

Article

Biochemical Conversion of Lignocellulosic Biomass from Date Palm of *Phoenix dactylifera* L. into Ethanol Production

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Abstract: Cellulosic fibers from date palm are among the most promising lignocellulose feedstock for biorefinery purposes. The world production is between 1.9 and 2.4 million t/year. Initially, a pretreatment with dilute-sulphuric acid of these fibers was performed using a response surface methodology, with temperature and process time as factors. The aim is to produce bioethanol from young and old fibers from date palm, *Phoenix dactylifera* L. Optimal thermochemical pretreatment conditions for both fibers palms were 220 °C in hydrothermal conditions (without acid); in these conditions pretreated young fibers presented a maximum content in holocelluloses of 45.18% and old fibers 61.97%. Subsequently, during the enzymatic hydrolysis a maximum yield of total reducing sugars (TRS) was reached, 46.32 g/100 g for pretreated dry young fibers and 48.54 g/100 g for pretreated dry old fibers. After enzymatic saccharification, hydrolysates were fermented by *Pachysolen tannophilus* (ATCC 32691) to ethanol, reaching yields ($Y_{E/TRS}$) of 37.94 g ethanol/100 g of TRS for young fibers and 35.84 g ethanol/100 g of TRS for old fibers. Globally, considering the full process, in the fermentation of the hydrolysates, a yield (Y_E) of 10.64 g ethanol/100 g of dry young fibers and 10.88 g ethanol/100 g of dry old fibers was reached.

Keywords: enzymatic hydrolysis; ethanol; fermentation; cellulosic fibers from date palm; *P. tannophilus*; pretreatment



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1. Introduction

Date palm *P. dactylifera* L. is one of the oldest crops existing in the arid zones of the Arabian Peninsula, North Africa and Middle East [1]. Cellulosic fibers are the byproduct of the date palm tree; it is the fibrous part that covers the trunk of the tree which is bonded to the stipe before the death of a palm frond [2]. In Tunisia, its annual quantity is about 30,460 t/year [3]. This substrate is used both crude and oxidized as a composite material [4]. Furthermore, this fiber is used to generate heat by combustion as well as for wood production; it is also employed as an energy resource for the wood industry [5]. Cellulosic fibers have not been used for bioenergy purposes through biochemical pathways; only its combustion evaluation in a fixed bed laboratory reactor and its comparison with sawdust was performed [6]. Therefore, the cellulosic fibers from date palm could be a potential raw material due to its lignocellulosic composition, which is why the purpose of this study was to produce bioethanol through biochemical pathways from both young and old cellulosic fibers of thermochemically pretreated *P. dactylifera* L. [7,8]. Different techniques of pretreatment were used including physical, chemical, physicochemical and biological in order to improve hydrolysis rates. There are different pretreatment techniques including physical, chemical (alkali, acid, organosolv and ionic liquid), physicochemical (steam explosion, liquid hot water, AFEX and CO₂ explosion) and biological [9–11]. In general, in these pretreatments, hemicellulose is hydrolysed, and cellulose and lignin

are modified, and the values achieved can be improved by the enzymatic hydrolysis. Enzymatic hydrolysis is carried out using cellulases that attack specific sites of the complex lignocellulose biomass structure which cannot be hydrolysed by only one enzyme. In fact, with the synergy of different enzymes (cellulases, endo-1,4- β -glucanases, exo-1,4- β -glucanases and β -glucosidases), cellulose can be hydrolysed into cellobiose, and afterwards β -glucosidases hydrolyse cellobiose into D-glucose [12].

Subsequently, the released monomeric sugars are converted to ethanol by yeasts. *Saccharomyces cerevisiae* is the most used microorganism for ethanol production by hydrolyzing cellobiose into hexoses. Other species of yeasts have also shown fermentation capacity for ethanol production from pentoses (D-xylose and L-arabinose) e.g., *Pichia stipitis*, *Candida shehatae*, *Candida guilliermondii* and *P. tannophilus*, but their ethanol yields are lower than when *S. cerevisiae* converts hexoses into ethanol [13]. Finally, it has been observed that the continuous process with cell recycling is one good technique for ethanolic fermentation, because this process reduces substrate inhibition and offers higher productivity of ethanol. It also reduces the toxic effects of the components highly concentrated in the medium [14].

In the lignocellulosic biomass pretreatment processes, in addition to sugars, other compounds are generated that inhibit subsequent ethanolic fermentation with yeasts. In this sense, the main compounds generated are: acetic acid, furfural, 5-hydroxy-methyl-furfural (HMF) and phenolic compounds [15,16]. These compounds cause an inhibition per substrate that decreases the specific rate of ethanol production and the yield in this bioproduct. If the stage prior to fermentation is an enzymatic hydrolysis instead of acid hydrolysis, this inhibition effect is less, as has been observed in previous works using *P. tannophilus* in the fermentation process and fragments of olive stones as substrate [17–19].

The aim of this work was to produce bioethanol from both young and old cellulosic fibers from date palm, *P. dactylifera* L., by a thermochemical pretreatment using a pressure reactor, followed by an enzymatic hydrolysis and a subsequent fermentation process by a non-traditional yeast, such as *P. tannophilus*, that is resistant to inhibitors production during the process [17,18].

2. Materials and Methods

2.1. Raw Materials and Mechanical Pretreatment

Two kinds of waste cellulosic fibers, derived respectively from young and old date palm trees (Figure 1) were collected in Tozeur, south of Tunisia. The raw materials were dried at $55\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ during 12 h in an oven type Borel, Mod. BLN300. After that, the samples were crushed by a hammer mill type Vert Saint Denkay, Mod. nf-6i-19ZSIII, in order to reduce fiber size and subsequently this material was sieved by selecting a specific particle range (\varnothing : 0.425–0.6 mm) using a sieve type. Retsch, mod. Vibro. Finally, the raw materials were stored in glass bottles until use.

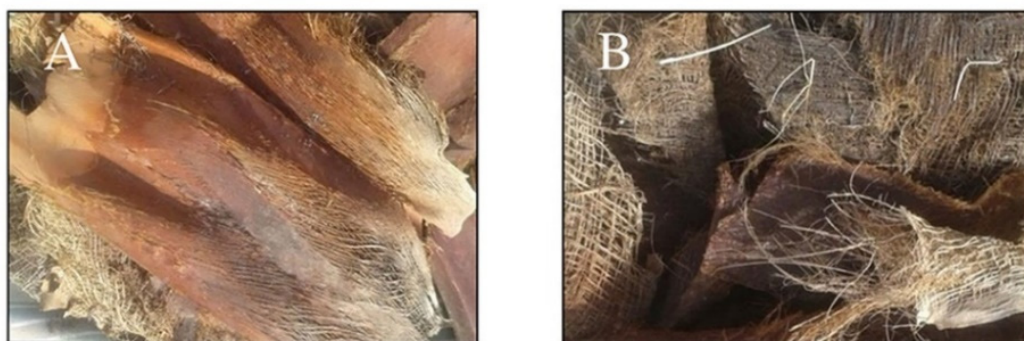


Figure 1. Lignocellulose material from *Phoenix dactylifera* L. young (A) and old (B), called in Tunisia 'lifs'.

