Fermentable Sugars from *Lupinus rotundiflorus* by Cumulative Pretreatments Using UV Light, Freezing, and Boiling in Alkaline Medium, Followed by Enzymatic Hydrolysis

J. Jesús Vargas-Radillo,[a] Mario A. Ruiz-López,[b] Ramón Rodríguez-Macías,[b] Lucía Barrientos-Ramírez,[a] Ricardo Manríquez-González,[a] Fernando Navarro-Arzate,[a] Eduardo Salcedo-Pérez,[a] and Fernando A. López-Dellamary Toral [a,*]

A pretreatment in tandem composed of sunlight or sun-like UV-irradiation, freezing-thawing, soda swelling, and boiling (never drying between treatments), was applied to a slurry of ground-up *Lupinus rotundiflorus,* followed by enzymatic hydrolysis. The effects were studied through an experimental design in which the factors were employed cumulatively to statistically evaluate the effect of each factor on enzymatic saccharification. Results showed that swelling and physical disarrangement of the lignocellulosic complex probably occurred with little or no delignification and soda consumption. The disarrangement of the cell wall and tissue structures generated by the combined effects of UV-light, freezing-thawing, soda swelling, and boiling contributed to a yield of up to 67.0% of fermentable sugars with respect to hydrolyzed material (82.8% of theoretical fermentable sugars). This yield was comparable to that obtained in control samples using Whatman No.1 paper, which produces a very high yield of fermentable sugars after hydrolysis. Finally, the acceptable overall results showed that improved saccharification of lignocellulosic materials by means of natural agents is feasible.

Keywords: Lignocelluloses; Saccharification; Hydrolysis; UV light; Sunlight; Freezing; Soda; Swelling

Contact information: a: Departamento de Madera, Celulosa y Papel, Universidad de Guadalajara, Km 15.5, Carretera Guadalajara-Nogales, Las Agujas, Nextipac, Zapopan, Jalisco, México, código postal 45220; b: Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Km 15.5, Carretera Guadalajara-Nogales, Las Agujas, Nextipac, Zapopan, Jalisco, México, código postal 45220; **Corresponding author*: *fernando.dellamary@cucei.udg.mx*

INTRODUCTION

Lignocellulosic biomass is a complex microstructured material, composed of varying proportions of lignin and hemicelluloses, which form an encapsulating matrix enclosing highly crystalline cellulose fibrils that are packed into bundles (Fengel and Wegener 1984) with scarce pore volume (Stocker 2008). This makes biomass sources difficult to deconstruct. Chemical (alkali, acid, *etc.*), physical (milling, high energy radiation, *etc.*), or biological (fungi or bacteria) pretreatments are some of the processes used to disrupt the lignin-hemicelluloses-cellulose interaction (Ishizawa *et al.* 2007), and to make it more accessible for enzymatic hydrolysis. Recent literature describes almost exclusively chemical and thermochemical processes, including dilute acid, steam explosion, organosolv and sulfite pretreatments (SPORL), ammonia-fiber expansion (AFEX), and ammonia-recycle percolation (ARP), to overcome recalcitrance of

lignocellulose (Zhu and Pan 2010). Some methods have the advantage of using relatively low temperatures, and the application of lime (LTA) (Wu *et al.* 2011), or concentrated phosphoric acid and ethanol (COSLIF) (Pedersen *et al.* 2011). Among physical pretreatments, microwave in combination with dilute acid and ultrasound (Alvira *et al.* 2010), electron beam irradiation (Sarkar *et al.* 2012), gamma-irradiation, steam explosion and hot water, have been found the most successful (Sharma *et al.* 2012). However, to date, there is not a final veredict on which pretreatments for lignocellulosic biomass are best for its conversion into fuels (Zhu *et al.* 2009).

Of special interest is the use of ultraviolet sun-like radiations, alone or combined with other pretreatments (Mamar and Hadjadj 1990). The use of UV energy is a process in which chemical compounds absorb photons from UV radiation leading to chemical transformations through cleavage of bonds and formation of free radicals, such as hydroxyl •OH (Laoufi *et al.* 2008), that are able to degrade lignin (Hwang and Lucia 2004). UV light has been applied together with other factors such as soda or lime at high concentrations (6 to 20%) and low pressures and temperatures (Mirhamadi *et al.* 2010), causing material swelling, as well as some lignin degradation. Photosensitizers (rose bengal, methylene blue, *etc*.) have also been added for singlet oxygen generation to induce lignin breakdown (Ruggiero *et al.* 1994). Hwang and Lucia (2004) applied a photochemical delignification treatment to kraft softwood pulp, at 10% sodium hydroxide (w/w), 0.5 % (w/w) rose bengal, and UV light irradiation (300 nm, 400 W) for a period of over five hours (and up to 22 h, to maximize the effects) at 45 °C. They found that each of the variables applied successively in cumulative form increased the delignification level to a maximum of 90% of lignin removal.

