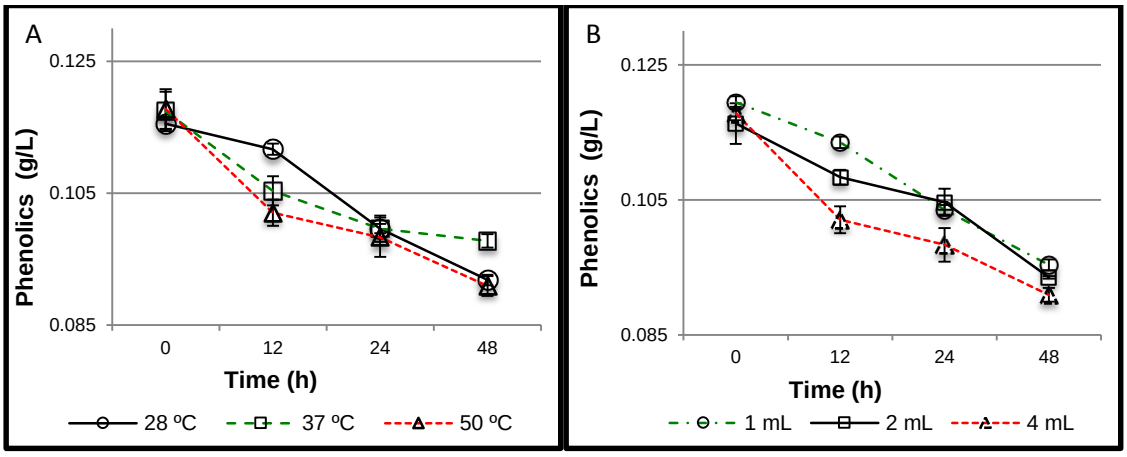

Fig. 3. Decrease of phenolic compounds present in the hydrolyzate when incubated with the enzyme extract and three different temperatures (A) and effect of the enzyme concentration in the reduction of phenolic compounds present in the extract (B). In all tests, an enzyme extract containing 8 and 79 AU/mL of MnP and laccase was used.

CONCLUSIONS

1. Alkaline pre-treatment favors lignin solubilization and hydrolysis of the hemicellulose present in the corn leaf. As a consequence, increased presence of xylose was observed, which is indicative of the preferential hydrolysis of the hemicellulose

2. Biological delignification significantly improved acid hydrolysis (9% more) of the cellulose and hemicellulose in the corn leaf.
3. The biological-chemical-biological treatment sequence improved the hydrolysis of cellulose and hemicellulose by 9% compared to the chemical treatment process.
4. The enzymatic extract of *Trametes* sp. 44 is capable of reducing the concentration of phenolic compounds present in the hydrolyzed of corn leaf.

REFERENCES CITED

Agbogbo, F., and Wenger, K. S. (2007). "Production of ethanol from corn leaf hemicellulose hydrolyzate using *Pichis stipis*," *J. Ind. Microbiol. Biotechnol.* 34(11), 723-727.

Aguiar, C.L. (2001). "Biodegradation of the cellulose from sugarcane bagasse by fungal cellulase," *Cienc. Tecnol. Aliment*. 3(2), 117-121

Alvira, P., Thomas-Pejó, E., Ballesteros, M., and Negro, M. J. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review," *Biores. Technol.* 101(13), 4851-4861.

Aziz, A. A., Das, K., Husin, M., and Mokhtar, A. (2002). "Effect of physical and chemical pre-treatments on xylose and glucose production from oil palm press fibre," *J. Oil Palm Res.* 14(2), 10-17.

Ballesteros, I., Negro, M., Oliva, J. M., Cabañas, A., Manzanares, P., and Ballesteros, M. (2006). "Ethanol production from steam-explosion pretreated wheat straw," *Appl. Biochem. Biotechnol.* 130(1-3), 496-508.

Binod, P., Sindhu, R., Singhania, R., Vikram, S., Devi, L., Nagalakshmi, S., Kurien, N., Sukumaran, R. K., and Pandey, A. (2010). "Bioethanol production from rice straw: An overview," *Biores. Technol.* 101(13), 4767-4774.

Biswas, A., Saha, B., Lawton, J., Shogren, R. L., and Willett, J. L. (2005). "Process for obtaining cellulose acetate from agricultural by-products," *Carbohyd. Polym.* 11(2), 1-4.

Chang, V. S., Nagwani, M., and Holtzapple, M. T. (1998). "Lime pretreatment of crop residues bagasse and wheat straw," *Appl. Biochem. Biotechnol.* 74(3), 135-159.

