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# Bioconversion of Moroccan Alfa (*Stipa Tenacissima*) by Thermomechanical Pretreatment Combined to Acid or Alkali Spraying for Ethanol Production

**I. Semhaoui[1,2], I. Zarguili[2], S.A. Rezzoug[1], T. Maugard[3], J.M.Q. Zhao[3], J. Toyir[2], M. Nawdali[2], Z. Maache-Rezzoug[1]**

*[1]Laboratoire des Sciences de l'Ingénieur pour l'Environnement, LaSIE, UMR CNRS 7356, Université de La Rochelle, Avenue Michel Crépeau, 17042 La Rochelle, France.*
*[2]Laboratoire de Chimie de la Matière Condensée, Research team: Procédés pour l'Energie et l'Environnement. Faculté Polydisciplinaire de Taza, Université Sidi Mohamed Ben Abdellah, Morocco.*
*[3]Equipe Approches Moléculaires Environnement-Santé, UMR CNRS 7266, LIENSs, Université de La Rochelle. France.*

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*zrezzoug@univ-lr.fr* ,
*Phone: +33 5 46 45 86 15*

## Abstract

Alfa is an interesting source for ethanol production; this abundant raw material has a high cellulose content. The aim of this study is to optimize the thermomechanical pretreatment conditions applied to Alfa (*Stipa Tenacissima*) fibers that maximize by enzymatic hydrolysis the conversion of biomass to fermentable sugars. The thermomechanical process used in this study involves subjecting the lignocellulosic biomass for a fixed time (30 min) to saturated steam pressure (0.7 MPa), followed by a flash decompression to vacuum pressure (50 mbar). The heat induced by saturated steam result in intensive vapor formation in the capillary porous structure of the plant material and the shearing forces due to the subsequent pressure release that damage the cellulosic biomass structure, making it more accessible to the attack by enzymes. Prior to pretreatment, the substrate was impregnated by spraying with $H_2SO_4$ and NaOH catalysts with concentration ranging from 0 to 15 wt%. Unwashed alkali and acid substrate were hydrolyzed using Celluclast 1.5 L (480 FPU/L). Acid catalyzed pretreatment of the biomass resulted in an efficient degradation of cellulose and hemicellulose by increasing recovery of reducing sugars before ($R_1$) and after enzymatic hydrolysis reactions and improving the specific surface area. $R_1$ varied from 0.88 g/L (uncatalyzed raw material) to 1.90 g/L (treated Alfa with 15 wt%). The maximum yield of reducing sugars of 95% was obtained at 5 wt% of sulfuric acid while the amount of sugars released after enzymatic hydrolysis was not affected by the presence of sodium hydroxide.

## 1. Introduction

The global rise in greenhouse gas emissions generated from fossil fuels use have harmful effects on the environment, it's related to the increase in energy demands accompanying the demographic development and lead to climate change concerns. In Morocco for example, the transport sector is the leading energy consumer with more than 41% of national final energy consumption, so it contributes to more than 23% of greenhouse gas emissions [1]. These reasons have prompted an interest in alternative sustainable biofuels from renewable resources such as lignocellulosic biomass [2]. Fuels based on lignocellulosic biomass, such as cellulosic ethanol, appear promising as alternative fuels for the transportation [3]. Lignocellulosic materials can be presented like agricultural residues, forest products or energy crops, and are characterized as sustainable, abundant, and environmentally friendly resource that can be converted into fermentable sugars for liquid transportation fuels. Alfa or esparto grass (S*tipa Tenacissima*) is a herbaceous perennial plant from the family of *Poaceae*. This lignocellulosic biomass is an endemic of the western Mediterranean, which grows on the semi-arid grounds of North Africa and South of Spain [4, 5]. It covers a large area estimated to about 3,186,000 ha in Morocco (middle and high Atlas), 4,000,000 ha in Algeria, 400,000 ha in Tunisia and 350,000 ha in Libya [6]. Alfa stem consists of strong, stiff and light cellulosic fibers which are used in several industrial sectors as the production of high quality papers and also traditionally sectors as manufacturing ropes and carpets [7]. As for other sources of lignocellulosic biomass, Alfa consists mainly of three natural polymers: cellulose, hemicellulose and lignin. These three polymers form a recalcitrant structure which makes difficult the enzymatic hydrolysis which is the main step of polysaccharides conversion into simple sugars. The pretreatment step is of prime importance for destructuring this lignocellulosic barrier and thus for the improvement of process efficiency. The pretreatment may be physical, chemical or physico-chemical nature [8]. Steam explosion is one of the most extensively studied pretreatment methods. The aim is to open the compact lignocellulosic plant cell walls causing hemicellulose degradation and lignin transformation and then increasing the potential of cellulose hydrolysis [9]. However, the hemicellulose removal and the lignin-carbohydrate matrix disruption are often incomplete during the steam explosion process [10]. In order to intensify the disruption of the lignocellulosic structure, a thermomechanical pretreatment called "D.I.C." process (in French: Détente Instantanée Contrôlée) was used for Alfa pretreatment. This physical process is close to steam explosion technology [11]. The difference is that the D.I.C process includes two additional steps: instauration of initial vacuum before injection of steam. This step, allows reducing the resistance of air facilitating the diffusion of steam into the product. Consequently, the time necessary to reach the steam equilibrium temperature is reduced. The second step consists to an abrupt decompression which carries out towards the vacuum (50 mbar) instead of atmospheric pressure as for steam explosion process. Due to the fast transformation and the adiabatic nature of water transition inside the product, the vaporization induces a rapid cooling. The temperature is quickly stabilized at an equilibrium temperature at the considered final pressure, limiting the reactions of degradations. This study aims to investigate the efficiency of acid ($H_2SO_4$) or alkali (NaOH) impregnation of Alfa prior to the thermomechanical pretreatment. Acid pretreatment is known to be more efficient for hemicellulose solubilization at high temperature, whereas alkaline pretreatment is more effective in removal of lignin [12]. The procedure adopted in this study is an impregnation by spraying and not by soaking as it is generally the case [13]. The process was carried out at moderate temperature (165 °C), corresponding to processing pressure of 0.7 MPa to limit the sugars loss and the formation of the inhibitory compounds for enzymatic hydrolysis and fermentation steps, but also contributing to reduce energy consumption. The structural and physicochemical properties were determined using FTIR spectrometry and BET measurements in order to correlate the performance of enzymatic hydrolysis with the intrinsic characteristics of the pretreated Alfa lignocellulosic substrate.

## 2. Materials and methods

### *2.1. Biomass material and chemicals*

Alfa fibers used in this work were collected from the region of Guercif, Oriental Morocco in October 2015. The samples were dried during 24h at 40°C, then cut manually into about 5-7cm long pieces and milled using a Gindomix (GM 200) Retsch crusher (7500xg) and then calibrated in a vibratory sieve shaker (Analysette 3Fritsch) to obtain particle sizes between1000 and 3150 µm. The chemicals were purchased from Sigma-Aldrich (France) anddeionized water was obtained through a Milli-Q system (Millipore,France).