However, in general, the pretreatment of lignocellulosic materials with artificial light alone (even when in excess, or when high-energy gamma rays are used) has been found to be ineffective, commonly slow, energy-intensive, and perhaps prohibitively expensive (Zheng *et al.* 2009). Therefore, other less expensive and more efficient systems are being sought. In this respect, sunlight provides the energy for most living processes and for biomass build-up; sunlight also causes the degradation of many compounds and materials when exposed to the outdoors. As a matter of fact, it is the main source of inexpensive and almost unlimited energy at our disposal. Solar radiation mainly includes visible (55.4% of sunlight), infrared (37.8%), and ultraviolet light (6.8%). Ultraviolet radiation (UVR) is the invisible sun energy in the wavelength range from 100 to 400 nm, of which portions are divided into UV-A (320-400 nm) and UV-B (280-320 nm). The latter has the right wavelength that can affect the cell wall lignocellulosic complex, although only 5% reaches the surface of the planet. UV-C radiation (100-280 nm) never reaches the earth. An energy balance on a sunny day determines that exposed materials would receive on average 1.5 to 3.0 W/m^2 of UV-B light. Ball (1995) reports a value of 2.5 W/m^2 of UV-B irradiance near the equator under clear, sunny skies. This energy could be used for cell wall deconstruction.

Water is an important factor affecting some fundamental chemical and mechanical properties of cellulosic fibers. For example, drying of fibers can result in irreversible pore collapse, capillary shrinking, and increased hydrogen bonding, thus resulting in reduced accesibility and chemical reactivity (Taherzadeh and Karimi 2008). On the other hand, it is well known that swelling with water increases the accessible area of cellulose to chemical agents (Taherzadeh and Karimi 2008). There are different states of water in the cell wall matrix with different physic-chemical characteristics. Below the fiber saturation point (25 to 30% moisture), primary bound water, or non-freezing water, is

found at the cellulose microfibril level, whereas secondary bound water, or freezing water, is located within the porous structure (Matthews *et al.* 2006; Hartley *et al.* 1992). Above of the fiber saturation point (60 to 70% moisture content), bulk water fills the cell lumens. Although this bulk is called free water, it is confined within the pores and/or interacting with fibril surfaces, thus possessing a depressed freezing point (Felby *et al.* 2008). Consequently, the manipulation of water by such methods as cavitation, freezing and thawing, and/or heating at moderate temperatures would affect the secondary bound water and the bulk water, hence improving cell wall disruption (Dohanyos *et al.* 1997).

Pekarovičová *et al.* (1991) reported the prehydrolytic pretreatment of cellulose (Whatman No.1) before enzymatic hydrolysis by swelling in aqueous NaOH (0.17 to 2.43 M) solutions and freezing at -10 to -50 °C. The authors found changes in cell wall morphology; such modifications are associated with primary layer dissolution as well as the formation of spiral-like cracks in the secondary cell wall S1 and S2 layers.

Therefore, it follows that water can greatly affect how lignocellulosic materials behave, as water is intimately related to them. Keeping the material always wet during treatments (never drying) and the freezing and thawing of water within the lignoellulosic structure, should produce advantageous physicochemical changes. Mechanical disruption would also occur, since water increases its volume as it freezes and forms sharp crystals.

Lupinus rotundiflorus, a leguminous herbaceous plant, which was used as the biomass in this study, is one of 80 endemic species of *Lupinus* that grow wild in Mexico (McVaugh 1987; Rzedowski and Rzedowski 2001). As it is with some other lupines elsewhere (*e.g.* Australia, Iceland, Poland, Spain, *etc.*), it might be feasible to establish it as a crop not only used mainly for animal feed, but for other alternative industrial uses as well, such as the isolation of alkaloids, proteins, lignin, and of course, carbohydrates as an energy source (Piotrowicz-Cieślak *et al.* 2003; Sánchez 2009).

The central goal of this research was to evaluate the effect of some natural or green pretreatments, applied in tandem with others that are well known and simple, on the yield of fermentable sugars after the enzymatic hydrolysis of a lignocellulosic material represented by ground *L. rotundiflorus* biomass.