2.2. Characterisation of Solid Fractions

The initial raw material, as well as the solid fractions samples from both thermochemical pretreatment and enzymatic hydrolysis, were analysed referring to standard protocols: moisture, insoluble acid lignin, holocellulose (cellulose and hemicellulose) and ash contents. All measurements were performed in duplicate.

2.2.1. Moisture and Volatile Matter

This parameter was determined by calculation of dry matter content for each of the samples using the standard TAPPI T11m-59 norm, evaluating the amount of water at 105 ± 1 °C during 48 h.

2.2.2. Ash Content (P_1)

This measure was performed following the standard TAPPI T211 norm, where an incineration process of the samples at 550 ± 5 °C during 3 h in a muffle oven Thermolyne®, Type 1300, is required to determine mineral content.

2.2.3. Acid-Insoluble Acid, AIL (P_3)

It was determined following the standard TAPPI T222 os-74 norm, according to which lignin content is defined as the insoluble fraction in 72% sulfuric acid (*w/w*).

2.2.4. Holocellulose Content (P_2)

Neutral detergent fiber (*NDF*) and Acid detergent fiber (*ADF*) were determined following the method described by Van Soest and Wine [20].

NDF is the insoluble fraction, corresponding to lignin, cellulose and hemicellulose, which was obtained by subjecting the sample into a slow ebullition in a neutral detergent solution [20].

ADF corresponds to the insoluble fraction, consisting mainly of lignin and cellulose, obtained after subjecting the sample into a slow ebullition in an acid detergent solution [20].

From the percentages of *NDF*, *ADF* and *AIL*, cellulose and hemicellulose fractions were determined. Holocellulose includes hemicellulose and cellulose fractions.

2.2.5. Determination of Residual Solids Content (*S*)

After the thermochemical pretreatment process, the obtained dry solid phase was weighed (on dry basis). This quantity referred to the amount of dry raw material initially pretreated represents the residual solids content (*S*).

2.2.6. Structural Characterization of Cellulosic Fibers from Date Palm Fibers by ATR-FTIR

The detection of functional groups, as well as the molecule vibration related to the composition and the structure of both young and old cellulosic fibers, was determined through the non-destructive method of Attenuated Total Reflectance (ATR)-Fourier transform infrared spectroscopy (FTIR) [21]. The spectrum was recorded in transmittance mode at a spectral range of 4000 and 400 cm^{-1} with an accumulation of 25 scans and a spectral resolution of 0.1 cm^{-1} , using an ALPHA FT-IR spectrophotometer with ATR-Platinum module, BRUKER®. The software used for spectrum acquisition was Opus 7.0.129 [22].

2.3. Characterisation of Liquid Fractions

The liquid fractions were characterised in terms of D-glucose, acetic acid, total reducing sugars concentrations and ethanol. All measurements were performed at least in duplicate.

2.3.1. D-Glucose Concentration (C_1)

D-glucose concentration was determined by an enzymatic-colorimetric method following the protocol detailed by Trinder [23], using the commercial kit Trinder GOD-POD.

2.3.2. Acetic Acid Content (C_3)

Acetic acid concentration was determined using an enzymatic-colorimetric method according to Bergmeyer and Möllering [24], using the enzymatic kit Cod. No 14826 of Boehringer Mannheim.

2.3.3. Total Reducing Sugars Determination (C_2)

Total reducing sugars determination was performed using DNS method according to the protocol described by Miller [25].

2.3.4. Ethanol Production

Ethanol concentration was determined using a commercial kit proposed by Beutler and Michal method [26].

2.4. Experimental Methodology

2.4.1. Thermochemical Pretreatment

A full factorial experimental design 2^k was employed in order to optimise thermochemical pretreatment conditions for young and old cellulosic fibers studying two factors, viz.: temperature (150 °C to 220 °C) and sulfuric acid concentration (0.00 M to 0.025 M) [27]. Eleven experiments were carried out, four of them performed in duplicate and a central one in triplicate. The responses were: residual solids content (S), ash measurement (P_1), holocellulose content (P_2), insoluble lignin (P_3), D-glucose concentration (C_1), total reducing sugars content (C_2) and acetic acid concentration (C_3).

The full factorial experimental design illustrates the different dilute acid pretreatment conditions (Figure 2). In order to determine the effect of the two factors on the seven responses, the regression coefficients C_r for each of the responses were calculated using Minitab® software, version 16.0.1, expressed by Equation (1) [28].

$$C_{rj} = \frac{1}{11} \left[\sum_{i=1}^{11} A_i Y_j \right] \quad (1)$$

where:

A_i —means either high (+) or low (−) level in experimental run i ,

Y_j —represents each of the response value for which C_{rj} is determined (where j varies from 1 to 7).

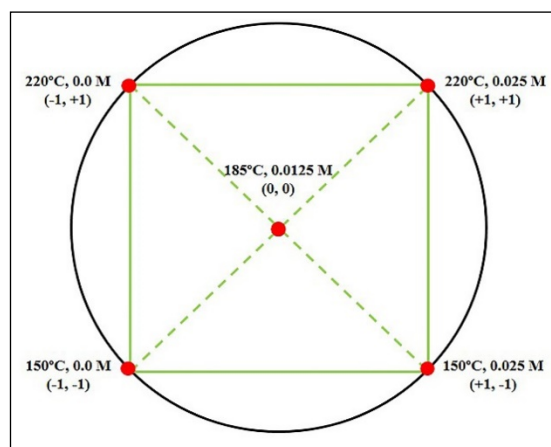


Figure 2. Factorial experimental design (square domain) and star points (circular domain). Each black dot represents a dilute acid pretreatment performed at the variable values indicated. The coded values are bracketed (−1 is the lowest value of a variable and +1 is its highest value) and the star points are distributed at a distance of 1.5 from central point.

If the regression coefficient has a positive sign, the correspondent factor therefore has a positive effect, and its high level will be held for the optimal experiment and vice versa [28]. Moreover, in order to study the relationship between the two factors and the seven responses, a first-order polynomial model exploring main effects of factors, was followed. The mathematical model for full factorial experimental design 2^k was expressed by Equation (2) [28].

$$Y = b_0 + \sum_{i=1}^k b_i X_i \quad (2)$$

where:

b_0 —constant coefficient,

b_i —linear coefficients,

X_i —factor,

k —number of factors ($k = 2$),

Y —predicted response value.