Cheng, Y., Zheng, Y., Wei Yu, C., Dooley, T., Jenkins, B., and VanderGheynst, J. (2010). "Evaluation of high solids alkaline pretreatment of rice straw," *Appl. Biochem. Biotechnol.* 162(6), 1768-1784.

Cruz, R. M. G., Rivera-Ríos, J. M., Téllez-Jurado, A., Maqueda, G. A. P., Mercado, F. Y., and Arana-Cuenca, A. (2012). "Screening for thermotolerant ligninolytic fungi with laccase, lipase and protease activity isolated in Mexico," *J. Environ. Manage.* 95(1), S256-S259.

Del Río, J. C., Speranza, M., Gutíerrez, A., Martínez, M. J., and Martínez, A. T. (2002). "Lignin attack during eucalypt wood decay by selected basidiomycetes: A Py-GC/MS study," *J. Anal. Appl. Pyrol.* 64(2), 421-431.

Dias, M. O. S., Ensinas, A. V., Nebra, S. A., Filho, R. M., Rossell, C. E. V., and Marciel, M. R. W. (2009). "Production of bioethanol and other bio-based materials from sugarcane bagasse: Integration to conventional bioethanol production process," *Chem. Eng. Res. Des.* 87(9), 1206-1216.

Dien, B. S., Jung, H. G., Vogel, K. P., Casler, M. D., Lamb, J. F. S., Iten, L., Mitchell, R. B., and Sarath, G. (2006). "Chemical composition and response to dilute acid pretreatment and enzymatic saccharification of alfalfa, reed canary grass, and switchgrass," *Biomass Bioenergy* 30(10), 880-891.

Du, W., Yu, H., Song, L., Zhang, J., Weng, C., Ma, F., and Zhang, X. (2011). "The promoting effect of bioproducts from *Irpex lacteus* on subsequent enzymatic hydrolysis of bio-pretreated cornstalks," *Biotechnol. Biofuels* 4(37), 3-8.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). "Colorimetric method for determination of sugars and related substances," *Anal. Chem.* 28(3), 350-356.

Esteghlalian, A., Hashimoto, A. G., Fenske, J. J., and Penner, M. H. (1997). "Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn leaf, poplar and switchgrass," *Biores. Technol.* 59(2-3), 129-136.

Gao, Z., Mori, T., and Kondo, R. (2012). "The pretreatment of corn leaf with *Gloeophyllum trabeum* KU-41 for enzymatic hydrolysis," *Biotechnol. Biofuels* 5(28), 1-11.

Garrote, G. (1999). "Hydrothermal processing of lignocellulosic materials," *Holz Roh Werkst.* 57(3), 191-202.

Gould, J. M. (2004). "Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification," *Biotechnol. Bioeng.* 26(1), 46-52.

Hatakka, A. I. (1983). "Pretreatment of wheat straw by white-rot fungi for enzymatic saccharification of cellulose," *Appl. Microbiol. Biotechnol.* 18(6), 350-357.

Herrera, A., Téllez-Luis, S., González-Cabriales, J., Ramírez, J. A., and Vázquez, M. (2004). "Effect of the hydrochloric acid concentration on the hydrolysis of sorghum straw at atmospheric pressure," *J. Food Eng.* 63(1), 103-109.

Howard, R. L., Abotsi, E., Rensburg, J. V., and Howard, S. (2003). "Lignocellulose biotechnology: Issues of bioconversion and enzyme production," *Afr. J. Biotechonol.* 2(12), 602-619.

Ingram, T., Rogalinski, T., Bockemühl, V., Antranikian, G., and Brunner, G. (2009). "Semi-continuous liquid hot water pretreatment of rye straw," *J. Supercrit. Fluids* 48(3), 238-246.

Isroi, R.M., Syamsiah, S., Niklasson, C., Nur, C.M., Lundquist, K., and Taherzadeh, J., (2011). "Biological pretreatment of lignocelluloses with white rot fungi and its applications: A review," *BioResources*. 6(4), 5224-5259.

Kamei, I., Hirota, Y., and Meguro, S. (2012). "Integrated delignification and simultaneous saccharification and fermentation of hard wood by a white-rot fungus, *Phlebia* sp. MG-60," *Biores. Technol.* 126, 137-141.

Kassim, E.A., and El-Shahed, A.S. (1986). "Enzymatic and chemical hydrolysis of certain cellulosic materials" *Agr. Wastes*. 17, 229-233.