EXPERIMENTAL

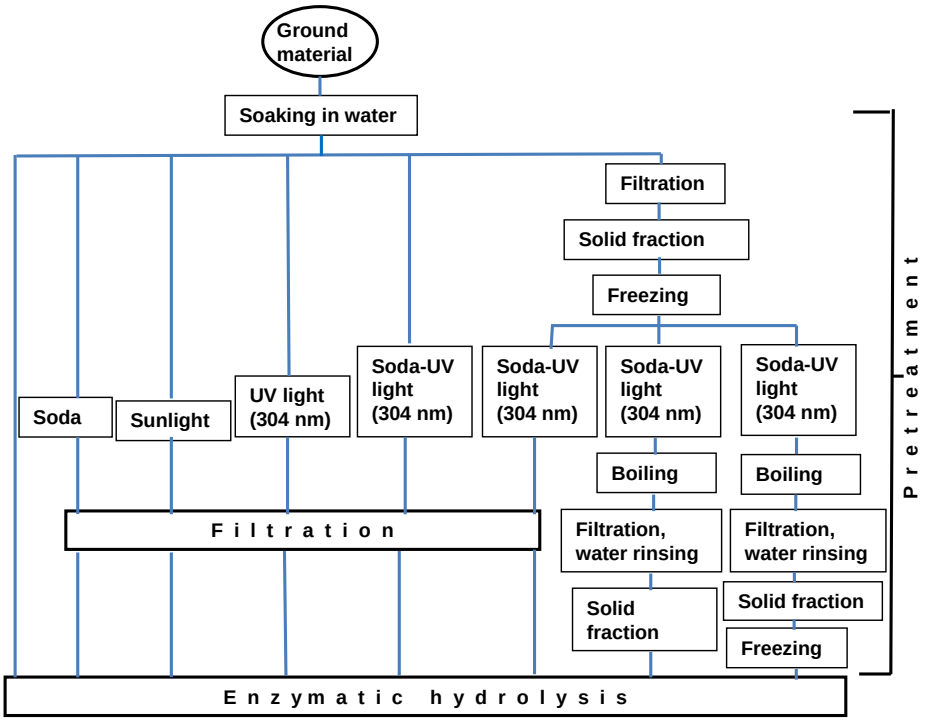
The schematic experimental procedure employed for the pretreatment and the saccharification is provided in Fig. 1.

Material

L. rotundiflorus, an annual herbaceous plant, was collected at 2320 m above sea level, near Atemajac de Brizuela, which is located 105 Km SW of Guadalajara, Mexico. Plant material (except foliage, which has lower carbohydrate and higher protein contents) was air-dried and ground with a Wiley mini- mill to a ≤0.4 mm particle size.

Pretreatments

Various pretreatments (in duplicate per sample),were applied either alone or in different sequences with cumulative effects (see Figs. 1 and 2). Experiments 1 through 3 were carried out with just one factor, whereas experiments 4 through 7 in Fig. 2 were carried out with two or more factors.



Pretreatment times: NaOH 30h, under artificial UV 30 h; sunlight 90 h; freezing 15 h; boiling 1 h; soaking in water 24 h
Experimental conditions: NaOH 15% (w/w), consistency 1%; T under artificial UV 45 °C; λ=304nm

Fig. 1. Schematic presentation of the individual and tandem pretreatment procedures followed by saccharification of *L. rotundiflorus* biomass

The different pretreatments were applied to a suspension of lignocellulosic material in water (2 L at 1% consistency) as follows: a) 15% (w/w) sodium hydroxide (S in Fig. 2) was added to reach pH 11-12, for 30 h; b) freezing experiments (F) were carried out in sealed polyethylene bags for 15 h at -14 ºC with samples which were previously immersed in water for 24 h, rinsed, and then filtrated (without squeezing); c) boiling (B) was performed for 1 h; d) UV irradiation was applied using a Rayonet carousel photochemical reactor (Southern New England Ultraviolet Co., Branford, CT) fitted with 16 UV lamps emitting a spectrum band centered at 304 nm with 21 watts at a flux of 10.01 W/m^2 for up to 30 h of to simulate long exposures to sunlight. The temperature reached 45 ºC by the heat generated from the UV treatment. It is known that a Rayonet reactor can provide a UV fraction simulating that of sunlight. The intensity of UV light from the Rayonet source at 304 nm is considered to be three times the intensity of UV-B sunlight and treatment times were proportional to the intensity of each source (sunlight or lamps). Therefore it would be expected that similar effects are produced from either energy sources. Hence, the effect of sunlight on biomass could be feasibly imitated in the laboratory spending less time, under controlled conditions. Reference samples were treated under sunlight during March and April from 9:00 to 17:00 h in shallow trays with mechanical agitation for 90 h and 1% of consistency (labeled as Sun 90 h in Figs. 2 and 3). This time of the year was chosen since it provides cloudless skies and very strong

sunlight in Guadalajara. Initial pH, final pH, Klason lignin, biomass yield, and reagent consumption were determined as control parameters.

