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High Solid and Low Cellulase Enzymatic Hydrolysis of Cardoon Stems Pretreated by Acidified γ-Valerolactone/Water Solution

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Abstract: Lignocellulosic biomass is a nonedible matrix that can be efficiently exploited as feedstock in an integrated biorefinery after a proper pretreatment. An organosolv pretreatment using an acidified γ-valerolactone (GVL)/water solution was proposed to improve the cellulose enrichment and enzymatic saccharification of cardoon (*Cynara cardunculus* L.) stems. At the optimal pretreatment condition (140 °C, 0.6 GVL/water, and 2.24% H_2SO_4), xylan was efficiently removed from the cardoon, and up to 50% of its content was recovered in the aqueous fraction, while 86% of the cellulose was retained in the solid fraction. The resulting cardoon pulp showed a cellulose content of 91.5% and an enzymatic digestibility of 100%. An overall glucose production of 37.17 g/100 g raw material (90% theoretical maximum) was obtained using high solid loading (20% *w*/*w*) and a high enzyme dosage (60 FPU/g cellulose). At a low enzyme dosage, glucose concentrations of 169 g/L and 210 g/L were achieved using 10 FPU/g cellulose and 20 FPU/g cellulose, respectively. Therefore, an organosolv pretreatment can be an effective process for producing cellulose-enriched pulp with enhanced enzymatic digestibility from cardoon stems, providing a promising option for green lignocellulosic biorefineries that aim to produce high concentrations of glucose with low cellulase addition.

Keywords: γ-valerolactone/water delignification; cellulose enrichment; high solid loading enzymatic hydrolysis; Cynara cardunculus; low cellulase addition; microwave-assisted extraction (MAE)

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1. Introduction

Over the last few years, growing attention has been focused on biofuel and biochemical production using renewable biomass as feedstock [1]. The goal is to reduce fossil fuel dependence to mitigate the negative environmental effects caused by their exploitation [2]. Lignocellulose is an abundant, low-cost type of biomass, as it is the main component of the plant cell wall, and its total amount accounts for 30–50% of the total dry weight of plants [3]. Lignocellulose is found in a wide variety of agricultural, forestry, municipal, and industrial waste, as well as in energy crops that grow in marginal lands [4]. In particular, cardoon is a multipurpose and versatile Mediterranean crop adapted to seasonal changes with a wide spectrum of potential applications due to its added value as a rich source of fibers, oils, and bioactive compounds [5]. For crops receiving around 500 mm annual rainfall, the aboveground biomass was reported in the range of 10–20 Mg ha^{-1} $year^{-1}$ (dry weight) with a stem fraction equal to 30% [6]. Therefore, cardoon stems (CSs) could be an important source of lignocellulose for the production of monosaccharides, which can be further used to produce not only biofuels and biochemicals but also high-quality lignin for added-value applications [6–8]. Lignocellulose can be converted to fermentable sugars through enzymatic hydrolysis, which uses cellulase to hydrolyze the cellulose [9]. However,

lignocellulose is recalcitrant to enzymatic attack since its three main components (cellulose, hemicellulose, and lignin) are arranged in a quite complex and tight structural network. Therefore, a pretreatment is generally required before performing enzymatic hydrolysis in order to remove the noncellulose component and enhance cellulose accessibility to enzymes, which results in improved saccharification [10,11].

These aspects are of great relevance in the context of a green lignocellulosic biorefinery, where low cellulase loadings and high cellulose loadings are required for the reduction of process cost, making the whole saccharification process economically viable [12–14].

An organosolv (OV) process could represent an economically viable and sustainable way to overcome biomass recalcitrance [15]. OV is a chemical pretreatment that employs either pure or aqueous mixtures of different organic solvents, such as methanol, ethanol, acetone, acetic acid, glycerol, and γ-valerolactone (GVL). Interestingly, the organic solvents previously mentioned can be derived from renewable sources [16].

OV pretreatment solubilizes lignin and hemicellulose, yielding a cellulose-rich residue. By adding a small amount of acid catalyst to an OV pretreatment mixture, cellulose with a low degree of polymerization is obtained, enhancing the hydrolysis rate [17]. Thanks to lignin removal, the nonspecific binding among cellulase and lignin is avoided, and a low dosage of enzymes can be employed during the enzymatic hydrolysis process [18]. Therefore, the cellulose-rich residue obtained after an acidified OV pretreatment is an adequate matrix for high solid loading saccharification. After an OV pretreatment, the lignin and the hemicellulose are recovered as technical lignin and monosaccharides, respectively, and the solvent can be easily regenerated [19].