### *2.2. Enzymes*

Celluclast 1.5 L, the enzyme concentrate used for cellulose hydrolysis was commercial *Trichoderma reesei* cellulase preparation contains endo-glucanases, exo-glucanase*s*, cellobiohydrolases and b-glucosidases. This preparation was a brownish liquid with density of approx. 1.20 g/mL and contained 191 mg protein/mL (calculated by Smith assay [14]). The cellulasic activity of concentrate was 96 FPU/mL. One unit of FPU is defined as the amount of enzyme which releases 1 µmol of glucose equivalents from Whatman n°1 filter paper in 1 min. Optimum conditions of activity were between 4.5 and 6 for pH and 50-60 °C for temperature [15].

### *2.3. Dilute-acid or alkali spraying method*

Aqueous solutions of dilute $H_2SO_4$ and NaOH at concentration ranging from 0 to 15 wt% was sprayed at room temperature over the Alfa stems (20g) through a nozzle, creating a mist above the solid. These levels were fixed basing on the literature [16, 17] that reported that concentrations used during spraying as an impregnation technique are more important than those for soaking method. The quantity of sprayed solution was adjusted to reach a final moisture content of 40 wt% (40 g $H_2O$/100 g dry). The acid-sprayed Alfa fibers were mixed manually in sealed bags to ensure a homogenous distribution and stored at 4 °C for 48 h prior to DIC hydrothermal pretreatment (section below) to promote the impregnation process.

### *2.4. Thermomechanical pretreatment*

The detailed procedure and equipment of thermomechanical pretreatment have been described previously [18]. Briefly, as described in Figure 1, the experimental setup is composed of: a processing reactor (Figure 1a) where the sample was treated at high temperature/high steam pressure, a vacuum system which comprises mainly a stainless-steel vacuum tank (Figure 1b) with a volume (1600 L), 133-fold greater than that of the reactor (12 L), a vacuum pump (fig.1d), and a steam generator (fig.1c) supplying steam into the reactor.

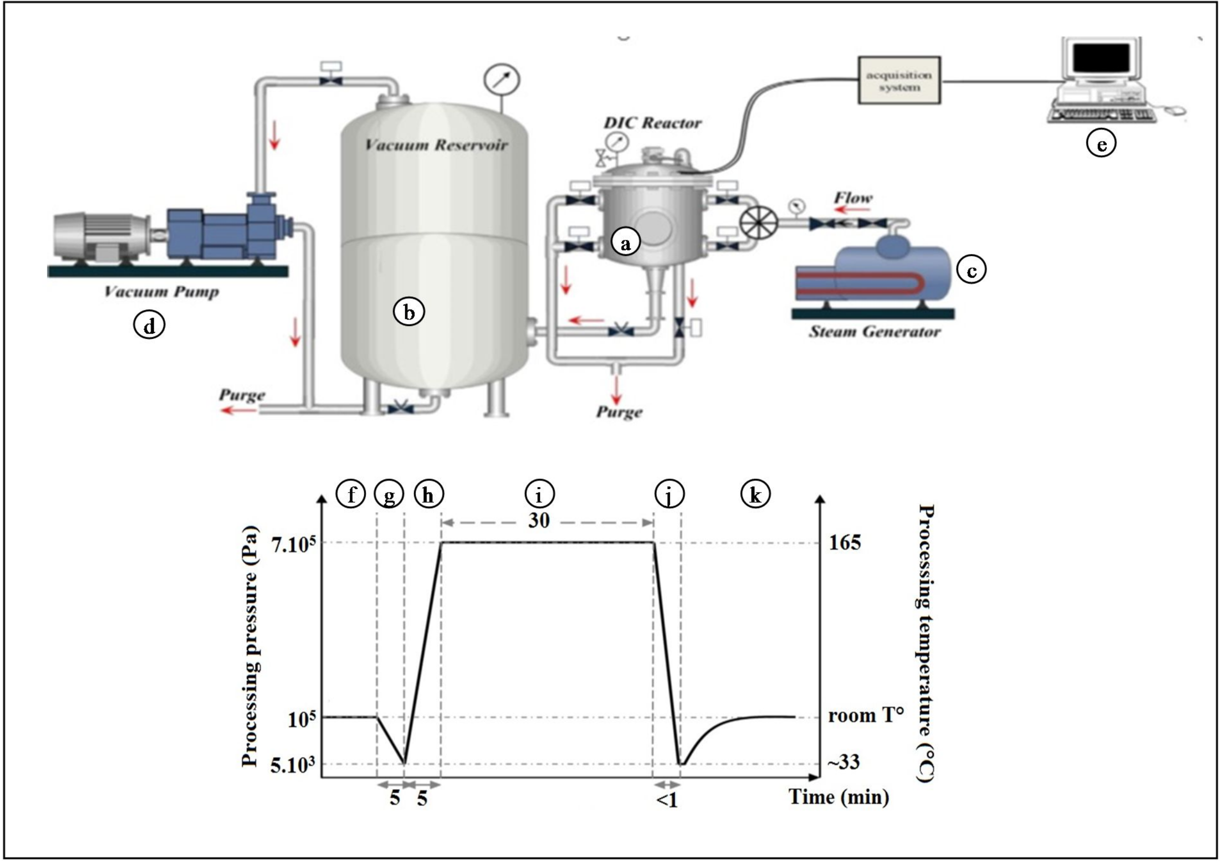

**Figure 1.** Experimental set-up of DIC process and typical pressure-temperature-time profile.

The acid-sprayed Alfa (20 g) at moisture content of 40 g $H_2O$/100 g dry matter was introduced into the reactor at atmospheric pressure (Figure 1f). A reduced pressure of 5 kPa was then created in the reactor (Figure 1g), contributing to acceleration of the transfer phenomenon, associated to the simultaneous heat and mass transfer in the material. The pretreatment was initiated and controlled by steam injection which is maintained at fixed pressure (0.7 MPa) and processing time (30min) (Figure 1h and 1i). The temperature rises rapidly from room temperature to steam equilibrium temperature. Main heating is the result of the transfer of latent heat of steam condensation during direct contact between biomass and saturated steam. After this period, there is no heat transfer and the exchange is dominated by the absorption of condensed steam [19]. The increase in moisture content depends on the amount of condensed water which is linked to pressure level and processing time [20]. The pressure was then released by an abrupt decompression towards a vacuum (5 kPa) (Figure 1j). After reducing pressure, the atmospheric air was injected to return to atmospheric pressure to retrieve the sample (Figure 1k). Alfa was recovered at the output of reactor, with moisture around of 400 g $H_2O$/100 g dry matter, due to water retention of steam condensation during hydrotreatment. Pretreated Alfa samples were dried in oven at 40 °C for 24 h and stored at room temperature for subsequent enzymatic hydrolysis to avoid any degradation.

### *2.5. Severity parameter calculation*

The severity of the thermomechanical pretreatment under diluted-acid conditions was analyzed using the combined severity factor (CS). CS is an index that integrates changes in temperature, time and acidity into a single value, which facilitates comparisons of different conditions [20, 21]. It was defined as:

$$CS = log(R_0) - pH \; (1) \quad \text{with} \quad R_0 = t \times EXP\left(\frac{T-100}{14.75}\right) (2)$$

Where t (min), T (°C), 14.75 and 100 are respectively, the residence time, process temperature, empirical parameter related to activation energy value and the reference temperature. pH in Eq. (1) was defined as the acidity of pretreated solid fraction, measured in aqueous solution after decantation of solid material, according to the protocol defined bellow.