Enzymatic Hydrolysis

Hydrolysis experiments, performed in duplicate, were carried out in stoppered 100 mL Erlenmeyer flasks in a thermostated shaker at 50 ± 3 °C and 140 rpm. Wet pretreated samples equivalent to 1 g of dry matter were placed in 50 mL of citric acid/sodium citrate buffer at pH 4.8 (2%, w/v) containing 100 μg/mL tetracycline hydrochloride and 100 μg/mL chloramphenicol (antibiotics used to avoid microbial contamination).

An enzyme mixture was prepared from commercial enzymes, consisting of: cellulase from *Trichoderma reesei* (Celluclast 1.5 L, Sigma Co., St. Louis, MO, 66 FPU/mL); cellobiase from *Aspergillus niger* (Novozyme 188, Sigma, 400 β-glucosidase CBU/mL); and xylanase from *Trichoderma longibrachiatum* (Sigma, 1 β-xylanase U/mg). Enzymatic hydrolysis was carried out at cellulase activities of 11 FPU/g dry basis (db), 20 CBU/mL cellobiase, and 2.5% (w/w) of xylanase. Thus, each flask contained 1.0 gdb pretreated material, 0.27 mL of Celluclast, 0.05 mL of Novozym 188, 0.025 g of xylanase, and the antibiotics as described previously.

Experiments were conducted at different times (6, 12, 24, or 48 h). At the end of each hydrolysis period, samples were chilled on ice to stop the hydrolysis, filtered, and the filtrate assayed for fermentable sugars. Saccharification, which represents hydrolysis efficiency (for all fermentable sugars), was calculated using Equation 1,

$$Saccharification = \frac{[Reducing \quad sugars](V)(d)100}{m(1.1)}, \quad \% \qquad (1)$$

where [*Reducing sugars*] is the final fermentable sugars concentration in mg/mL, *V* is the volume of the hydrolyzate (mL), *d* is dilution ratio of the hydrolyzate, *m* is the weight of initial biomass (oven dry basis), 1.1 is the glucose/anhydroglucose mass ratio (180/162), and % represents the sugar yield in respect to pretreated raw material.

Also included in the hydrolyzed samples were: a) Whatman No. 1 filter paper (highly pure cellulose) to measure the efficiency of the enzyme blend; b) raw material without any pretreatment as reference sample, and c) samples treated with sunlight as previously described.

Analytical Methods

Sodium hydroxide consumption was evaluated by titration with standard hydrochloric acid solution.

Cellulase and cellobiase activities were measured with the Filter Paper Unit method (FPU) and Cellobiase Unit method (CBU) according to the International Union of Pure and Applied Chemistry (IUPAC) procedure (Ghose 1987).

Total reducing sugars in the biomass enzymatic hydrolysate was done by the DNS method (Miller 1959), using an UV-Vis spectrophotometer. To calculate the concentration of reducing sugars, a calibration curve was constructed using 10 different concentrations of D-glucose (0.2-2.0 mg/mL, $R^2 = 0.997$).

Holocellulose (Wise *et al.* 1946), Klason lignin (TAPPI Test Method T 222), and extractives (TAPPI Test Method 264) were determined as described in the references (TAPPI 2000).