The dilute acid pretreatment experiment step was performed in a thermo-reactor of 2 L, type Parr[®] series 4522 (Moline, IL, USA), the latter offering the possibility of being loaded up to 0.5% of its capacity. In fact, it was loaded up with a reaction mixture at 10.0% of dry matter of young or old cellulosic fibers solid/liquid fraction about 1/10, 25 g fiber and 0.25 L H₂SO₄ solution.

Under agitation conditions of 250 rpm, the heating of reactor load was started until reaching the predetermined maximum temperature, which was maintained for 5 min. Subsequently, fast cooling was performed at the same time as decompression was carried out. Then, the reactor was covered with ice in order to lower its temperature to 25 °C, which was usually reached in 30 min. These operating conditions were selected in previous works [7,29].

Once the pretreatment is finished, the solid fraction was separated from the liquid by vacuum filtration; after water-washing, the solid fraction was dried and characterised by the methods described in Section 2.2. The liquid fraction was characterised in relation to D-glucose, TRS and acetic acid concentrations, according to the methodology indicated in the Section 2.3.

Otherwise, it is important to study the combination of the variables reaction time and temperature in a single parameter to correlate the severity of pretreatment with its efficacy; the parameter R_o , Equation (3), combines the effects of both variables in the auto-hydrolysis process of the different fractions that make up the lignocellulose material [30].

$$R_o = \int_0^t e^{\frac{T(t)-T_r}{w_g}} dt \quad (3)$$

where:

T_r —reference temperature (100 °C, base temperature for an initial process time $t = 0$), °C,

$T(t)$ —function that relates temperature to residence time, °C,

t —residence time in reactor, min,

w_g —fixed parameter ($RT_r^2/E_a = 14.75$, where R is the universal gas constant, and E_a is the apparent activation energy).

During the pretreatment with dilute acid, the severity factor ($\text{Log } R_o$) was determined using Mathematical 80 software, version 8.0.4.0, in order to evaluate the area of ‘time versus experiment temperature’ graphic using Simpson method.

Optimal conditions of the thermochemical pretreatment of young and old cellulosic fibers from date palms, which showed the higher percentage of holocellulose and minimum content in lignin, were then used for the subsequent enzymatic saccharification and fermentation processes.

2.4.2. Enzymatic Hydrolysis

The solid residues of both pretreated cellulosic fibers from date palm were enzymatically hydrolysed. Assays were performed in two batch reactors of Pyrex-glass jacketed, stirred-tank type, of 2 L capacity using citrate buffer solution (0.05 M and pH = 4.8). The enzymes used were cellulases, “Celluclast 1.5 L” commercial preparation of *Trichoderma reesei*, and β -glucosidase, “Novozym[®] 188” commercial preparation of *Aspergillus niger*, both provided by Novo Nordisk[®] Bioindustrial S.A. (Madrid, Spain). The cellulase activity of “Celluclast 1.5 L” was measured by filter paper assay and the activity was expressed in terms of Filter Paper Units (FPU) per gram of substrate. The enzymatic activity was 20 FPU per gram of substrate (cellulase activity) and 10 International Units (IU) per gram of substrate (β -glucosidase activity). Cellulase activity was determined following the standard procedure recommended by the Commission on Biotechnology, IUPAC [31]. During enzymatic saccharification, optimal conditions followed for young cellulosic fibers were 33.33 FPU of cellulases and 336.8 IU of β -glucosidase, and 36.6 FPU of cellulases and 356.4 IU of β -glucosidase for old cellulosic fibers. These experiments were carried out at 50 °C, during 72 h and under stirring conditions of 450 rpm. These operating conditions of the enzymatic hydrolysis processes were selected in previous works [7,29]. A sample of 2 mL was taken every 8 h for three days, in order to analyse TRS, D-glucose and acetic acid concentrations. Finally, the solid fraction was separated from the liquid by vacuum filtration and centrifugation using refrigerated centrifuge Jouan, Mod. MR 18 22. After, the solid fraction was washed, dried and characterised. On the other hand, the liquid hydrolysate was conducted to a fermentation process.

2.4.3. Fermentation

The non-traditional yeast, *P. tannophilus* ATCC 32691, was stored at 15 °C in 0.1 L test tubes on a sterilized solid culture medium that contained yeast extract (3 g/L), malt extract (3 g/L), casein peptone (5 g/L), D-xylose (10 g/L) and agar-agar (20 g/L). At the beginning of each experiment the microorganisms were inoculated under sterile conditions into glass test tubes and cotton closures containing the solid culture medium described above. These tubes were kept in an incubator at 30 °C for 72 h to obtain cells at the same growth stage for every experiment. Then, the yeast was resuspended into 100 mL of sterile ultrapure water until reaching an initial concentration of 0.1 g/L. Assays were performed in two discontinuous reactors, stirred-tank type, of 2 L of capacity. Experiments were carried out at 30 °C, pH = 4.5 and under stirring conditions of 450 rpm [32]. The hydrolysates (0.5 L) were enriched with the following nutrients: yeast extract (2 g/L), casein peptone (1.8 g/L), (NH₄)₂SO₄ (1.5 g/L), MgSO₄·7H₂O (1 g/L), KH₂PO₄ (1 g/L) [33]. During the fermentation, liquid samples of 5 mL were collected at different time intervals for being analysed in ethanol production and sugar consumption (D-glucose and TRS). In the fermentation processes D-glucose, D-xylose, L-arabinose, D-galactose, D-mannose and D-fructose concentration were also determined by high-performance-liquid-ionic-chromatographic (HPLIC) using an amperimetric detector, Dionex ICS-3000 (Sunnyvale, CA, USA).

3. Results and Discussions

3.1. Physico-Chemical Characterisation of Crude Fibers

The main component in both types of cellulosic fibers from date palm is the cellulose, then lignin and hemicelluloses (Table 1). In young cellulosic fibers, it was achieved a cellulose content of 57.42%, and 62.31% for old cellulosic fibers. These results were similar to those cited in a previous research [34]. In fact, old cellulosic fibers were more lignified, at 21.12%, than young ones, at 13.44%.

Table 1. Physicochemical characterisation of young and old cellulosic fibers.

Compounds	Young Fibers	Old Fibers
Moisture (%)	10.60 ± 0.19	8.19 ± 0.11
Cellulose (%) *	57.42 ± 1.09	62.31 ± 0.98
Hemicellulose (%) *	19.63 ± 1.59	5.80 ± 0.44
Lignin (%) *	13.44 ± 0.48	21.12 ± 1.13
Ash (%) *	3.32 ± 0.14	4.06 ± 0.30

* Percentages on dry basis.