GVL is a green solvent that exhibits an excellent dissolving efficiency for lignin [20]. GVL is obtained from lignocellulosic-derived sugars, and due to its high boiling point and hypotoxicity, it is an excellent candidate for lignocellulosic biomass delignification [21]. Recently, Gelosia et al. obtained a cellulose-rich pulp through deconstruction of biomass in a GVL/water solution at mild temperatures (150 °C) using diluted concentrations of sulfuric acid (0.2 M). The pretreated substrate was enzymatically hydrolyzed at low solid loading and high cellulase loading with a glucose yield of 30.17 g per 100 g of raw material (89% of the maximum theoretical yield) [22]. Notably, the cellulose digestibility was higher than other cardoon pretreatment processes, such as acid-catalyzed steam explosion (70%) [23], diluted sulfuric acid (68% and 78%) [24,25], and steam explosion (64%) [26], and it was even higher than a similar OV process based on the use of an ethanol/water mixture as a pretreatment solvent (72%) [27].

In the present study, a microwave-assisted OV pretreatment using a GVL/water solution with the addition of 0.2 M H_2SO_4 was proposed to obtain cellulose-rich pulp with improved enzymatic digestibility from CSs. The OV process was optimized by means of the design of experiment (DoE), which generated response surface models of the three responses (cellulose enrichment, cellulose yield, and cellulose digestibility) as a function of two factors (temperature and GVL concentration in water). This first part is a confirmatory analysis of a recent study performed by the same authors [28]. Finally, high solid loading (up to 20%) enzymatic hydrolysis at relatively low cellulase addition (10 and 20 FPU/g cellulose) was performed for the evaluation of the overall glucose production.

2. Materials and Methods

2.1. Materials

The *Cynara cardunculus* L. (var. *altilis* DC) used in this work was provided by Matrica SpA (Porto Torres, Italy). The plant stems were ground using a laboratory ultracentrifuge mill (RETSCH, Haan, Germany) and a rotary blade mill (RETSCH, Haan, Germany) to obtain two different types of particles with a diameter <0.5 and <1 mm for characterization and OV pretreatment, respectively. These particle sizes were obtained using a 0.5 mm sieve and a 1 mm sieve. The moisture content was measured using an HB43-S Halogen Classic Plus Analyzer (Mettler Toledo, Columbus, OH, USA). Monosaccharide concentrations were analyzed using ultra-high performance liquid chromatography (HPLC) with a Dionex

UltiMate 3000 system (Thermo Fisher Scientifc, Sunnyvale, CA, USA) equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA). The flow rate and temperature were set at 0.6 mL/min and 50 °C, respectively. The refractive index detector, an ERC RefractoMax 520 (Thermo Fisher Scientific, Waltham, MA, USA), was set at 50 °C. The mobile phase was a 0.01 N H_2SO_4 water solution.

An Ethos One Microwave Digestion System (Milestone Inc. Srl, Sorisole (BG), Italy) was used for microwave-assisted OV pretreatment. The solvents used were GVL (Sigma-Aldrich, Saint Louis, MO, USA) and deionized water obtained from an ELGA Purelab Option system (ELGA LabWater, High Wycombe, UK). Enzymatic hydrolysis was performed inside a thermostatic incubator shaker (KS 4000i control, IKA®, Staufen, Germany) using a Cellic® CTec2 enzymatic cocktail with a filter paper activity of 250 filter paper cellulase units (FPU)/ mL (Merck, Darmstadt, Germany).

2.2. Design of Experiment Approach

A DoE was generated using Minitab17 statistical analysis software (Minitab, Coventry, UK). The full factorial design consisted of 3 levels (−1, 0, and +1) for 2 factors: temperature and concentration of GVL (*w*/*w*) in the mixture used for the OV process. The reaction conditions were based on preliminary work performed by the same authors [28]. In particular, the upper and lower levels and the central value of the factors considered are given in Table 1.

Table 1. Full factorial design factors and levels used for the cardoon stem fractionation process.

Factor	−1	0	+1
Temperature	140	150	160
GVL/water (*w*/*w*)	0.55	0.65	0.75

The models resulting from the experimental tests were used to predict the optimal conditions for maximum cellulose recovery and enrichment in the CSs.

2.3. Microwave-Assisted OV Pretreatment

The pretreatment was performed with 2.5 g of 1 mm ground CS and 24.43 g GVL/water mixture in a PTFE vessel. Based on previous work, the pretreatment time was set for 30 min (10 min cooling), and 0.571 g of 98% (*w*/*w*) H_2SO_4 was added as a catalyzer to the mixture (final acid concentration of 2.24% *w*/*w*) [28]. The CSs were pretreated at different temperatures and GVL/water mixtures following the experimental design generated.