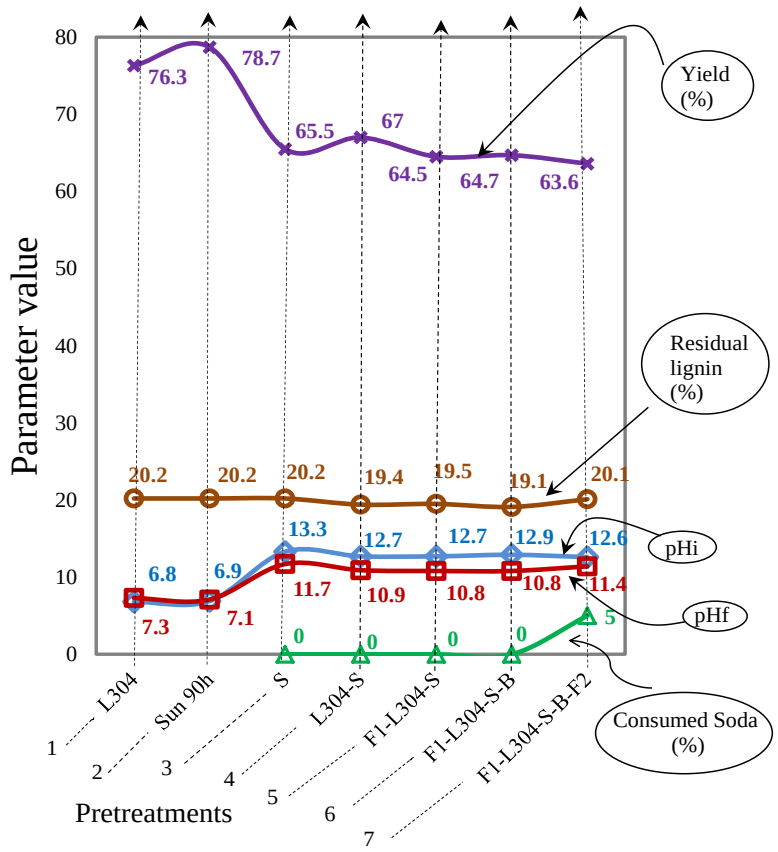
RESULTS AND DISCUSSION

Material

The main components of the cell wall of *L. rotundiflorus* were 62.6% holocellulose, 15.8% lignin (20.4% lignin after removing extractives), and 22.6% extractives.

Pretreatments

The results of the seven pretreatments are shown in Fig. 2.



L304 = Light, 304 nm; S= Soda; L304-S = Light-Soda; F1-L304-S = Frozen-Light-Soda; F1-L304-S-B=Frozen-Light-Soda-Boiling; F1-L304-S-B-F2= Frozen-Light-Soda-Boiling-Frozen. Consistency:1%;Treatment Time: 30 h; Soda Charge: 15%; Frozen Time 15 h.

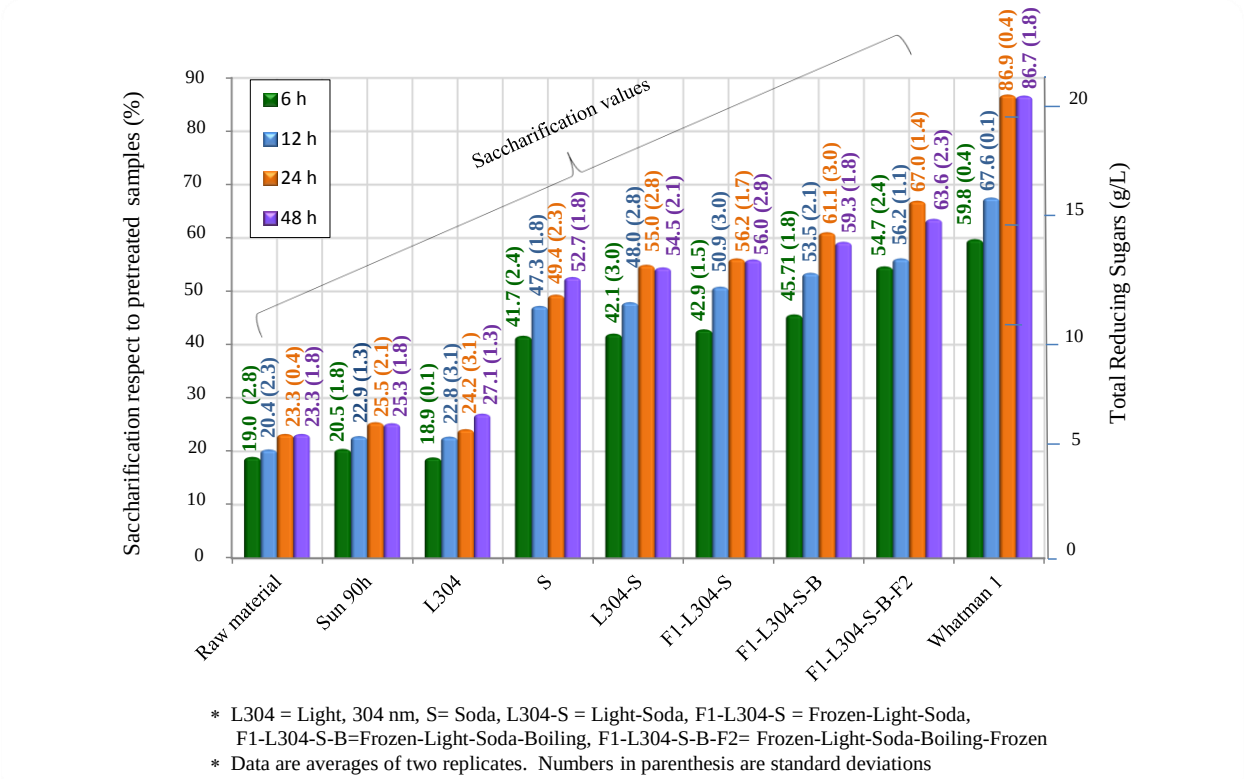
Fig. 2. Results of the individual and cumulative pretreatments on *L. rotundiflorus* samples