### *2.6. Experimental procedure*

The experimental procedure is outlined in Figure 2. The Alpha biomass was impregnated by spraying before steam pretreatment, either by dilute $H_2SO_4$ or NaOH. The interest of impregnation by spraying over the immersion method is that it requires low volumes of acid. This technique allows avoiding additional steps of separation between liquid and solid phases as well as the recycling of acid residue or their neutralization. As the quantity of sprayed solution is adjusted to have final moisture content of 40 wt% the method generates no liquid phase.

### *2.7. Analysis*

#### *2.7.1. Moisture content*

The moisture contents of Alfa were determined by drying samples in an oven at 105 °C for 24 h, according to the A.F.N.OR standard method [22].

#### *2.7.2. Compositional analysis of Alfa*

The compositional analysis of native Alfa fibers was carried out according to the NDF (Neutral Detergent Fiber) and ADF (Acid Detergent Fiber) analysis procedure developed by Van Soest and Wine [23]. The NDF solution is kept at neutral pH, while the ADF solution contains 1.00 N sulfuric acid. After refluxing for 1h in a boiling aqueous surfactant solution, the solids were filtered, rinsed several times with water and acetone and dried. The remaining biomass is weighed, and reported as either a fractional value remaining (%). Acid Detergent Lignin (ADL) procedure was performed on samples after ADF procedure. ADL uses concentrated (72% w/v) sulfuric acid to dissolve any remaining structural carbohydrates, leaving insoluble lignin. The difference between ADF and ADL is often used as an estimate of cellulose, while the difference between NDF and ADF is often used as an estimate of hemicelluloses content [23].

#### *2.7.3. pH measurements*

The pH of untreated and pretreated Alfa was measured by a pH probe attached to a pH meter (InoLab® pH 720, WTW). Approximately 0.1 g of dry material was added to distilled water with a solid/liquid ratio of 1:20. The suspension was stirred mechanically for 5 min and pH measured in the liquid phase after decantation of the solid phase.

#### *2.7.4. Specific surface area*

The measurements of specific surface area of Alfa were determined by widely used method of adsorption of krypton using BET method. After have been oven-dried at 40 °C, the samples(0.3 g) were degassed during 36 h at room temperature in order to minimize structural changes. The BET surface area was determined at a relative pressure of about 0.999 using a Micromeritics ASAP2020 analyzer (Micromeritics Instrument, USA).

#### *2.7.5 Fourier transform infrared spectroscopy (FTIR)*

FTIR analyses were carried out using a Nicolet I5S spectrophotometer equipped with ID7 attenuated total reflectance accessory (ATR-ID7). The spectra were recorded at wave numbers from 400 to 4000 $cm^{-1}$ with 4 $cm^{-1}$ resolution. An average of 32 scans was recorded for each spectrum. All experiments were performed in triplicate. The results were analyzed using OMNIC software.

### *2.8. Enzymatic hydrolysis process*

Celluclast-1.5 L (480 FPU/L) was added to 50 mM citrate-phosphate buffer (pH ¼ 4.6) and then mixed with the substrate (10 g/L). The experiments were carried out in 100 ml Erlenmeyer flasks containing 10 mL total reaction volume (buffer enzyme mixture). The flasks were sealed and incubated in a rotary shaker at 600 rpm at 50 °C for 24 h. To follow the hydrolysis, a flask was withdrawn at different times and the liquid phase (hydrolyzate) immediately heated for 5 min in a boiling water bath to precipitate the proteins and prevent further hydrolysis. The mixture was then centrifuged at 14000 rpm for 2 min to remove solids. The cellulose hydrolysis yield of samples was determined by the 3.5-dinitrosalicylic acid (DNS) method [15].

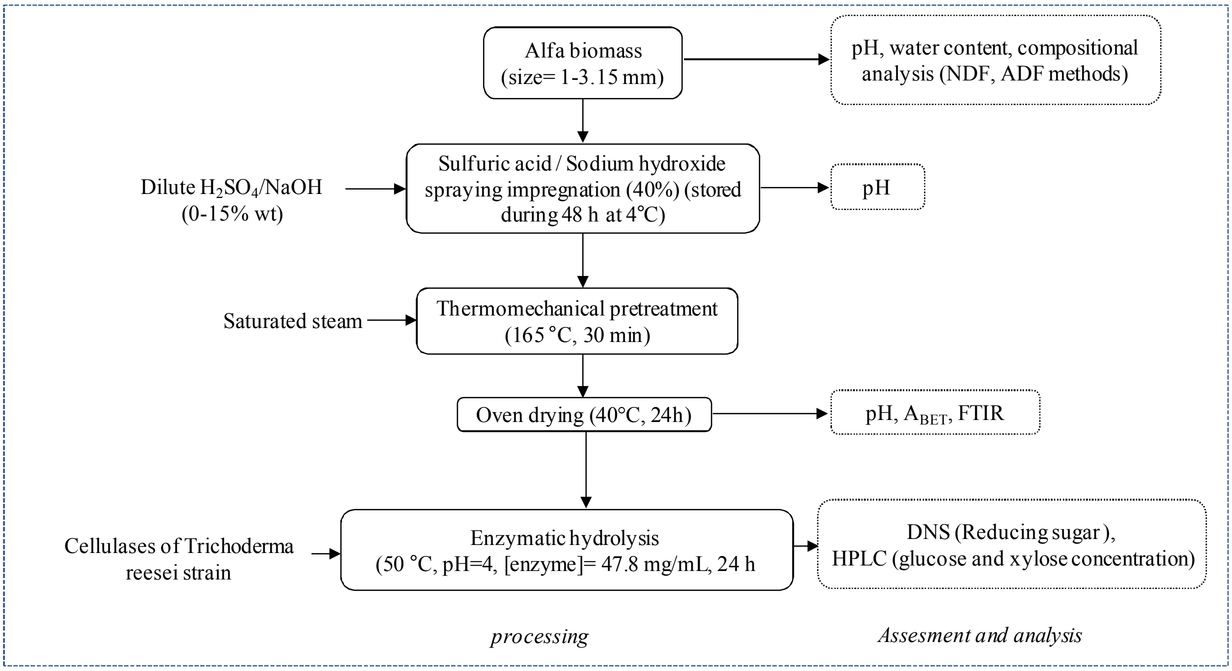

**Figure 2**. Flow chart for the acid or alkali catalyzed combined to DIC process pretreatment.

### *2.9. HPLC analyses*

After enzymatic hydrolysis of the biomass, glucose, xylose and arabinose, concentrations in the hydrolysates were determined using an HPLC system from Agilent (1100 LC and differential refractometer, Waters model 410), with a Transgenomic ICE-ORH-801 column (300 x 6.5 mm, Interchim) eluted with sulphuricacid/water (0.1% v/v) at room temperature and at a flow rate of0.6 ml/min.

## 3. Results and discussion

### *3.1. Feedstock compositional analysis*

The characterization of raw Alfa was determined by The NDF and ADF analysis methods and the results are presented in Table 1.