Keller, F., Hamilton, J., and Nguyen, Q. (2003). "Microbial pretreatment of biomass," *Appl. Biochem. Biotechnol*. 105-108, 27-41

Kirk, T. K., Croan, S., Tien, M., Murtagh, K. E., and Farrell, R. L. (1986). "Production of multiple ligninases by *Phanerochaete chrysosporium:* Effect of selected growth conditions and use of a mutant strain," *Enzyme Microb. Technol.* 8(4), 27-32.

Kumar, P., Barrett, D. M., Delwiche, M. J., and Stroeve, P. (2009). "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Ind. Eng. Chem. Res.* 48(8), 3713-3729.

Lenihan, P., Orozco, A., O´Neil, E., Ahmad, M. N. M., Rooney, D. W., and Walker, G. M. (2010). "Dilute acid hydrolysis of lignocellulosic biomass," *Chem. Eng. J.* 156(2), 395-403.

López, F., Alfaro, A., Caparros, S., García, M. M., Pérez, A., and Garrote, G. (2008). "Aprovechamiento energético e integrado por fraccionamiento de biomasa lignocelulósica forestal y agroindustrial. Caracterización de hemicelulosas, celulosas y otros productos del fraccionamiento," *Bol. Inf. CIDEU* 5, 7-19.

López-Miranda, J., Soto, N. O., Rutiaga, O. M., Medrano, H., and Arévalo, K. (2009). "Optimización del proceso de obtención enzimática de azúcares fermentables a partir de aserrín de pino," *Rev. Int. Contam. Ambient.* 25(2), 95-102.

Lu, X., Zhang, Y., and Angelidaki, I. (2009). "Optimization of H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content," *Biores. Technol.* 100(12), 3048-3053.

Manjarres, K., Piñeros, Y., and Rodríguez-Sandoval, E. (2011). "Evaluación del complejo enzimático producido mediante el co-cultivo de *Aspergillus* sp. y *Trichoderma* sp. en fase sólida sobre residuos de palma," *Biagro* 23(1), 19-26.

Martínez, E., Villareal, M., Almeida, J. B., Solenzal, A., Canilha, L., and Mussatto, I. (2002). "Uso de diferentes materias primas para la producción biotecnológica de xilitol," *Cienc. Tecnol. Aliment.* 3(5), 295-301.

Mendoça, R., Guerra, A., and Ferraz, A. 2002. "Delignification of *Pinus taeda* wood chips treated with *Ceriporiopsis subvermispora* for preparing high-yield kraft pulps," *J. Chemical Technol. Biotechnol.* 77(4), 411-418.

Mendoza, E. M., Andrio, E. E., Juárez, G. J. M., Mosqueda, V. C., Latournerie, M. L., Castañón, N. G., López, B. A., and Moreno, M. E. (2006). "Contenido de lisina y triptofano en genotipos de maíz de alta calidad proteica y normal," *Universidad y Ciencia* 22(2), 153-161.

Miller, G. L., Blum, R., Glannon, W. E., and Burton, A. L. (1960). "Measurement of carboxymethylcellulase activity," *Anal. Biochem.* 2(1), 127-132.

Mojovic, L., Nikolic, S., Rakin, M., and Vukasinovic, M. (2006). "Production of bioethanol from corn meal hydrolyzates," *Fuel* 85(12-13), 1750-1755.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., and Ladisch, M. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass," *Biores. Technol.* 96(6), 673-686.

Mukherjee, R., and Nandi, B. (2004). "Improvement of in vitro digestibility through biological treatment of water hyacinth biomass by two *Pleurotus* species," *Int. Biodeterior. Biodegrad.* 53(1), 7-12.

Müller, H. W., and Trösch, W. (1986). "Screening of white-rot fungi for biological pretreatment of wheat straw for biogas production," *Appl. Microbiol. Biotechnol.* 24(2), 180-185.

Okano, K., Kitagawa, M., Sasaki, Y., and Watanabe, T. (2005). "Conversion of Japanese red cedar (*Cryptomeria japonica*) into a feed for ruminants by white-rot basidiomycetes," *Anim. Feed Sci. Technol.* 120(3-4), 235-243.

Oleskowicz-Popiel, P., Lisiecki, P., Holm-Nielsen, J. B., Mette, A. B., and Thomsen, H. (2008). "Ethanol production from maize silage as lignocellulosic biomass in anaerobically digested and wet-oxidized manure," *Biores. Technol.* 99(13), 5327-5334.