The final pH (pH_f) of the slurries after alkaline pretreatment was in the range 10.8 to 11.7, noting that it was somewhat lower compared to the initial pH (pH_i) of the samples (12.6 to 13.6). This reduction probably was not caused by soda consumption by delignification, but caused by the extraction of hemicelluloses. Only treatment 7 showed slight soda consumption. This was probably due to more hemicellulose extraction, but not due to delignification of the material. Yields were markedly lower, at around 65.0%, after treatments involving soda, due to hemicelluloses and extractives released from the biomass. Lignin content (residual lignin in Fig. 2) remained relatively constant and similar to the initial lignin value of the raw material (20.4%). Qualitatively, swelling was

observed as the main pretreatment effect, in contrast to almost nil delignification. Moreover, the yield was 76.3% after UV pretreatment without alkali (L304).

Enzymatic Hydrolysis

The effect of the seven pretreatments on enzymatic hydrolysis (saccharification) is shown in Fig. 3.



∗ L304 = Light, 304 nm, S= Soda, L304-S = Light-Soda, F1-L304-S = Frozen-Light-Soda, F1-L304-S-B=Frozen-Light-Soda-Boiling, F1-L304-S-B-F2= Frozen-Light-Soda-Boiling-Frozen

∗ Data are averages of two replicates. Numbers in parenthesis are standard deviations

Fig. 3. Fermentable sugar yield of *L. rotundiflorus* hydrolysates obtained with different pretreatments, after distinct elapsed hydrolysis times. Total reducing sugars (g/L) concentration is read on the right axis, whereas saccharification (%) with respect to pretreated raw material is read on the left axis.

As it can be seen in Fig. 3, the increase in fermentable sugars yield depended upon each one of the variables applied successively, in addition to the hydrolysis time. High initial rates of hydrolysis and acceptable reaction times (24 h) were observed, which is a sign that the crystallinity was lowered (Zhu 2005). The yield reached a maximum after 24 h and did not increase significantly thereafter in most cases, especially since the enzyme activity decreased. The cause of this decay could be explained by the inhibition of exoglucanase *via* strong binding of its catalytic domain to the cellulosic fibers (Xiao *et al.* 2004), along with increased sugar concentrations (Hodge *et al.* 2008). The reference sample (Raw material, Fig. 3) exhibited a low saccharification value of 23.3%, whereas samples pretreated either with UV light alone (L304) or sunlight (Sun 90 h) produced around 25%. Soda (S) was the highest individual factor that affected the yield of fermentable sugars, with 49.4% after 24 h of hydrolysis (this value was twice as much saccharification as the raw material). This individual pretreatment not only disrupted the cell wall by swelling, dissolving hemicelluloses and decreasing cellulose crystallinity (Xiao *et al.* 2001) as is well known, but also disrupted the intermolecular bonding

between the xylans with lignin and the xylans with other hemicellulosic components. Altogether, the total porosity was probably increased, thereby extending the internal surface of the lignocellulosic matrix, rendering it more accessible to the enzymes. In addition, it would be feasible to reuse the NaOH solution in this process, since it is barely consumed, thus its economy and environmental impact would not be so critical (Mirhamadi *et al.* 2010).

However, the main interest focused on finding the individual contributions of UV light at 304 nm (L304), pre-freezing (F1), boiling (B), and post-freezing (F2) on the overall pretreatment (*e.g.* B effect in multiple treatments F1-L304-S-B-F2). ANOVA analysis showed that L304, F2, and B factors were statistically significant at the 95% level, F2 (p = 0.000) > B (p = 0.0028) > L304 (p = 0.0200), with the p -value less than 0.05. The F1 (p = 0.0704) was not statistically significant at the level examined. Figure 3 depicts the contribution of every treatment to sacharification. It may be observed that the only treatment that did not produce an accumulated effect was an initial freezing. For example after 24 h of hydrolysis, the UV-light followed by soda treatment (L304-S), hydrolysis increased 5.6% in comparison to only soda (S). Evidently, initial freezing (F1-L304-S) was ineffective, showing only a 1.2% increment. When boiling was added (F1-L304-S-B), a 4.9% gain was obtained. Remarkably, final freezing (F1-L304-S-B-F2) produced 5.9% more sugars. It is well known that heating improves the effect of swelling of the cell wall caused by soda treatment (Taherzadeh and Karimi 2008), while F2 could fragment the cell wall by mechanical disruption of fiber cells due to swelling and tearing by ice formed from secondary water and bulk water. This should result in enlarged porosity and decreased overall crystallinity in the cellulosic component, thus allowing an improved access to the remaining crystalline region in cellulose.