**Table 1**. Chemical composition of Alfa biomass based on dry matter.

| Components | Mass fraction (%) | | |
|---|---|---|---|
| | Present study | Bouiri et al.[24] | Zaafouri et al.[25] |
| Water content | 8.95 ± 0.67[a] | - | - |
| Dry matter | 91.92 ± 0.50 | - | - |
| Extractives | 15.09 ± 0.30 | 6.59 | 13.52 |
| Cellulose | 63.38 ± 1.58 | 47.63 | 49.09 |
| Hemicelluloses | 9.29 ± 0.50 | 22.15 | 18.20 |
| Lignin[b] | 12.23 ± 0.80 | 17.71 | 16.25 |
| Ash | 3.33 ± 0.02 | 5.12 | 11.41 |

[a] Standards deviations were calculated from triplicates.

[b] Acid Insoluble Lignin.

The Alfa biomass contains 91.9% mass fraction of total solids and 8.9% of water. The composition of the organic matter in the biomass, based on dry mass, showed the presence of 72.67% of hollocellulose (cellulose and hemicelluloses) which were slightly higher than the literature reported data [24, 25]. However, the amount of lignin content of Moroccan Alfa was lower than those of cited works originating from Tunisia and Algeria. This can be explained by the fact that the total lignin includes acid soluble and insoluble lignin while that our reported value represents only the insoluble fraction. Typical lignin contents of herbaceous species and agricultural residue are in the range of [10-20%][26]. Glucose, which is basically derived from cellulose of plant cell walls and xylose the major of hemicelluloses constituent, represent the main carbohydrates in the Alfa feedstock. The high concentration of polysaccharides provides to Alfa *Stipa Tenassicima* a real potential to be integrated as a raw material for bioethanol production.

### *3.2. Physico-chemical characterization of* Alfa *Stipa Tenassicima biomass*

The DIC pretreatment combined to dilute sulfuric acid or sodium hydroxide by spraying was applied in order to cause beneficial structural changes in Alfa lignocellulosic substrate. The induced changes were analyzed to further investigate the factors effectively influencing enzymatic hydrolysis.

### *3.2. 1. Assessment of pretreatment efficiency*

**Table 2**. Combined severity factor (CS), specific surface area ($A_{BET}$), pH values and enzymatic hydrolysis parameters measured on pretreated Alfa by DIC process at acid conditions.

| Run | $HH_2O_4$ Concentration (wt%) | Acid conditions | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ${}^{a}$pH | ${}^{b}$pH | CS | $A_{BET}$ ($m^2/g$) | Enzymatic Hydrolysis Conversion (g/L) | | Yield Hydrolysis (24h) % |
| | | | | | | $R_1$ | $R_2$ | |
| 1 | 0 | 5.99 ± 0.04 | 4.80 ± 0.01 | -1.41 | 0.263 ± 0.012 | 0.88 | 2.87 | 45.3 |
| 2 | 2 | 3.59 ± 0.07 | 3.79 ± 0.01 | -0.40 | 0.373 ± 0.014 | 0.89 | 3.94 | 62.1 |
| 3 | 3 | 2.99 ± 0.15 | 3.47 ± 0.01 | -0.07 | 0.450 ± 0.005 | 0.97 | 4.60 | 72.5 |
| 4 | 5 | 2.66 ± 0.02 | 2.71 ± 0.04 | 0.68 | 0.751 ± 0.021 | 1.31 | 6.02 | 95.0 |
| 5 | 7 | 2.45 ± 0.05 | 2.60 ± 0.02 | 0.79 | 0.963 ± 0.013 | 1.33 | 5.39 | 85.1 |
| 6 | 10 | 2.20 ± 0.01 | 2.31 ± 0.01 | 1.08 | 1.366 ± 0.013 | 1.44 | 5.28 | 83.3 |
| 7 | 12 | 2.12 ± 0.03 | 2.15 ± 0.07 | 1.24 | 1.457 ± 0.013 | 1.72 | 3.48 | 54.9 |
| 8 | 15 | 2.05 ± 0.04 | 2.09 ± 0.01 | 1.30 | 1.613 ± 0.015 | 1.90 | 3.31 | 52.3 |
| Untreated | - | 5.99 ± 0.04 | - | - | 0.297 ± 0.026 | 0.83 | 2.03 | 32.1 |

*$R_1$ and $R_2$ are the reducing sugars concentration (g/L) at 0 h and 24 h of enzymatic hydrolysis, respectively.*
*${}^{a}$pH and ${}^{b}$pH are pH value measured before and after thermomechanical pretreatment, respectively.*
*The average and standard deviation of pH, $A_{BET}$ were calculated by three replications.*

Tables 2 and 3 show the pH variation, combined severity factor (CS), surface area ($A_{BET}$), reducing sugars concentration, and overall sugar yield released after enzymatic hydrolysis of pretreated Alfa at various conditions (acid and alkali). The data of untreated Alfa are also listed for comparison. The pH values measured before and after DIC treatment show no significant variation for the acid-impregnated samples. However, pH values decreased either for non-impregnated or alkali-impregnated samples after thermomechanical pretreatment. According to Aita and Kim [27], at high temperature, hydrogen ions are generated by auto-ionization reaction of water. The treatment process can induce hemicelluloses solubilization and hydrolysis of the acetyl groups. Acetic acid is a by-product of this reaction, which further reduces the pH and catalyzes the hydrolysis.

In acid conditions, the severity factor increased with increasing of sulfuric acid concentration used in spraying solution, and thus with low values of pH, since temperature and processing time were fixed at 165 °C and 30 min, respectively. In order to isolate the effect of pretreatment on physicochemical changes in biomass (hemicelluloses solubilization), the severity factor is often adopted by authors to compare the effectiveness of treatments [28].

From table 2, it can be seen that the lowest pH values of acidic-pretreated Alfa biomass was of 2.09, corresponding to severity factor (CS) of 1.3. This value was obtained with 15 wt% $H_2S0_4$ spraying solution remains relatively moderate compared to those cited in the literature for acid-catalyzed hydrothermal pretreatment [29].

**Table 3.** Combined severity factor (CS), specific surface area ($A_{BET}$), pH values and enzymatic hydrolysis parameters measured on pretreated Alfa by DIC process at alkaline conditions.

| Run | NaOH Concentration (wt%) | Alkali conditions | | | | | |
|---|---|---|---|---|---|---|---|
| | | $^a$pH | $^b$pH | $A_{BET}$ ($m^2/g$) | Enzymatic Hydrolysis Conversion (g/L) | | Yield Hydrolysis (24h) % |
| | | | | | $R_1$ | $R_2$ | |
| 1 | 0 | 5.99 ± 0.04 | 4.80 ± 0.01 | 0.263 ± 0.012 | 0.88 | 2.87 | 45.3 |
| 2 | 2 | 8.84 ± 0.02 | 5.63 ± 0.04 | 0.304 ± 0.000 | 0.68 | 1.44 | 22.7 |
| 3 | 3 | 8.94 ± 0.03 | 5.74 ± 0.06 | 0.325 ± 0.000 | 0.64 | 1.85 | 29.2 |
| 4 | 5 | 9.18 ± 0.02 | 6.40 ± 0.14 | 0.206 ± 0.000 | 0.68 | 2.09 | 33.0 |
| 5 | 7 | 9.61 ± 0.03 | 6.42 ± 0.01 | 0.164 ± 0.006 | 0.68 | 2.16 | 34.0 |
| 6 | 10 | 10.05 ± 0.03 | 6.84 ± 0.01 | 0.195 ± 0.002 | 0.62 | 2.87 | 45.3 |
| 7 | 12 | 10.17 ± 0.03 | 7.14 ± 0.03 | 0.212 ± 0.002 | 0.70 | 3.15 | 49.7 |
| 8 | 15 | 10.48 ± 0.02 | 7.76 ± 0.05 | 0.210 ± 0.002 | 0.79 | 3.5 | 55.1 |
| Untreated | - | 5.99 ± 0.04 | - | 0.297 ± 0.026 | 0.83 | 2.03 | 32.1 |