However, the high percentage of lignin presents a real problem for access of lignocellulolytic enzymes into the interior lignocellulose structure [35]. Furthermore, the low percentage of mineral material in both types of cellulosic fibers explains that the raw material has a low capacity to produce ash during its combustion. Cellulosic fibers from date palm are the richest lignocellulose biomass in terms of cellulose compared to other biomasses, like: rosemary [36], olive stone [37], and olive pomace [38], which are more lignified than cellulosic fibers from date palm. It is also richer in cellulose content than wheat straw [39] and sugarcane bagasse [40].

Thus, this high content of cellulose of the cellulosic fibers from date palm waste makes it a potential source of cellobiose and fermentable monomeric sugars, which could be liberated during pretreatment and enzymatic hydrolysis steps, in order to produce bioethanol. In general, low concentrations of lignin, particularly in the cellulosic fibers from date palm, decrease the liberation inhibitors that block the activities of enzymes and microorganisms, during saccharification and fermentation processes, respectively [41].

3.2. Structural Characterisation of Cellulosic Fibers from Date Palm Fibers by ATR-FTIR

In order to track the structure of both young and old cellulosic fibers of '*P. dactylifera* L.' ATR-FTIR spectroscopy was employed to explore functional groups and molecular vibration detection (Figure 3). This method has the ability to identify or characterise both the organic and inorganic components of a sample. The idea behind this analysis method is that no two molecules that are structurally different will produce the same infrared spectrum, which is useful in positively identifying samples. Different samples have different compositions, resulting in various infrared absorption band intensities at different wavenumbers, which allows for the characterization and comparison of samples. In base of previous research bibliographic review [21,22,42], band identification was performed.

Many bands were associated with lignin for young cellulosic fibers from date palm (Figure 3, green lines); the bands located in the regions 3518 cm⁻¹ and 3697 cm⁻¹ were assigned to O-H stretching vibration. Indeed, another region at 2970 cm⁻¹ was attributed to C-H stretching vibration of lignin. On the other hand, the presence of extractive compounds in young cellulosic fibers from date palm was proved by stretching vibration bands of the functional groups C-O, C-C or C=C, and C=O observed at 1271 cm⁻¹, 1529 cm⁻¹ and 1730 cm⁻¹, respectively. Furthermore, the functional group associated with the free ester of hemicellulose of young cellulosic fibers from date palm was highlighted through the vibration bands located at 1726 cm⁻¹. Moreover, C-H bending vibration was shown at 1285 cm⁻¹, which was an indicator of the crystalline cellulose presence in young cellulosic fibers.

In relation to the spectrum obtained from old cellulosic fibers (Figure 3, yellow lines), it shows many functional groups of lignin located at different wavenumbers. In fact, bands located at 3578 cm⁻¹, 1665 cm⁻¹, 1583 cm⁻¹, 1513 cm⁻¹, 1467 cm⁻¹, 1280 cm⁻¹ and 835 cm⁻¹ correspond to O-H stretching vibration, C=O stretching vibration (unconjugated), aromatic ring and C=O stretching vibrations, C-C and C=C stretching vibrations, C-H deformation vibration, aromatic ring vibration of guaiacyl lignin, and O-H or C-H deformation of lignin, respectively. Moreover, the functional groups of extractives of old cellulosic fibers from date palm are shown through the vibration bands located at 1730 cm⁻¹, 1510 cm⁻¹ and 1271 cm⁻¹, which were attributed to C=O, C-C+ C=C, and C-O stretching vibrations, respectively. Likewise, hemicellulose and crystalline cellulose of old cellulosic fibers from date

palm are highlighted through their C=O stretching vibration bands (ketone/aldehyde) and C-H bending vibration bands, respectively, which were shown at 1726 cm^{-1} and 1280 cm^{-1} , in each case. Thus, the conclusions about chemical structure characterisation obtained from ATR-FTIR spectroscopy confirm the lignocellulose nature of both young and old cellulosic fibers. In fact, these results are similar to those reported from ATR-FTIR spectroscopy for date palm '*P. dactylifera* L.' leaflets and rachis in a hydrothermal pretreatment of this feedstock studied in order to enhance enzymatic digestibility and bioethanol potential [43].

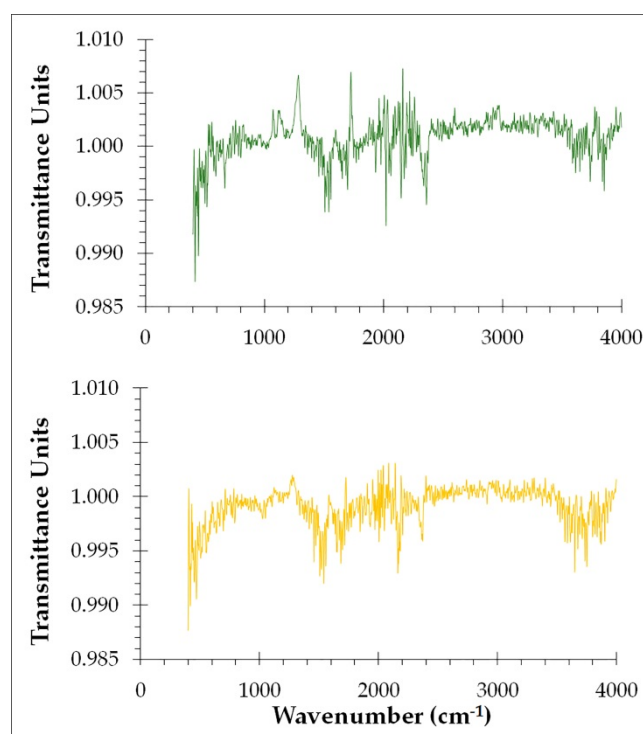


Figure 3. Attenuated Total Reflectance (ATR)-Fourier transform infrared spectroscopy (FTIR) spectrums of young (—) and old (—) lif fibers.

Similarly, in a previous study to improve bioethanol production from corn stover [44], the results reported from FTIR spectra were similar to those obtained during this study. The same tendency was shown from FTIR spectroscopy when greenhouse residues were used as raw material to improve methane production [45].

3.3. Thermochemical Pretreatment of Cellulosic Fibers from Date Palms

After thermochemical pretreatment, dry solid residues were characterised by the methods described in 'Characterisation of solid fraction', determining percentages in residual solids (S), ash (P_1), holocelluloses (P_2) and insoluble lignin (P_3) for both young and old cellulosic fibers from date palms (Tables 2 and 3, respectively). In relation to hydrolysates obtained (liquid fraction), they were analysed in D-glucose (C_1), TRS (C_2), and acetic acid (C_3) for both types of pretreated cellulosic fibers from date palm (Tables 2 and 3).

Table 2. Full factorial design matrix in the optimisation of thermochemical pretreatment conditions for young cellulosic fibers. Experimental responses (S , P_1 , P_2 , P_3 , C_1 , C_2 , C_3) and $\text{Log } R_0$.