Patel, S. J., Onkarappa, R., and Shobha, K. (2006). "Study of ethanol production from fungal pretreated wheat and rice straw," *Inter. J. Microbiol.* 4(2).

Pedersen, M., and Meyer, A. (2010). "Lignocellulose pretreatment severity relating pH to biomatrix opening," *New Biotechnol.* 27(6), 739-750.

Reid, I. D. (1985). "Biological delignification of aspen wood by solid-state fermentation with the white-rot fungus *Merulius tremellosus*," *Appl. Environ. Microbiol.* 50(1), 133-139.

Quintanar, G. S., Arana-Cuenca, A., Mercado, F. Y., Gracida, R. J. J., and Téllez-Jurado, A. (2012). "Effect of particle and aeration on the biological delignification of corn straw using *Trametes* sp. 44," *BioResources* 7(1), 327-344.

Salaber, J., and Maza, F. (1972). "Bagasse black liquor recovery," *Filtr.* 9, 55-77.

Sidiras, D., and Koukios, E. (2004). "Simulation of acid-catalysed organosolv fractionation of wheat straw," *Biores. Technol* .94(1), 91-98.

Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D., and Osborne, J. A. (2007). "Comparison of chemical pretreatment methods for improving saccharification of cotton stalks," *Bioresource Technol.* 98, 3000-3011.

Singleton, V. L., Orthofer, R., and Lamuela-Raventos, R. M. (1998). "Analysis of total phenols and other oxidation substrates and antioxidiants by means of Folin-Ciocalteu reagent," *Method Enzymol.* 299, 152-178.

T222 om-88 (1988). "Acid-insoluble lignin in wood and pulp," Approved by the Chemical Properties Committee of the Process and Product Quality Division, TAPPI, p. Al.

Taherzadeh, M. J., and Karimi, K. (2008). "Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A Review," *Int. J. Mol. Sci.* 9, 1621-1651.

Talebnia, F., Karakashev, D., and Angelidaki, I. (2009). "Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation," *Biores. Technol.* 101(13), 4744-4753.

Vázquez, M., Oliva, M., Téllez-Luis, S., and Ramírez, J. A. (2006). "Hydrolysis of sorghum straw using phosphoric acid: Evaluation of furfural production," *Biores. Technol.* 98 (16), 3053-3060.

Viñals, V., Maciel, I., Batista de Almeida, J., and Nápoles, A. I. (2006). "Métodos de purificación de hidrolizados de bagazo de caña de azúcar para la obtención de xilitol," *Cienc Tecnol. Aliment.* 5(2), 129-134.

Wan, C., and Li, Y. (2012). "Fungal pretreatment of lignocellulosic biomass," *Biotechnol. Adv.* 30(6), 1447-1457.

Yang, B. Y., and Montgomery, R. (2007). "Alkaline degradation of invert sugar from molasses," *Biores. Technol.* 98(16), 3084-3089.

Yu, H., Zhang, X., Song, L., Ke, J., Xu, C., Du, W., and Zhang, J. (2010). " Evaluation of white rot fungi assisted alkaline/oxidative pretreatment of corn straw undergoing enzymatic hydrolysis by cellulase," *J. Biosci. Bioeng*. 110, 660-664.

Zadrazil, F., and Puniya, A.K. (1994). "Influence of carbon dioxide on lignin degradation and digestibility of lignocellulosics treated with *Pleurotus sajor-caju*," *Int. Biodeterior. Biodegrad*. 33, 237-244.

Zhao, J., and Xia, L. (2009). "Simultaneous saccharification and fermentation of alkaline-pretreated corn leaf to ethanol using a recombinant yeast strain," *Fuel Process. Technol.* 90(1193-1197), 1193-1197.

Zhao, X., Peng, F., Cheng, K., and Liu, D. (2009). "Enhancement of the enzymatic digestibility of sugarcane by alkali-peracetic acid pretreatment," *Enzyme Microb. Technol.* 44(1), 17-23.

Zhao, Y., Wang, Y., Zhu, J. Y., Ragauskas, A., and Deng, Y. (2007). "Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature," *Biotechnol. Bioeng.* 99(6), 1320-1328.

Zhao, X., Zhang, L., and Liu, D. (2007). "Comparative study on chemical pretreatment methods for improving enzymatic digestibility of crofton weed stem," *Biores. Technol.* 99, 3729-3736.

Article submitted: December 25, 2013; Peer review completed: April 22, 2014; Revised version received: August 26, 2014; Further revisions: September 21, 2014; Accepted: September 22, 2014; Published: September 29, 2014.