*$R_1$ and $R_2$ are the reducing sugars concentration at 0 h and 24 h of enzymatic hydrolysis, respectively .$^a$pH and $^b$pH are pH value before and after hydrothermal pretreatment respectively.*

Negative values of CS (Table 2) corresponded to low processing conditions for a pH measured after treatment greater than about 3 and sulphuric acid concentration not exceeding 3 wt%. It is clear that the pH, temperature and processing time are the main factors affecting the severity of pretreatment and thus on enzymatic digestibility of lignocellulosics [30,31]. However, other parameters can also influence the accessibility of the biomass to enzymatic attack as the exchange surface. During the pretreatment, the lignocellulosic complex matrix should be broken in order to reduce the crystallinity of cellulose and increase its amorphous fraction, most suitable form for enzymatic attack.

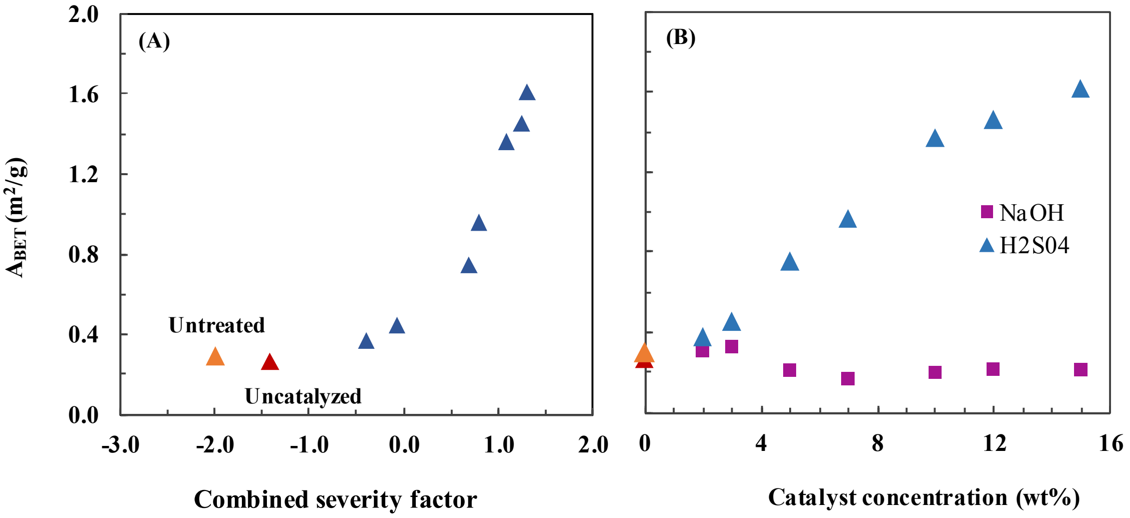

**Figure 3**. Effect of the severity conditions (A) and catalyst concentration (B) on specific surface area of treated Alfa.

As enzymatic hydrolysis takes place by direct physical contact between enzymes and cellulose, therefore the increase of specific surface area is among the main objectives of all pretreatment processes. Exchange surface of cellulose is known as cellulose accessibility has been consistently recognized as one of the most critical factors affecting the enzymatic hydrolysis yield [8,32-33]. The access of cellulases to cellulose in plant cell walls is limited by the available channels for their penetration. According to Chen and Liu [9], pretreatment by solubilization of the hemicelluloses and/or lignin helps to improve the enzymatic hydrolysis by increasing the surface area of the biomass. Acid is more efficient in hemicelluloses solubilization at high temperature, whereas alkaline pretreatment is more effective for removal of lignin [11].

The Alfa biomass pretreatment in acid conditions improved the specific surface area. $A_{BET}$ is positively correlated with the combined severity factor (Figure 3A).The highest surface area was observed for pretreated Alfa with 15 wt% of sulfuric acid (CS=1.30), which was 1.61 $m^2/g$, approximatively 5 times that of the untreated Alfa, which was equal to 0.30$m^2/g$. Comparison of changes in $A_{BET}$ after pretreatment of biomass is shown in Figure 3 for sulfuric acid and sodium hydroxide with [0-15] wt% of catalyst. The sulfuric acid can solubilize hemicelluloses, mainly xylan and thus increase the pore sizes in the residue. Pei et al [34] reported that about 96% of the hemicelluloses was removed for corn stover treated at 180 °C for 20 min at pH 2.0. Removal of hemicelluloses increases the mean pore size of the substrate and therefore increases the accessibility and the probability of the cellulose to become hydrolyzed. Maache-Rezzoug et al [11] and Alvira et al [35] showed an increase in $A_{BET}$ area values after combined dilute-acid-catalyzed and pretreatment of wheat straw, from 0.76 $m^2/g$ for untreated substrate to 1.07-1.68 $m^2/g$ for treated straw. The highest value was obtained at most severe condition (CS = 1.17). However, there was no significant change in specific area for sodium hydroxide pretreated Alfa (Figure 3B). In alkali treatment, hydroxyl ions are responsible for delignification. Three groups of reactions can take place: fragmentations, degradation and dissolution, and condensations of lignin causing the clogging of the pores [36]. Solubilized lignin removal requires a large amount of water for washing. This step has not been applied in our study in order to avoid generating chemical waste. This can probably explain the lack of increase of $A_{BET}$ on the treated Alfa. $A_{BET}$ values remained identical to that of the untreated substrate despite the increase in NaOH concentration.