Runs	T (°C)	$[\text{H}_2\text{SO}_4]$ (M)	$\text{Log } R_0$	S (%)	P_1 (%)	P_2 (%)	P_3 (%)	C_1 (g/L)	C_2 (g/L)	C_3 (g/L)
1	150.0	0.0000	2.69	93.00	2.92	31.81	61.53	0.26	0.94	0.00
2	220.0	0.0000	4.82	56.51	3.34	30.83	70.08	0.67	3.17	0.21
3	150.0	0.0250	4.87	69.83	2.18	29.44	59.05	0.34	3.98	0.03
4	220.0	0.0250	6.58	58.77	1.90	9.96	82.13	0.31	1.03	0.02
5	150.0	0.0000	3.64	99.23	2.66	29.70	60.43	0.16	1.44	0.00
6	220.0	0.0000	3.63	64.57	3.31	45.18	58.30	0.23	1.61	0.02
7	150.0	0.0250	5.94	77.38	3.90	25.68	58.72	0.29	1.56	0.18
8	220.0	0.0250	6.04	54.82	2.60	18.47	78.14	0.34	0.88	0.03
9	185.0	0.0125	6.07	89.65	1.43	10.61	82.17	0.22	1.00	-
10	185.0	0.0125	5.54	91.48	1.87	13.05	79.32	0.30	0.77	-
11	185.0	0.0125	6.07	96.36	1.98	13.35	81.15	0.21	0.88	-

$\text{Log } R_0$ (Severity factor), S (Residual solids content), P_1 (Ash percentage), P_2 (Holocellulose content), P_3 (AIL), C_1 (D-glucose concentration), C_2 (TRS concentration), C_3 (Acetic acid concentration).

Table 3. Full factorial design matrix in the optimisation of thermochemical pretreatment conditions for old cellulosic fibers. Experimental responses (S , P_1 , P_2 , P_3 , C_1 , C_2 , C_3) and $\text{Log } R_0$.

Runs	T (°C)	$[\text{H}_2\text{SO}_4]$ (M)	$\text{Log } R_0$	S (%)	P_1 (%)	P_2 (%)	P_3 (%)	C_1 (g/L)	C_2 (g/L)	C_3 (g/L)
1	150.0	0.0000	6.53	92.12	4.61	49.61	28.45	0.02	0.68	0.25
2	220.0	0.0000	6.80	62.46	1.70	61.97	37.22	0.10	2.06	0.39
3	150.0	0.0250	4.30	73.49	2.04	23.94	64.66	0.26	0.74	0.27
4	220.0	0.0250	5.58	59.37	4.98	19.68	74.65	0.62	1.87	0.27
5	150.0	0.0000	5.10	90.92	7.28	67.00	27.49	0.02	1.61	0.23
6	220.0	0.0000	6.65	62.57	3.74	58.08	39.08	0.08	1.89	0.56
7	150.0	0.0250	7.24	70.34	4.73	39.18	54.26	0.55	0.98	0.28
8	220.0	0.0250	6.59	61.50	4.71	13.23	77.58	0.06	1.17	0.31
9	185.0	0.0125	6.23	95.26	1.25	17.80	78.09	0.03	0.83	0.30
10	185.0	0.0125	6.11	94.16	2.66	17.52	75.46	0.04	0.75	0.32
11	185.0	0.0125	6.97	94.58	2.22	17.35	70.07	0.03	0.74	0.25

$\text{Log } R_0$ (Severity factor), S (Residual solids content), P_1 (Ash percentage), P_2 (Holocellulose content), P_3 (AIL), C_1 (D-glucose concentration), C_2 (TRS concentration), C_3 (Acetic acid concentration).

In the case of young cellulosic fibers, higher values of severity factor ($\text{Log } R_0$) were reached due to severe conditions (greater concentrations of sulfuric acid and higher temperatures during pretreatment). Furthermore, as the pretreatment temperature was increased, lower residual solids content (S) values were determined (Table 2); therefore, this implied a higher percentage of solids solubilisation. This fact could be attributed to the hemicelluloses hydrolysis. In relation to the percentage of insoluble lignin (P_3), it was higher in severe conditions; in fact, the maximum percentage of AIL, 82.13%, was achieved when $\text{log } R_0 = 6.58$ (220 °C, H_2SO_4 0.05 M). The minimum percentages in holocelluloses were reached at 185 °C and H_2SO_4 0.0125 M in the central point of experimental design (10.61%, 13.05% and 13.35%), Table 2; however, a minimum in holocelluloses, close to 10.0%, was reached at 220 °C and H_2SO_4 0.025 M. According to the full factorial experimental design matrix, Figure 2, the maximum concentrations of TRS, 3.17 g/L and 3.98 g/L, were determined at 220 °C and without sulfuric acid, and 150 °C and H_2SO_4 0.025 M, respectively, Table 2.

During old cellulosic fibers thermochemical pretreatment, the higher values of residual solids content (95.26%, 94.16% and 94.58%) were achieved at 185 °C and concentration of sulfuric acid 0.0125 M, Table 3; these medium conditions could be considered severe.

In general, when the pretreatment was carried out at fixed of H_2SO_4 concentration, the percentage in holocellulose decreased as the temperature was increased; percentages of holocellulose contents were maximum at 185 °C and H_2SO_4 0.0125 M in the central point (17.80%, 17.52% and 17.35%) (Table 3). On the other hand, logically, contrary behavior was observed respect to insoluble lignin content; in fact, percentage of IAL increased at higher temperatures. Moreover, at 220 °C and hydrothermal conditions (0.0 M H_2SO_4), the percentage of holocelluloses and lignin was 61.97% and 37.22%, respectively (Table 3).

These data proved that severe conditions of thermochemical pretreatment enhanced the digestibility of the complex carbohydrates in order to produce monomeric sugars. In fact, the major concentration of reducing sugars, 2.06 g/L, was achieved in the thermochemical pretreatment carried out at 220 °C and H₂SO₄ 0.0 M (Table 3).

Maximum percentages in holocellulose were reached in hydrothermal conditions, at 220 °C for young pretreated cellulosic fibers (45.18%) and 150 °C for old pretreated cellulosic fibers (67.00%) (Tables 2 and 3, respectively). The minimum percentages of insoluble lignin were achieved in hydrothermal conditions, at 220 °C for young pretreated (58.30%), and 150 °C in case of old pretreated cellulosic fibers (27.49%). Fang et al., in at hydrothermal pretreatment of the date palm *Phoenix dactylifera* L. leaflets and rachis, determined at 210 °C the highest lignin content in pretreated leaflets (64.95%) and in pretreated rachis (45.95%) [43].

The maximum concentration of acetic acid, 0.56 g/L, was reached for old fibers at 220 °C and H₂SO₄ 0.0 M (severity factor = 6.65), while D-glucose concentration in these conditions was very small, 0.08 g/L.