The F2 effect was notably larger than F1, making the expansion and disruption produced by ice at the end more effective. This may be explained by the previous cumulative disruption of the cell wall**.** Although pre-freezing might be obviated, the mechanical disruption it causes in the cell wall and tissue organization should prepare the lignocellulosic material for further processing. UV light alone had no significant consequence, but combined with the other factors, a synergistic effect was achieved (67.0% saccharification at 24 h of hydrolysis, an 82.8 % sugars yield in respect to the pretreated raw material).

This was a remarkable achievement, since the results were similar to the hydrolysis of Whatman No. 1 paper (86.9%), which is an almost pure hydrolysable carbohydrates source. This paper, very high in cellulose content, is considered the standard to which other materials should be compared. It is surprising that the accumulated treatment achieves hydrolysis yields comparable to those of almost pure cellulose by significantly disrupting the cell wall of the lignocellulosic material, with almost no lignin removal. A fact that influenced this excellent yield is that the pretreated sample was never dried prior to the hydrolysis step. Water that is in between fibers and individual fibrils and bundles has a positive effect on the enzymatic digestibility, keeping an open structure that is more amenable to hydrolyitic activity (Taherzadeh and Karimi 2008). A mass balance (pretreatment/enzymatic hydrolysis) was calculated for treatment F1-L304-S-B-F2 after 24 h of hydrolysis. Total sugars yield was 56.7 g (41.9 g sugars after hydrolysis step plus 14.8 g sugars, mostly hemicelluloses, extracted in the pretreatment steps); 20.7 g of non-hydrolyzable material (15.8 g of lignin plus 4.9 g of recalcitrant polysaccharides), and 22.6 g of extractives, per 100 g of original raw biomass (oven dry basis).

On the other hand, the effect of pretreatment with sunlight was similar to that obtained in the photochemical reactor with artificial UV light at 304 nm (Sun 90 h *vs.* raw material in Fig. 3). Sunlight UV is capable of initiating photochemical changes (ASTM-USDA 2000); however, although sunlight (Specially its UV-B) has the power (73-97 kcal/mole) to cleave some carbon-oxygen bonds found in lignin (dissociation energy is about 89 Kcal/mol according to Williams 2005), it does not have enough energy to break most of the other covalent bonds found in lignin. So, sunlight or artificial UV-B alone does not have enough energy to significantly degrade the lignocellulosic complex, and in this way, increase the hydrolysis rate significantly. Notwithstanding, it is worthy to speculate that photons inflicted enough derangement so as to significantly increase the effect of subsequent treatments.

To qualitatively evaluate the effect of the treatments on the morphology of the lignocellulosic material, two representative samples were chosen, one with a very mild treatment and the second one after a full sequence of treatments. In Fig. 4, microphotographs (optical microscope with polarized light, Axioskop 40 model Zei 55) of the L304 sample (mild treatment) and the F1-L304-S-B-F2 sample (fully treated) are compared.

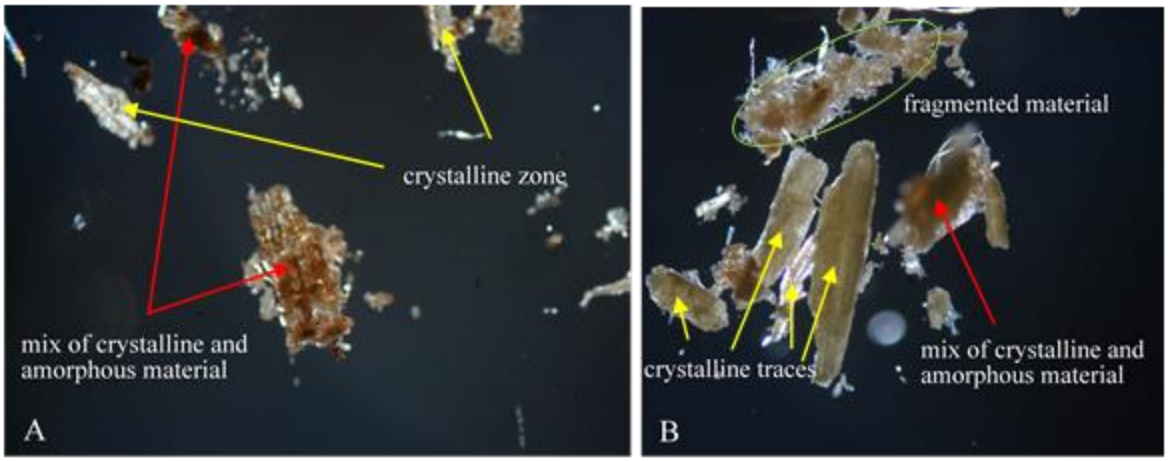


Fig. 4. Microphotographs under polarized light of *L. rotundiflorus* samples, 50X. A) L304 pretreatment and B) F1-L304-S-B-F2 pretreatment