### *3.2. 2. Effect of pretreatment on enzymatic hydrolysis efficiency*

Alfa pretreated with DIC process after spraying by diluted sulphuric acidic and sodium hydroxide (NaOH) solutions at different concentrations as well as the untreated and uncatalyzed substrates were subjected to enzymatic hydrolysis. Hydrolysis enhancement observed after the pretreatment was different between the acid and alkali catalyst. As shown in Figure 4A, the recovery of reducing sugars before starting of enzymatic hydrolysis reactions (0 h) depends on acidic concentration. The maximum and minimum $R_1$ values (Table 2) varied from 0.88 g/L (uncatalyzed raw material) to 1.90 g/L (treated Alfa with 15 wt%). A partial solubilization of sugars after acid spraying and steam pretreatment of Alfa was obtained, corresponding to the degradation of cellulose and hemicelluloses via autohydrolysis reactions, as observed by Panagiotopoulos et al [37]. The partial carbohydrate solubilization to its monomeric form may occur during acid pretreatment mainly consisting of hemicelluloses fraction; hemicelluloses having more susceptibility to this effect as it presents features like low molecular weight, more amorphous zone and low degree of polymerization. The concentrations of reducing sugars released after 24 h of enzymatic hydrolysis following a combined sulfuric acid spraying and DIC treatment of Alfa substrate ($R_2$), showed a linear increase in the range of acid concentration of (2-5) wt%. At 5% sulfuric acid, the reducing sugar concentration reached the maximum value of 6.02 g/L corresponding to overall yield of 95%, which was approximately 3 times that of untreated biomass. This hydrolysis efficiency is greater than observed by Zaafouri et al. [25] on sulfuric acid soaked Alfa at 3% (v/v) and treated at 120 °C for 30 min. As already shown, $A_{BET}$ has increased with increasing acidic concentration, we can expect an improvement of sugars release. Kumar and Wyman [38] showed that the hydrolysis rate is controlled by enzymes adsorption onto cellulose, which is conditioned by enzyme accessibility to cellulose active sites and thus by the available biomass surface area. Therefore the reduction of the concentration of reducing sugars observed at high sulfuric acid concentration of ($\geq 7$) is probably due to the degradation of sugars, corresponding to CS > 0.8.

However, no release of reducing sugar was observed at 0 h of enzymatic hydrolysis on the Alfa previously sprayed with sodium hydroxide and treated by DIC (Figure 4B). $R_1$ values were identical to that of untreated biomass. The presence of sodium hydroxide did not affect the amount of sugars released after 24 h of enzymatic hydrolysis. The comparison between substrates pretreated values (Table 3) at different concentrations with uncatalyzed showed no improvement but rather a decline up to 7 wt% of NaOH. This result is in agreement with the $A_{BET}$ values of the alkali-pretreated Alfa, since no enhancement was observed in specific surface area of pretreated substrate.

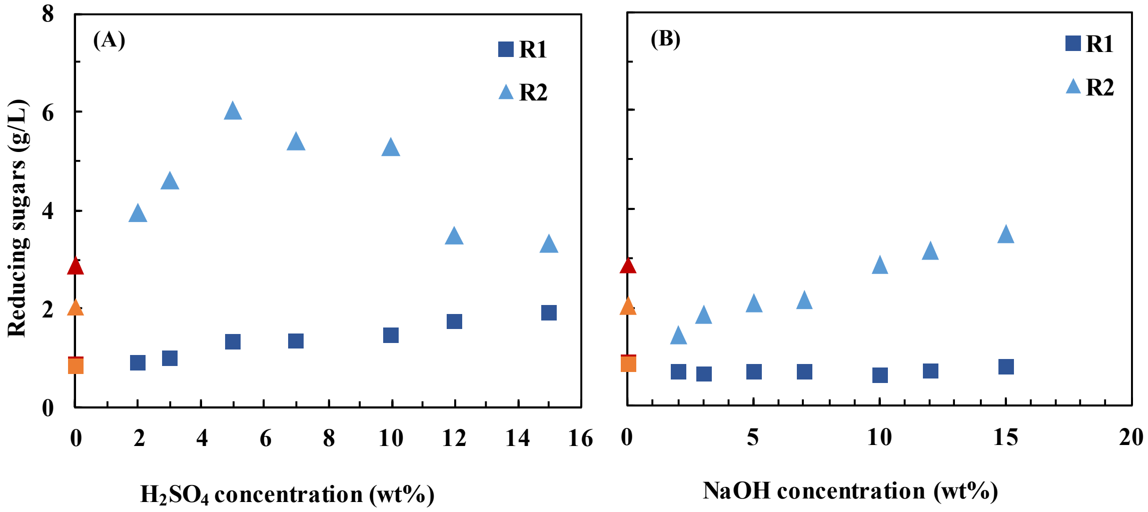

**Figure 4**. Effect of catalyst concentration on reducing sugar release at 0 h ($R_1$) and 24 h ($R_2$) of enzymatic hydrolysis. (A): $H_2SO_2$ and (B): NaOH. The red and orange colors correspond to the uncatalyzed and untreated Alfa, respectively.

In general, yields or concentrations of reducing sugars released following both the pretreatment and enzymatic hydrolysis processes are an indicator of pretreatment efficiency for ethanol bioconversion. Figure 5 shows the enzymatic hydrolysis yields of reducing sugar released of untreated Alfa and those treated at different concentrations of catalyst as well as the uncatalyzed substrate. The maximum overall yield of reducing sugars of 95% was obtained at 5 wt% of sulfuric acid and 165°C for 30 min as processing conditions for the thermomechanical DIC pretreatment. Despite the potential of Alfa for bioethanol production, a few works are reported in the literature. In recent study, three successive steps were used consisting in pretreatment with dilute $H_2SO_4$ (3%) at 120 °C during 30 min, enzymatic saccharification by a commercial enzyme (AP2) and fermentation [25]**.** The yield of reducing sugars was about 41.27% of the theoretical maximum from which 88.1% were converted into alcohol. Figure 5 illustrates also the low improvement of hydrolysis yields as the concentration of sodium hydroxide has increased.

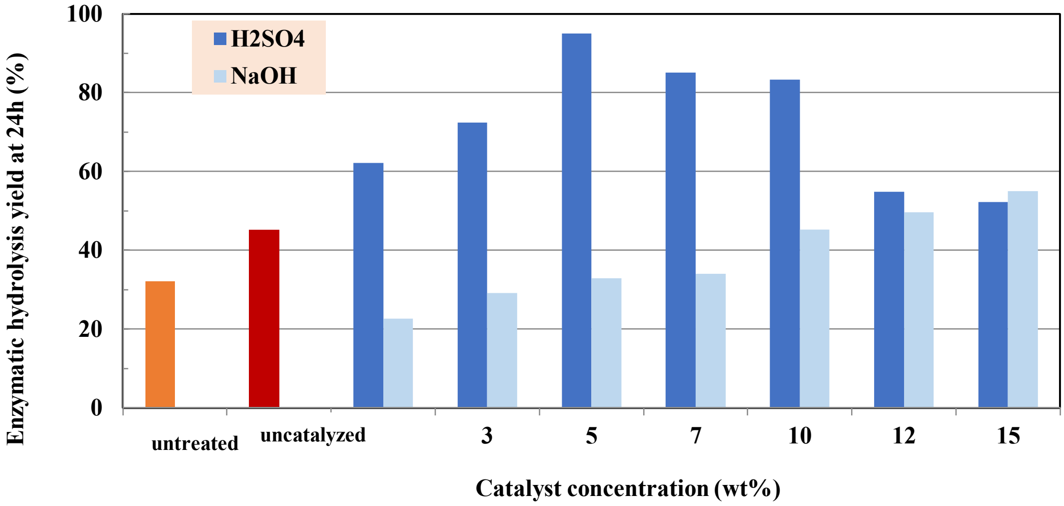

**Figure 5**. Enzymatic hydrolysis yield as function of catalyst concentrations.

### *3.2. 3. HPLC results*

The results of concentration analysis of glucose, xylose and arabinose released after enzymatic hydrolysis of the Alfa biomass are given in table 4. It can be seen that concentrations the favors the maximum arabinose released was 5% in acid conditions and 3% in alkaline conditions.