The relationship between each response (log R_0 , S , P_1 , P_2 , P_3 , C_1 , C_2 and C_3) and each factor (temperature and sulfuric acid concentration) was expressed referring to the coded model described in section ‘Experimental methodology’. Tables 4 and 5 show the empirical equations for each response and the adequate optimal conditions for the thermochemical pretreatment of young and old cellulosic fibers. By way of example, the adjustments made for the fraction of residual solids and holocellulose contents are represented in Figures 4 and 5, respectively.

Table 4. Empirical equations for each response and adequate optimal conditions for the thermochemical pretreatment of young cellulosic fibers (°C, M).

Response	Empirical Equations	Optimal Conditions (T , [H ₂ SO ₄])
Percentage of residual solids	$S = 153.2 - 0.4T - 525.1[\text{H}_2\text{SO}_4]$	150 °C, 0 M
Ash content	$P_1 = 3.1 - 0.0T - 16.5[\text{H}_2\text{SO}_4]$	150 °C, 0 M
Holocellulose content	$P_2 = 38.3 - 0.0T - 539.7[\text{H}_2\text{SO}_4]$	220 °C, 0 M
Acid-insoluble lignin content	$P_3 = 34.308 + 0.175T + 277.0[\text{H}_2\text{SO}_4]$	220 °C, 0.025 M
D-glucose concentration	$C_1 = -0.0226 + 0.0018T - 0.4[\text{H}_2\text{SO}_4]$	220 °C, 0 M
Total reducing sugars	$C_2 = 2.3455 - 0.004395T + 2.9[\text{H}_2\text{SO}_4]$	150 °C, 0.025 M
Acetic acid concentration	$C_3 = 0.01125 + 0.00025T + 0.3[\text{H}_2\text{SO}_4]$	220 °C, 0.025 M

Table 5. Empirical equations for each response and adequate optimal conditions for thermochemical pretreatment of old cellulosic fibers (°C, M).

Response	Empirical Equations	Optimal Conditions (T , [H ₂ SO ₄])
Percentage of residual solids	$S = 136.8 - 0.35T - 433.7[\text{H}_2\text{SO}_4]$	150 °C, 0 M
Ash content	$P_1 = 6.07 - 0.013T - 8.7[\text{H}_2\text{SO}_4]$	150 °C, 0 M
Holocellulose content	$P_2 = 70.0 - 0.0T - 1406.0[\text{H}_2\text{SO}_4]$	220 °C, 0 M
Acid-insoluble lignin content	$P_3 = 4.18 + 0.19T + 1389.1[\text{H}_2\text{SO}_4]$	220 °C, 0.025 M
D-glucose concentration	$C_1 = -0.0008 + 0.0T + 12.7[\text{H}_2\text{SO}_4]$	220 °C, 0.025 M
Total reducing sugars	$C_2 = -0.57 + 0.01T - 14.8[\text{H}_2\text{SO}_4]$	220 °C, 0 M
Acetic acid concentration	$C_3 = 0.019 + 0.002T - 3.0[\text{H}_2\text{SO}_4]$	220 °C, 0 M

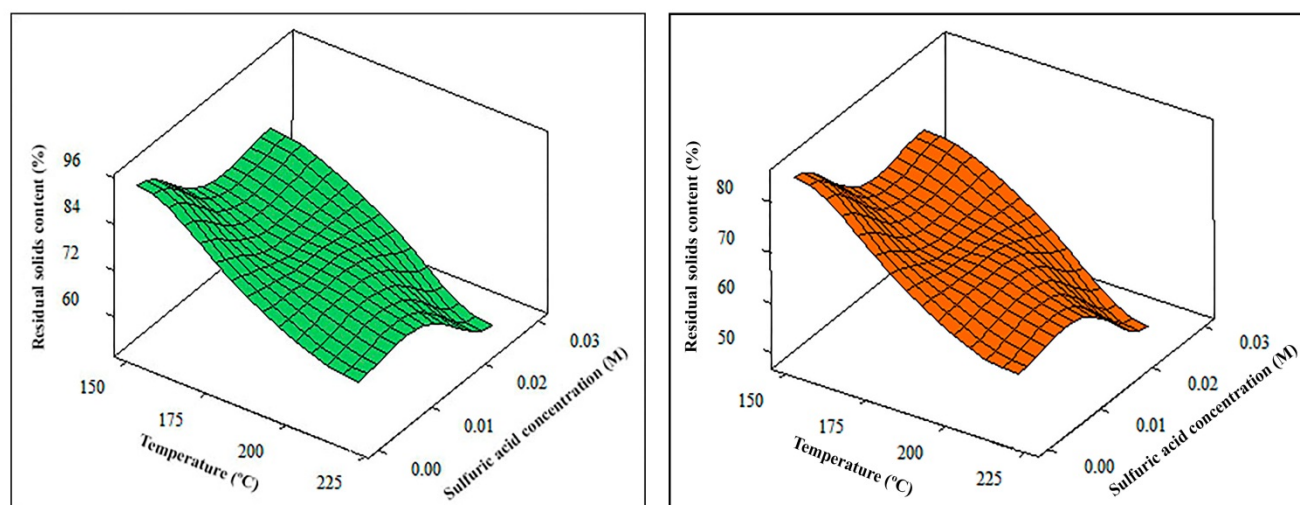


Figure 4. Response superficial for the solid recovery percentage in function of sulfuric acid concentration and temperature for young (■) and old (■) life during thermochemical process.

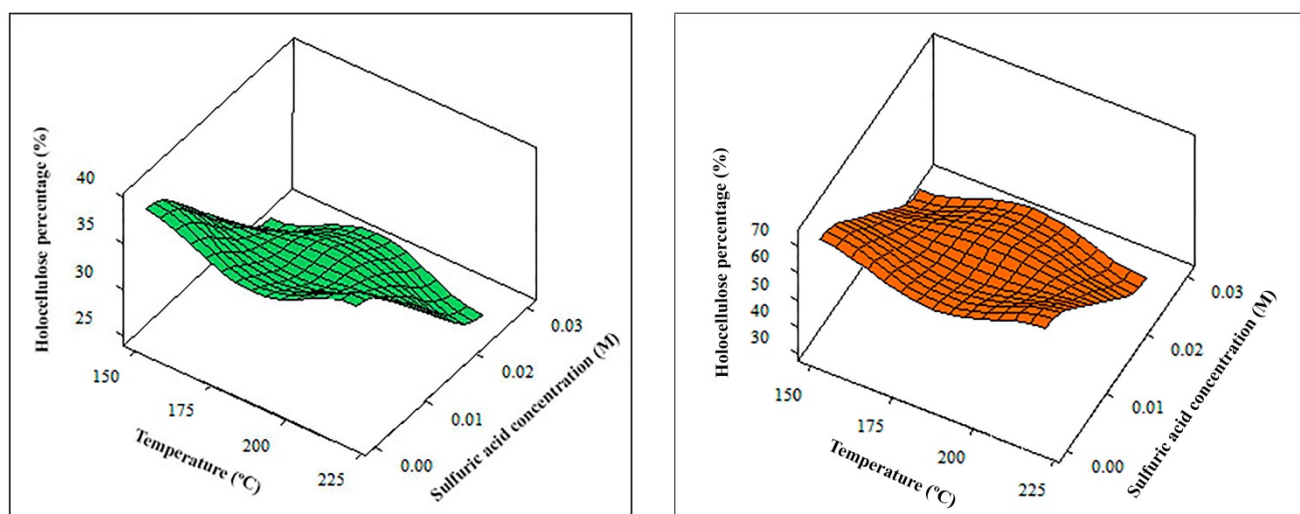


Figure 5. Holocellulose content in function of sulfuric acid concentration and temperature for young (■) and old (■) cellulosic fibers from date palm during thermochemical process.