Under polarized light, the amorphous material was appreciably opaque, more homogeneous and light-brown, while the more crystalline material was seen bright-white or bright-reddish-brown with white speckles and with more texture and even well-defined. The L304 microphotograph (A) showed a more crystalline heterogeneous material, while the F1-L304-S-B-F2 sample (B) showed a less crystalline material with more abundant, homogeneous, and continuous amorphous regions. This was physical evidence of the profound disarrangement of this lignocellulosic substrate after the pretreatment sequence, which was not so evident in the chemical analysis (lignin content, for example), but is evident in the accessability to the hydrolytic enzymes.

Similar results have been previously described. Mirahmadi *et al.* (2010) used ground spruce and birch for bioethanol and biogas production through mild alkaline pretreatment (7% soda, -15 °C to 100 °C, 2h) followed by enzymatic hydrolysis. They found that although no significant change in lignin content was observed (27% lignin in

original wood, compared to 25.6% residual lignin in pretreated birch at 50 °C), the pretreatment improved the yield of bioethanol. This enhancement was attributed both to the considerable decrement in hemicellulose content (28% in the original to less than 17.5%, in pretreated birch), and the reduction in the crystallinity of cellulose. In a more related study (Zhao *et al.* 2008), it is reported that the success of the pretreatment of spruce wood to improve enzymatic hydrolysis appears to result predominantly from the looser structure and smaller wood bundles that allow penetration of cellulolytic enzymes. Another reason given by the authors was the possible cleavage of lignin–carbohydrate bonds and increased pore volume. Both of these papers are in general agreement with our results, although the experiments were not precisely the same. They do not include UV-light (sun-like) treatment, and in the freezing experiment performed by Zhao *et al.*, urea in high concentration was used. The explanation and interpretation by both groups of the improved enzymatic hydrolysis without significant delignification is the same as ours: The improved accessibility to enzymatic activity produced by the disruption and deconstruction of the cell wall structure. Moreover, Zhao *et al.*, suggests as we do, in our conclusion, that it would be possible to take advantage of subfreezing temperatures in cold winter climates by leaving samples outdoors overnight during winter, as they did, in the Madison, WI area.

CONCLUSIONS

1. As it is well known and as expected, NaOH solution at moderate temperature (45 °C) was the most effective individual pretreatment; there was a 26.1% increased yield of fermentable sugars in comparison with that obtained from the raw material without any pretreatment after 24 h of enzymatic hydrolysis. However, the additional steps, in order of decreasing statistical significance: Post-freezing (F2), boiling (B), UV light (L304), and pre-freezing (F1), improved the yield another 17.6% with respect to the moderate alkaline treatment. This cumulative pretreatment (F1-L304-S-B-F2) produced the best yield of 41.9% of fermentable sugars after the enzymatic hydrolysis, with respect to the untreated raw material (67.0 % with respect to pretreated material).
2. Water plays an important role in the structure of lignocellulosic fibers. Therefore, water in combination with other factors such as UV light, alkaline media, and boiling may be utilized to alter the inaccessible barrier of lignocellulosics. Boiling should increase the disorganization of the already disrupted structures. Freezing and thawing of the water inside the fibers should pave the way for enzymes to degrade cellulose. After melting, the water presumably formed swelled hydrated entities at the molecular level with the disrupted lignocellulosic complex, keeping a more open organization, thus facilitating enzymatic digestion. This would be a reason to maintain the material always never-dried between treatments.
3. Additionally, UV light, resembling the light that radiates from the sun, was shown to be capable of modifying the cell wall to a sufficient extent, synergistically with other vectors, so that it produces an easy-to-hydrolyze material. The use of sodium hydroxide, key to the process, should be of little concern, since very small quantities are consumed and it might be recycled multiple times. All these factors would contribute to the economy of the process.

4. These treatments, as part of the full sequence, might be highly beneficial in geographical regions with winters with subfreezing temperatures periods for the freezing steps, and enough sunny skies during other seasons for UV-light (sun-light) steps.
5. Aqueous treatments such as freezing and never-drying, together with sunlight (UV light) and natural and abundant green chemistry resources, could be used to boost the deconstruction of the cell wall, thus improving saccharification of residual plant materials to give promising yields in the production of biofuels or biorefining.

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