**Table 4**: Recovery of Glucose, Xylose and Arabinose in solid fraction of pretreated Alfa by HPLC analysis.

| Concentration (wt%) | Acid conditions |  |  | Alkaline conditions |  |  |
|---|---|---|---|---|---|---|
|  | Glucose (g/L) | Xlyose (g/L) | Arabinose (g/L) | Glucose (g/L) | Xlyose (g/L) | Arabinose (g/L) |
| 0 | 2.75 | 0.23 | 1.69 | 2.75 | 0.23 | 1.69 |
| 3 | 4.14 | 0.50 | 1.57 | 3.33 | 0.13 | 2.71 |
| 5 | 4.83 | 0.73 | 1.46 | 2.43 | 0.17 | 1.72 |
| 7 | 7.84 | 1.34 | 2.09 | 3.02 | 0.32 | 1.93 |
| 10 | 6.04 | 0.86 | 1.97 | 3.30 | 0.40 | 1.66 |
| 12 | 5.36 | 0.58 | 1.56 | 3.59 | 0.61 | 1.93 |
| 15 | 4.94 | 0.88 | 1.90 | 4.51 | 0.80 | 2.15 |
| Untreated | 2.15 | 0.00 | 1.10 | 2.15 | 0.00 | 1.10 |

Figure 6 displays the individual sugar release (xylose and glucose) as a function of catalyst concentration for acid and alkali pretreatment determined using HPLC analysis. A continuous increase in the concentration of xylose and glucose released (Figure 6A) was observed when the acid concentration increased up to 7 wt%. Similar trend as that observed for glucose analyzed by DNS as well as the decrease that reflects the degradation of sugars in the presence of high acid concentration. A decrease in sugar release (glucose+ xylose) was noticed with a simultaneous continuing increase of surface area. This result suggests that, at these pretreatment conditions, sugar release is independent of accessible surface area. Indeed, a molecular degradation process implying pentose and hexose reaction with sulfuric acid may take place leading to products such as: furfural from xylose or arabinose, HMF from glucose as reported by Sharma et al [39]. A slight improvement of glucose released in the presence of sodium hydroxide (Figure 6B). The quantity of sugars obtained after 24 h of enzymatic hydrolysis was 4.5 g/L at 15 wt% and that of uncatalyzed Alfa was 2.7 g/L.

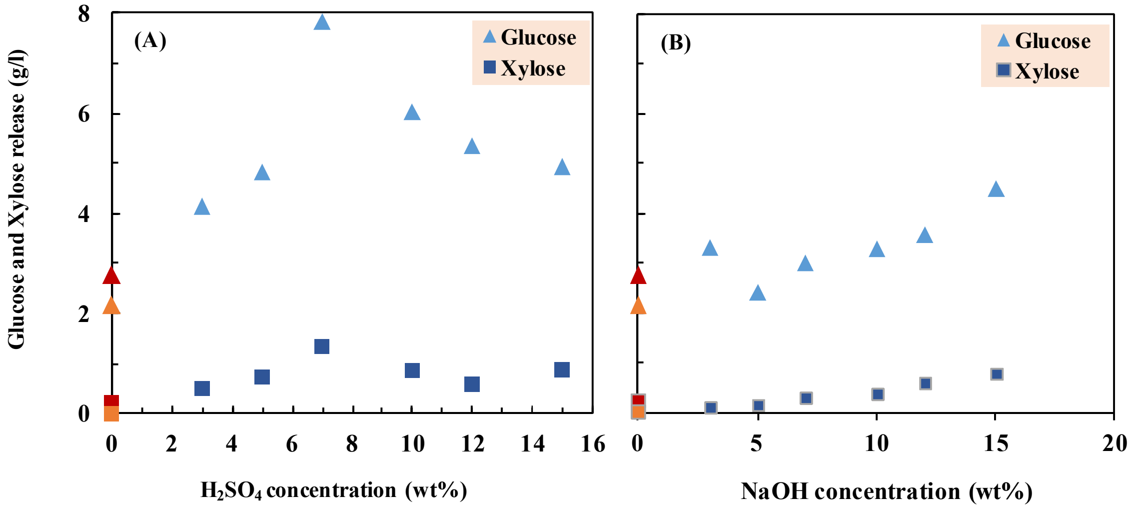

**Figure 6.** Glucose and xylose release after 24 h of enzymatic hydrolysis as function of sulfuric acid (A) and hydroxide concentration (B) concentrations.

### *3.3. Structural characterization of Alpha fibers by spectroscopy FTIR*

The chemical structure of cellulose, hemicelluloses and lignin constituents present in Alfa fibers were analyzed by FTIR spectroscopy. These constituents spectra were used to investigate the shifts in functional groups that occurred as a result of the physicochemical pretreatment on the fibers. The infrared spectra of the untreated and three pretreated samples are shown in Figure 7. The general patterns are close to the literature [40, 41]. A first comparison of the different spectra indicates that the two absorption bands between 720 and 730 $cm^{-1}$ are important in native Alfa conversely to pretreated samples for which one of the two absorption bands disappeared (730 $cm^{-1}$) and the other (720 $cm^{-1}$) had a weaker intensity. The absorption bands between 950 $cm^{-1}$ and 700 $cm^{-1}$

called the anomeric region is indicative of β-glycosidic linkages in holocellulose [42] suggesting that the pretreatment was efficient for cellulose and hemicelluloses autohydrolysis.