After OV pretreatment, the vessel content was vacuum-filtered using a Büchner flask to separate the solid cellulose pulp (CP) fraction from the GVL/water acid solution. This liquid fraction was collected and quantified. The CP was then washed using the same volume and concentration of GVL/water solution used in the pretreatment to ensure maximum lignin removal. Finally, the CP was washed using 350 mL deionized water to ensure GVL removal for proper enzymatic hydrolysis. The CP was washed with another 50 mL fresh deionized water, which was then analyzed by HPLC to confirm the absence of GVL.

The aqueous fraction was separated from the collected liquid fraction using a saturating amount of NaCl, and the monosaccharide content was analyzed by HPLC after a proper dilution for reducing the salinity. The optimal condition samples were washed using 350 mL of 45 mM citrate buffer to ensure pH maintenance in the high solid loading enzymatic hydrolysis.

2.4. Enzymatic Hydrolysis

Enzymatic hydrolysis was performed in a 100 mL Duran bottle using the CP resulting from the OV process. In order to ensure pH maintenance and avoid bacterial activity, the CP was further washed with 45 mM citrate buffer, to which was added a specific volume of

sodium azide (NaN_3) solution as a bacteriostatic agent, for 1 h at 40 °C with a solid:liquid ratio of 1:10.

The CP obtained from each DoE run underwent enzymatic hydrolysis with a solid loading of 1% (*w*/*w*) with a 50 mL reaction volume. The cellulase loading was 60 FPU/g cellulose to provide an indication of the maximum enzymatically accessible cellulose content. An amount of 0.2 mL sodium azide (5%) solution was added as a bacteriostatic substance. A solution of 45 mM buffer citrate (pH 5) was used for pH maintenance and reaching the reaction volume. The enzymatic hydrolysis process was performed at 50 °C for 72 h under agitation at 200 rpm.

The enzymatic hydrolysis of CP obtained from optimal conditions was performed using three different solid loadings (5, 12.5, and 19% *w*/*w*) and enzyme concentrations: low dosage (10 FPU/g cellulose), medium dosage (20 FPU/g cellulose), and high dosage (60 FPU/g cellulose). The final mass of the reaction given by sum of CP, buffer, sodium azide solution, and cellulase was equal to 10 g. During the test, 100 µL samples were taken from the reaction solution at intervals of 24 h to analyze the monosaccharide concentration by HPLC. Samples were only taken after the solutions achieved complete liquefaction, and the achievement time differed from sample to sample.

After enzymatic hydrolysis, all the hydrolysates were separated from the residual solid fraction by filtering through a Büchner vacuum flask and then diluted in 100 mL water before HPLC analysis for monosaccharide concentration.

2.5. Laboratory Analytical Procedure

All the characterizations were conducted in triplicate following the procedure of the National Renewable Energy Laboratory (NREL, Golden, CO, USA) analytical methods for biomass [29]. The raw material (RM) (water content of 5.18% *w*/*w*) was characterized by analyzing cellulose, xylan, lignin, extractives, pectin, acetyl groups, and ash content, and the result are shown in Table 2. The CP was characterized in terms of cellulose, xylan, and lignin content. By acid hydrolysis with H_2SO_4 of the solid samples (RM and CP), C5 and C6 monosaccharides were obtained from cellulose and xylan polymers, respectively. After appropriate dilution, the monosaccharide contents were analyzed by HPLC and corrected for anhydrous factors of 0.88 (C5) and 0.90 (C6). The solid residue from the acid hydrolysis was used to calculate the acid-insoluble lignin after removing the ash content by gravimetric analysis. Equation (1) was used to calculate cellulose recovery (CY) after the OV pretreatment:

$$CY = C_{CP}/C_{RM} \quad (1)$$

where C_{CP} is the cellulose content (g) in CP after the OV pretreatment, and C_{RM} is the cellulose content (g) in RM before the OV pretreatment.

Table 2. *Cynara cardunculus* L. composition before the OV pretreatment.

Cellulose (g/100 g RM)	Xylan (g/100 g RM)	Acetyls (g/100 g RM)	Lignin (g/100 g RM)	Extractives (g/100 g RM)	Ash (g/100 g RM)
37.28 ± 0.36	15.33 ± 0.09	2.38 ± 0.03	22.63 ± 2.00	9.47 ± 0.36	6.42 ± 0.21

The following Equation (2) was used to calculate the enzymatic hydrolysis yield (HY):

$$HY = G_h \times V \times 0.9/C_{CP} \quad (2)$$

where G_h is the glucose concentration (g/L), and V is the volume (L) of the hydrolysate.