Comparing the results obtained for the pretreatment of young and old cellulosic fibers with other lignocelluloses feedstock, it is possible to conclude that our substrate is the best one to conduct the subsequent enzymatic saccharification. In this sense, the solid fractions of the olive stones pretreated at 220 °C with a solution of sulfuric acid 0.025 M, are composed by 56.8% of lignin and 17.4% of holocelluloses [37]. On the other hand, rosemary fibers, pretreated at 150 °C and with 0.025 M of sulfuric acid, generated 17.0% of lignin and 81.0% of holocelluloses [36].

As a result of the previous experiments of both young and old cellulosic fibers, the optimal pretreatment conditions prior to the enzymatic hydrolysis are 220 °C without acid; it is justified due to the significant amount of holocellulose under operation conditions (Tables 2 and 3). These results are similar to those obtained in a previous research refereed to hydrothermal pretreatment of date palm *Phoenix dactylifera* L. leaflets and rachis [42]; in this case, the process was carried out under optimal conditions of hydrothermal pretreatment (210 °C for 10 min), these being the best conditions to enhance enzymatic digestibility and later bioethanol production.

3.4. Enzymatic Hydrolysis

Pretreatment is a step to improve the enzymatic digestibility of highly crystallised lignocellulosic biomass [46]. Thus, in order to produce D-glucose, an enzymatic hydrolysis of the solid fractions from the pretreatment step has been carried out, which was performed under optimal conditions at 220 °C and H₂SO₄ 0.0 M (maximum TRS concentrations). During the enzymatic hydrolysis, liquid samples were taken in order to analyse the concentrations of TRS and D-glucose, for both young and old cellulosic fibers (Figure 6); moreover, acetic acid concentration was determined for each type of cellulosic fibers.

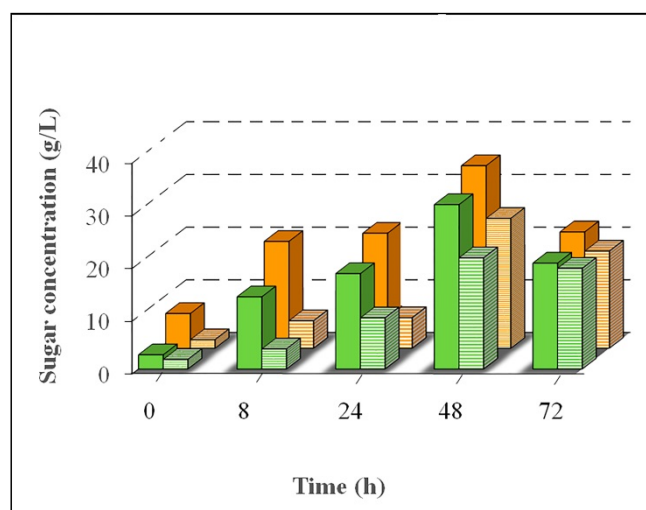


Figure 6. Total reducing sugar concentration (non-striped shape) and D-glucose concentration (striped shape) during enzymatic hydrolysis of young (■) and old (■) lufs, at 50 °C, and 72 h under stirring conditions of 450 rpm.

At the beginning of enzymatic hydrolysis TRS concentration increased. After a 48 h process, TRS concentrations decreased for both pretreated young and old cellulosic fibers (Figure 6). In fact, in the case of young cellulosic fibers, initial TRS concentration, 2.7 g/L, increased to 31.2 g/L at 48 h and decreased to 20.1 g/L after 72 h of enzymatic hydrolysis (Figure 6). The same tendency was observed in D-glucose concentration for both young and old cellulosic fibers (Figure 6). During enzymatic hydrolysis of pretreated old cellulosic fibers, initial D-glucose concentration, 1.6 g/L, increased until reaching 24.6 g/L at 48 h; from this point of the process a decrease of D-glucose concentration was observed (18.4 g/L at 72 h), Figure 6.

These diminutions can be due to the inhibitors (among them acetic acid and furfural) released during the pretreatment stage, or could it be due to microbial (contaminant) activity, such as an unwanted fermentation of D-glucose. The inhibitors presence has a negative influence in the process since it reduces the activity of cellulases and, with it, the production of D-glucose [47].

During enzymatic hydrolysis, maximum TRS yields (Y_{TRS}) were reached at 48 h for both pretreated cellulosic fibers from date palms, 0.46 g TRS/g dry pretreated young fiber, and 0.49 g TRS/g dry pretreated old fiber. Furthermore, maximum D-glucose yields (Y_G) were determined at 48 h for both young and old cellulosic fibers (0.31 g D-glucose/g dry pretreated young fiber and 0.35 g D-glucose/g dry pretreated old fiber, respectively).

During enzymatic hydrolysis, acetic acid concentration increased until reaching 2.5 g/L and 2.1 g/L at 72 h for young and old cellulosic fibers, respectively.

3.5. Fermentation Process

Fermentation assays of hydrolysates of pretreated young and old cellulosic fibers, derived from the enzymatic hydrolysis, were carried out by *P. tannophilus* yeast. The

concentrations of D-xylose, L-arabinose, D-mannose and D-galactose were determined at the beginning and end of the fermentation processes. Their concentrations were very small and at the end of the fermentation process it was found that they were completely consumed except for D-xylose and L-arabinose (Table 6).

Table 6. Sugar concentrations during the fermentation processes of young and old hydrolysed fibers.

Hydrolysed Fibers	Time (h)	D-Glucose (g/L)	L-Arabinose (g/L)	D-Xylose (g/L)	D-Galactose (g/L)	D-Manose (g/L)
Young	0	38.10	0.41	0.62	0.13	0
	71	0.32				
	96		0.40	0.56	0.13	0
Old	0	33.61	0.41	0.61	0.13	0
	71	2.05				
	96		0.41	0.56	0.14	0

During the fermentation of the hydrolysates by *P. tannophilus* yeast, ethanol was produced until reaching a maximum concentration, 12.40 g/L for young fibers and 10.20 g/L for old fibers, after 6 h and 7 h of fermentation. After that, there was a decrease in ethanol concentration, which could be explained by the evaporation of this product as well as its possible consumption by the yeast (metabolic routes).