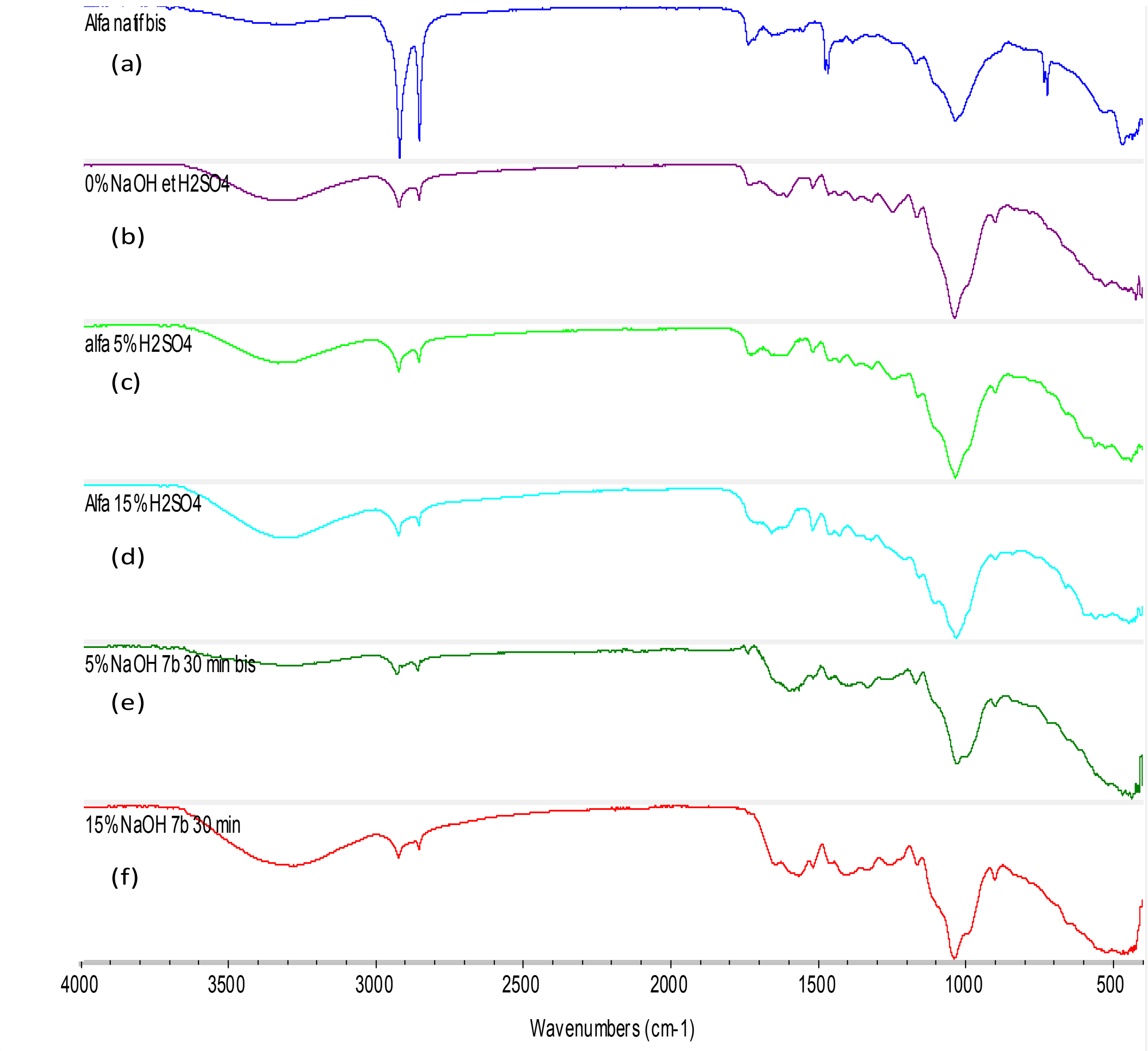

**Figure 7**. FTIR spectra of untreated and pretreated Alfa at different concentrations of $H_2SO_4$ spraying solutions: (a) unpretreated; (b) pretreated without catalyst; (c and d) pretreated with 5 wt% and 10 wt% $H_2SO_4$; (e and f) pretreated with 5 wt% and 10 wt% NaOH.

The characteristic peaks at 900 $cm^{-1}$ and especially at 1465 $cm^{-1}$ were designated as the C-H bending of amorphous and crystalline cellulose. The absorbance band at 900 $cm^{-1}$ is present in untreated and in pretreated samples but that at 1465 $cm^{-1}$ is strongly present only in the FTIR spectra of untreated Alfa. A higher intensity of the bands around 1380 $cm^{-1}$ and 1166 $cm^{-1}$ was observed for untreated Alfa indicating a presence of more cellulose since these bands are earmarked to the vibration of C-C and C-O characteristics of cellulose skeleton [43]. The peak at 1470 $cm^{-1}$ is attributed to the C-H bending or C-O stretching frequencies of hemicelluloses [44]. This peak clearly disappeared for pretreated Alfa fibers indicating that the degradation occurred due to the physicochemical pretreatment even without catalysts. The peak at 1654 $cm^{-1}$ is due to the presence of bonded water in hemicelluloses which usually show a strong affinity with water molecules [45]. This peak was almost identical for samples treated with 5 wt% $H_2SO_4$ sprayed solution and untreated one indicating a small removal of bonded water from hemicelluloses in these conditions. For the sample treated with 15 wt% $H_2SO_4$ the peak at 1654 $cm^{-1}$ is not visible. The band at 1735 $cm^{-1}$ is attributed to the acetyl and uronic ester group attached to hemicelluloses carboxylic ester group and p-coumaric acid of lignin. This peak is present in both untreated and pretreated samples with 5 wt% $H_2SO_4$ spraying solution but disappeared for samples treated with 15 wt% $H_2SO_4$

indicating hemicelluloses degradation (autohydrolysis). The characteristic peak at 1030 $cm^{-1}$ was assigned to the aromatic C-H in-plane deformation and C-O stretching in primary alcohol (guaiacyl) of lignin. This large peak is identical in all spectra indicating that no reaction of lignin took place. Moreover, the prominent bands between 2848 and 2917 $cm^{-1}$ assigned as stretching of hydroxyl groups correspond to C-H stretching of cellulose [6]. It is clear that these two peaks are prominent for untreated sample compared to pretreated ones. The huge absorbance band at 3300 $cm^{-1}$ assigned to elongation vibration of hydroxyl groups (alcohol and phenols) present in cellulose, hemicelluloses and lignin [40].

From Figure 7, it appears that these peaks are not discriminating due to the high and probably similar proportion of lignin in untreated and pretreated Alfa. FTIR spectra also indicated that structural modifications occurred for alkali treated samples as indicated by the absence of some characteristic peaks of aromatic compounds for samples sprayed with NaOH before thermomechanical pretreatment. For example, the intensity of the bands at 1740 $cm^{-1}$ which correspond to C=O stretching of carbonyl, carboxyl and acetyl groups present in hemicelluloses and lignin [46] are visible for untreated and pretreated sample without NaOH and more or less for the samples pretreated with 5 wt% and. This peak completely disappeared for the samples sprayed with solution at 15 wt% NaOH. The same remark for the band at 1514 $cm^{-1}$ assigned to aromatic syringyl units of lignin. These two last affirmations seem to be in contradiction with the results obtained for specific surface area in section 3.2 since no change of $A_{BET}$ was observed for all samples sprayed with NaOH before the thermomechanical pretreatment. The reason is probably due to the fact that the samples were not washed after the thermomechanical pretreatment, limiting the accessibility of enzymes and thus inducing low yields of sugars released.

## Conclusions

The results showed the interesting potential of Alfa *Stipa Tenassicima* as lignocellulosic feedstock for the production of bioethanol in North Africa. Combined dilute-acid-catalyzed and DIC pretreatment has improved the performance of the enzymatic hydrolysis of the substrate conversely to alkali-catalyzed conditions. These results were positively correlated with $A_{BET}$ which was improved in acid conditions while there was no significant change in specific surface area for sodium hydroxide pretreated Alfa. The maximum overall yield of reducing sugars of 95% (corresponding to 6.02 g/L) was obtained at 5 wt% of sulfuric acid and 165°C for 30 min as processing conditions for the thermomechanical pretreatment. Beyond this concentration, the yield of reducing sugars decreased due to probable cellulose degradation. Increasing in the concentration of xylose and glucose released was observed by HPLC when the acid concentration increased up to 7 wt%. Furthermore, FTIR spectra indicated that structural modifications occurred for both acidic or alkali spraying before the thermomechanical pretreatment. The results were correlated to $A_{BET}$ for the samples pretreated after $H_2SO_4$ spraying but not with those pretreated with NaOH. Then, the pretreatment showed promising results in acid condition and need a further optimization for alkali conditions. Moreover, economic and technical feasibility study is needed to evaluate the suitability of the process for commercial application.

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