The overall yield (OY) expressed as grams of glucose per 100 g RM was calculated by Equation (3):

$$OY\ (g) = 100 \times C \times CY \times HY/0.9 \quad (3)$$

where C is the cellulose content (g) in 1 g RM.

3. Results and Discussion

3.1. Characterization of CP and Aqueous Fraction Obtained from OV PreTreatment

The chemical characterization of CPs obtained after the OV pretreatment showed interesting findings. The xylan was efficiently hydrolyzed, so its content in the CP was negligible, ranging from 0% to 1%. The lignin content was very low in all the samples due to its solubilization in the GVL phase. As a result, the cellulose content in the CP ranged from 68% to 93% with a medium and median value of 86% and 89%, respectively.

In eight samples, the cellulose recovery was higher than 70%. Therefore, after an optimization process, CP with a high cellulose content without sacrificing cellulose recovery was technically achievable.

These results showed that the acidified GVL/water solution can be efficiently employed for biomass delignification at mild process temperatures. The process temperature supports lignin solubilization in GVL [30], while the acid catalyst in the aqueous fraction promotes the hydrolysis of xylan [31,32].

Delignification improved the accessibility of cellulose to cellulase due to the removal of the spatial obstacle formed by both lignin and hemicellulose [33]. As a matter of fact, the saccharification performed at high enzyme dosage (60 FPU/g cellulose) and low solid loading (1% w/w) showed an HY higher than 93% for all CPs, meaning the cellulose was completely accessible to cellulase action. This enzymatic hydrolysis conversion efficiency is an extraordinary result, since it was higher than ones that have been obtained using several pretreatment technologies, such as alkali (HY $\approx$ 80%), acid (HY $\approx$ 76%), steam explosion (HY = 70–90%), liquid hot water (HY $\approx$ 85%), and ammonia fiber expansion (HY = 80–90%) [34]. Meng et al. studied the effect of GVL on cellulose, showing that its digestibility was highly increased due to crystallinity reduction and augmented porosity promoted by the solvent [35].

This result is encouraging for performing tests at low enzyme dosages and high solid loading, since the only variables able to reduce the HY should be the mass transport limitation and the end-product inhibition [36,37].

A complete table with all the figures for cellulose enrichment, recovery, and digestibility is provided in the Supplementary Material.

Table 3 shows xylan-derived xylose concentration and cellulose-derived glucose concentration of the aqueous fraction obtained after separation from GVL by the addition of salt. The concentrations were used to calculate the masses of cellulose (CW) and xylan (XW) recovered in the aqueous fraction respective to their initial contents (Table 2).

Table 3. Xylan-derived xylose and cellulose-derived glucose concentrations in the aqueous fraction. CW and XW are the mass of cellulose and xylan, respectively, recovered in the aqueous fraction respective to their initial contents in the CSs.

T	GVL/Water (w/w)	CW	XW	[Xylose] g/L	[Glucose] g/L
150	0.65	13.99%	19.80%	10.06	16.90
160	0.75	1.96%	2.85%	2.00	3.28
150	0.55	5.33%	57.22%	22.77	5.04
150	0.65	11.75%	27.27%	13.81	14.16
140	0.55	4.56%	53.16%	21.01	4.29
150	0.65	8.05%	37.14%	18.96	9.77
140	0.65	6.54%	41.98%	21.33	7.90
160	0.55	5.63%	59.22%	23.34	5.28
140	0.75	14.14%	11.94%	8.44	23.78
150	0.75	14.35%	11.80%	8.44	24.42
150	0.65	11.77%	33.66%	16.93	14.09
150	0.65	8.07%	41.92%	21.27	9.74
160	0.65	7.59%	38.25%	19.20	9.06

The total monosaccharide concentration ranged between 25 and 30 g/L and showed high variability in term of chemical species (i.e., xylose and glucose), especially among the

replicated treatment points (central points of the DoE). The high variability was probably due to operator error during manual recovery of the aqueous fraction from the treated GVL/water solution.

The pretreatment conditions caused the degradation of xylose into unwanted products, such as furfural and formic acid [38]; therefore, xylan recovery was below 60% for all samples. These degradation products, although being inhibitors of microbial growth, were found to be present in concentrations far below the tolerance limit value [39].

Due to the low and variable xylan recovery values, this output was not used for modelling the experimental response. Despite these figures, the addition of salt is essential for recovering the lignin-containing GVL solution, which can be further treated by CO_2 extraction for GVL recycling and lignin precipitation, as demonstrated by Luterbacher et al. [40]. In addition, the same authors showed that a 12% (*w*/*w*) saline solution of monosaccharides could be used as a substrate for ethanol-producing yeast.