If the complete process is considered, from the initial raw material to the bioproduct production, ethanol yields (Y_E) were 10.64 g ethanol/100 g of dry fiber for young fibers and 10.88 g ethanol/100 g of dry fiber for old fibers (Figure 7). These values are similar to those obtained for ethanol yields in the fermentation of olive stones by *P. tannophilus* [37].

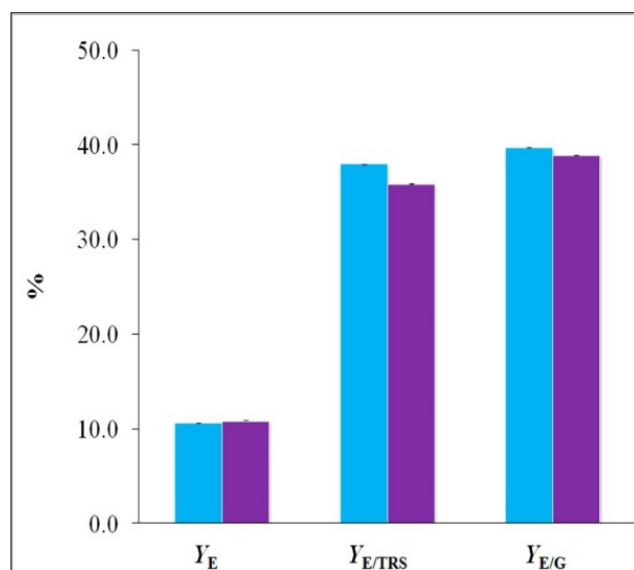


Figure 7. Ethanol yields in relation to initial raw material (Y_E) and overall ethanol yields respect TRS and D-glucose consumption ($Y_{E/TRS}$, $Y_{E/G}$), for young (■) and old (■) fibers during fermentation process.

Ethanol yields referred to TRS ($Y_{E/TRS}$) and D-glucose consumption ($Y_{E/G}$) were 37.94 g ethanol/100 g TRS and 39.68 g ethanol/100 g D-glucose, respectively, for young cellulosic fibers from date palm; in case of old cellulosic fibers, it was reached 35.84 g ethanol/100 g TRS and 38.90 g ethanol/100 g D-glucose. It should be noted that these obtained yields are high, and considering the theoretical yield (or Gay-Lussac yield) referred to D-glucose (0.511 g ethanol/g D-glucose) the $Y_{E/G}$ for young fiber represents 77.7% of the theoretical yield and for old fiber 76.1%. In previous studies that referred to microbial fermentation of juice extracted from sugarcorn plants in order to produce ethanol [48], it was reached similar values in ethanol yield. In fact, sugarcorn juice used in the study

contained a maximum of 145 g/L carbohydrates, with sucrose, D-glucose and D-fructose accounting for 80% of the sugars. In the fermentation of this juice by *S. cerevisiae* it was determined similar values, 45.6 g/L ethanol in 72 h (ethanol yield = 0.41 g ethanol per g carbohydrates).

Nowadays, there are some studies that improve D-xylose assimilation by yeasts which is the more abundant component in plant cells, which can lead to higher bioethanol productivity [49].

This study proves that biofuel yield from date palm waste via fermentation is high, as recent research also shows [50].

4. Conclusions

Lignocellulose fibers have physical and thermal advantages for industrial application in energy production. In fact, this research highlights that waste of date palm tree *P. dactylifera* L. could be an alternative to produce second-generation bioethanol.

Therefore, this byproduct should be developed for green energy production. The optimal conditions for thermochemical pretreatment were 220 °C without addition of sulfuric acid for both young and old cellulosic fibers. In general, D-glucose generation was easier in young fibers, and under higher severity factors. Maximum concentration of acetic acid generated was 0.56 g/L for the thermochemical pretreatment of old cellulosic fibers carried out at 220 °C and without acid (severity factor = 6.65), while D-glucose concentration in these conditions was very small, 0.08 g/L.

After 48 h of enzymatic hydrolysis, a maximum concentration of TRS, 31.2 g/L and 34.6 g/L for young and old cellulosic fibers was achieved, respectively; maximum TRS yields (Y_{TRS}) were reached at 48 h for both pretreated cellulosic fibers: 0.46 g TRS/g dry pretreated young fibers, and 0.49 g TRS/g dry pretreated old fibers.

During the ethanolic fermentation of the hydrolysates by *P. tannophilus* (ATCC 32691), a yield (Y_E) of 10.64 g ethanol/100 g of dry fiber for young cellulosic fibers and 10.88 g ethanol/100 g of dry fiber for old cellulosic fibers was reached.

These results obtained in this study show that cellulosic fibers from date palm would be an alternative waste with great potential for ethanol production.

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Abbreviations

ADF	Acid detergent fiber
AFEX	Amonia Fibre Explosion
ATCC	American Type Culture Collection
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
DNS	3,5-Dinitrosalicylic acid
FPU	Filter Paper Cellulase Units
AIL	Acid-Insoluble Lignin
NDF	Neutral detergent fiber
TAPPI	Technical Association of Pulp and Paper Industry
TRS	Total Reducing Sugars

Variables

A_i	Means either high (+) or low (−) level in experimental run i
C_1	D-glucose concentration [g/L]
C_2	Total reducing sugars content [g/L]
C_3	Acetic acid concentration [g/L]
C_r	Regression coefficient
E_a	Apparent activation energy (78.4 kJ/mol)
k	Number of factors ($k = 2$)
Log R_o	Severity factor
P_1	Ash measurement [%]
P_2	Holocellulose content [%]
P_3	Lignin content [%]
R	Universal gas constant [0.008314 kJ/(mol K)]
R_o	Severity
S	Residual solids content [%]
T_r	Reference temperature for an initial process time $t = 0$ (100 °C)
$T(t)$	Function that relates temperature to residence time [°C]
X_i	Factor in Equation (2)
Y	Predicted response value
Y_E	Ethanol yield respect initial substrate [g/g]
$Y_{E/G}$	Ethanol yield respect D-glucose consumption [g/g]
$Y_{E/TRS}$	Ethanol yield respect TRS consumption [g/g]
Y_G	D-glucose yield [g/g]
Y_j	Response value for which C_{rj} is determined (where j varies from 1 to 7) [% , g/L]
Y_{TRS}	Total reducing sugar yield [g/g]
b_o	Constant coefficient
b_i	Linear coefficients
t	Residence time in reactor [min]
w	Weigh [g]
w_g	Fixed parameter (14.75)
\emptyset	Particle diameter [mm]

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