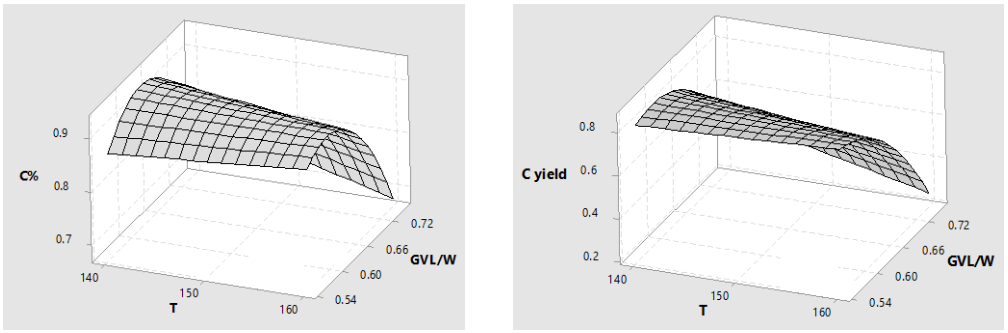
3.2. Optimization of Cellulose Recovery and Enrichment

The cellulose content in the CP (C%) and CY values obtained at the end of the runs were analyzed by RSM methodology using backward elimination with a significance level of 0.1. The linear regression equations expressed in uncoded units (Equations (4) and (5)) explain the total deviance in the C% and CY responses with an R2 (adjusted) of 98% and 89%, respectively.

$$C\% = -5.798 + 0.01925\ T + 17.35\ GVL/W - 10.174\ GVL/W \times GVL/W - 0.03239\ T \times GVL/W \quad (4)$$

$$CY = -9.07 + 0.0373\ T + 24.99\ GVL/W - 13.62\ GVL/W \times GVL/W - 0.0644\ T \times GVL/W \quad (5)$$

The residuals of the regression model were normally distributed and random. The model equations (Equations (4) and (5)) were used to create a surface plot with the two variables (GVL concentration and temperature) on the x- and y-axes and a continuous surface that represents the fitted response values on the z-axis (Figure 1a,b). The two response surfaces are curved because the models contained quadratic terms that were statistically significant (Equations (4) and (5)). The ANOVA and the coded coefficient of the two models are provided in the Supplementary Material.



(**a**) (**b**)

Figure 1. Response surface of the DoE. The factors are temperature and GVL/water solution concentration; in (**a**), the response is cellulose content (C%); in (**b**), the response is cellulose recovery yield (CY).

The highest values of C% and CY were in the mid-lower edge of the plots (Figure 1a,b), which corresponds with low values of GVL concentration. The highest GVL concentration and temperature produced cellulose carbonization (the collected samples showed a dark color), reducing the cellulose enrichment and, concurrently, increasing the acid-insoluble residue. The same authors already observed this phenomenon in a previous work, where at the harsher condition, a carbonized sample showed a cellulose content equal to 0 [28]. Since the acid concentration was calculated based on total solution weight, the more concentrated

the GVL/water solution is, the more the acid concentration in the water fraction increases. A higher acid concentration combined with improved catalytic activity in the GVL-rich reaction environment caused cellulose loss in terms of solubilization and carbonization [41].

However, as shown in previous work [28], an acid concentration of at least 2% w/w is fundamental to obtain highly digestible cellulose. Given this acid concentration, it seems that maximum C% and CY were achieved approximately at a GVL/water concentration of 0.6, since lower concentrations did not efficiently remove lignin, reducing the cellulose enrichment (Figure 1a).

The treatment temperature had little effect on C% and CY values, especially at lower GVL concentrations, where higher temperatures caused a slight drop in cellulose enrichment and recovery. At increasing GVL concentrations, high temperatures had a greater magnitude on response values, producing CP with very low C% and CY values.

Equations (4) and (5) were used by Minitab to predict the optimal solution for simultaneously maximizing the two responses (C% and CY), and the result is shown in Table 4.

Table 4. Factor settings for the optimal solution and composite desirability. The goals was to maximize CY and C%.

Factor Settings	Response	Composite Desirability	Fit	SE Fit	95% CI
140 °C; 0.6 GVL/water (w/w)	CY	0.97	0.83	0.037	(0.74; 0.91)
	C%		0.93	0.0069	(0.91; 0.94)

A composite desirability value near 1 means that the combination of proposed factor level settings satisfied very well the goals defined for the two responses (i.e., maximum value). Indeed, a C% between 91% and 94% and a CY between 74% and 91% can be obtained by performing an OV treatment at the optimal solution conditions. These findings were experimentally confirmed by running an OV pretreatment of CSs at the suggested conditions in triplicate. The samples obtained (named CSopt) underwent chemical characterization and enzymatic hydrolysis, and the average outcomes are shown in Table 5. The treated pulp was almost entirely made up of cellulose (91.49%), and the remaining fraction was lignin, since the xylan was completely removed. The cellulose recovered after the treatment (CY) was 86.04%, and complete saccharification (100%) was observed. This proved that the predictions of the mathematical model were consistent with the experimental observations.

Table 5. Cellulose pulp and aqueous fraction characterization after the OV pretreatment with optimized factor settings. CW and XW are the mass of cellulose and xylan, respectively, recovered in the aqueous fraction respective to their initial contents in CSs.

Sample	CY	C%	HY	CW	XW	[Xylose] g/L	[Glucose] g/L
CSopt	86.04% ± 3.29	91.49% ± 2.38	100% ± 1.55	5.05% ± 0.45	51.88% ± 2.08	26.16 ± 0.65	6.19 ± 0.44

In addition, the analysis of the CSopt aqueous fractions showed that about 5% of the initial cellulose was solubilized, meaning the total loss of cellulose due to treatment conditions was lower than 5%. The xylan reached a less satisfying result in terms of recovery, since 50% of the initial content was lost as unwanted by-products. In any case, the carbohydrates recovered in the aqueous fraction were in monosaccharidic form at a concentration of about 32 g/L. Due to the saline environment, the aqueous fraction could be employed in industrial fermentation with halophilic microorganisms. In a recent work, Kucera et al. produced polyhydroxyalkanoates (PHAs) from *Halomonas halophila* using different carbon sources, including xylose [42]. Otherwise, the aqueous fraction could be used in hydrothermal processing as it is or after a desalination process [43,44].

The positive findings of the CSopt sample confirmed and enhanced the results obtained in previous work by the same authors demonstrating the great potential of the GVL-OV process in the production of enriched and highly digestible cellulose pulp from CSs [28].

The CY and C% of CSopt were comparable to those obtained with other biomasses treated with GVL/water mixtures. Yang et al. used a GVL/water mixture to treat a hardwood bleached kraft pulp, obtaining CY and cellulose contents of 76.8% and 92.2%, respectively [45]. Pulp with a cellulose content of 88.8% and a CY of 82.1% was obtained by Trevorah et al. by treating *Eucalyptus obliqua* sawdust in an acidified GVL/water mixture (50/50) at 150 °C for 85 min [46]. The complete saccharification of the CSopt sample was achieved thanks to the GVL/water mixture that facilitated cellulose deconstruction better than other OV/water mixtures, such as ethanol- and acetone-water [47], and to the acid catalyst that decreased the cellulose degree of polymerization [15].

3.3. High Solid and Low Cellulase Enzymatic Hydrolysis

The CSopt samples underwent nine enzymatic hydrolysis runs using different solid and enzymatic loadings to approach the industrial condition. Figure 2 shows that every sample achieved an HY higher than 70% after 120 h, demonstrating the possibility to also hydrolyze the CP with low enzymatic and high solid loadings. Samples 7, 8, and 9, which possessed the highest enzymatic loading (60 FPU/g cellulose), were used as a reference and showed an HY over 96%, with sample 7 completely hydrolyzed (HY equal to 100%). Different glucose concentrations were achieved in the final hydrolysate, from 43 and 48 g/L (sample 1) to 235.94 g/L (sample 9), as shown in Figure 3.

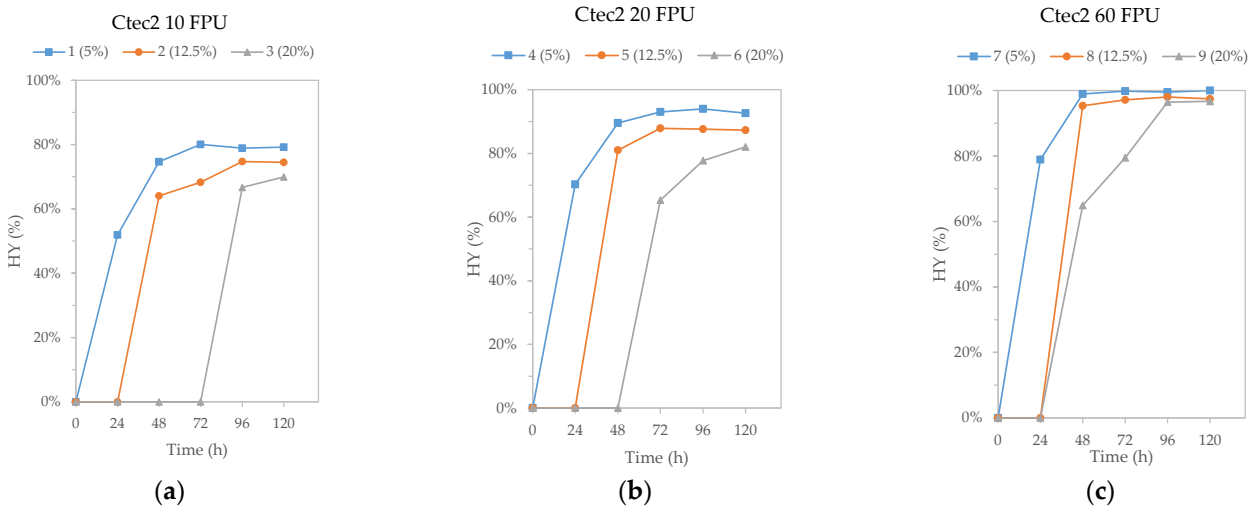


Figure 2. Enzymatic hydrolysis yield (HY) of CSopt saccharification using different solid loadings (5%, 12.5%, and 20% *w*/*w*) at low cellulase (Ctec2) loading (10 FPU/g cellulose, (**a**)), medium cellulase (Ctec2) loading (20 FPU/g cellulose, (**b**)), and high cellulase (Ctec2) loading (60 FPU/g cellulose, (**c**)).

As expected, with the same SL, higher glucose concentration and HY were achieved in the samples with higher enzymatic loading (Figures 2 and 3). All the runs that were performed with a solid loading of 5% showed a glucose concentration lower 60 g/L (Figure 3a) and the highest HY (Figure 2). Glucose concentration higher than 100 g/L and 150 g/L was achieved using solid loading of 12.5% and 20%, respectively. These results demonstrated that the concentration of glucose increased correspondingly with the increase in solid content (Figure 3b,c). Notably, the concentration of glucose obtained at 20% SL was not 4-fold higher than that at 5% SL, meaning the HY decreased with increasing SL (Figure 3).

The reduction in enzymatic efficiency was due to the so-called "high solid effect". Since the lignin content was drastically reduced by the OV pretreatment, this effect was mainly caused by end-product inhibition and mass transfer limitation. Mass transfer limitation, due to the small quantity of free water among the fibers of the biomass and their tendency to build interjunctions, affects the hydrolysis process through poor contact between the enzymes and substrate, reducing the HY [37]. Therefore, by using a more

efficient agitation system, such as a mixer, for viscous liquids, a higher HY and glucose concentration could be achieved [48].

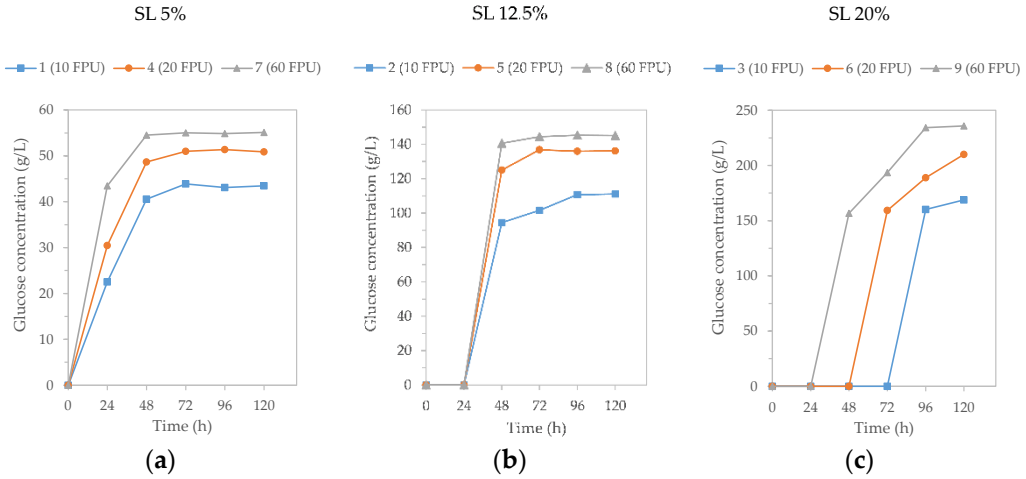


Figure 3. Glucose concentration (g/L) during enzymatic saccharification of CSopt using different cellulase loadings (10, 20, and 60 FPU/g cellulose) at low solid loading (SL of 5% *w*/*w*, (**a**)), medium solid loading (SL of 12.5% *w*/*w*, (**b**)), and high solid loading (SL of 20% *w*/*w*, (**c**)).

Since cellulase accounts for a large portion of the production cost of glucose [23], low enzyme and high solid (20% *w*/*w*) loadings facilitate the economic feasibility of lignocellulosic biorefinery [49,50]. In this regard, samples 3 (10 FPU/g cellulose) and 6 (20 FPU/g cellulose) showed interesting findings, since the glucose concentration at 120 h was 169 g/L and 210 g/L, respectively (Figure 3c). In addition, samples 3 and 6 showed (Figure 2b,c) an HY (70% and 82%, respectively) that was slightly higher than that obtained on average with other agricultural residues pretreated differently and hydrolyzed with high solid loadings and low FPU values [12]. A good compromise between cellulase dosage and solid loading was achieved by sample 5. Thanks to medium solid loading (12.5% *w*/*w*) and FPU value (20 FPU/g cellulose), at 72 h, sample 5 produced a glucose concentration of 137 g/L with an HY of 87%.

Finally, Table 6 shows the OY obtained from CSopt using different enzymatic hydrolysis conditions, which was essential for evaluation of the biomass conversion process proposed in this article [49]. Extended time to achieve the final OY was required for increased solid loading and decreased cellulase addition (Figure 3).

Table 6. Overall yield (OY) obtained for the CSopt sample with different enzymatic hydrolysis conditions.

Csopt Sample Number	Solid Loadings (%)	Cellulase Loading (FPU/g Cellulose)	OY (g Glucose/100 G RM)
1	5	10	30.44
2	12.5	10	28.63
3	20	10	26.89
4	5	20	35.60
5	12.5	20	33.55
6	20	20	31.52
7	5	60	38.43
8	12.5	60	37.46
9	20	60	37.17

As discussed above, the maximum OY was obtained with sample 7 (38.43 g glucose/100 g RM), which underwent enzymatic hydrolysis with low solid loading (5% *w*/*w*) and high enzymatic dosage (60 FPU/g cellulose). Interestingly, at this FPU value, samples with higher solid loadings (8 and 9) achieved an OY of about 37 g glucose per 100 g RM, meaning the previously mentioned high solid effect was less pronounced for high enzyme

loading. Therefore, when subjected to enzymatic hydrolysis with high cellulase addition, the CSopt sample showed an OY of about 90–92% of the maximum theoretical yield (41.4 g glucose/100 g RM). These figures are extraordinary results for herbaceous biomass, such as cardoon. For example, Ballesteros et al. [24] achieved an OY of 31.4 g glucose/100 g raw material (corresponding to 85% of the theoretical maximum) after pretreating the cardoon by dilute sulfuric acid and performing enzymatic hydrolysis with a solid loading of 2% *w*/*v*. Nevertheless, at the same pretreatment condition, only 6.5 g xylose/100 g RM (corresponding to about 38% of the theoretical maximum) was recovered. Vergara et al. [27] obtained a cellulose-enriched pulp, recovering over 92% of the initial cellulose after pretreating the cardoon by an ethanol/water mixture. In the best hydrolysis condition, only 58% of the cellulose-enriched pulp was converted to glucose.

Due to degradation of the enzymatic hydrolysis performance at low and medium enzyme dosages at the highest solid loading level, the OY value showed a reduction. For example, samples 3 and 6 attained a final OY equal to 65% and 76% of the theoretical value, respectively. These figures are still decent, especially considering the high glucose concentration obtained at the end of enzymatic hydrolysis.

4. Conclusions

The present work proposed an efficient pretreatment for CSs using an acidified GVL/water solution able to produce a cellulose-enriched pulp (91.5%) easily hydrolyzed by a low dosage of cellulase at a high solid loading. As a result, the CSs can be used as feedstock to produce hydrolysates at high glucose concentrations. Glucose production of 37.17 g/100 RM (90% of the maximum theoretical value) was achieved with high solid loading (20%) and high enzyme dosage (60 FPU/g cellulose). Enzymatic hydrolysis showed a slightly lowered performance (70% and 82%) when the CS pulp was hydrolyzed at high solid loading with low cellulase addition. In this scenario, high glucose concentrations (169 g/L and 210 g/L) were attained using low (10 FPU/g cellulose) and medium (20 FPU/g cellulose) enzyme dosages, respectively. Enzymatic hydrolysis efficiency could be improved using a better agitation system, such as a stirred reactor with an impeller, for viscous fluids.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en15072600/s1, Table S1: Cellulose yield (CY), cellulose content (C%), and digestibility (EH) of pulp after the OV pre-treatment at different conditions.

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